NONSEGMENTED NEGATIVE STRAND VIRUSES

Paramyxoviruses and Rhabdoviruses

Edited by

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1984

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Preface

Negative strand viruses include several families of large, enveloped RNA viruses. They are responsible for many important common or exotic, human and animal diseases, including influenza, mumps, measles, rabies, canine distemper, some human encephalitis, and hemorrhagic fevers. Four previous symposia have been held on negative strand viruses. Three were organized by Drs. R. D. Barry and B. W. J. Mahy and were held in Cambridge, England, in 1969, 1973, and 1977. A fourth symposium was held in St. Thomas, Virgin Islands, in 1980, and was organized by Drs. D. H. L. Bishop and R. W. Compans.

Research on the molecular biology of negative strand viruses has continued over the last years at an accelerated pace and was recently reviewed at a fifth symposium held September 11–17, 1983, at Mariner's Inn, Hilton Head, South Carolina. To focus the subject matter, the proceedings of this symposium are being published in two volumes. One volume concerns the three negative strand virus families with segmented RNA genomes: arenaviruses, bunyaviruses, and orthomyxoviruses. This volume contains papers on negative strand virus families with nonsegmented genomes: paramyxoviruses and rhabdoviruses.

Rhabdoviruses and paramyxoviruses, although evidently different in their biological, biochemical, and structural properties, do share certain basic organizational features such as gene product arrangements and transcriptional signals. Thus studies of the two virus families are complementary in nature and advances in the field have developed in parallel. The papers in this volume reflect such developments and illuminate the various stages in the strategy of negative strand virus infections: adsorption, penetration, mRNA transcription, translation, RNA replication, morphogenesis, virus release. Also addressed are questions concerning the biology of virus infection and host response.

We would like to acknowledge the sponsorship of the symposium by the University of Alabama in Birmingham, and financial support provided by the following organizations: Amicon; Biocell Laboratories; Elsevier/North Holland; Hoffmann–La Roche, Inc.; Imperial Chemical Industries; New Brunswick Scientific Company, Inc.; Pitman-Moore, Inc.; Rheem Manufacturing Company, Inc.; Vangard International, Inc.; and Wheaton Instruments.

We also wish to thank all of the participants who contributed to the success of the meeting and the authors who have provided an excellent and timely series of manuscripts. Our special thanks are also due to the following participants who served as chairmen of the scientific sessions: Drs. G. M. Air, M. A. Bratt, J. M. Dalrymple, N. J. Dimmock, S. U. Emerson, A. Flamand, C. Y. Kang, E. D. Kilbourne, D. W. Kingsbury, H.-D. Klenk, D. Kolakofsky, W. G. Laver, R. A. Lazzarini, D. P. Nayak, M. B. A. Oldstone, P. Palese, M. Schubert, and G. W. Wertz.

Finally, we would like to thank Betty Jeffrey and Denice L. Montgomery, both of whom worked cheerfully and extraordinarily hard in organizing the symposium and in preparing these volumes for publication.

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Contents of *Segmented Negative Strand Viruses*

Genome Structure, Transcription, and Genetics

Gene Expression, Protein Synthesis, and Protein Modification

Biology

ANALYSIS OF THE SENDAI VIRUS GENOME BY MOLECULAR CLONING

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A special focus of this laboratory is the study of the control mechanisms of negative strand RNA viral transcription and replication. We have demonstrated that the VSV leader region contains at least two signals, a nucleation site for leader chain encapsidation and a genomic transcription termination site, both of which are functionally keyed to the presence of the VSV nucleocapsid (N) protein (1,2,3). Control of transcription and replication in Paramyxoviruses and in Bunyaviruses is likely to be more complicated, as these viruses produce multiple leader RNAs (4,5), and each also codes for nonstructural proteins which have been suggested to function in such a control capacity (6,7,8).

The C protein of Sendai virus is particularly interesting because, although it is barely detectable in the mature virion, it is present in infected cells and can be synthesized _in vitro_ using mRNA from infected cell cytoplasm (6,9). The C protein has been shown to have a unique peptide map (6,10), and although annealing studies have shown that esentially the entire Sendai genome is transcribed into viral mRNAs (11,12), no viral mRNA corresponding to the small size (22kd) of this protein has been reported. The finding that C protein is among the _in vitro_ translation products of mRNAs cosedimenting on sucrose gradients with that for the 79kd viral P protein (9) suggested that the C mRNA might be part

---

of a larger structure. Since the 15kb coding capacity of
Sendai would allow for any of several possibilities – tandem
or overlapping cistrons, or even spliced mRNA, however un-
likely for this cytoplasmic virus – cloning of the viral
genome was essential for determining its organization.

CLONING AND MAPPING OF THE 3´ PROXIMAL THIRD OF THE SENDAI
VIRUS GENOME

     The molecular cloning of the Sendai virus genome has
been described by Dowling et al.(13).   Briefly, methyl-
mercury-denatured virion 50S RNA was reverse transcribed
using calf thymus pentamers as primers, the second DNA strand
was synthesized by boiling at pH8 and incubating with the
Klenow fragment of DNA polymerase, and the mixture was
treated with S1 nuclease to generate ds-DNA. These viral-
specific DNAs were cloned into the PstI site of pBR322 by
poly dC/poly dG tailing, and tet^R amp^S transformants were
isolated and screened by the method of Grunstein and Hogness
(14) using as probes partially alkali digested, radiolabelled
Sendai 50S RNA. To order the genome, clones containing viral
3´ end sequences were selected using ^{32}P – *in vitro* virion
polymerase RNAs as probes, since these RNAs should favor 3´
end gene products(4,9)– whence the designation of these
clones as S(Sendai), L(leader) or N(NP). A series of overlap-
ping clones selected by these probes was provisionally or-
dered by a combination of dot blotting, primer extension and
restriction mapping. The DNA sequence of one clone was found
to match the RNA sequence of the genome from position 22 of
the 3´ end onwards, thus establishing the orientation of the
series.   Positive selection or hybrid arrest of translation
of the viral proteins using restriction fragments from these
clones annealed to CsCl pellet RNA from Sendai virus infected
cells then revealed the gene order to be 3´-NP-P+C-M.
     Giorgi et al.(15) have determined by DNA sequencing of
the P+C region of the genome that P and C are translated in
overlapping reading frames from a single mRNA. Here we
report the addition to our series of a further clone which
contains the termination site of the M gene, as shown in the
updated genomic map (Figure 1).

IDENTIFICATION OF A CLONE CONTAINING THE END OF THE M GENE

     A rough calculation of the coding capacity required for
the NP (59kd), P+C (79kd) and M (35kd) proteins, about 4800
bp, suggested that the end of the M gene would lie beyond the
clones mapped in Dowling et al.(13). To find a further clone
which would contain this site, duplicates of the Grunstein-

Figure 1. Map of the 3′ proximal third of Sendai virus. Bars denote the clones used to determine the map. The restriction sites shown are sufficient to establish the alignment of the clones, but not all sites have been marked.

D = DdeI
R = RsaI
P = PstI
H = HaeIII
f = HinfI
E = EcoRI
A = AvaI
III = Hind III

Hogness filters used originally to screen for viral-specific
clones were therefore re-screened with nick-translated (16)
probes made from the excised viral-specific PstI inserts of
these clones. Those colonies which were positive to the SL-2
probe and negative to a mixed probe made from the inserts of
all clones more 3′ proximal than SL-2 were picked for fur-
ther analysis, and a preliminary PstI restriction digest was
performed on minilysates of these colonies to determine the
sizes of the viral-specific inserts in their plasmids (data
not shown). Twelve clones containing the largest inserts
were then grown in one-liter cultures, and the plasmids were
extracted (17) and isolated by centrifugation in CsCl.

      These DNAs were then subjected to a combination of dot
blot and detailed restriction analysis, as described in Dow-
ling et al.(13). Plasmid DNA (0.5ug) from these and other
clones was dotted identically onto four nitrocellulose fil-
ters (18), and hybridized in turn, under formamide annealing
conditions (19), with nick-translated probes made from their
excised PstI inserts. Figure 2 panel A shows the results
using a mixed probe specific for 3′ proximal sequences
through SN-5, panel B using SL-2 as a probe, and panel C
using the clone 11/15-9 (upper right corner of these grids -
the numbering system of this series of clones derives from
their Grunstein blot number and location). Nine new clones
were strongly positive to SL-2. Those at grid positions 3-8
and 5-1 (this clone corresponds to 12/9-7 on the map) were
identified as lying to the left side of SL-2 because they
were also positive to the mixed probe. A gap between 12/9-7
and the clone at grid position 1-8 (corresponding to 11/15-9
on the map) was indicated since 12/9-7 (grid position 5-1)
did not hybridize to the 11/15-9 probe (Figure 2, panel C).
The clones were next aligned by digestion with several
restriction enyzmes. Two of the ethidium bromide stained gels
are shown in Figure 3. Insert bands which are unchanged by
PstI digestion are identified as internal fragments of the
clone. The clone 11/15-9 apparently contained only internal
restriction fragments (e.g. the 135bp Rsa-Rsa band) which
were also contained within SL-2, whereas another strongly
selected clone (12/1-10 on the map, circled on the grid)
clearly contained no internal restriction fragments in common
with SL-2. The presence uniquely in 12/1-10 of the large 370
bp Hinf-Hinf fragment further suggested that this clone might
lie well beyond the end of SL-2, and thus the map layout
would be as shown in Figure 1.

      Comparison of the restriction patterns established the
probable insert orientations and boundaries of the mapped
clones, and a small (~30bp) Hinf-Hinf fragment common to SL-2
and 12/1-10 was eventually found by radiolabelling (not
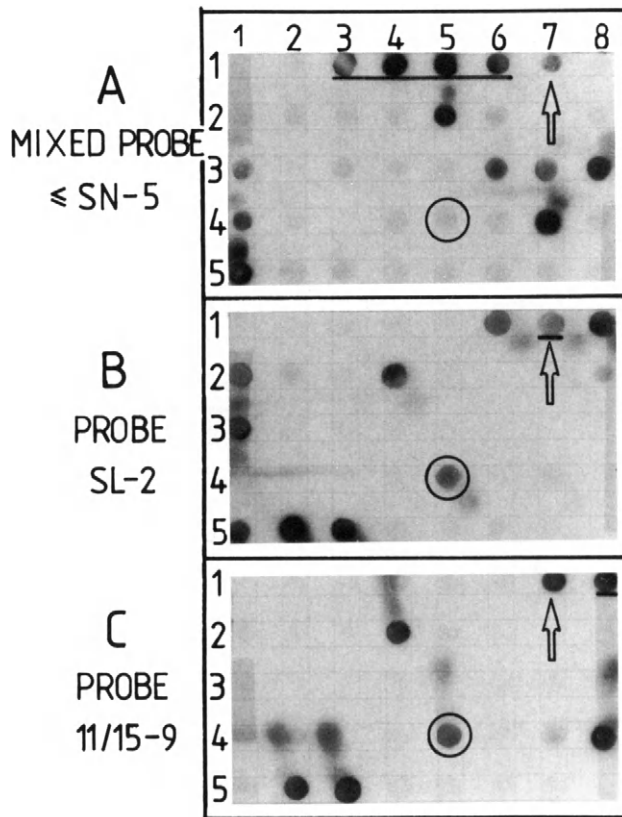
Figure 2. Dot blot analysis of SL-2 related clones. Clones used as probes are underlined. The grid position of SL-2 is marked with an arrow, that of clone 12/1-10 is circled. Control DNAs from E coli B and pBR322 are at grid positions 1-1 and 1-2 (upper left).

shown). Maxam-Gilbert (20) sequencing from the Hind III site common to SL-2 and 12/1-10 has revealed the genome sequence 5´- AUUUAUUCUUUUUGAAUCCC -3´, located just beyond the G-C tail of SL-2. This sequence appears near the end of a sequence of 700 continuous bases in SL-2 and 12/1-10, in which there is a single open reading frame (unpublished, work in progress), and contains the conserved Sendai polyadenylation signal determined by Gupta and Kingsbury (21). It would also code for a mRNA beginning Cap-AGGG---, a sequence also found at the start of the viral NP, P+C and M genes (15,21).
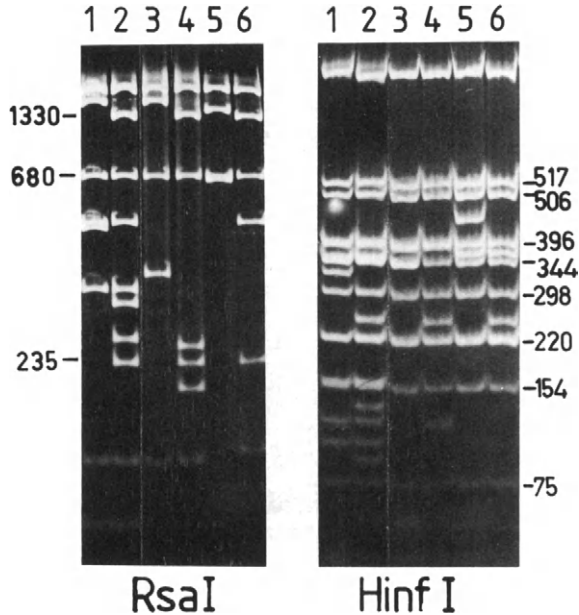
Figure 3.   Restriction analysis of SL-2 related clones. Three-microgram aliquots of SL-2 (lanes 1,2), 11/15-9 (lanes 3,4) and 12/1-10 (lanes 5,6) were digested with either RsaI or HinfI alone (odd-numbered lanes), or with these enzymes plus Pst I (even-numbered lanes).

This sequence must thus represent the end of the M gene and the start of the next.

REMARKS
    Ordering the Sendai virus genome by the overlapping clone technique may appear cumbersome, but it has the virtue of giving a continuous sequence at intercistronic regions. It furthermore allows us to control for spliced mRNAs by S1 mapping and primer extension studies (15), and restriction fragments for these studies are relatively easy to isolate from the smaller clones.   There are pitfalls in this technique: for example, poly G-C tailing may create spurious restriction sites at the ends of a clone.   The resulting spurious restriction fragments may then make ordering of the clone difficult.   Such a spurious Dde I restriction fragment was actually present in 11/15-9 (data not shown).
    We are presently trying to patch over a different sort

of pitfall: the location of the glycoprotein genes. Although
the Sendai genome was provisionally mapped some time ago by
UV inactivation (22), the order reported (3´NP-F-M-P-HN-L)
differs both from that reported (23) for NDV (3´ NP-P-(F+M)-
HN-L) and from the map determined in our studies. As discus-
sed by Dowling et al.(13), the assumption of a single entry
promoter made in these UV studies may not hold true for
Sendai virus. If, for example, a separate internal promoter
were responsible for the relatively high molar rate of F
protein synthesis in Sendai virus (22,24), this gene could
now occupy any position on the genome after M. By means of
our cumbersome technique, we have assembled two further
clones, accounting for about 1800 bp of new sequence.
Hopefully these clones should provide enough information to
identify the next gene.

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NON-CODING REGULATORY SEQUENCES OF THE SENDAI VIRUS GENOME[1]

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LEADER RNA TEMPLATES

Of the 15,000 bases in the Sendai virus genome, it appears that less than 2% are dedicated to regulatory purposes. At the genome's 3' end, there is a leader RNA template about 50 bases long, where RNA synthesis initiates and where the RNA-synthesizing enzyme complex may be committed to proceeding in the replicative mode, producing a full-length antigenome (1; Re, Morgan, Gupta, and Kingsbury, this volume). Alternatively, the enzyme complex may act as a transcriptase, obeying the sequences that signal termination of leader RNA synthesis and initiation and termination of virus mRNAs. At the 5' end of the genome, there is encoded a homolog of the positive strand leader RNA template (1,2). In complementary form at the 3' end of the anti-genome, this negative strand leader RNA template would serve as the initiation site for synthesis of genome replicas; the termination signal for the negative strand leader may permit early abortion of genome replication under conditions unfavorable for extending the chain, such as a temporary unavailability of proteins to encapsidate it (3).

In our studies of the terminal and internal regulatory sequences of the Sendai virus genome (2,4,5), we have seen several homologies with the regulatory sequences of the other main model of the nonsegmented negative strand RNA viruses, vesicular stomatitis virus (VSV). As shown in Fig. 1, the 46 base VSV positive strand leader RNA template and the 51 base Sendai virus positive strand leader RNA tem-

Sendai virus:
 - 3'-<u>UGGUUUGUUCUC</u>UUC<u>UUU</u>G<u>C</u>ACAU<u>A</u>CCUUAU<u>AUAUU</u>ACUUC<u>AAUCUG</u>UCCU...
 + 3'-<u>UGGUCUGUUCUC</u>AAA<u>UU</u>CU<u>C</u>UAUA<u>A</u>AUAAGA<u>AAAUU</u>UAAAAG<u>AACAG</u>AAGA...
                  | | | | |
                  10   20   30   40   50
VSV: | | | | |
 - 3'-<u>UGCUUCUG</u>UUUG<u>UUUGGU</u>AAUAA<u>UAGUAAUUUU</u>CCGAGU<u>CCUCU</u>UU...
 + 3'-<u>UGCUUCUG</u>GUGU<u>UUUGGU</u>CUAUU<u>UUUUAUUUUU</u>GGUGUU<u>C</u>UC<u>C</u>CAG...
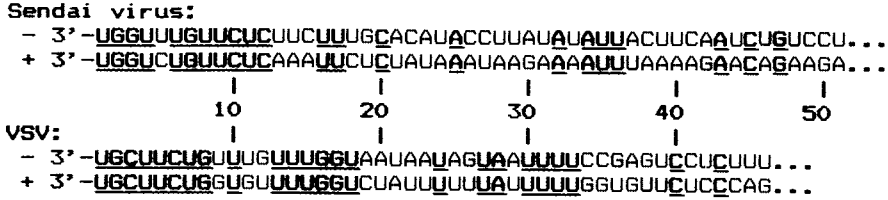
Base Composition: A+U (%)

Sendai virus:	–	67
	+	75
VSV:	–	67
	+	63

FIGURE 1. 3'-terminal nucleotide sequences of Sendai virus (Enders strain) and VSV (Indiana) genomes (–) (Re, Morgan, Gupta, and Kingsbury, this volume; 13) and antigenomes (+) (2,14).

plate are rich in the bases A and U (especially U), reflecting homologies (and an evolutionary relationship) between the two viruses, most marked in their first 20 bases (5).   Even more striking are the homologies between the sequences of positive and negative strand leader templates within each virus genome (6), providing a common initiation signal recognizable by a single RNA polymerase complex (7).

SEQUENCE SIGNALS AT GENE JUNCTIONS

     Conceptually and functionally, we can divide the base sequences at the junctions of Sendai virus genes into three regions: the end (E) of the upstream gene, an unexpressed intergenic (I) sequence, and the sequence that signals the start (S) of the next downstream gene (Fig. 3).   The E sequence of each Sendai virus gene comprises a tetranucleo-tide, 3'-AUUC, followed by (U)$_5$ (4).   The tetranucleotide appears to represent the termination signal for transcrip-tion and the (U)$_5$ is the signal for initiating 3'-terminal poly(A) addition. Reexamination of our data reveals another consensus, a U residue two bases upstream from 3'-AUUC (Fig. 3).   However, despite clear homologies with the VSV genome in the terminating tetranucleotide and oligo(U) sequence, nothing comparable to this penultimate U exists in the VSV genome (8,9).

Upstream gene	Next gene	Sequence
leader	NP	3'...AAA UCCCAGUUUCAUAGG...
NP:48	P(Fₒ)	...GAA UCCCACUUUCAAGUA...
P(Fₒ):45	M	...GAA UCCCACUUUCUUUAA...
M:47	Fₒ(P)	...GAA UCCCUAUUUCAGGGA...
Fₒ(P):44	HN	...GAA UCCCACUUUCACUCC...
HN:46	L	...GGG UCCCACUUACCCUUC...
VSV Consensus		...GA UUGUCNNUAG...

FIGURE 2.   Intergenic and mRNA start signals of Sendai virus (Enders strain).   Numbers in the first column designate the gene-terminal oligonucleotides sequenced previously (4). Uncertainties in the order of the P and F₀ genes are indicated by parentheses.

SEQUENCES OF THE I AND E REGIONS

The following steps were taken to determine the sequences downstream from each gene-terminal E region. First, upstream sequences were determined. mRNAs extracted from infected cells were reverse-transcribed using a radio-labeled phasing primer, 5'-(dT)$_{10}$dCdT.   Treatment of the products with restriction endonucleases capable of cleaving single stranded DNA (10) yielded several electrophoretically separable 5'-labeled cDNA fragments.   Each fragment had a 5'-proximal sequence identical to one of the E sequences (4).   A synthetic oligodeoxynucleotide primer complementary to a sequence slightly upstream from each E sequence was then synthesized.   Each primer, labeled at its 5' terminus, was separately used in the reverse transcription of virus genomes, for sequence determination by dideoxynucleotide (11) and chemical cleavage (12) methods.
Next to the (U)$_5$ polyadenylation signal, we found the trinucleotide 3'-GAA in four cases and GGG once (Fig. 2). The 3'-AAA that follows the leader template sequence (Re, Morgan, Gupta and Kingsbury, this volume) also has homology with these trinucleotides.   Next, the pentanucleotide 3'-UCCCA was found in five of six Sendai virus genes, a U being substituted for the A in one case.   After a single hetero-geneous nucleotide, the tetranucleotide 3'-UUUC appeared in five of six genes, an A being substituted for U in the third position of one gene.   Thereafter, four of six genes had a consensus A, which may also contribute to the mRNA start signal (Fig. 2).
We believe that the 3'-GAA and its congeners represent the intergenic (I) sequence and that mRNA start (S) signals begin at the next U.   This conclusion was supported by

Consensus Sequence
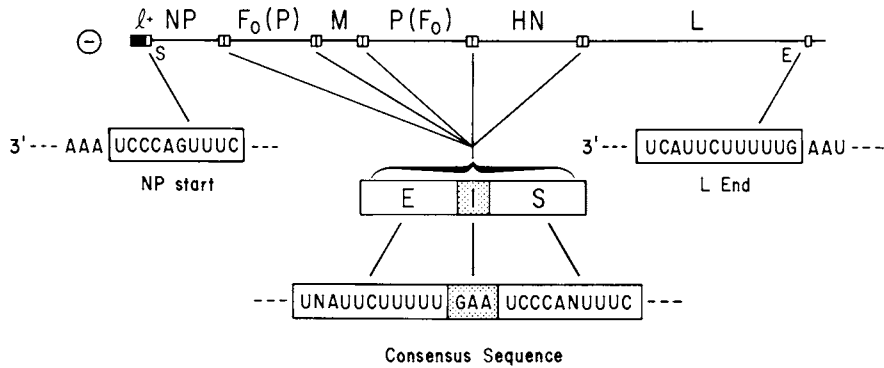
FIGURE 3.   Diagram of the Sendai virus genome, the locations of its transcriptional regulatory signals and their nucleotide sequences.   E, I and S refer to mRNA end, intergenic and start signals, respectively.   Uncertainties in the order of the P and F_0 genes are indicated by parentheses.

sequencing the 5' termini of several viral mRNAs (data not shown; D. Kolakofsky, personal communication).   These assignments are clearly analogous to the VSV transcriptional control signals, which have 3'-GA as the I sequence, followed by a consensus pentanucleotide S signal starting with U, then by a heterogeneous dinucleotide and a consensus 3'-UAG (Fig. 2).

THE COMPLETE SET OF SIGNALS

These sequences may represent the entire complement of nucleotides, about 200, that regulate the expression of the remaining 15,000 nucleotides in the virus genome (Fig. 3). The organization of the Sendai virus genome is so similar to that of the VSV genome, not only in gene order, but in the strong homologies between regulatory sequences, that there can be little doubt that these representatives of the para-myxovirus and rhabdovirus families have evolved from a common ancestor (4).
However, a point of divergence between Sendai virus and VSV is in their S signals; although organized in a similar manner, there were few base homologies between them.

Perhaps mRNA initiation (or, more properly, reinitiation) signals do not make stringent demands on the recognition faculties of the viral RNA polymerase. In contrast, strong homologies in the E and I regions of both viruses underscore the special significance of transcription termination for the nonsegmented negative strand RNA viruses. The same template must be used both for transcription and replication and it is essential that the viral RNA-polymerizing enzyme can be instructed to observe those termination signals strictly during transcription and ignore them absolutely during replication. Perhaps this feat is so difficult that alternative solutions have not been readily generated by the evolutionary process.

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STRUCTURE OF A COMPLETE CLONE OF THE SENDAI VIRUS NP GENE[1]

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CONSTRUCTION OF THE CLONE

We have been generating molecular clones of Sendai
virus genes to determine the primary structures of the pro-
teins they encode and to perform in vitro genetic manipula-
tions (1). Using virus-specific mRNA templates, we obtained
cDNA clones representing sequences of the genes for the NP,
M and P proteins, but none of the clones contained much more
than half of any of those genes (1).

A modified procedure has now provided a full-length
clone of the NP gene. As before, the mixture of mRNAs taken
from Sendai virus-infected cells was used as template for
reverse transcription primed by oligo(dT). However, this
time, we selected the largest transcripts by centrifugation
in alkaline sucrose gradients (2), tailed them with
oligo(dC) and synthesized the second strand with reverse
transcriptase primed with oligo(dG)$_{10-12}$ (3). The largest
double-stranded DNA molecules were selected in neutral
sucrose gradients (2) and inserted into the PstI site of
pBR322 (1). NP-specific clones were identified by blot
hybridization with radiolabeled DNA from two shorter NP
clones (2). One of the colonies contained a plasmid, desig-
nated pNP3, with an NP insert about 2000 base pairs long,
congruent with the expected size of the NP gene (4).

IDENTIFICATION OF 5' AND 3' GENE TERMINI

Base sequences at the termini of pNP3 were determined
both by labelling the PstI sites with [^{32}P] dideoxy ATP and

by labelling the 3' termini of internal restriction sites
with the Klenow fragment of Escherichia coli DNA polymerase
I.   The sequence 5'-AGGGT at the 5' terminus of the clone,
followed, after a space of one nucleotide by 5'-AAAG, corre-
sponds to the initiation consensus sequence described for
all Sendai virus mRNAs (Gupta, Re and Kingsbury, this
volume).   Downstream, the sequence corresponded to that of
virus genomic RNA (Re, Gupta, Morgan and Kingsbury, this
volume), confirming that the gene represented in the pNP3 is
NP, which occupies the first position in the genetic map of
Sendai virus (4).
     At its 3' terminus, the insert contained sequences
almost identical to those previously described in a smaller
NP clone that embraced the viral mRNA termination and
poly(A) initiation signals (1).   However, there was a minor
discrepancy: the last 3 bases, 5'-AAG, preceding the poly(A)
tract, were absent (Fig. 1).   This may have occurred because
the oligo(dT) primer slipped past the G residue that marks
(in complementary form) the end of the mRNA stop signal (5),
but it is also possible that the AAG sequence had not been
transcribed by the viral RNA polymerase when it made the
mRNA template of the cloned DNA.   At any rate, coupled with
the apparent size of the insert, the terminal sequence data
indicate that pNP3 contains the entire coding sequence of
the NP protein.

RESTRICTION MAPPING AND SEQUENCING

     Fig. 1 also depicts a restriction map of pNP3 and the
regions that we have sequenced at least once (indicated by
black segments).   So far, we have sequence data on more than
1600   nucleotides,   leaving   less   than   200   completely
uncharted.   The positions of the restriction sites were
determined by electrophoretic restriction mapping (6) and
confirmed by sequence data.
     We have identified four potential initiation codons
within the first 310 nucleotides of the gene, at positions
65, 201, 204 and 305.   The first AUG appears to initiate the
correct NP reading frame, but further sequencing data will
be necessary to confirm this.
     The availability of a complete NP clone sets the stage
for enhancing our understanding of NP structure   and for
obtaining expression of the gene in either prokaryotic or
eukaryotic cells in studies of NP functions.   In particular,
the hypothesis that NP protein abundance is the regulatory
switch between viral RNA replication and transcription (7,8)
can be tested.

Clone pNP3 :   5' ........AGGGTCAAAGTATCCACCCTG...   ...GCTTCAAGGTACTTGATCCGTAGT(A)$_{12}$...

Virus genome : ...GATTTTAGGGTCAAAGTATCCACCCTG...   ...GCTTCAAGGTACTTGATCCGTAGTAAG(A)$_5$...

FIGURE 1. Restriction map of clone pNP3. Restriction sites were determined as described in the text. Only sites actually used to obtain nucleotide sequence information are shown. Black zones represent regions that have been sequenced at least once; the stipled area represents a region that has not yet been examined. The 5' and 3' terminal-sequences of the insert and of the corresponding cDNAs deduced from RNA sequence data (Gupta, Re and Kingsbury, this volume) are also shown.

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cDNA CLONING, MAPPING AND TRANSLATION OF TEN RESPIRATORY SYNCYTIAL VIRUS mRNAs[1]

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Human respiratory syncytial (RS) virus, the prototype of the pneumovirus genus of the paramyxovirus family (1), is an important agent of respiratory tract disease. The RS virus genome is a single negative strand of encapsidated RNA that is transcribed in infected cells into polyadenylated mRNAs (2-6). As described here, we have used molecular cloning techniques to characterize the genome and gene products of RS virus strain A2. Some of this work has been described elsewhere (5).

cDNA cloning. cDNAs were copied from mRNAs from virus-infected HEp-2 cells and cloned using the Escherichia coli plasmid pBR322 (5). Viral-specific clones were catagorized by reciprocal dot blot hybridization and by hybridization to reverse transcripts of viral mRNAs into ten families that lacked detectable cross-hybridization (5, unpublished data).

RNA (Northern) Blots. cDNA clones representing the ten different families were radiolabeled by nick translation and hybridized to RNA blots prepared from intracellular RS viral RNAs.
As shown in Fig. 1, all ten clones hybridized with genomic RNA (RNA 8), confirmation of their viral-specificity. One clone each hybridized with RNAs 4, 5 and 7 (Fig. 1, lanes 4, 5 and 7, respectively), three clones hybridized at the position of RNA gel band 1 (lanes 1a, 1b and 1c), two with RNA 2 (lanes 2a and 2b), and two with RNA 3 (3a and 3b). In hybrid-selected translation experiments (described below), each of these clones selected mRNAs encoding different viral proteins. This demonstrated that each of the ten clones represents a different,

unique viral mRNA.   Thus, RNA gel band 1 contained three uni-
que mRNAs (designated, in order of increasing size, RNAs 1a,
1b and 1c), and RNA gel bands 2 and 3 each contained two unique
mRNAs (mRNAs 2a, 2b, 3a and 3b).

RNAs A-H and 6 each hybridized with two or three different
clones (Figs. 1 and 2).   This suggested that these RNAs were
nonunique di and tricistronic transcripts, an interpretation
that was supported fully by mol. wt. comparisons (Fig. 2).

Transcriptional Map.   On the premise that the poly-
cistronic RNAs A-H and 6 were generated by readthrough tran-
scription of adjacent genes, their sequence homologies and
mol. wts. were interpreted to prepare a viral transcriptional
map (Fig. 2).   This analysis did not, however, determine the
number of viral promoters, the direction of transcription, or
the relationship between the two groups of genes in the tran-
scriptional map.
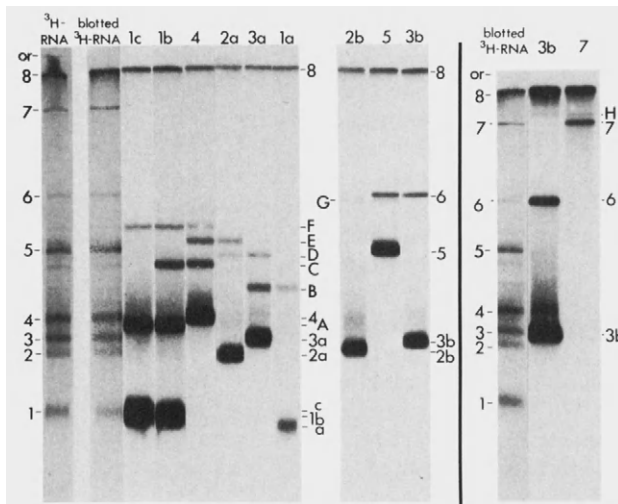
Figure 1. Hybridization of cDNA clones with RNA (Northern)
blots.   Intracellular (^{3}H) viral RNAs were separated in repli-
cate lanes by electrophoresis in 1.5% agarose-urea gels.   One
lane was analyzed by fluorography (lane marked "^{3}H RNA") and
the remaining lanes were transferred to diazobenzyloxymethyl
paper.   Two strips were analyzed by fluorography ("blotted ^{3}H
RNA").   The remaining strips were analyzed by autoradiography
following hybridization with cDNA clones of viral RNAs 1a, 1b,
1c, 2a, 2b, 3a, 3b, 4, 5 and 7 (as indicated over each lane).

Therefore, transcriptional mapping was also performed by
exposing virus-infected cells to UV light and quantitating the
rates of inactivation of the viral genes. The transcription
of surviving genes was monitored by the incorporation of (^{3}H)
uridine or (^{32}P) phosphate into RNA in the presence of cyclo-
heximide (to block replication of undamaged genomes) and
actinomycin D. Individual transcripts were quantitated by gel
electrophoresis and by hybridization to replicate dot blots of
the ten viral cDNA clones. In some experiments, cycloheximide
was omitted and the replication of surviving genomic RNAs was
quantitated by gel electrophoresis. The target mol. wt. for
UV-inactivation of genome replication was assumed to be the
estimated genome mol. wt., 5.6 x 10^6. This provided a standard
for calculating target mol. wts. for individual genes (Table
1). As shown in Table 1, the target mol. wts. for the ten
viral genes increased cumulatively in the order 1c < 1b < 4
2a < 3a < 1a < 2b < 5 < 3b < 7. The target mol. wt. for
gene 1c alone was similar to its gene size, suggesting that it
alone lay close to a promoter. The simpliest interpretation
of these data is that the ten viral genes are transcribed from
a single promoter in the sequence 1c -1b -4 -2a -3a -1a
-2b -5 -3b -7. In addition, the target size analysis pro-
vided evidence that genes 1a and 2b are adjacent in the map;
the proximity of these two genes had not been determined by
analysis of polycistronic RNAs.
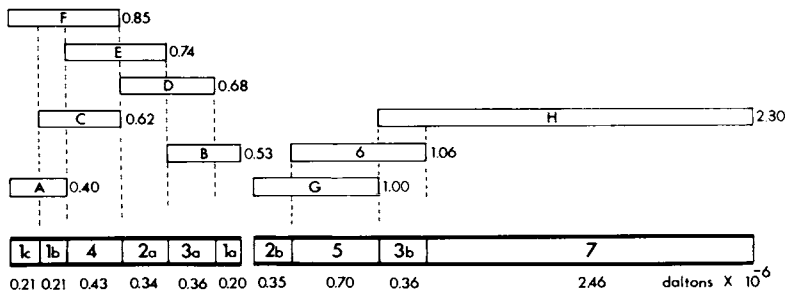
FIGURE 2. Transcriptional map for RS virus. RNAs 1-7
and A-H are drawn overlapping based on sequence homologies
determined as shown in Fig. 1. The RNAs are drawn in pro-
portion to their estimated mol. wt. minus the mol. wt. of
125 polyadenylate residues (7). Their adjusted mol. wts. (x
10^{-6}) are indicated.

TABLE 1
UV TARGET SIZES FOR RS VIRAL GENES

GENE	UV TARGET MOL. WT. $(\times 10^{-6})$ [a]	GENE MOL. WT. $(\times 10^{-6})$ [b]	CUMULATIVE GENE MOL. WT. $(\times 10^{-6})$ [c]
1c	0.36 [d]	0.21	0.21
1b	0.44 [d]	0.21	0.42
4	0.82 [d]	0.43	0.85
2a	1.20 [d]	0.34	1.19
3a	1.24 [d]	0.36	1.55
1a	1.43 [d]	0.20	1.75
2b	1.81 [d]	0.35	2.1
5	2.69 [e]	0.70	2.8
3b	2.97 [d]	0.36	3.16
7	6.4 [e]	2.46	5.62

[a] Target mol. wt. for an individual gene = (D_{06} for RNA replication D_0 for individual genes) x 5.6 x 10^6. D_0 is the dose of radiation that results in 37% survival.
[b] Calculated by subtracting the mol. wt. of 125 polyadenylate residues (7) from the mRNA mol. wt.
[c] Sum of the gene mol. wt. plus the mol. wt. of preceding genes based on the gene order 1c, 1b, 4, 2a, 3a, 1a, 2b, 5, 3b, 7.
[d] Quantified by dot blot hybridization; average of three experiments.
[e] Quantified by gel electrophoresis; average of two experiments.

Polypeptide Coding Assignments of the RS virus mRNAs.
cDNA clones representing nine of the ten unique viral mRNAs
were used to purify mRNAs by hybridization-selection (8) for
translation in vitro. SDS-gel electrophoresis of the (^{35}S)
methionine-labeled translation products (Fig. 3) determined
the following polypeptide coding assignments: RNA 1a, 9.5
kilodalton (K) protein (lane 1a); RNA 1b, 11K protein (lane
1b); RNA 1c, 14K protein (lane 1c); RNA 2a, nucleocapsid
phosphoprotein P (lane 2a); RNA 2b, 32K and 36K proteins (lane
2b); RNA 3a, matrix protein M (lane 3a); RNA 3b, 24K protein
(lane 3b); RNA 4, major nucleocapsid protein N (lane 4); and
RNA 5, 59K protein (lane 5). The coding assignment of RNA 7
was not tested, but is assumed by size considerations to be
the L protein. The 32K and 36K proteins appeared to be related
based on limited digest peptide mapping (not shown). Comparison of the hybrid-selected products with polypeptides extracted
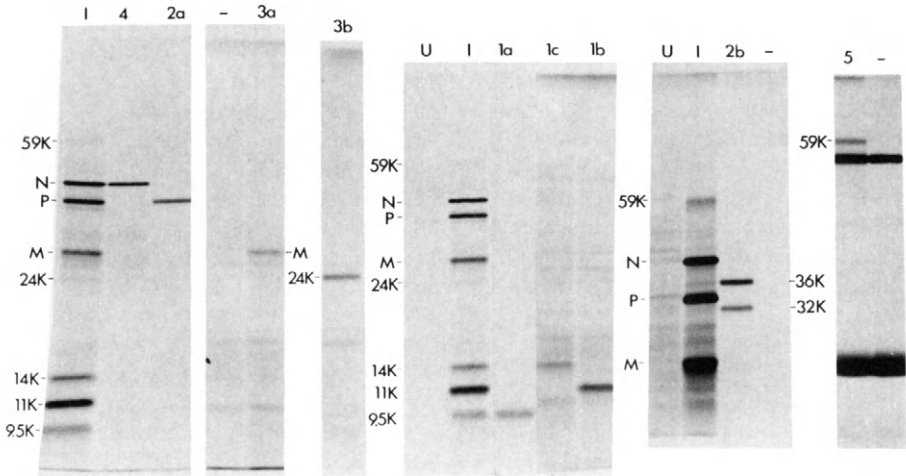
Figure 3. Polypeptide coding assignments of the RS virus mRNAs. mRNAs were purified by hybridization-selection (8) using cloned cDNAs of RNAs 1a, 1b, 1c, 2a, 2b, 3a, 3b, 4 and 5. Wheat germ extracts (lanes 1a, 1b, 1c, 2a, 2b, 3a, 3b and 4) or reticulocyte lysates (lane 5) were programmed with the selected mRNAs (as indicated) or with no added mRNA (lanes marked "-") or with total mRNA from uninfected ("U") and virus-infected ("I") actinomycin D-treated cells. The (^{35}S) methionine-labeled translation products were analyzed by electrophoresis in SDS-polyacrylamide gels. Fluorograms of the fixed, dried gels are shown.

from infected cells by gel electrophoresis and limited digest peptide mapping showed that the 9.5K, 11K, 14K, 24K, M, P and N proteins had authentic counterparts of the same size (not shown). In contrast, the 36K and 59K proteins lacked detectable counterparts of the same size in vivo (not shown) and therefore are candidates to be the unprocessed polypeptide moieties of the viral F and G glycoproteins.

SUMMARY

Cloned cDNAs of viral mRNAs were used as hybridization probes to (i) analyze RNA blots of intracellular viral RNAs,

and (ii) purify (hybrid-select) homologous mRNAs for trans-
lation <u>in vitro</u>. These analyses demonstrated the existence of
ten unique viral mRNAs and identified their sizes and poly-
peptide coding assignments. The available data indicated that
each mRNA encodes a single polypeptide chain. Thus, the mRNA
and polypeptide products of ten RS viral genes have been
identified.

   A transcriptional map for RS virus was determined by two
independent methods: (i) analysis of polycistronic read-
through transcripts, and (ii) UV-inactivation studies. Results
with both methods supported fully the interpretation that the
ten viral genes are transcribed from a single promoter site
in the sequence (designated by mRNA number with the encoded
protein indicated in parenthesis): 1c (14K), 1b (11K), 4 (N),
2a (P), 3a (M), 1a (9.5K), 2b (36K), 5 (59K), 3b (24K),
7 (L).

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STRUCTURAL ANALYSIS OF HUMAN RESPIRATORY SYNCYTIAL VIRUS GENOME

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Human respiratory syncytial virus (RS virus), a pleomorphic enveloped RNA virus that replicates in the cytoplasm is a leading causative agent of serious lower respiratory disease among infants and children (1). Although previously classified as a paramyxovirus, it is distinct in its morphology and lack of hemagglutinin and neuraminidase activities. In spite of its importance as a human pathogen in early life, detailed studies relating to the nature of its organization and expression have only recently begun, primarily because of its poor growth in tissue culture and virus instability. We have explored the structure of this virus by use of recombinant DNA techniques, and this report is a summary of some preliminary observations.

The genome of RS virus consists of a single 5000 kDal RNA species of negative polarity. Several workers (2, 3) have identified 7 or 8 virus coded polypeptides consistent with the earlier genetic analysis of ts mutants that identi- fied 7 or 8 nonoverlapping complementation groups (4). Work from our laboratory has identified at least 8 polypeptides that are present in the extracellular virus that have apparent molecular masses of 160, 84, 68, 46, 36, 28, 22 and 18 kDal. The viral origin of these polypeptides was confirmed by specific immunoprecipitation with rabbit RS antiserum. The 84 and 68 kDal proteins are envelope glycoproteins, readily solubilized by nonionic detergents. One of these (84 kDal) probably represents the major envelope glycoprotein (G), the equivalent of the paramyxovirus HN protein. Under reducing conditions of electrophoresis, the 68 kDal glycoprotein is replaced by two proteins of 49 and 16 kDal. This is analogous to the paramyxovirus fusion factor (Fo) that is synthesized as a precursor and subsequently cleaved proteolytically into two subunits (F_1 and F_2) held together by disulfide bonds. The other proteins include a 160 kDal protein (L, probably the viral polymerase by analogy with paramyxoviruses), major nucleocapsid protein (NC, 46 kDal), phosphoprotein (36 kDal), matrix protein (M, 28 kDal), and two nonstructural proteins (NS_1 and NS_2, 22 and 18 kDal). Poly (A) containing RNA from infected cells was translated in

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vitro yielding proteins corresponding to NC, P, M and the two
nonstructural proteins (Fig. 1, Lane 8). Occasionally, a 59
kDal polypeptide was detected. Huang and Wertz have shown
that a viral mRNA 2200 bases long is translated into a
protein of 59 kDal (5). Whether this represents a precursor
of the fusion factor is not clear.

CONSTRUCTION AND CHARACTERIZATION OF cDNA CLONES

    In order to analyze the different transcriptional units
of RS virus, a cDNA library was constructed in E. coli (HB
101) using plasmid pBR322 as a vector. The strategy used
avoided the traditional self-priming reaction for the second
strand synthesis and nuclease S_1 treatment to remove the
hairpin (6). Discrete sized single stranded (ss) cDNA were
used separately for constructing double stranded (ds) cDNAs.
A library of 2900 tetracycline resistant and ampicillin
sensitive transformants was screened by hybridization to ^{32}P
labeled poly (A) RNA from infected cells. Seventy-seven % of
the transformants gave a positive reaction and from this
group a sublibrary of 75 transformants was selected on the
basis of size of the cDNA inserts. Several rounds of hybrid
selection of viral mRNAs using recombinant DNA immobilized on
nitrocellulose filters and subsequent cell-free translation
of selected mRNAs allowed us to identify 4 different classes
of recombinants that encoded the RS viral NC, P, M or NS_2
gene (Fig. 1). Certain recombinants hybrid selected mRNA
that yielded on translation P protein and a smaller protein
comigrating with a viral nonstructural protein NS_1 (Fig. 1,
lane 5, bottom arrow). Similarly, another recombinant
plasmid hybrid selected RNA that yielded on translation M
protein and a smaller protein comigrating with another
nonstructural protein (Fig. 1, lane 4).   Two dimensional
tryptic fingerprinting and partial protease V8 cleavage
digestions were utilized to confirm that the translation
products were of viral origin. Several independent
recombinant plasmids with cDNA inserts adequate in size to
accomodate the coding sequence of each of the genes were thus
identified (7).
    The cDNA library was further screened in duplicate by
hybridization with ^{32}P labeled cDNA synthesized in vitro
using infected or uninfected cell poly (A) RNAs as templates.
Approximately 85% of the recombinants reacted only with cDNA
prepared from infected cell RNA. Recombinants hybridizing to
the virus specific cDNA were also screened by hybridization
with radioactively labeled genomic RNA extracted from
intracellular nucleocapsids. The genomic RNA was either 3'
end labeled by the RNA ligase reaction or 5' end labeled by
the polynucleotide kinase reaction after fragmentation and
dephosphorylation.
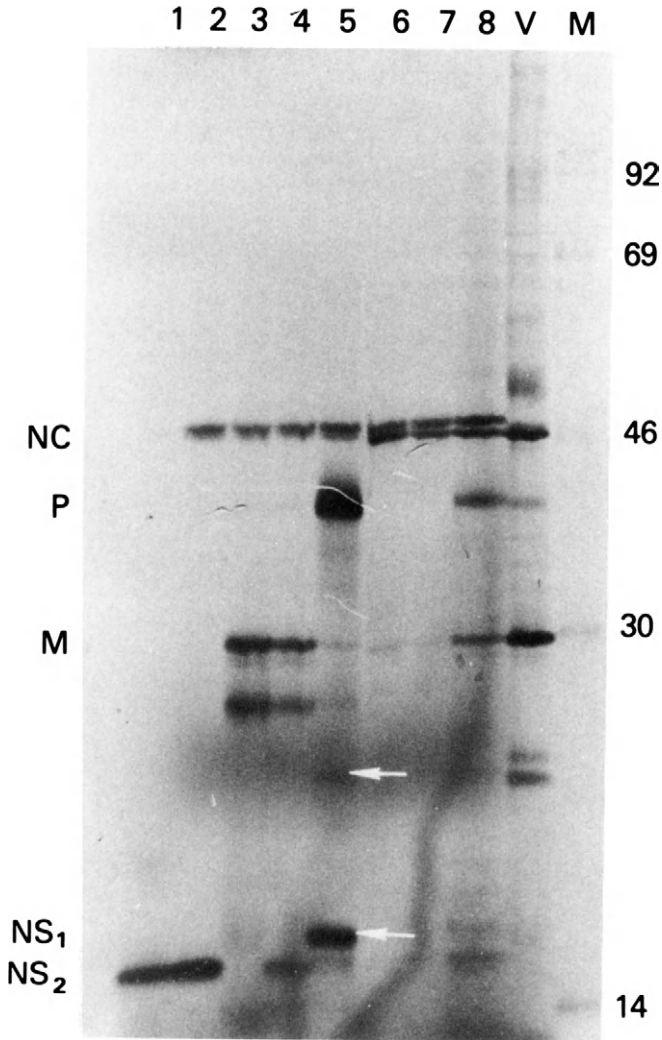
Fig. 1. Cell-free translation products of mRNAs from RS virus-infected cells selected by hybridization to RS virus cDNA plasmids. The plasmids used were pRSB$_8$ (lane 1), pRSC$_6$ (lane 2), pRSB$_7$ (lane 3), pRSD$_3$ (lane 4), pRSC$_1$ (lane 5), pRSB$_{10}$ (lane 6), and pRSB$_{11}$ (lane 7). Translation products of polyadenylylated RNA from infected cells are displayed in lane 8. RS virion polypeptides labeled in vivo with [^{35}S]methionine are shown in lane V.

Subtractive screening eliminated recombinants belonging
to the classes described above. The remaining transformants
were grown in groups of five. Plasmid DNA was then radio-
actively labeled by nick translation and used to detect poly
(A) RNAs from infected cells. Two transformants (pRS$_4$ and
pRSA$_2$) hybridized with poly (A) RNAs 2200 and 1100 bases in
length, respectively (Fig. 2). pRS$_4$ had a 1000 bp cDNA
insert and hybridized to a mRNA twice its length. We have
since identified additional recombinants that contain larger
inserts. One of these (pRSA$_{14}$), hybridizes to pRS$_4$, contains
2000 bp of viral sequences and might represent a full-length
copy of the 2200 base mRNA. This species of mRNA was shown
to encode a polypeptide of 59 kDal (5).

M   pRSB$_{11}$ pRSA$_{11}$ pRSA$_3$ pRSC$_6$ pRS$_4$ pRS$_2$ pRSA$_2$

1375|
1078|
875|

603|

310|

Φx174   NC   M   P   NS$_2$ —   —   —
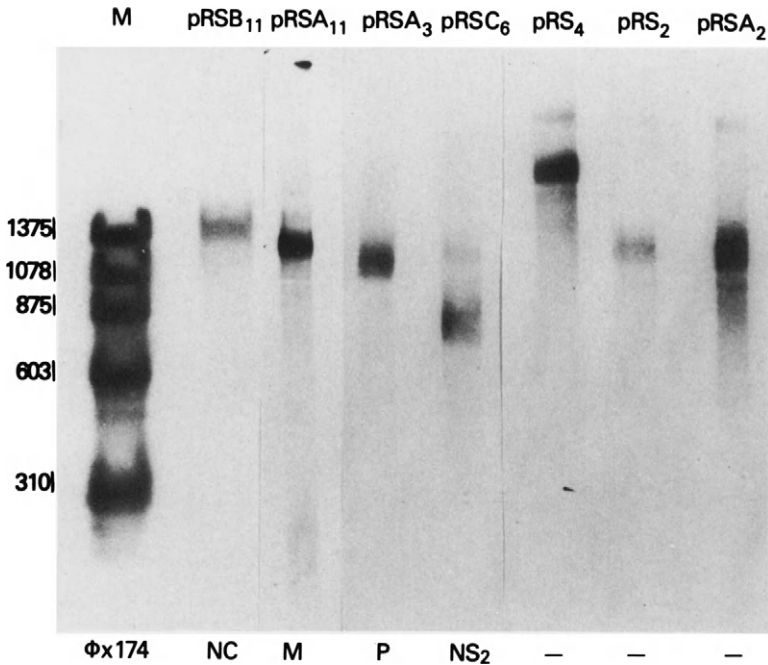
Fig. 2. Hybridization of recombinant plasmids to poly
(A) RNA from infected cells. Poly (A) RNA from infected
cells was electrophoresed on formaldehyde agarose (1.5%)
gels, transferred to nitrocellulose and hybridized to
radioactively labeled plasmid DNAs. The symbol (-) denotes
that the identity of the respective viral gene product(s) is
not known. Molecular weight markers are 5' end labeled Hae
III fragments of φx174 Form I DNA (M).

SIZE DETERMINATION OF VIRAL mRNAs

As illustrated in Fig. 2, recombinants encoding the RS viral NC, P, M or NS_2 hybridized to discrete sized poly (A) RNAs from infected cells. Three of these, encoding the NC, P or M gene had cDNA inserts almost identical in size to the RNAs with which they hybridized. However, recombinant ($pRSC_6$), that encoded a viral nonstructural gene, reacted with a poly (A) RNA about 200 bases shorter than the cloned RS viral sequence. All these cDNA inserts had a terminal stretch of A residues derived from the poly (A) tail of the viral mRNAs. By primer extension the recombinant plasmids ($pBRSB_{11}$ and $pRSA_{11}$) encoding the NC and M genes, respectively, were found to lack 6 and 7 nucleotides corresponding to the 5' end their respective mRNA (data not shown).

DNA SEQUENCE ANALYSIS

DNA sequence of the RS viral NC, M and NS_2 gene inserts genes was determined. A 1443 bp DNA sequence of the NC gene insert of plasmid $pRSB_{11}$ had a single open reading frame encoding a protein of 467 amino acids (Fig. 3). The other two reading frames were extensively blocked throughout. The 51 kDal NC protein was relatively rich in basic amino acids but poor in cysteine. There was no clustering of basic amino acid residues within specific domains (8). A recombinant ($pRSA_{11}$) encoding the matrix protein gene had 944 bp of RS virus sequence. There was a single long open reading frame potentially encoding a protein of 256 amino acids (Fig. 3). This 28717 dalton protein was relatively basic and moderately hydrophobic. There were two clusters of hydrophobic amino acids in the C-terminal third of the molecule. These might be involved in the interaction of matrix protein with virus altered plasma membranes. A second open reading frame encoding 75 amino acids and partially overlapping with the C-terminus of the matrix protein was also present (Fig. 3). Interestingly, certain M gene recombinants hybrid selected mRNA that yielded on translation M and a protein comigrating with a viral nonstructural protein (Fig. 1, lane 4). The biological significance of the second open reading frame is not clear at this time. The amino acid sequence of both the viral NC and M did not share homology with the known sequence of other viral capsid and matrix proteins, implying that RS virus has undergone extensive evolutionary divergence.
A recombinant plasmid ($pRSC_6$) encoding a viral nonstructural protein (NS_2) was also selected for sequencing. The viral insert of 1050 bp had two nonoverlapping reading frames capable of encoding proteins of 139 and 140 amino acids, respectively (Fig. 3). Since the molecular weight of

the protein obtained by cell-free translation of viral mRNA
hybrid selected by this plasmid agreed well with the
calculated molecular weight of the proteins encoded by either
reading frame, it is not clear whether one or both are used
for translation in vivo. Presently we are investigating this
question by a combination of 2D gel analysis of the
protein(s), primer extension to map the 5' end of the mRNA(s)
and hybrid arrested translation of mRNA(s) using restriction
fragments that span only one of the two reading frames. The
precise gene order of RS virus is not established, although
Collins and Wertz (9) have proposed a transcriptional map
wherein two viral nonstructural protein genes are 3'-proximal
on the genomic template. Also, it is not known whether an
untranslated leader RNA is synthesized prior to synthesis of
mRNAs as in the case of other negative strand RNA viruses.
If the 0 frame encodes NS_2 protein, we may have fortuitously
cloned a linked transcript containing this gene and upstream
sequences on the genomic template for a second nonstructural
protein; conversely, NS_2 protein might be encoded by the +2
frame.

The codon usage of the RS genes showed an inherent bias
against CG dinucleotide within the coding region similar to
the situation observed with VSV, influenza virus and
eukaryotic genomes (10). Each of the viral genes lack the
canonical eukaryotic polyadenylation signal AAUAAA upstream
of the 3' poly (A) tail. In addition, these genes lack
homologous sequences just upstream of the poly (A) tail,
unlike VSV and Sendai virus which have a conserved four
nucleotide sequence upstream of the poly (A) tail of each
transcript (11). The translation initiator codon AUG for
each gene is embedded within a canonical PXXAUGG sequence
seen around functional eukaryotic initiation sites.

Recombinant $pRSA_3$ contains a P gene cDNA insert that
hybridized to a viral mRNA of similar size. Certain
recombinants containing this gene hybrid selected viral
mRNA(S) that translate P and a protein comigrating with a
viral nonstructural protein (NS_1) (Fig. 1). This may be
analogous to Sendai virus, where P and C proteins are
translated from the same mRNA, either as a tandem
nonoverlapping dicistronic RNA or as a mRNA with overlapping
reading frames (12). We are presently sequencing cDNA
inserts encoding the P gene to determine whether this is the
case with RS virus.
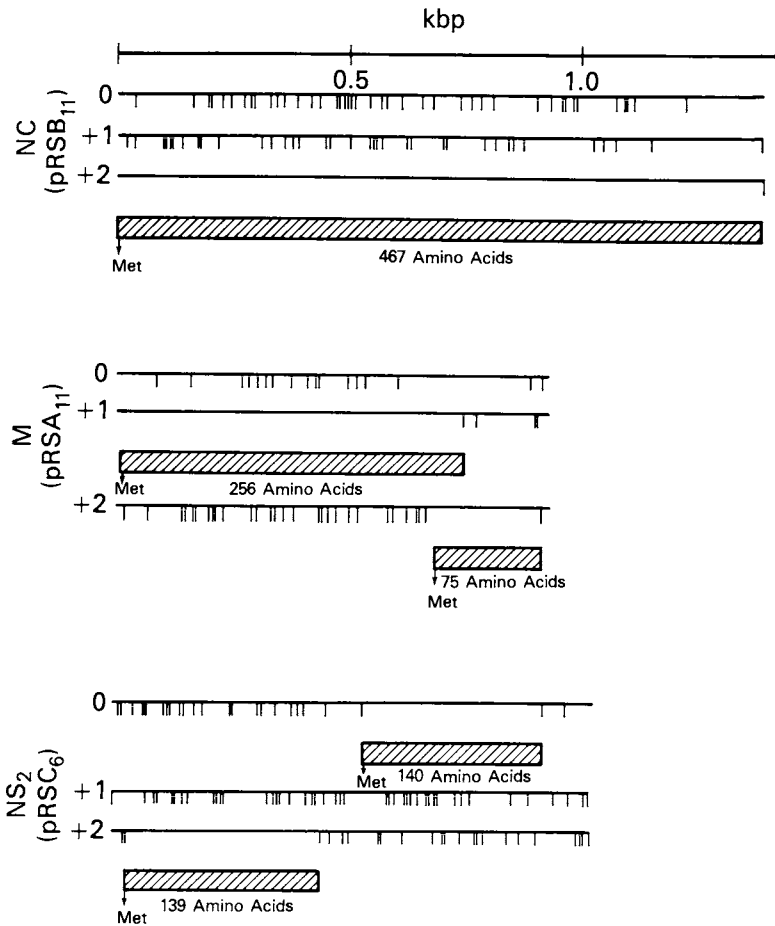
Fig. 3. Schematic illustration of open reading frames within the cDNA inserts of the recombinants encoding RS viral NC, M, or NS$_2$ gene. The lengths of the cDNA inserts are indicated in kbp. Each plasmid and the gene contained in it are identified on the left. The vertical lines denote stop codons. The hatched rectangles represent the open reading frames. The number of amino acids for each protein is shown below the rectangles.

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THE POLYMERASE GENE OF VSV

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The synthesis of translatable messenger RNAs of
Vesicular stomatitis virus (VSV) requires a great number of
different enzymatic activities. These include specific
binding of the polymerase to the ribonucleoprotein template,
initiation, polymerization, capping, methylation, polyadeny-
lation and specific termination. In addition, highly
conserved signal sequences are recognized by the polymerase
along the template.

In vitro transcription experiments demonstrated that,
besides the template, only the viral NS and L proteins are
required for transcription (1). Both are considered
dissimilar subunits of the polymerase complex. Possibly all
the functions described above are carried out by the
polymerase complex since they can not be separated from it.
Exogenous RNAs are not accepted as substrates for any of the
modification functions such as capping, methylation and
polyadenylation. It, therefore, appears that all of these
essential enzymatic activities are not only carried out on
the template, but that they also require nascent transcripts
as substrates. Because of its giant size, we expect that
most of the functions are associated with the L protein.

In this communication, we summarize our nucleotide
sequence analysis of the polymerase gene and the deduced
amino acid sequence of the L protein. The sequences will be
published elsewhere.

CLONING OF THE L GENE

As a first step on our way to dissect the individual
functions of the polymerase complex, we have isolated double-
stranded cDNA copies of the L gene. VSV genomic RNA (Indiana
serotype, Mudd-Summers strain) was reverse transcribed by
random priming. After second strand synthesis and oligo (dC)
tailing, the DNA fragments were inserted into the oligo (dG)
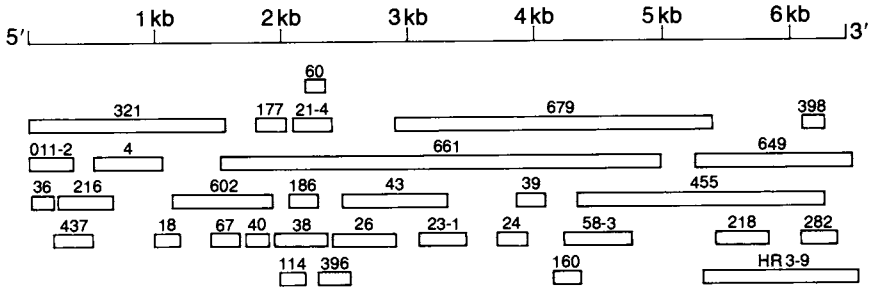
FIGURE 1. Map of 32 cDNA clones of the L gene which were partially or completely used to determine the nucleotide sequence of the L gene. The 5' terminus of VSV genomic RNA is indicated. The 3' end corresponds to the G and L boundary.

tailed Pst 1 restriction site of pBR322. E. coli HB101 cells were transformed and the tetracycline resistant colonies were characterized by filter hybridization.   5'-^{32}P-end labeled RNA fragments of defective interfering particle RNAs such as DI 011, T, T(L) and 611 which lack sequences from the smaller genes (2, Meier et al., this volume) were used as hybridization probes.   Approximately 1200 colonies   were analyzed and more than 500 cDNA clones of the L gene region were   identified   and   tentatively   mapped.   More   than   50 overlapping   cDNA   clones   covering   the   entire   gene   were precisely   mapped   by   hybridization   and   restriction   cuts. Figure 1 shows the map of 32 cDNA clones which were either partially   or   completely   used   to   determine   the   nucleotide sequence of the L gene.   The sizes of the inserts ranged from 100 bp to 3400 bp.

NUCLEOTIDE SEQUENCE ANALYSIS

Each   nucleotide   of   the   L   gene   was   sequenced,   on   the average,   5   times   in   plus   and   minus   sense   using   3 independently   derived   cDNA   clones   (3).   This   extensive sequence   analysis was   necessary   because   more   than   20 base differences between various cDNA clones from the same regions were   detected.   These   differences   included   not   only   base substitutions, but surprisingly, also single base deletions and insertions, changes which would clearly be lethal to the virus.   We   are   currently   investigating   whether   these biologically   very   interesting   frame   shift   mutations   are present in subpopulations of the VSV genomic RNA or whether they are simply a reflection of the infidelity of the reverse transcriptase.
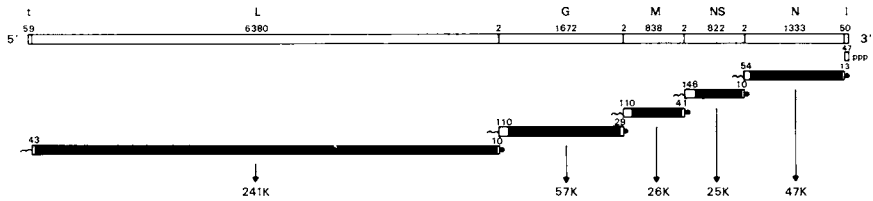
FIGURE 2.   The genome of VSV and its transcripts.   The
sizes of the leader region 1 (6, 7), the N, NS, M, G genes
(8, 9), the L gene (this report), the intercistronic regions
(4) and the 5' terminal extracistronic trailer region t (5)
are indicated.   The coding regions of each messenger RNA
(closed bars) and the sizes of the terminal untranslated
regions are as marked.   The calculated molecular weights of
the corresponding proteins are listed in kilodaltons.

Our nucleotide sequence analyses showed that the L gene
spans   6380   nucleotides,   starting   at   the   intercistronic
boundary of the G and L genes (position 4724 from the 3' end
of the genome) (4) and terminating at its polyadenylation
signal at position 60 from the 5' end of the genome (5).
These data, together with the published sequences of the N,
NS, M and G genes, complete the entire nucleotide sequence of
VSV Indiana.   The genome consisting of the leader region (6,
7), the 5 genes (8, 9, this report), the four intercistronic
regions (4) and the 5' terminal trailer region (5) is 11,162
nucleotides long (Figure 2).   The L gene alone comprises
57.2% of the total genome.

TRANSLATION OF L MESSENGER RNA

There   is   no   evidence   for   post-transcriptional   size
alterations of VSV messages such as splicing.   The ribosome
binding site (10) and the polyadenylation site of L messenger
RNA (5) have been described earlier.   Therefore, the amino
acid sequence of the protein can be deduced from the primary
structure of the L message.   Figure 3 shows the translation
of (+) sense L message as well as the corresponding (-) sense
genomic RNA in three reading frames.   In (+) sense, only
three regions were detected which are at least 70 nucleotides
in length and contain an AUG start codon.   It is obvious that
reading frame No. 2 encodes the L protein.   Although we found
a region in the genomic RNA, B, which has an open reading
frame   and   could   potentially   code   for   a   peptide   with   a
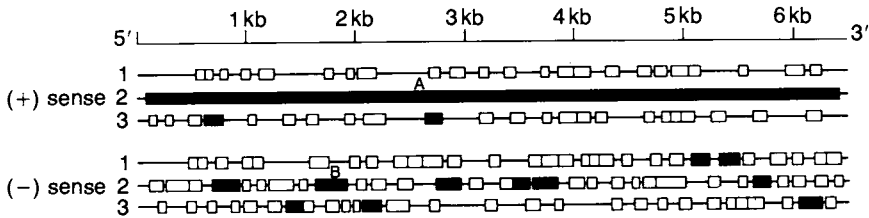
FIGURE 3. Translation of the L messenger RNA (+) and the L gene (-) sequence in all 3 reading frames. Regions without a translational stop codon and at least 70 nucleotides in length are boxed. Closed boxes mark regions containing the translational start codon AUG. A corresponds to the open reading frame for the L protein. B represents the second largest potential coding region.

molecular weight of 10,000 daltons, there is no evidence that it is actually used. Most importantly, (-) sense translatable transcripts from (+) strand templates are highly unlikely.

The L messenger RNA contains a single open reading frame which starts with the first AUG 11 nucleotides from the 5' end of the message -- consistent with the reported ribosome binding site (10) -- and terminates with UAA leaving 43 nucleotides before the poly (A) tail untranslated (Figure 2). The L message contains the least number (53) of noncoding nucleotides of all VSV messages. The total number of encoded amino acids is 2109 which gives the L protein a calculated molecular weight of 241,000 daltons. Its size exceeds earlier estimations which ranged from 160,000 to 230,000 daltons (11).

The total coding region of the VSV genome is 93.9% leaving 6.1% for the untranslated terminal message regions, the leader and the trailer regions. From this coding capacity, the L protein comprises 60.4% compared to 6.3% for the NS protein which is approximately one tenth the size of L.

AMINO ACID COMPOSITION OF THE L PROTEIN

The amino acid composition of the L protein as deduced from the nucleotide sequence of the L message is listed in Table 1. The amounts of basic, acidic, polar and nonpolar amino acids are summarized. The L protein is a basic protein, but less basic than the M protein which contains 17.1% basic and 11.4% acidic amino acids (9). Unlike the NS, M and G proteins (8, 9), L does not exhibit large clusters of amino acids of similar characteristics, i.e., charged,

TABLE 1.   Amino acid composition of the VSV L protein. The amino acid sequence of the L protein was deduced from the nucleotide sequence of L messenger RNA.

ala	92	leu	219
arg	124	lys	129
asn	97	met	60
asp	120	phe	93
cys	34	pro	98
gln	70	ser	178
glu	112	thr	122
gly	127	trp	42
his	64	tyr	70
ile	155	val	103

Total: 2109 aa

Basic	15.0%	Acidic	11.0%
Polar	31.9%	Nonpolar	42.1%

hydrophobic. Although the overall amounts of basic, acidic, polar and nonpolar amino acids are almost identical to the PB2 protein of influenza virus (12), the relative amounts of the individual amino acids are quite different.

Comparison of the nucleotide sequence and amino acid sequences of L and the P proteins of influenza virus (12, 13), which combined are roughly the size of L, did not reveal any homology. In addition, computer analysis of the sequence to all nucleotide sequences in the Genbank Data Bank in Los Alamos as well as the Protein Sequence Data Base of the National Biomedical Research Foundation in Washington, D.C., did not reveal any significant similarities to other sequences or proteins.   The total number of nucleotides screened was about 2 x 10^6, including the sequences of many proteins which interact with nucleic acids.   The L protein has a unique sequence.   A comparison to the L proteins of other rhabdoviruses or paramyxoviruses awaits nucleotide sequence information from these viruses.

The VSV L gene represents the first large polymerase gene of a negative strand virus to be cloned and sequenced. This work completes the cloning and sequencing of the VSV Indiana genome.   Despite the massive size of the gene, our sequence analysis suggests that only one protein is encoded. The molecular weight of 241,000 daltons makes L a perfect candidate for a multifunctional protein.   With the final

assembly of the L gene and its insertion into an expression
vector, we anticipate to unravel the functional domains of
the protein involved in transcription and replication and
study its interactions with the nucleocapsid template and the
NS protein.

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CLONING AND SEQUENCING OF M mRNA OF
SPRING VIREMIA OF CARP VIRUS

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Spring Viremia of Carp Virus (SVCV) is a fish
Rhabdovirus structurally similar to vesicular stomatitis
virus (VSV). As shown in Fig. 1 like VSV, SVCV has three
major polypeptides, a glycoprotein G, a nucleocapsid protein
N and a matrix (or membrane) protein M, and two minor
proteins L and NSI. In addition, another minor protein, a
second phosphoprotein NS2, which is an easily distinguished
viral protein, is also present. It is of interest that the
SVCV virion proteins are similar both in type, size and
relative abundance to those of VSV, except that (1) the two
SVCV NS phosphoproteins are easily resolved, (2) the SVCV G
polypeptides are larger than the 67×10^3 VSV G and SVCV M
polypeptide is slightly smaller than the 27×10^3 VSV M.
We have shown previously that although these two
viruses are serologically distinct, they can synthesize mRNA
species with similar 5' termini either in vitro or in vivo
(1). Our recent studies on the 3' termini sequence of SVCV
genome shows that the first 20 nucleotides are exactly
similar to that of VSV Indiana and New Jersey serotypes
(Unpublished data).
Since SVCV and VSV were recovered from two diverse
sources, fish and mammals respectively, we were interested
to know the structure and evolution of the viral membrane
protein, particularly, whether there are any homology
between their membrane protein M. To approach the question
of M protein homology, we have extended our sequence
analyses to the mRNA encoding the M protein from SVCV. In
order to do this, we isolated a cDNA clone of mRNA encoding
the protein. We have compared the predicted amino acid
sequences of this M protein with that of VSV M protein (San
Juan strain) determined previously by Rose et.al.(2).
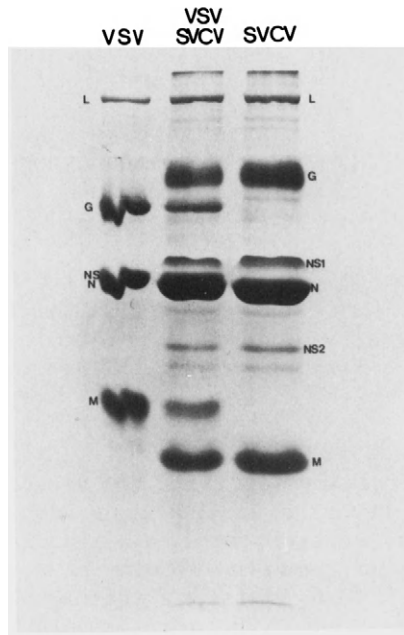
FIGURE 1.   The autoradiogram of a polyacrylamide gel showing a comparison of [^{14}C]-leucine labeled VSV proteins and [^{3}H]-leucine labeled SVCV proteins.
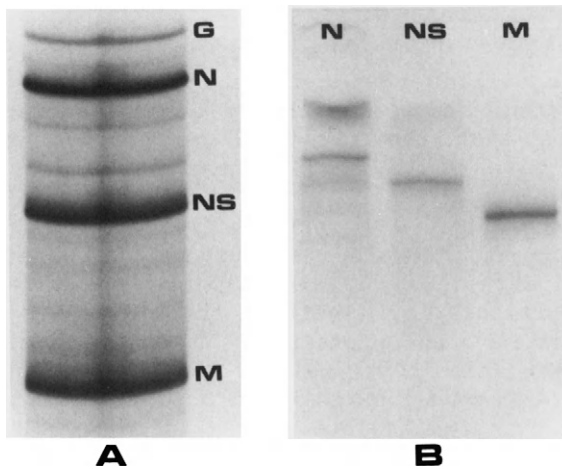
FIGURE 2. Autoradiogram of denatured gel showing cDNAs synthesized from polyA selected (A) total mRNA's of SVCV (B) individual mRNA's of SVCV.

The Strategy for Cloning SVCV mRNAs. To prepare cDNA we have used total mRNA from infected cells. PolyA tailed RNA were selected on oligo(dT) cellulose column and cDNA were synthesized using oligo(dT)$_{12\ 18}$ primer and reverse transcriptase. After boiling off the RNA template in formamide, the individual first strand cDNA were purified through a denatured gel as shown in Fig. 2A. To confirm the size of each cDNA the individual mRNA, were isolated in agarose gel containing 10mM methyl mercury hydroxide (3) and were used for cDNA synthesis as shown in Fig. 2B. Eluted purified cDNA were then polydA tailed at the 3' end and second strand were synthesized using oligo(dT)$_{12\ 18}$ primer. Double stranded cDNA were treated with nuclease S1 and repaired with Klenow's fragment and were ligated to Pvu II cut blunt end of pBR322. Hybrid plasmids were used for E. Coli. transformation and ampicillin resistant colonies were selected. Positive clones were screened by colony hybridization techniques using ^{32}P-labeled viral RNA probes.

Identification of the Cloned SVCV mRNAs. In order to identify the cDNA clones of SVCV mRNAs, we use the dot blot hybridization technique of Thomas (4) with slight modifications. Individual mRNA species that were isolated from agarose gel, are spotted (1-3µl) onto dry nitrocellulose paper that has been already saturated with 20 x SSC. The nitrocellulose paper containing the dots were then baked for 2 hours at 80°C under vacuum, then prehybridized for 8 hours at 42°C, and then hybridized for overnight with ^{32}P labeled, Hinf I digested plasmid DNA probe. After washing, the nitrocellulose paper was exposed at -70°C for 1 hour with intensity screen. The results are shown in Fig. 3. This clone was only hybridized to SVCV viral RNA, total mRNA and individual M mRNA. Clearly, this clone contained DNA capable of hybridizing specifically to the M mRNA.

DNA Sequencing. DNA sequencing was carried out by standard techniques using different restriction endonucleases as shown in Fig. 4. The distances and direction sequenced are shown by arrows. All sequence analyses were done by the Maxam & Gilbert method (5).

The Sequence of the Cloned M mRNA. The cloned M cDNA was sequenced. The complete nucleotide sequences of cDNA of M messenger RNA and the predicted amino acid sequences of M protein are shown in Fig. 5. Excluding poly A tail, the entire sequence is 710 bp long, encoding a protein of 223 amino acids. The amino acid composition of SVCV M protein

and VSV M protein are shown in Table 1.   SVCV M protein has
a calculated size of 25,599 daltons while VSV M protein has
a molecular weight of 26,064.
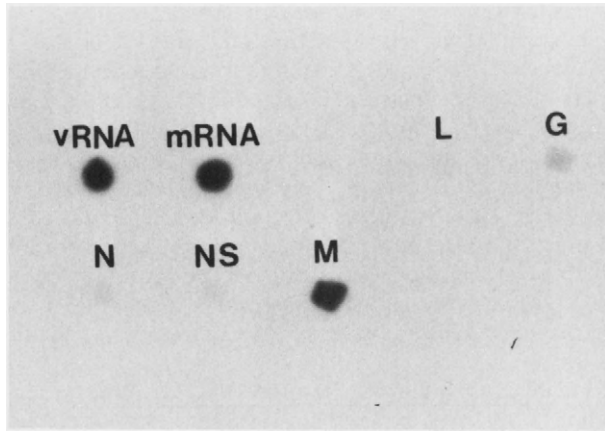
FIGURE 3.   Dot blot hybridization of SVCV RNA species
with ^{32}P-probes prepared from cDNA clone.
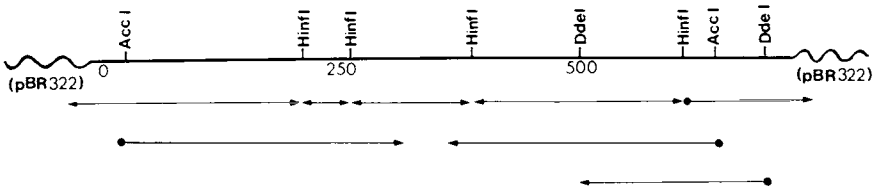
FIGURE 4.   The strategy employed to sequence the cloned
M mRNA.   Sites for restriction enzymes that were used for
sequencing   are   shown.   The   distances   and   directions
sequenced are shown by arrows.

```
        M  S  T  L  R  K  L  F  G  T  K  K  S  K  G  T
GAACAGACATCATGTCTACTCTAAGAAAGCTCTTTGGAACCAAGAAGTCAAAAGGTACTC
      10        20        30        40        50        60

P  P  T  Y  E  E  T  L  A  T  A  P  V  L  M  D  T  H  D  T
CTCCCACTTATGAGGAGACACTGGCGACTGCGCCAGTGTTAATGGATACTCATGATACTC
      70        80        90       100       110       120

H  S  H  S  L  Q  W  M  R  Y  H  V  E  L  D  V  K  L  D  T
ACTCCCACTCACTGCAGTGGATGAGGTATCATGTTGAATTGGACGTAAAATTGGATACAC
     130       140       150       160       170       180

P  L  K  T  M  S  D  L  L  G  L  L  K  N  W  D  V  D  Y  K
CCTTAAAAACGATGTCGGATCTTCTCGGACTCCTGAAAAATTGGGATGTAGATTACAAAG
     190       200       210       220       230       240

G  S  R  N  K  R  R  F  Y  R  L  I  M  F  R  C  A  L  E  L
GTTCTAGGAACAAGCGTAGATTCTACAGATTGATCATGTTCCGTTGTGCGTTAGAACTCA
     250       260       270       280       290       300

K  H  V  S  G  T  Y  S  V  D  G  S  A  L  Y  S  N  K  V  Q
AGCATGTATCGGGAACATACTCTGTTGACGGGTCGGCTTTGTACTCTAACAAGGTGCAAG
     310       320       330       340       350       360

G  S  C  Y  V  P  H  R  F  G  G  M  P  P  F  R  R  E  I  E
GGAGTTGTTATGTGCCTCATCGATTCGGTCAAATGCCTCCTTTCAAGAGGGAGATCGAGG
     370       380       390       400       410       420

V  F  R  Y  P  V  H  Q  H  G  Y  N  G  M  V  D  L  R  M  S
TCTTTAGATACCCAGTACATCAACATGGATACAACGGGATGGTAGATCTGAGAATGTCGA
     430       440       450       460       470       480

I  C  D  L  N  G  E  K  I  G  L  N  L  L  K  E  C  Q  V  A
TCTGTGACCTAAACGGGGAAAAGATAGGCCTCAATTTGTTGAAAGAGTGTCAGGTAGCAC
     490       500       510       520       530       540

H  P  N  H  F  G  R  Y  L  E  E  V  G  L  E  A  A  C  S  A
ACCCCAACCATTTCCAAAAATATCTGGAAGAAGTGGGGCTGGAAGCAGCCTGTTCAGCCA
     550       560       570       580       590       600

T  G  E  M  I  L  D  M  T  F  P  M  P  V  D  V  V  P  R  V
CAGGAGAATGGATTCTTGATTGGACATTTCCTATGCCAGTAGACGTGGTGCCTCGCGTTC
     610       620       630       640       650       660

P  S  L  F  M  G  D  •••
CTTCCCTGTTCATGGGAGATTAAATTGAGATTGATGTTCGCTGAGATATG
     670       680       690       700       710
```

FIGURE 5.   The nucleotide and corresponding amino acid
sequence of the SVCV M mRNA as deduced from the sequence
data of cDNA clone.   Nucleotides are numbered below their
respective residues.   Amino acids are centered over the
corresponding nucleotide triplets.

TABLE 1 - The amino acid composition of SVCV
M protein and VSV M protein.

Amino acid	No. of residues in:	
(abbreviation)	SVCV[a]	VSV[b]
Ala (A)	8	17
Arg (R)	12	10
Asn (N)	7	6
Asp (D)	13	13
Cys (C)	5	1
Gln (Q)	6	3
Glu (E)	11	13
Gly (G)	15	15
His (H)	10	8
Ile (I)	5	11
Leu (L)	25	17
Lys (K)	15	21
Met (M)	10	11
Phe (F)	9	14
Pro (P)	13	16
Ser (S)	14	18
Thr (T)	13	11
Trp (W)	4	3
Tyr (Y)	10	12
Val (V)	17	9

[a] Total number of residues, 223:Molecular weight. 25,599.
[b] Total number of residues, 229:Molecular weight. 26,064.

The Hydropathic Pattern of SVCV and VSV M Protein.
When the hydropathic pattern of M proteins of SVCV and VSV
were compared, no significant characteristics were observed.
However, as shown in Fig. 6, both proteins have clusters of
positive charges in similar positions. Both proteins are
rich in basic amino acids and contain highly basic amino
terminal domains. When the sequences of the two proteins
were compared visually, as shown in Fig. 7, it was observed
that there were about 63 identities which comprises of 28%
and there were six gaps. Although the standard deviation is
not known at the present time, it is quite clear that there
is high homology between the two M proteins.
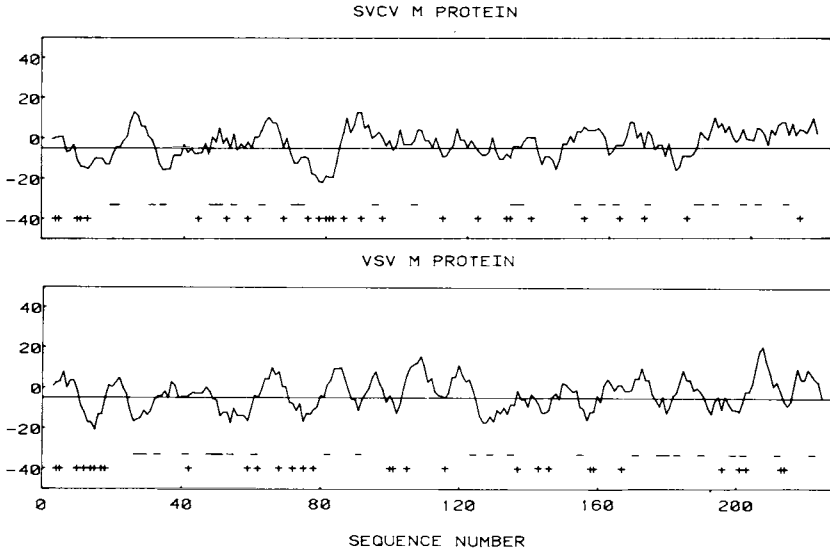
FIGURE 6.   The hydropathic pattern of SVCV and VSV M proteins.

SVCV MSTLRKLFGT   KKSKGT PPIYEETLATAPVL   MDTHDT
 VSV MSSLKKILGLKGKGKKSKKLGIAPPPYEEDTSMEYAPSAPIDSYFGVDFMDTYDP

SVCV HSHSLQWMRYHVELDVKLDTPLKTM SDLLGLLKNWDVDYKGSRNKRRFYRLIM
 VSV NQ PNQLRYEKFFTVKMTVRSNPFTYSDVAAAVSHWDHMYIGMAGKRPFYKILA

SVCV FRCALELKHVSGTYSVDGSALYSNKVQGSCYVPHRFGQMPFFKREIEVFRYPVHQ
 VSV ELGSSNLKATPAVLADQGQPFYHTHCEGRAYLPHRMGKTPFMLNVPEHFRRPFNI

SVCV HGYNGMVDIQMSICDLNGEKIGLNLLKECQVAHPNHFQKYLEEVGLEAACSATGE
 VSV GLYKGTIELTMTIYDDESLEAAPMIWDHFNSSKFSDERFKALMFGLIVEKKASGA

SVCV WILDWTFPMPVDVVPRVPSLFMGD
 VSV WVLDSISHFK

FIGURE 7.   Comparison between the predicted sequences of VSV M protein and SVCV M protein.   The identities between the two sequences are shown by boxed areas.

Rose, et al., (6) have suggested previously both VSV M protein and Influenza virus M protein begins with the sequence Met-Ser-X-Lys. It is notable that SVCV M protein also begins with a similar sequence. From these data it seems that certain amino acid residues are highly conserved in these sequences and although SVCV and VSV were recovered from two diverse sources, it is quite likely that the two viruses might have originated from a common ancestor among the Rhabdoviridae family.

ACKNOWLEDGEMENTS

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CHARACTERIZATION OF MEASLES VIRUS RNA[1]

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MEASLES VIRUS RNA

Measles virus, a paramyxovirus, is a pathogen known to cause acute and persistent infections in man. The measles virus polypeptides have been identified, however, there is considerable variation in the literature reports of the size of the measles virus RNA. Estimations of the molecular weight have been from 4.5×10^6 to 6.4×10^6 using sucrose gradient sedimentation(1), electron microscopy(2), estimation from the structure of nucleocapsids(3), and from agarose or acrylamide gels under denaturing conditions(4,5). In this report we have determined the size of measles virus RNA using both morphological and biochemical techniques.

Virus Growth and Purification. Vero cells were grown in roller bottles (1600 cm^2) and at confluency infected at 1 pfu/cell with the Lec strain of measles virus. The infectious virus was twice plaque purified and used after one passage. Measles virus was purified from the supernatant fluid at 72 hours post-infection as previously described(6). Cell material was retained for nucleocapsid preparation using the following procedure. The cell pellet was washed 2x with PBS and disrupted by the addition of 1% Triton X-100. After a 30 minute incubation, the nuclei were pelleted at 1000 g for 20 minutes. The supernatant was layered onto a discontinuous gradient of 6 ml 25%, 4 ml 30%, and 2 mg 40% CsCl in PBS and centrifuged on an SW27 rotor at 25,000 rpm for 1.5 hours. The nucleocapsid band present in the 30% CsCl region was removed, diluted in PBS and layered on a second gradient composed of 4 ml each of 20%, 25%, 30%, 35%, and 40% CsCl in PBS and centrifuged at 4°C in an SW rotor at 24,000 rpm for 16 hours. The nucleocapsid band was removed, diluted

with PBS and pelleted at 40,000 g for 30 minutes. The
nucleocapsid pellet was resuspended in 1.0 ml of 0.01 M
Tris-HCl, pH 8.0, 0.1 M NaCl and 1 mM EDTA prior to RNA
extraction.

     RNA Extraction. Purified measles virus and nucleocapsid
were diluted 1:1 with TNE buffer (0.01 M Tris-HCl, pH 7.5,
0.1 M NaCl, 0.001 M EDTA). Virions were disrupted with 0.1%
2-mercaptoethanol and 1.0% SDS. The suspension was extracted
with an equal volume 90% phenol/chloroforum and the organic
phase was reextracted with TNE buffer. The pooled aqueous
phase was mixed with 2.5 volume ethanol and RNA precipitated
at -20°C. The RNA was pelleted at 16,000 g for 30 minutes at
4°C and resuspended in sterile distilled water.

     Electron Microscopy of Measles Virus, Nucleocapsid and
RNA. Purified nucleocapsid and viral proteins were negatively
stained using 1.0% sodium phosphotangstate and examined in a
Phillips EM 300 electron microscope. Electron micrographs
were made of measles virus RNA molecules after treatment with
DMSO and glyoxal(7). Internal standards for the RNA
measurements were pBR322 DNA (partially cleaved by Pst 1) and
intact mengo virus RNA. Nucleic acid lengths were measured
on prints (3x enlarged) using a Hewlett-Packard 9874A
digitizer coupled to a Tektronix 4051 graphics computer.

     Agarose Gel Elecrophoresis Under Denaturing Conditions.
Agarose gel electrophoresis of RNA in 10 mM methylmercury
hydroxide was done using the method of Lehrach et al(8) with
minor modifications. Horizontal slab gels were run in a
BioRad mini electrophoresis apparatus and 1-3 ug of RNA
samples used in each well. After electrophoresis at 35mA at
room temperature for 3 hours, the gels were stained in 2 ug/ml
ethidium bromide and photographed under shortwave UV light.
     Agarose gel electrophoresis of RNA denatured in glyoxal
and DMSO was performed using the method described by McMaster
and Carmichael(9). Gels were either blotted onto
nitrocellulose or stained in acridine orange and photographed
as previously described.

     In Vitro Synthesis of cDNA Probes and RNA Blot Analysis.
RNA isolated from purified measles virus preparations was
electrophoresed in 1% low melting temperature agarose gel
containing 10 mM methylmercury hydroxide. The gels were
stained with ethidium bromide and the measles viral RNA band
was excised. The RNA was extracted and used as a template
for cDNA synthesis. Cellular ribosomal RNA was also used as
a template for cDNA synthesis. The cDNA probles were
synthesized as described by Leung et al(10). The technique

for RNA blot analysis used was similar to that described by
Thomas(11).

RESULTS

    RNA extracted from purified measles virus was denatured
in 10 mM methylmercury hydroxide or in glyoxal and DMSO. A
typical gel profile is shown in Figure 1.
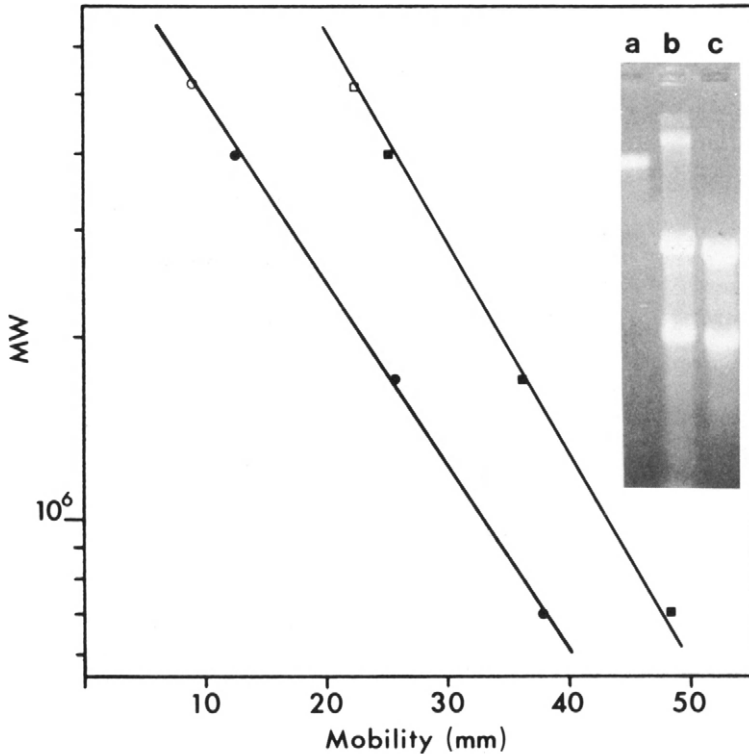
    FIGURE 1.   Electrophoresis of RNA Under Denaturing
Conditions in Agarose Gels. A typical gel of RNA preparations
in a methylmercury hydroxide agarose gel (a-Vesicular
stomatitis virus RNA; b-Measles virus RNA; and c-Ribosomal
RNA). The results of the mobility versus molecular weights
of RNA have been plotted for methylmercury hydroxide (●——●)
and DMSO and glyoxal (■——■) gels.

    The profile shows measles virus RNA with three major RNA
bands, two of which co-migrate with host cell ribosomal 28S
and 18S RNA. The largest RNA species is believed to represent

measles virus RNA. In order to obtain molecular weight
estimations of the measles virus RNA, three RNA markers, i.e.
vesicular stomatitis virus (VSV) RNA (4.0×10^6), 28S (1.7×10^6) and 18S (7×10^5) ribosomal RNA. The results obtained
are shown in Figure 1. The molecular weight of measles viral
RNA from these studies was estimated to be 5.2×10^6. RNA
extracted from purified measles virus preparations and
analyzed in DMSO and glyoxal denaturing gels was also
compared to RNA markers. By this method, the molecular
weight of measles virus RNA was 5.2×10^6 (Figure 1).

    Electron microscopy measurements of measles virus RNA
spread after treatment with DMSO and glyoxal showed a good
deal of fragmentation, but the longest molecules were 5.1 um.
An internal standard of pBR322 DNA (4362 base pairs, 1.47 um
in length) was used (Figure 2). Mengo virus spread under the
same conditions was 2.5 um in length and has a known
molecular weight of 2.56×10^6. Using these internal
standards, the measles virus RNA molecular weight determined
in this manner was 5.2×10^6. Thus the results by electron
microscopic measurements confirmed the results obtained by
denaturing agarose gel electrophoresis.
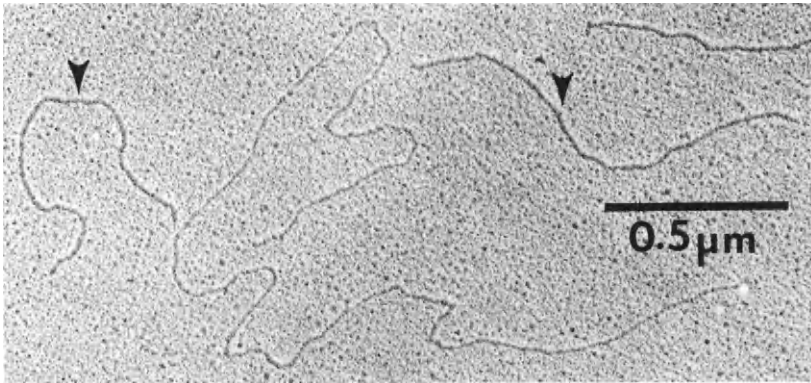
    FIGURE 2. Electron Micrograph of Intact Measles Virus
RNA. Arrows indicate pBR322 cleaved by Pst I which was used
as an internal standard (length 1.47 um). Intact measles RNA
with a length of 5.1 um is shown.

    Since the 28S and 16S ribosomal RNA species co-purify
with measles virus RNA, it is important to eliminate the
possibility that large RNA species (5.2×10^6) was not a
ribosomal precursor RNA, e.g. the 45S RNA. This possibility
was ruled out by the following experiment, RNA extracted from
VSV, measles and measles nucleocapsids was denatured in

glyoxal and DMSO and electrophoreses in agarose. The gel was blotted onto nitrocellulose paper and probed with radio-labelled cDNA prepared from RNA extracted from uninfected Vero cells. The cDNA probe hybridized to the cellular 28S and 18S RNA species and a few minor bands, but did not hybridize to the RNA isolated from VSV, measles virus, or purified nucleocapsid. When the measles RNA band was excised from methylmercury gels and used for cDNA synthesis, this probe hybridized to measles virus RNA from purified virus and nucleocapsid, but not to VSV RNA or rRNA (data not shown).

CONCLUSION

     The molecular weight of measles virus RNA has been difficult to accurately determine. The molecular weight estimations have varied from 4.5 x 10^6(5) to 6.4 x 10^6(3). Using either purified virus or nucleocapsid preparations as the source of measles virus RNA, a molecular weight to 5.2 x 10^6 was determined by both biochemical and morphological techniques. Although some smaller species of RNA were observed on the electron micrographs of measles virus RNA extracted from nucleocapsid, the largest samples had a molecular weight of 5.2 x 10^6, the same length as the largest species of RNA seen in electron micrographs of RNA extracted from whole virus. Our results are in agreement with the molecular weight estimations recently reported by Dunlap et al(2).

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THE CLONING OF MORBILLIVIRUS
SPECIFIC RNA[1]

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Measles virus (MV) and canine distemper virus (CDV), both morbilliviruses have been associated with several degenerative neurological diseases such as subacute sclerosing panencephalitis and old dog encephalitis. These diseases have been suggested to involve a persistent infection by these morbilliviruses. In order to understand the persistent infection fully, it is necessary to identify the factors which are responsible for initiation and maintenance. Several models have been proposed to explain the phenomenon in cell culture such as the selection of virus mutants, the modulation of matrix protein synthesis and the selection of defective interfering particles. Few studies have been made of the molecular basis of persistence due to the low levels of gene expression in the persistently infected cell. However, virus gene specific radiolabelled cDNA probes could overcome this problem allowing the quantification of changes in gene expression during the initiation and course of a persistent infection. Furthermore, control of gene expression at the transcriptional and translational level could be distinguished.

THE PURIFICATION AND CHARACTERISATION OF MORBILLIVIRUS MESSENGER RNA

The most abundant and readily obtainable morbillivirus specific RNA species in the infected cells are the mRNA's. Thus, they were the template of choice for cDNA synthesis. The mRNA's of the morbilliviruses are probably monocistronic and have been shown to posses polyadenosine (polyA) tails (1).
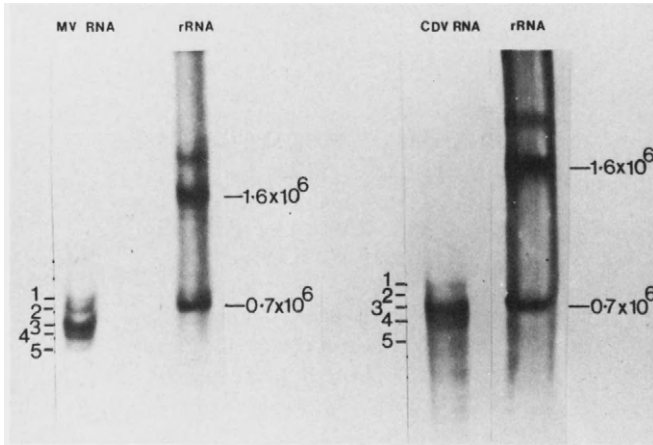
FIGURE 1.   MV and CDV infected cell polyA$^+$ RNA
fractionated in the presence of CH_3HgOH.
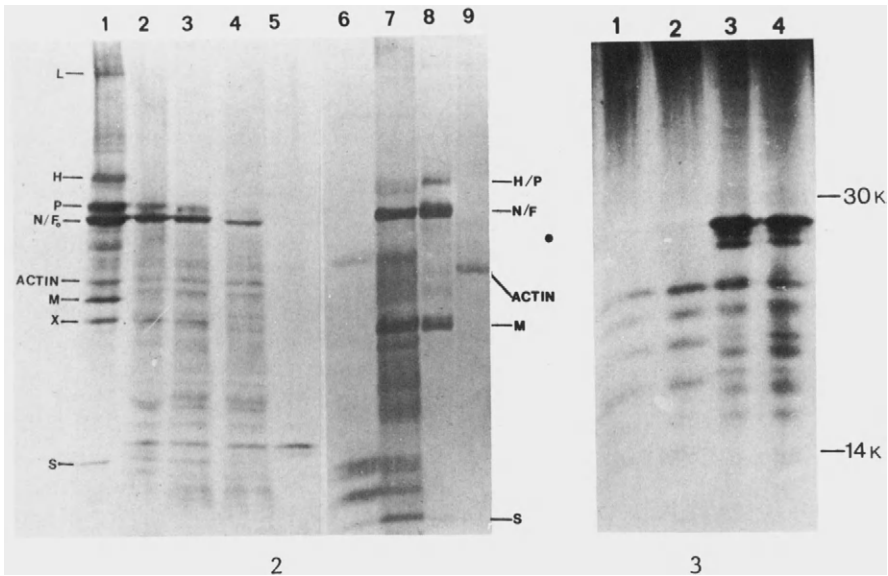
FIGURE 2 (left).   The products of in vitro translation
of MV and CDV polyA$^+$ RNA.   Lane (1) MV in vivo proteins;
(2) proteins directed by RNA harvested at 21 hpi; (3) 19 hpi;
(4) 17 hpi; (5) and (6) zero RNA control; (7) CDV in vitro
and (8) CDV in vivo proteins.

FIGURE 3 (right).   The products of limited proteolytic
cleavage of MV X (lanes 1,2) and P (lanes 3,4) proteins with
V8 protease synthesized in vivo (1,3) and in vitro (2,4).

They code for seven major viral proteins: L (Large; mol.wt.
180K), H (Haemagglutinin; 80K), P (Polymerase; 70K), N
(Nucleocapsid; 60K), F (Fusion; 60K), M (Matrix; 40K) and
S (Small; 20K) (reviewed in 2).   Figure 1 shows the principal
polyA containing RNA species synthesized in MV and CDV
infected Vero cells during the latter part of a lytic infect-
ion, in the presence of sufficient actinomycin D to inhibit
>90% of host cell RNA synthesis. The total, infected cell
RNA was obtained by the method of Glisin and coworkers (3),
enriched for polyA RNA by oligo-dT-cellulose affinity
chromatography and fractionated in agarose gels in the
presence of methylmercury (II) hydroxide. These gels
indicate the presence of five major RNA species in the
approximate mol. wt. range of $0.7-1.0 \times 10^6$ in MV infected
cells and five species in CDV infected cells, which do not
comigrate with the MV mRNA's, but are in the same mol. wt.
range. The mol. wts. of the MV mRNA species are similar to
those reported (4). However, as has been noted (5), the
maximum coding capacity of these molecules is in excess of
that required to produce the MV specific proteins. Similar
investigations of the labelled polyA$^+$ RNA of mock infected
cells show a different pattern of bands with mol. wts. in
the range of $0.3-1.2 \times 10^6$, with a specific activity five
times less than the infected cell polyA$^+$ RNA.
      To characterize more fully the virus specific mRNA
species in the total polyA$^+$ RNA population the ability of
this RNA to direct the synthesis of proteins in a cell free
translation system was examined (Fig. 2). MV infected cell
polyA$^+$ RNA directed the synthesis of six major proteins
having approx. mol. wts. of 70K, 60K, 45K, 37K and 20K.
These have been identified by limited proteolysis with V8
protease as P, N, actin, M, X and S respectively, where X
is considered to be a derivative of P (earlier also identified
in vivo), as it shares several major peptides with the P
protein (Fig. 3). CDV infected cell polyA$^+$ RNA directed the
synthesis of four major proteins which have been identified
as the P, N, M and S proteins. In the case of MV the cell
free translation of M specific mRNA was highly variable
between preparations. This was not the case for CDV.
Neither CDV nor MV infected cells polyA$^+$ RNA directed the
synthesis of L protein, nor have nonglycosylated forms of
the H or F proteins been detected. However, in both
systems virus specific proteins appear to be the major
products in in vitro translation of infected cell mRNA
populations .

THE SYNTHESIS Aⁱ CLONING OF MORBILLIVIRUS cDNA

   Single stranded cDNA (sscDNA) was synthesized by reverse
transcription of oligo-dT primed infected cell polyA$^+$ RNA.
The average yield of sscDNA per µg of RNA template was 100 ng.
By sizing on alkaline sucrose gradients or in denaturing gel
systems, the sscDNA was found to be a heterogeneous population
of molecules ranging from 200->1100 nucleotides in length.
This material was cloned using the procedure described by Cann
and coworkers (6) whereby sscDNA/mRNA hybrids, immediately
after reverse transcription, were directly extended at their
3' termini with an oligo-dC tail using terminal transferase
in the presence of Co^{++}. The tailed hybrids were reannealed
to Pst1 cut pAT153 plasmid which had its 3' termini extended
with an oligo-dG tail of similar length to the dC tails of
the hybrids (30-40 nucleotides). The putative recombinant
plasmids were then used to transform Escherichia coli K12
(HB 101 or ED8767) (7). Putative insert carrying bacteria
were selected using the insertional inactivation of the pAT153
ampicillin resistance gene as indicator. The proportion of
transformed colonies with an inactivated ampicillin gene
varied from 60-25% depending upon the extend of the initial
Pst1 digestion of the vector. Colony hybridisation (8) with
sscDNA probes (specific activity approx. 4.5 x 10^8 cpm/µg),
reverse transcribed from uninfected Vero cell or morbilli-
virus infected cell polyA$^+$ RNA, selected out those colonies
that carried infection specific sequences. Of those bacteria
which yielded a reproducible signal approximately one quarter
were infection specific. About 60% of these plasmids
contained inserts greater than 300 base pairs (bp) which
could be released by restriction with Pst1.

THE CHARACTERIZATION OF MORBILLIVIRUS SPECIFIC CLONES

   Of 25 MV infection specific clones, 5 have been shown to
carry cDNA inserts greater than 600 bp (pMV1: 1kbp; pMV11:
1kbp - both with internal Pst1 sites -; pMV5: 800 bp;
pMV14: 750 bp; pMV9: 600 bp). Of the 15 CDV infection
specific clones examined 3 have large Pst1 releasable inserts
(pCDV2: 1450 bp; pCDV6: 800 bp; pCDV7: 400 bp). When clones
pMV1-6 and pCDV2,6,7 were challenged with a cDNA probe,
prepared by reverse transcription of mumps virus infected
Vero cell polyA$^+$ RNA, no signal was obtained which suggested
that they are MV or CDV virus specific and not derived from
paramyxovirus induced cell specific mRNA's. Cross hybrid-
isation studies are presently underway to establish sequence
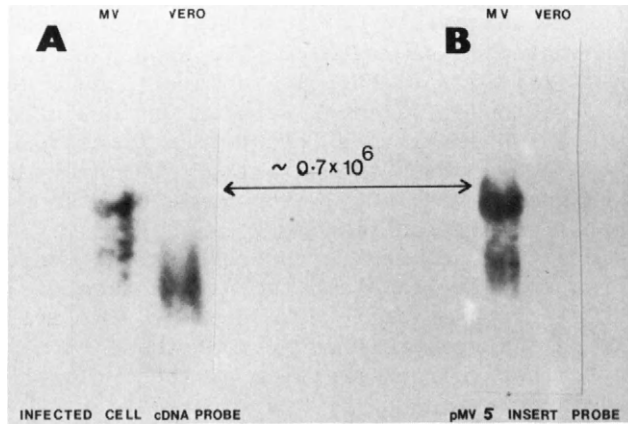
Figure 4. MV and Vero cell RNA immobilized on DBM paper
and probed with (a) total MV infected cell cDNA probe
(b) nick translated pMV5 insert DNA probe.

homologies between the clone banks in our possession derived
from two strains of mumps virus, MV and two plaque morphology
mutants of CDV.

Probing the 25 MV specific clones with nick-translated
purified insert of pMV5, lit up two other related clones,
pMV2 and pMV3, both of which carry small inserts (200-300 bp).
Further work of this type to group the remaining 22 clones
is currently in progress.

To demonstrate that the clones were complementary to
viral mRNA's, polyA$^+$ RNA immobilized on DBM paper (9) after
northern blots has been probed. Figure 4A shows the results
obtained when MV infected cells polyA$^+$ RNA and Vero cell
polyA$^+$ RNA were challenged with a MV infected cell cDNA probe.
Both types of RNA yielded a signal. However, the hybridis-
ation patterns were quite distinct. This demonstrated that
both types of RNA had been successfully transferred and
immobilized and also, that there appeared to be extra RNA
species in the infected cell RNA population. Figure 4B shows
the results when this RNA was challenged with a nick-trans-
lated pMV5 insert probe. A band was apparent which
corresponded closely to the position of band 3 (Fig. 1). No
hybridisation to Vero cell RNA could be detected (even at
very long exposure times) at the stringency conditions used
(0.1 x SSC at 65°C). To position the hybridisation signals
with respect to MV mRNA bands (Fig. 1) a ^{3}H-uridine labelled
infected cell polyA$^+$ RNA sample was fractionated in the

in the original gel before northern blotting. This was cut
off and fluorographed separately. The band pattern and
separation was similar to Fig. 1. However, the electro-
phoretic separation differences between various mRNA species
appeared to be too small to allow an identification to be
made of the band to which the insert of pMV5 hybridised.
Similar experiments with pCDV2 probes yielded the same type
of interpretation difficulties.
      Therefore, this communication reports the isolation of
morbillivirus specific mRNA and the preparation of a number of
MV and CDV infection specific cDNA clones. The evidence
obtained to date suggests strongly that these clones are virus
specific. Further characterisation of these clones is
currently being carried out by hybrid arrest or hybrid
selected translation experiments.

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SPECIFICITY OF INTERACTION OF L, NS, AND M PROTEINS WITH N-RNA COMPLEX OF VESICULAR STOMATITIS VIRUS IN A HETEROLOGOUS IN VITRO RECONSTITUTION SYSTEM

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The transcribing nucleocapsid of vesicular stomatitis virus (VSV) consists of the N protein-RNA complex associated with two dissociable proteins: L and NS (1). An efficient reconstitution system for in vitro transcription was developed by Emerson and Wagner (2), where addition of dissociated L and NS proteins to the purified N-RNA complex resulted in faithful transcription of the genome RNA. Transcription products included the leader RNA and five 5' capped, 3'-polyadenylated mRNA species coding for the five VSV structural proteins (3). Although both L and NS are needed for transcription (4), the precise role(s) of each protein in this process is not yet established.

In order to study specificity of interaction of the L and NS proteins with the N-RNA complex, in the present studies we have used two serotypes of VSV i.e., Indiana (VSV$_{IND}$) and New Jersey (VSV$_{NJ}$). Although these two viruses are serologically distinct, previous studies have shown evidence of relatedness based on cross-reactive group-specific antigen (ribonucleoprotein core, RNP) (5). Moreover, it was shown that L protein of VSV$_{NJ}$ could be utilized in vivo in the transcription of a defective particle obtained from a heat-resistant mutant of VSV$_{IND}$ (HR-DI) which lacks the L protein gene (6). Also of interest are the observations that wild-type VSV$_{NJ}$ can complement the transcription defect of a VSV$_{IND}$ Group I mutant (7); VSV-NJ interferes with the replication of VSV$_{IND}$ (8) and HR-DI IND can cause heterotypic interference with VSV$_{NJ}$ in coinfected cells (9). These results clearly indicate that interaction of L and NS proteins with heterologous N-complex probably occurs in vivo despite the fact that corresponding heterologous template in a reconstitution experiment failed to complement the serotype-specific proteins to restore transcription in vitro (10). The N genes of VSV$_{IND}$ and VSV$_{NJ}$ share considerable sequence homology (D. Rhodes, unpublished observations) in addition to coding for a common leader RNA (11) and 5'-terminal hexanucleotide on each of their mRNAs (12). It was, therefore, of interest to reexamine the

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specificity of interaction of L and NS proteins with heterologous N-RNA complex.

HETEROLOGOUS RECONSTITUTION SYSTEM.

The L and NS proteins of VSV_{IND} (Mudd-Summers strain) and VSV_{NJ} (Ogden strain) were purified from virions disrupted with detergent in the presence of 0.4 M NaCl. The RNP containing the L and NS proteins were removed from the G and M proteins by centrifugation (13). The L and NS proteins were then dissociated from the RNP in 0.8 M NaCl and further fractionated by chromatography on phosphocellulose column as described by Emerson and Yu (4). As shown in Fig. 1, (#1) purified NS protein (Indiana) free from L protein was recovered as flow through while the L protein that eluted at 1M NaCl contained only a trace amount of NS protein (#2). The band migrating slightly faster than the NS protein in both fractions was found to be N protein by Western blot using N-specific antibody. The L and NS proteins of VSV_{NJ} were purified similarly and had an elution profile the same as shown in Fig. 1. The N-RNA complexes of both the serotypes were prepared as described previously (13). In a typical reconstitution system, the NS protein (#1) was transcriptionally inactive. The #2 fraction retained some activity but addition of #1 stimulated transcription by 3 to 4-fold.
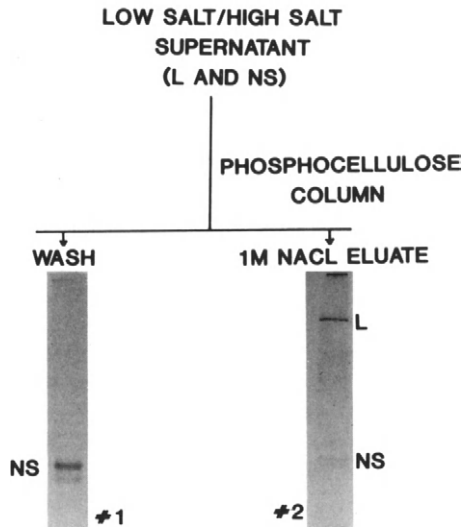
Fig. 1. Separation of L and NS proteins of VSV. Purified virus of both the serotypes were disrupted with Triton X-100 and L and NS

proteins were purified as described earlier (13). The proteins were further separated by chromatography on a phosphocellulose column (4).   The wash (#1) and IM NaCl eluate (#2) were analyzed by polyacrylamide gel electrophoresis and proteins were stained by silver reagent.

Fig. 2 shows the specificity of interaction of L and NS proteins of VSV_{NJ} with N-RNA complex of VSV_{IND} based on their ability to synthesize RNA in vitro.   The RNA products were analyzed by polyacrylamide gel electrophoresis.   The L and NS proteins of VSV_{NJ} were active as shown by their ability to synthesize RNA with homologous N-RNA complex (Lane A).   In contrast, the proteins failed to synthesize mRNA and leader RNA when added to N-RNA complex of VSV_{IND} (Lane B). In sharp contrast, when L and NS proteins of VSV_{NJ} were added together with purified NS_{IND} (#1, Fig. 1) RNA synthesis ensued as evidenced by the synthesis of leader RNA and mRNA which stayed at the origin (Lane D).   Virtually no RNA product was synthesized when NS_{IND} was added alone to N-RNA_{IND} (Lane C). These results clearly indicate that L and NS_{NJ} can replace the L_{IND} function, and subsequent addition of NS_{IND} was required for mRNA synthesis.
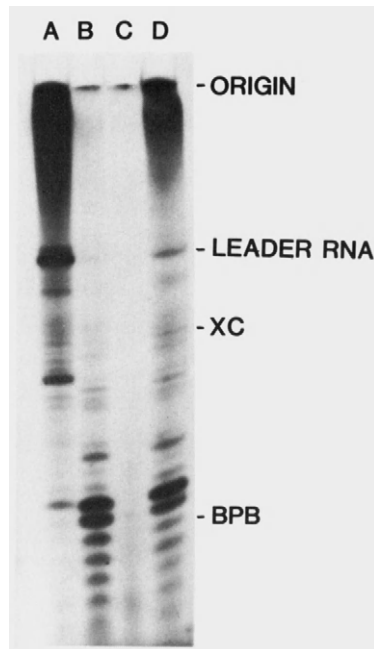
Fig. 2. RNA synthesis by in vitro reconstituted system. Purified fractions#1 and #2 (Fig. 1) were added to N-RNA complex under RNA synthesis conditions, and products were analyzed by electrophoresis in a 20% polyacrylamide gel followed by autoradiography (13). (A) $(L+NS)_{NJ} + $ N-RNA_{NJ}; (B) $(L+NS)_{NJ} + $ N-RNA_{IND}; (C) $NS_{IND} + $ N-RNA_{IND}; (D) $(L + NS)_{NJ} + NS_{IND} + $ N-

RNA_{IND}. Positions of leader RNA, xylene cyanol (XC) and bromophenol blue (BPB) dyes are shown.

Fig. 3 Lane B shows that in a partial reaction (14) containing only ATP and α-^{32}P CTP, $(L+NS)_{NJ}$ were capable of synthesizing AC (oligo I) and AAC and AACA (oligo II) on the N-RNA_{IND}, i.e., similar to the homologous components (Lane A). In contrast, NS_{IND} failed to synthesize significant oligomers when reacted with its homologous template (Lane C). Addition of NS_{IND} together with $(L+NS)_{NJ}$ to the N-RNA_{IND} (Lane D) did not increase oligonucleotide synthesis. (Compare Lanes B & D). From the results shown in Figs. 2 and 3, it can be concluded that L_{NJ} could bind to N-RNA_{IND} and initiate RNA chains but homologous NS_{IND} could interact with L_{NJ} for subsequent chain elongation and continued RNA synthesis.
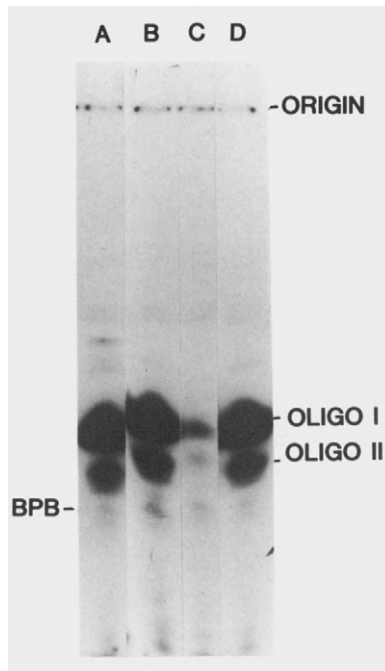
Fig. 3. Initiation of RNA synthesis in the reconstituted system. Synthesis of oligonucleotides AC (oligo I) and AAC, AACA (oligoII) was carried out in the presence of ATP and α-^{32}P CTP and products analysed by polyacrylamide gel electrophoresis (14). (A) $(L+NS)_{NJ}$ + N-RNA_{NJ}; (B) $(L+NS)_{NJ}$ + N-RNA_{IND}; (C) NS_{IND} + N-RNA_{IND}; (D) $(L+NS)_{NJ}$ + NS_{IND} + N-RNA_{IND}.

In contrast to the above results, when N-RNA_{NJ} was used in a similar reconstitution sysem with $(L+NS)_{IND}$, different results were obtained. $(L+NS)_{IND}$ was able to bind to N-RNA_{NJ} and initiate RNA chains similar to that shown in Fig. 3, but NS_{NJ} was unable to synthesize RNA when added to the mixture (data not shown). These

results indicated that NS_{NJ} lacks the ability to bind to L_{IND} to synthesize RNA chains, whereas as shown above, NS_{IND} can specifically interact with L_{NJ}. In order to further confirm this specific interaction between L_{NJ} and NS_{IND}, we studied the capacity of NS_{IND} to replace NS_{NJ} in a homologous reconstitution system. The results of such an experiment is shown in Fig. 4. The $(L+NS)_{NJ}$ (#1, Fig. 1) in a homologous reconstitution system was capable of synthesizing mRNA (Lane A), and addition of homologous NS_{NJ} (#2) stimulated mRNA synthesis by 3-fold (lane B). When NS_{NJ} was replaced by NS_{IND}, identical stimulation of mRNA synthesis occurred (lane C). These results demonstrate that NS_{IND} could specifically interact with L_{NJ} to synthesize mRNA. Thus, NS_{IND} and NS_{NJ} share common reactive sites that are involved in interaction with L_{NJ}, but interaction of NS_{IND} with L_{IND} is mediated by a different site not shared by NS_{NJ}.
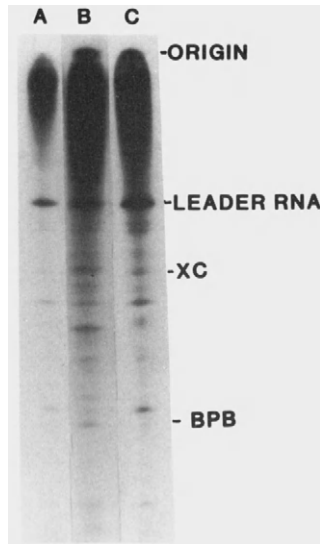
Fig. 4. Effect of NS_{IND} in VSV_{NJ} reconstituted system. RNA synthesis was carried out using template N-RNA$_{NJ}$ and (A) L_{NJ}(#2); (B) L_{NJ}(#2) + NS_{NJ} (#1); (C) L_{NJ}(#2) + NS_{IND}; products analysed by electrophoresis in a 20% polyacrylamide gel.

SPECIFICITY OF INTERACTION OF M PROTEIN WITH HETEROLOGOUS RNP.

It has been previously shown that the matrix protein (M) interacts strongly with RNA at low ionic condition with virtual

cessation of RNA synthesis in vitro (13).   To study whether this
interaction was serotype specific, we purified a mixture of G and M
proteins from VSV_{IND} and VSV_{NJ} and studied their effect on RNA
synthesis in heterologous systems. As shown in Table 1, M_{IND} was
capable of inhibiting transcription efficiently (90%) when added to
RNP_{NJ}, although at a slightly higher concentration compared to
homologous inhibition.   Similarly, M_{NJ} interacted with RNP_{IND}
although at a lower efficiency (60%).   These results indicate that
the M protein of both the serotypes share homologous regions that
are specifically involved in binding to the RNP and inhibiting
transcription.

TABLE 1

Inhibition of Transcription in Vitro by M Protein

	M_{IND}[1] (µg)			M_{NJ} (µg)	
	10	30	60	30	60
RNP_{IND}	80%	97%	N.D	48%	76%
RNP_{NJ}	N.D	76%	90%	61%	75%

(1)   Fraction containing M and G proteins were prepared from
      purified VSV_{IND} and VSV_{NJ} (13); they correspond to M_{IND} and
      M_{NJ}, respectively.   Indicated amounts were added to purified
      RNP fraction (10 µg) of both serotypes and inhibition of RNA
      synthesis in vitro was assayed as described (14).   N.D; not
      determined.

In order to further confirm these findings, we raised monoclonal
antibodies against purified M_{IND} and M_{NJ} using the procedure
described previously (15). A total of 72 and 31 hybridomas for M_{IND}
and M_{NJ}, respectively, were obtained. Using a micro ELISA for M
protein antibody, it was found that 22 of 72 M_{IND} hybridomas
reacted specifically with M_{IND} and 50 with both M_{IND} and M_{NJ}.
Only one of 31 M_{NJ} hybridomas was specific for M_{NJ} and 30 reacted
with both M_{IND} and M_{NJ}. A typical micro ELISA for M proteins of
both the serotypes is shown in Table 2. These results indicate that
M_{IND} and M_{NJ} must share common epitopes which are involved in
specific interactions with the N protein.

Table 2

Micro ELISA for M Protein Antibody[1]
(0.D at 410 mµ)

M_{IND} Antibody Hybridoma #	Antigen		M_{NJ} Antibody Hybridoma #	Antigen	
	M_{IND}	M_{NJ}		M_{IND}	M_{NJ}
35	1.27	0.13	26	0.08	0.64
44	1.22	1.25	19	1.24	1.22
60	0.19	0.81	16	0.41	0.04

[1]   M and G proteins were isolated from virions and M protein was
further purified by Con A-Sepharose column and used to raise
monoclonal antibodies according to the method described
previously (15). Supernatant fluids from different hybridomas
were used for micro ELISA using purified M protein as antigen
and β-galactosidase conjugated anti-mouse IgG as the screening
reagent.

Conclusion.   Although VSV_{IND} and VSV_{NJ} are serologically
distinct, considerable homology exists between the N genes, leader
RNAs and some specific domains on the genome RNAs. The above
results, in addition, indicate that the interaction of L and NS proteins
with heterologous template is also quite specific. We have shown
that L_{NJ} could complement L_{IND} only in the presence of NS_{IND} in
the in vitro reconstitution system. Thus, it seems that NS_{IND} has
specific domains that recognize L_{NJ}.   In contrast NS_{NJ} lacks
specificity to interact with L_{IND}. These results would clarify some
of the previous observations, such as replication of $HR-DI_{IND}$ in the
presence of VSV_{NJ} (6) and complementation of VSV_{IND} group I
mutant with VSV_{NJ} (7).   Nucleotide sequence determinations of
$L_{IND,NJ}$ and NS_{NJ} genes would help locate regions of homology
within the corresponding genes. The above results also give insight
into the possible role of L and NS proteins in the transcription
process.   The L protein appears to be involved in the initiation
process, while NS protein mediates chain elongation and continued
RNA synthesis. The precise mode by which L and NS interact with
each other and with the template N-RNA remains to study. The M
protein of both serotypes appears to possess common regions that
interact with heterologous RNPs. The complete nucleotide sequence
of M_{NJ} gene and further studies with monoclonal antibodies should
determine which regions in the M proteins are involved in that
interaction.

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DOES MODIFICATION OF THE TEMPLATE N PROTEIN PLAY A ROLE IN REGULATION OF VSV RNA SYNTHESIS?[1]

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INTRODUCTION

Vesicular stomatitis virus (VSV) RNA synthesis, as well as that of other negative-strand RNA viruses takes place on ribonucleoprotein (RNP) templates, and is regulated via specific termination events (1). What controls termination is crucial for understanding the overall regulation of virus growth in lytic and persistent infections. VSV transcription templates contain six major termination sites corresponding to the ends of the leader gene and the five virus structural genes. To achieve the first step in replication, termination at each of these sites must be suppressed to allow genome-size plus strand synthesis. This switch is dependent on new viral protein synthesis in vivo (2). However, the coupling of replication to translation can be circumvented in vitro using purified virus in the presence of base analogues (3,4,5). Kolakofsky and colleagues have recently provided some evidence for a model whereby suppression of termination in vivo may be achieved by binding of soluble N protein to nascent leader RNA chains (6,7). The initiation of the helical RNP assembly process is thus viewed as being sufficient to prevent termination at the leader RNA sites. Our recent studies with a unique class of VSV mutants, called pol R, suggest that antitermination at leader sites may be governed by modification of template-associated N protein subunits (8,9,10). We shall briefly summarize our results here and discuss a model incorporating these ideas.

PROPERTIES OF POL R VSV MUTANTS

We have documented several changes in the nature of
transcripts synthesized in vitro by purified pol R VSV
particles as compared to wild-type virus. Most important
among these are 1) a large increase (~7X) in readthrough
transcripts spanning the plus strand leader-N gene junction
(10), 2) a quantitatively similar increase in readthrough of
the minus strand leader RNA termination site (measured on DI
templates) (8,10), 3) an increased resistance to inhibition by
replacement of ATP by AMP-PNP during initiation of synthesis
(Perrault and McClear, in preparation), and 4) a decreased
sensitivity to virion M protein inhibition (McClear and
Perrault, unpublished). Other minor changes include 1) an
approximately 60% decrease in the accumulation of plus strand
leader RNAs (9,10), 2) a two-fold increase in initiation at
the 3' end of the standard template relative to total RNA
synthesized (10), and 3) a significant increase in the size
and heterogeneity of poly(A) tails on otherwise normal mRNAs
which are also abundantly synthesized (9). The importance of
the readthrough property of pol R VSV is enhanced by the fact
that this phenomenon is specific for leader RNAs since no such
increase was detected for the junctions between structural
genes (9). It should also be emphasized that the phenotype of
pol R VSV represents a major change in behaviour for the virus
polymerase at least in vitro. All productive VSV RNA
synthesis apparently begins at the 3' end of the templates
(11), and mRNAs are produced in decreasing amounts as a
function of the distance from this 3' end. The leader RNA
termination sites, only 46-48 nucleotides from the entry
sites, are therefore the most frequent termination signals
encountered by the polymerase.
    Wild-type virus also reads through the leader RNA
termination sites in vitro under standard conditions, but the
frequency of such readthrough (~12%) versus pol R1 VSV (~83%)
clearly suggests a shift from a transcription mode to a
replication mode in the latter (10). At the present time, we
do not understand why most pol R in vitro readthrough
transcripts terminate heterogeneously only 300 to 800 bases
from the 3' terminus, instead of continuing synthesis until
the end of their templates. This perhaps indicates that a
second function is needed for sustained elongation beyond the
critical leader junction. This second function would then
presumably be operative when elongation is carried out in the
presence of base analogues since full-length plus strands are
synthesized in vitro under these conditions by wild type
virus (3).
    The properties of the two independently isolated pol R1
and pol R2 viruses are qualitatively similar but show distinct

quantitative differences (Perrault, unpublished). Although
these mutants were obtained following cycles of heat
inactivation of virus lysates, they are not heat resistant
compared to wild-type. In vivo, their growth is somewhat
restricted (~10-50% of wild type yields). Viral RNAs in
mutant-infected cells show characteristics similar to those
seen in vitro but less dramatically so. We have recently
detected unencapsidated pol R readthrough transcripts in vivo
and are currently examining their properties (Perrault,
unpublished).

     From our point of view, the most unexpected aspect of the
pol R mutants concerns the mapping of the mutation responsible
for this interesting phenotype. In vivo construction of
chimeric DI composed of wild-type RNA template and pol R1
proteins, or mutant template RNA and wild-type proteins,
allowed us to test whether the readhtrough phenotype was due
to sequence changes at the sites of leader RNA termination,
or due to changes in a protein(s) involved in termination.
The answer was clearly a protein change (8,10). We then
attempted in vitro reconstitution of polymerase activity using
purified RNP templates (standard and DI) and solubilized
polymerase proteins (L+NS). Again, the answer was clear.
Readthrough of plus strand or minus strand leader termination
sites partitioned with the highly purified RNP template
fractions (10). These results very strongly implicate the N
protein of pol R viruses in the readthrough activity.

     Despite the limitations inherent in studying non-
conditional mutants, we were able to independently confirm
that the properties of the pol R mutants are due to an N
protein alteration. We found a qualitatively and
quantitatively similar isoelectric charge shift in the N
protein of both pol R mutants (10). Additional comparisons
with wild-type virus revealed only two other protein changes,
one in M for pol R1, and one in NS for pol R2. These results
indicate that the N protein charge shift common for both
independent mutant isolates is in all likelihood responsible
for most or all of phenotypic changes seen in pol R virus.

TEMPLATE MODIFICATION MODEL OF VSV RNA SYNTHESIS

     The specificity and magnitude of the changes in
termination of RNA synthesis seen in pol R viruses argue that
an important element controlling the balance between
transcription and replication has been altered. Whether N
protein alone, or N protein in concert with other viral
and/or host determinants, controls this switch is not yet
clear. What our studies point out, however, is that
assembled VSV RNP templates, containing only N protein can
carry the information necessary to direct the polymerase
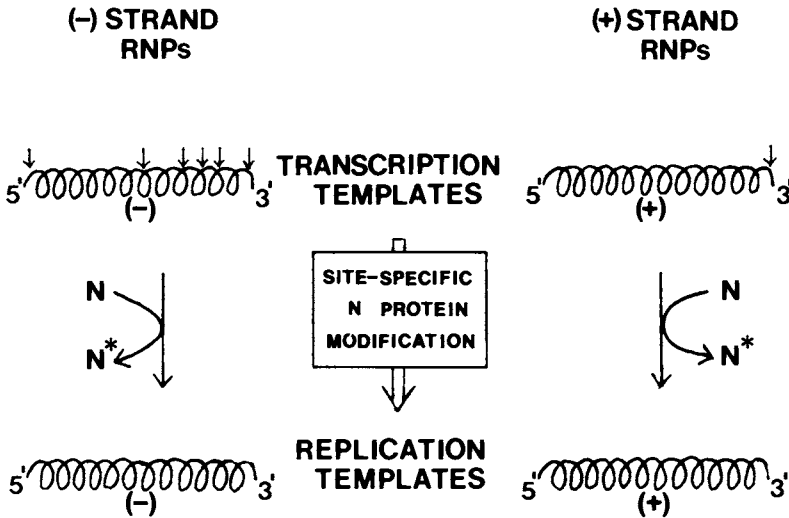
FIGURE 1.   A model for the control of VSV transcription
and replication by site-specific N protein modification. The
six termination sites on minus strand RNP templates (arrows)
correspond to the ends of the leader gene and the five
structural genes. Only one termination site, that giving
rise to the minus strand leader RNA transcript, is found on
the plus strand standard RNP template (also found on both
plus and minus strand DI RNP templates). We propose that one
or more N protein subunits at these sites are specifically
modified (N>N*) in vivo before the viral polymerase can
replicate or readthrough the templates.

towards a transcription mode (termination) or a replication
mode (antitermination). The shift towards the replication
mode in the pol R mutant RNPs, solely as a result of an N
protein mutation, in turn suggests that the simplest way to
carry out this switch is by modification of the template
protein. We have therefore recently proposed that the VSV
RNP transcription templates, normally found in virions

(both standard and DI), are modified in vivo to replication
templates whose structure allows readthrough of the leader
termination sites (10). We further suggested that this change
may involve a post-translational modification of the N protein
(see figure 1). This proposal, of course, does not rule out
a need for additional factors to achieve successful and
sustained replication in vivo such as a pool of soluble N
protein capable of initiating assembly of nascent replication
products. It may be that concurrent assembly is normally
needed to suppress termination sites beyond that of the
leader gene on the minus strand genome template.
    How can we explain the pol R mutant phenotype in the
context of this model? The difference in readthrough activity
between wild-type and mutant virus is not so much a qualita-
tive difference but a quantitative difference (~12% vs 83%
readthrough). This suggests that both types of viruses
contain the two postulated forms of RNPs in different ratios.
The amino acid change in the pol R mutant N protein could
either affect the rate and/or extent to which the conversion
of transcribing RNPs to replicating RNPs occurs in vivo, or
affect the assembly mechanism which selects transcription-
type RNPs for packaging in virions. It should be noted here
that structural differences between virion and cellular RNPs,
based solely on N-N or N-RNA interactions, have been
documented before (12).
    Our studies have not yet identified what kind of N protein
modification could be involved. The multiple isoelectric
species of N protein we and other have observed in wild-type
VSV (10,13) strongly suggest that this protein is post-
translationally modified. However, all of the four sub-
species we detected were shifted to the anode in the pol R
viruses, and the relative difference in charge between these
species within wild-type or mutant viruses remained the same
(10). So, the putative charged amino acid substitution in the
mutant N protein does not appear to affect this particular
post-translational modification, at least superficially.
Some other modification may therefore be involved in
regulating the activity of the templates. One possibility is
that the switch from transcription to replication RNPs invokes
changes in only a few of the 1500-2000 N protein subunits on
each template. This idea of site-specific modification of
the template (see figure 1) transpired from results of our in
vitro reconstitution experiments. As mentioned previously,
readthrough products synthesized on pol R1 standard RNP
templates appear to terminate non-specifically, i.e., at a
large number of sites ~300 to 800 bases from the 3' end.
Curiously, this very heterogeneous pattern of termination is
reproduced (band for band on acrylamide gels) whether we
examine transcripts from unfractionated pol R1 standard virus,

or reconstituted reactions with either homologous pol R1
solubilized enzyme or heterologous wild-type solubilized
enzyme (10). Thus, the pol R1 RNP standard templates behave
as if the termination sites are already "tagged" in the
assembled virion (the same phenomenon is also seen with pol
R1 DI templates and to a more limited extent with wild type
standard and DI templates). Such behaviour could be explained
by modifications of N protein subunits at selected sites along
the template which act as termination signals. The abnormal
pol R mutant N protein might then allow "illegitimate" or
incorrect modification of sites on its RNP templates.

     Lastly, we have not yet addressed the question of which
entity might be responsible for N protein modifications. It
seems likely to us that this function is carried out normally
by a virally-coded polypeptide. The aforementioned
observation that full-size plus strands can be synthesized in
the absence of new viral protein synthesis with purified
virions in the presence of base analogues, suggests that this
is the case and that the modification can also be carried out
in vitro under special conditions. The NS phosphoprotein and
the M protein would seem to be logical candidates for the
modifying function since they are known to bind directly to
the RNP template and, in the case of NS, at sites close to
both plus and minus leader RNA termination sites (14,15,16).
Perhaps, the modification can also be carried out or
complemented by host cell factors under different conditions.
A role for host cell factors in VSV replication is well
established (17) and a direct or indirect effect in the
reaction responsible for the switch from transcription to
replication is a distinct possibility.

ACKNOWLEDGMENTS

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BINDING STUDIES OF NS1 AND NS2
OF VESICULAR STOMATITIS VIRUS[1]

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INTRODUCTION

Vesicular stomatitis virions (VSV) are composed of five virally coded proteins: N, NS, M, G, and L. Of these, N, NS, and L are all needed for transcription (1). The template for transcription contains the virion's negative strand RNA tightly encapsidated by N protein. This template, when combined with column-purified L and NS in the presence of nucleotides, $MgCl_2$ and D.T.T., is capable of _in vitro_ transcription.

The NS protein is a phosphoprotein which exists in several different phosphorylated forms. DEAE-cellulose chromatography used by Kingsford and Emerson (2) separates virion NS into two species called "NS1" and "NS2." The NS2 is more highly phosphorylated than NS1. Each of these NS species when run on urea-SDS polyacrylamide gels separates into two closely migrating bands (TOP and BOTTOM) which differ in the extent of phosphorylation. The NS BOTTOM is the more highly phosphorylated species. Urea-acetic acid gels and isoelectric focusing gels also separate species of NS differing in phosphorylation (3,4).

VSV is thought to encode proteins capable of functions necessary for its own replication and transcription including initiation, elongation, capping, and methylation enzymes. Since there are only five proteins to supply all

of these functions, the assumption is made that some if not
all of the proteins play a multifunctional role in the life
cycle of the virus. Because the NS protein exists in
different phosphorylated states, it is a good candidate for
such a multifunctional protein.

    Kingsford and Emerson (2) have examined transcriptional
activity of NS1 and NS2 and found only NS2 is active in _in_
vitro transcription. Kingsbury (5) employed bacterial
alkaline phosphatase to dephosphorylate NS on virion
nucleocapsids. These treated nucleocapsids were found to
lack transcriptional activity. Several approaches follow
which further examine this correlation between the state of
phosphorylation of NS and its activity in transcription.

RESULTS

    L protein is unable to bind to template in the absence
of NS protein (6), but it has not been shown whether NS1,
NS2, or both are required for L binding. Two simple
explanations for the inability of NS1 to function in a
reconstituted transcription system are that NS1 does not
bind to template or is unable to promote the binding of L
protein. Therefore, VSV virions were examined to determine
if both NS1 and NS2 are bound to the nucleocapsid core. The
virions were solubilized in low salt and detergent to remove
G and M proteins and any other proteins which were unbound
or loosely associated with nucleocapsids. The nucleocapsids
were separated from the free proteins by centrifugation.
These purified nucleocapsids were then treated with high
salt, 3.5 M urea, and detergent to release L and NS. Again
free proteins were separated from the template by
centrifugation. These freed proteins were analyzed for NS1
and NS2 content (see Fig. 1). As is seen, both NS1 and NS2
are associated with nucleocapsid cores purified from
virions.

    Next, virions were solubilized with high salt, 3.5 M
urea, and detergent, and NS1 and NS2 were purified by the
method of Kingsford and Emerson (2). Each protein was then
assayed for its ability to bind to purified template (N and
RNA) (see Fig. 2). Both NS1 and NS2 rebind to template.
The rebinding of both NS species reached saturation at
approximately the same level. This level of saturation is
very close to that of whole NS (which is a mixture of NS1
and NS2) on intact virions (7).
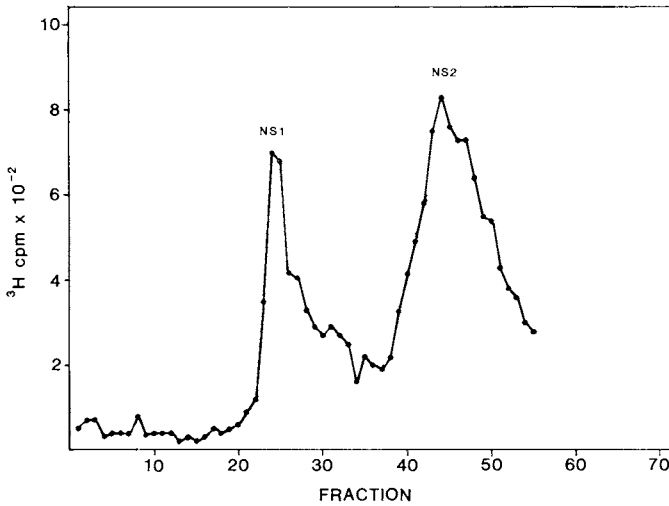
Figure 1. DEAE cellulose chromatography of NS protein. VS virions were solubilized in 0.25 M NaCl, 1% Triton X-100, 10 mM Tris 7.2, and 5 mM D.T.T. Nucleocapsids were separated from solubilized proteins by centrifugation in a SW50.1 rotor for 3 hours at 40K rpm. The nucleocapsids were spun onto a 200 µl pad of 100% glycerol overlaid by 1 ml of 20% glycerol. The nucleocapsid band was removed along with the 100% glycerol and diluted to 12.5 mls with 10 mM Tris 7.2. This was added to 12.5 mls of high-salt, urea-solubilizing solution (1.44 M NaCl, 7 M urea, 1% Triton X-100, 20% glycerol, 20 mM Tris 7.2, and 10 mM D.T.T.). Again the mixture was spun in a SW50.1 rotor for 3 hours at 40K rpm. This time the supernatant was pooled and dialyzed overnight against 0.1 M NaCl column wash (0.1 M NaCl, 0.2% Triton, 25% glycerol, 0.2 mM D.T.T., and 10 mM Tris 7.2). The DEAE column chromatography was performed as published by Kingsford and Emerson (2). 0.5 ml fractions were collected, and 50 µl fractions were analyzed by liquid scintillation spectrophotometry.
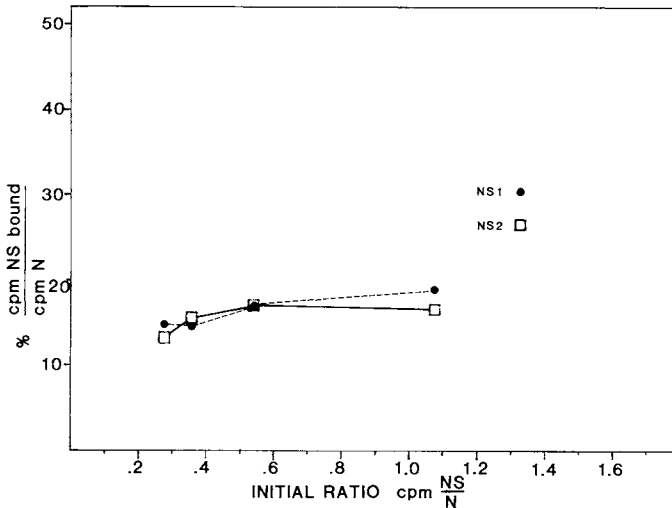
Figure 2. NS1 and NS2 were purified as outlined by Kingsford (2). Peak fractions were pooled and dialyzed against 0.1 M NaCl column wash. Purified template was prepared as described by Mellon and Emerson (7). An equal number of ^{3}H leucine counts of NS1 or NS2 were added to a reaction mix (yielding a final concentration of 0.057 M NaCl, 2% Triton, and 10 mM Tris 7.2). To this were added decreasing amounts of template. Rebinding was allowed to occur for 30 minutes at 31°C. The reaction was then layered onto 200 µl of 30% glycerol overlaid on 20 µl of 100% glycerol. This was spun for 2 hours at 40K rpm in a SW50.1 rotor. The supernatant containing unbound proteins and the 30% glycerol were pulled and discarded. The template plus rebound NS was recovered from the 100% glycerol pad and was pooled and diluted with PAGE sample buffer without glycerol and run on 12.5% polyacrylamide gels (see Kingsford and Emerson [2]). The gels were fixed and stained. The NS and N bands were cut out and eluted with NCS tissue solubilizer from Amersham and quantitated by liquid scintillation spectrophotometry.
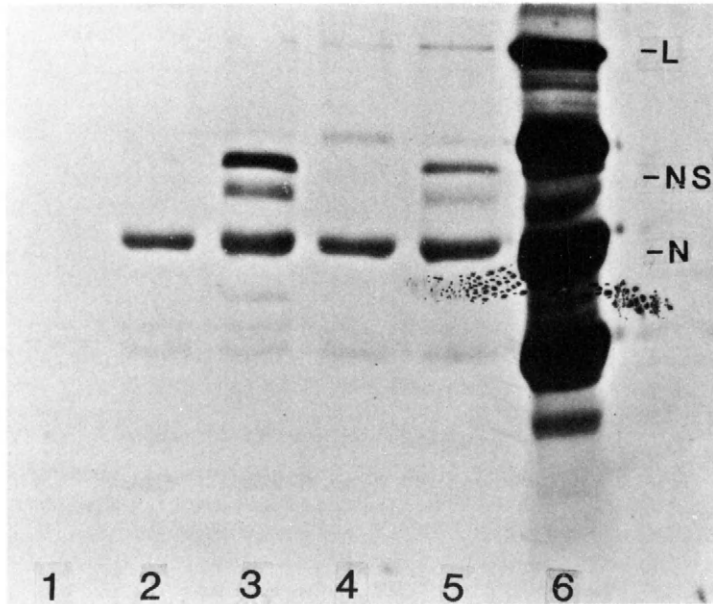
Figure 3. Autoradiograph of PAGE of rebound L with NS1
or NS2 and template. Rebinding reactions were set up as in
Figure 2 but the final salt concentration was 0.3 M NaCl.
After allowing rebinding to proceed for 1 hour on ice, the
reaction was spun as in Figure 2 (the 30% glycerol contained
0.3 M NaCl). The templates and rebound proteins were
dialyzed against 10 mM Tris 7.4. The samples were
lyophilized to dryness and resuspended in sample buffer and
run on 12.5% SDS polyacrylamide gels. The gels were fixed,
stained, and enhanced before drying and autoradiography.
Lane 1 is a reaction of L alone. Lane 2 is L + template.
Lane 3 is whole virion NS (NS1 and NS2) + L + template.
Lane 4 is NS1 + L + template. Lane 5 is NS2 + L + template.
Lane 6 is VSV. All proteins are labeled with S^{35}
methionine.

     Since both NS1 and NS2 are bound to nucleocapsids in
virions and both can rebind to purified template, templates
with either NS1 or NS2 bound to them were examined to see
whether one or both species were required for L protein
binding. Reconstitutions were set up with purified L, NS,
and templates. Rebinding of L occurred whether NS1 or NS2
was used (see Fig. 3).

SUMMARY

     The state of phosphorylation of a protein can affect
protein charge and/or conformation and thus alter
protein-protein or protein-nucleic acid interactions. The
difference in phosphorylation of NS1 and NS2 could affect
charge and/or conformation of NS and result in NS2 being the
only transcriptionally active form in vitro. We have
attempted to find the reasons why NS2 is active in
transcription and NS1 is not.

     We have shown that both NS1 and NS2 can rebind to
template. They both rebind to the same level of saturation
as NS in intact virions. Also we found NS1 and NS2 both can
promote the binding of L. Therefore, the functional
difference between NS1 and NS2 does not reflect the
inability of NS1 to interact with either the template or L
protein.

     Footprinting experiments from Jack Keene's lab showed
NS protein interacts with the virion RNA between bases 16
and 30 from the 3' end (8). Sue Emerson has shown that the
entry site for the transcriptase is at the 3' end of the
virion. These data suggest that NS interaction specifically
with these bases might constitute an active promoter region.
We therefore are attempting to footprint NS1 and NS2 bound
to templates to see if NS2 but not NS1 interacts with these
same bases.

     In vitro data indicate that the template is the
limiting transcription factor in virions. In other words,
an excess of polymerase components (L and NS) are packaged
in virions. We can postulate that, although NS1 and NS2 are
bound to templates in virions and both can rebind to
templates in vitro, this binding in the case of NS1 is a
nonspecific, transcriptionally inactive packaging
phenomenon. Perhaps only NS2 is capable of specific and
transcriptionally active binding.

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DYAD-SYMMETRY IN VSV RNA MAY DETERMINE THE AVAILABILITY OF THE RNA-POLYMERASE BINDING SITE.[1]

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VSV RNA SYNTHESIS

After infection of cells, the VSV polymerase initiates RNA synthesis at the 3' end of the genomic negative strand. This may result either in replication of RNA or in transcription. Subsequently the polymerase generates more negative strands by interaction with the 3' end of the complementary positive strand. In addition to the specific polymerase binding sites near the termini (1, 2), sequences further into the genome are thought to be important for the efficiency of replication (3, 4). This is based upon studies on the replication of defective interfering (DI) particles, where different sequence rearrangements result in their amplification at the expense of standard virus (5).

The nature of polymerase-RNA interactions during the replication of VSV is unknown. Aside from sequence specificity, secondary structures may be implicated. Possible stable or quasi-stable secondary structures at the 3' ends of the positive and the negative RNA strands were sought by using computer analysis with special reference to the presentation of the polymerase binding site. The secondary structures are discussed in relation to possible binding of the N, NS and M proteins of VSV which modulate polymerase activity during RNA synthesis. The possible secondary RNA structures at the 3' ends of three related defective interfering particles are also presented. Although our interpretations are based mainly on

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theoretical considerations, such sequence analysis points to
the necessity for quantitative binding studies between VSV RNA
and proteins.

SOURCES OF VSV SEQUENCES

All RNA sequences are of the Indiana serotype of VSV
determined by McGeoch and Dolan (8) and by Schubert et al.
(9). Sequences from defective interfering (DI) particles were
from Schubert et al. (3) as confirmed by Rao and Huang (4).
These DI sequences relate to standard viral RNA sequences
in the following way. The first 60 nucleotides at the 3' end
of the negative strand of DI 011 are identical to the 3' end
of the positive strand of standard VSV. DI 0.45 (also known
as DI-T(L)) and DI 611 have shorter 3'-end sequences (48
nucleotides) that are identical to the 3' ends of the posi-
tive strands. DI-T contains even a shorter portion of comple-
mentary sequences since it has only 45 nucleotides at its 3'
end that are identical to sequences on the positive strand.
All of the DI RNAs retain a portion of the L gene.

COMPUTER ANALYSIS

The SEQ program from the Molgen group at Stanford (10)
was used for the computer analysis. Parameters were initially
set to maximize the yield of dyad-symmetries, or stems, and
then narrowed to yield the most significant ones based on
estimates of the stability of these secondary structures
according to Tinoco et al. (11). The most stable stem and loop
structures give the lowest free energy values, Δ G, in kilo-
calories per mole.

SECONDARY STRUCTURES AT THE 3' END OF THE NEGATIVE STRAND RNA OF STANDARD VSV.

Since secondary structure might influence replication,
possible stem and loop structures were looked for at the 3'
end of VSV RNA. Figure 1 shows the secondary structures that
might form with the first 77 nucleotides at the 3' end of the
minus strand. Significant secondary structures were not
obtained with only the first 50 nucleotides. There were two
principal hairpin structures a and b. Each contained loops of
12 nucleotides. The one with the longest stem had two possible
stem endings, a and a'. Structure a ran from nucleotide 10
through nucleotide 72 while structure a' started with the
first nucleotide. The a structures were slightly more stable
than those of b.

```
        10        15   G-U   20         25       30      35  C   G  A  G   U
   3'  U-U-G-U-U-U-G ⟋  A-A-U-A-A-U  A  G-U-A-A  U-U-U-C                   C  40    G=-7.5    a
   5'  A-A-C-A-G-A-C A U U-U-G A-U-U-A  C  U-G-U-U-A-A-A-G                   C
          70      65        60        55        50
                                             ↑           U  U  C  U
                           THE N-GENE                   45


         5          15
   3'  U-G-C U U C U-G-U-U-U-G U U U G G U  A-A-U-A-A-U  A  G-U-A-A  U-U-U-C         C  40    G=-8.7    a'
   5'  A-C U G U A A-C-A-G-A-C A U U U-U-G A-U-U-A  C  U-G-U-U-A-A-A-G               C
          75        70      65        60        55        50
                                                    ↑        U  U  C  U
                                                            45
                           THE N-GENE

                                                    ↓
                                        50        55  A  U  U  A  G  60    G=-6.0    b
   -POLYMERASE BINDING SITE-⋀⋀⋀-U-G-A  A  A-U-U-G-U-C                    U
   5'  A-C-U  G  U-A-A-C-A-G                                             U
          75           70                    A                          U
                                               C  A  U  U
                                              65
```

Figure 1. Possible secondary structures formed by the 3'
end of the negative strand RNA of standard VSV. The polym-
erase binding site (1) is indicated by a straight line in a
and a'.

All three structures failed to place the polymerase bind-
ing site in a hairpin loop. Rather, the site, as indicated by
a dark line, was enclosed within stems of a and a', or free,
as in b. Note that the hexamer 3'UAGUUU 5' was also part of
the stem structure in a and a' but that the configuration in
structure b presented the hexamer in the loop.

SECONDARY STRUCTURES AT THE 3' END OF THE POSITIVE STRAND RNA OF STANDARD VSV.

Figure 2 shows three mutually exclusive hairpin loop for-
mations c, d, and e formed by the first 55 nucleotides at the
3' end of the positive strand. Two of them, c and e, con-
tained the polymerase binding site in a loops of 33 and 25
nucleotides, respectively. The smallest hairpin loop, d, had
the least significant G value. Here the polymerase binding
site was free and not in the loop or stem. Note that the loop
closings contained at least one G-C pair. The hexamer 3' UAG-
UUU 5' was free as a single strand in structures c and d but
only partially in e.

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$$G = -6.3$$

c
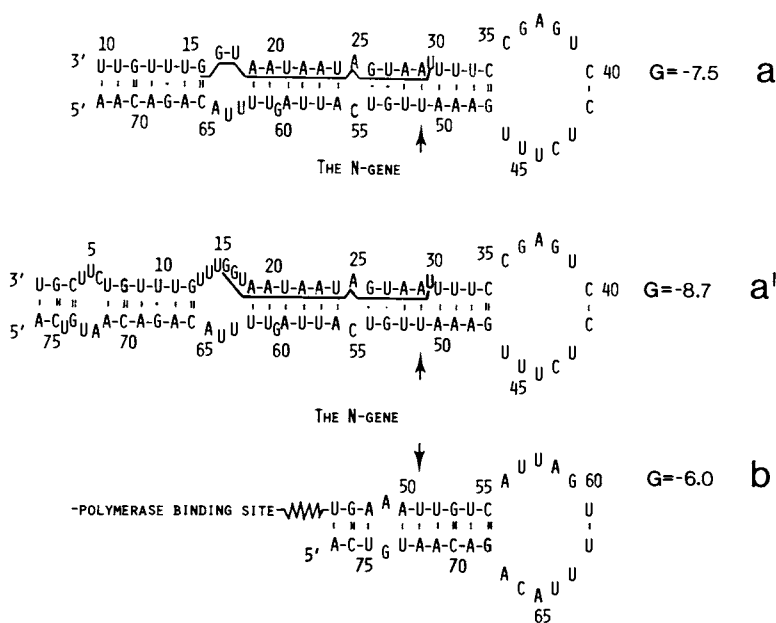
$$G = -1.4$$

d

$$G = -6.8$$

e
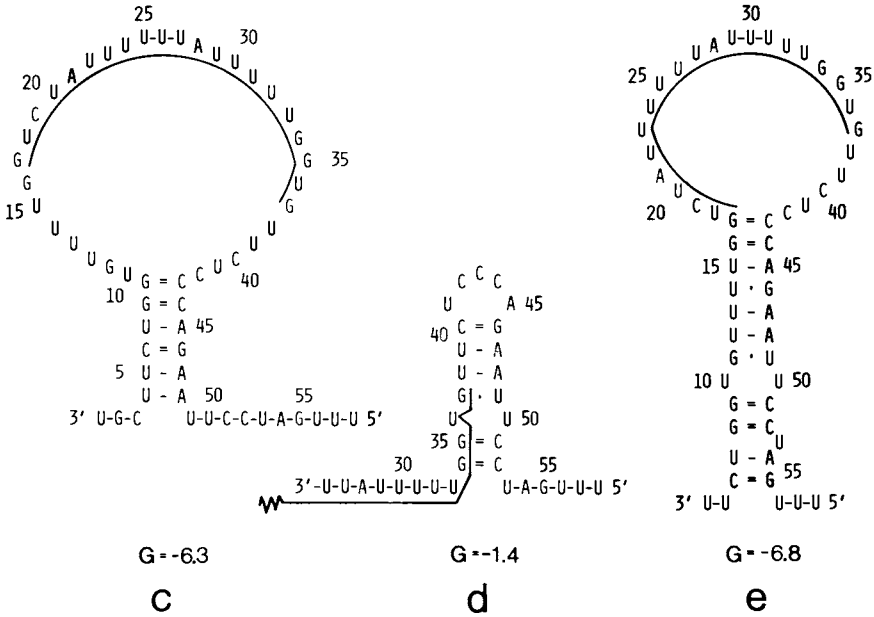
Figure 2. Mutually exclusive possible secondary struc-
tures formed by the 3' end of the positive strand of standard
VSV. The polymerase binding site (2) is indicated by a
straight line.

SECONDARY STRUCTURES OF THE 3' END OF THE NEGATIVE STRAND RNA
OF DI PARTICLES.

Secondary structures at the 3' ends of the RNA of VSV DI
particles were similar to those of the 3' end of the positive
strand RNA of standard VSV, except for variations introduced
by shorter sequence identities. The RNA of DI 011 at the 3'
end, identical for 60 nucleotides to the 3' end of the posi-
tive strand, formed the same secondary structures as those
shown in Figure 2. The RNAs of DI 611 and DI 0.45 formed the
same size loops, containing 33 or 25 nucleotides, as the posi-
tive strand of standard VSV but their stems differed (Figure
3).   The RNA of DI-T had the least stable structure (Figure
4). These low, negative G values may be improved if sequences
internal to the complementary termini were included in the
analysis.   Unfortunately, such sequences have yet to be
defined.

```
                    25
              U U U U-U-U A U U  30
         20 U A             U  U
            C                   U
            U                     U
            G                      U
            G                       G 35
         15 U                      U
             U                    G
              U                  U
               U G             U  U
               10 G = C C U C  40
                  G = C
                  U - A 45
                  C = G
                 5 U - A
                  U - A
                 C
                 __G_=_(C)___
                  U - A 50
                  3'    5'
```

```
                              C C
                           U  C    A 45
                        40 C = G
                           U - A
                           U - A
                           G|=(C)
                      ___U|-A 50___
                    35 G|     5'
                 30   G|
                 U-U-U-U-U/
              ~~~ 3'
```

```
                    30
              U A U-U-U U U
           25 U         U  G G 35
              U              U
              U               G
              A                U
           20 U                C U 40
              C U G = C C U
                  G = C
              15 U - A 45
                 U · G
                 U - A
                 U - A
                 __G_=_(C)___
              10 U - A 50
                 G    A
                 G    X
                 U    C
                 3'   5'
```

f G=-5.5   g G=-0.6   h G=-4.6
--------   --------   ---------
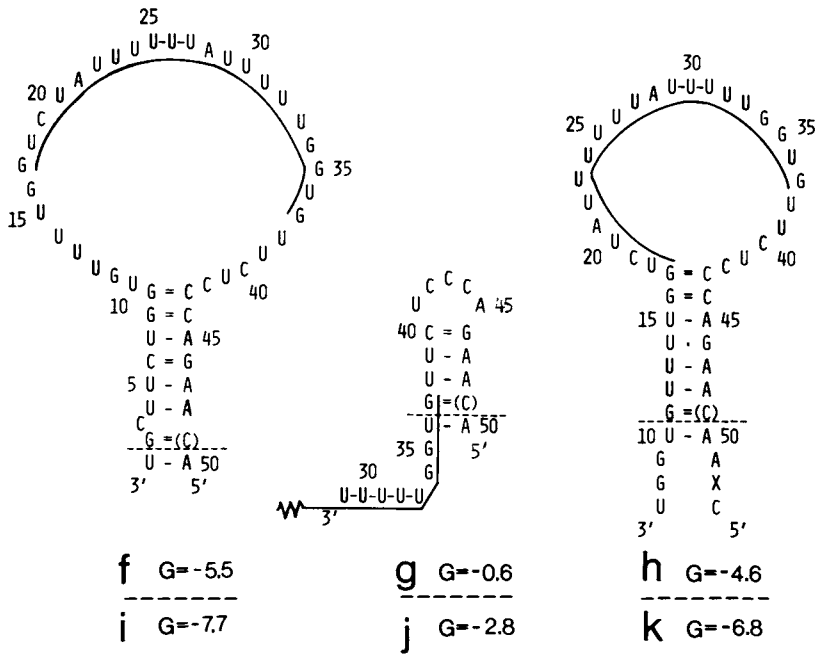i G=-7.7   j G=-2.8   k G=-6.8

Figure 3. Possible secondary structure of the 3' end of
the negative strand RNA of DI 0.45 and DI 611 particles.
Structures f, g, and h are those above the respective dotted
lines and are derived from DI 0.45. Structures i, j, and k
extend beyond the dotted lines and are derived from DI 611.

     Despite such instability, all of the RNA termini of DI
particles closed their loops with at least one G-C bond simi-
lar to the loops formed by the positive strand of standard
VSV. The small hairpin loop structure, m, might form, depend-
ing on what X represents. Structure i, interestingly, started
from the first nucleotide and had the greatest free energy of
formation among the DI RNA sequences and was comparable in
stability to the secondary structures formed at the 3' end of
the genomic negative strand. Despite the differences in the G
values between DI RNAs and positive strand VSV RNA, the large
stable loops, containing the polymerase binding sites, were
common to both.

THE INFLUENCE OF ALTERED SEQUENCES ON SECONDARY STRUCTURE.

     The influence of two known site mutations (12) on the
secondary structures presented here was considered. At posi-
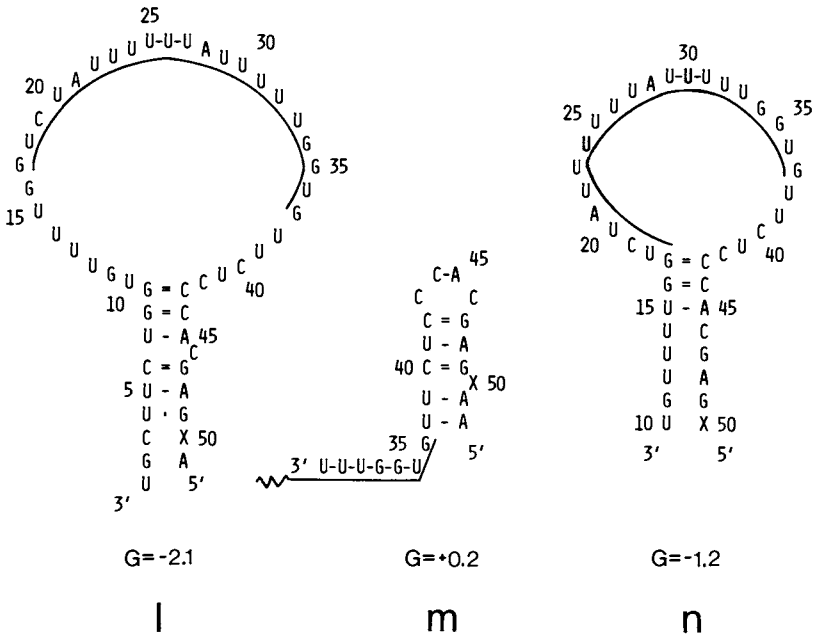tion 37, A is exchanged with U, and at position 48, A is

Figure 4. Possible secondary structures of the 3' end of the negative strand RNA of DI-T particles.

exchanged with C at the 3' end of the negative strand of VSV Indiana.   Placing these in Figure 2 showed that the first of the mutations was located in the loop of a and a' and, thus, had no influence on the stability of the secondary structure. The mutation at position   48, however, prevented closing of the hairpin loop of structure a and a' (11, 13).   Structure b remained principally unaltered (Δ G = -5.2).

INTERPRETATION OF DATA.

The most stable structures of the negative strand "closed up" the polymerase binding site in a stem. In contrast the structures of the positive   strand   presented   the   polymerase binding site in an "open" hairpin loop.   Therefore, these ana-lyses support the hypothesis that there   may   be   differential affinities in the binding of polymerase by the negative strand and its complement, resulting in the asymmetrical synthesis of plus and minus strands during VSV replication (5). Although one cannot be sure that stem structures actually prevent polymerase attachment or, alternatively, that the presentation of polymerase binding sites in loops facilitates polymerase

binding, such assumptions have been suggested for the interaction of the tobacco mosaic virus RNA with its coat protein (15). Also, Peattie et al. (16) identified a "bulged" double helix in an RNA-protein contact site of _Escherichia coli_ 5S RNA and found that this bulging appeared to be conserved throughout evolution. One of the stems of DI 0.45 and DI 611, _f_ and _i_, looks very similar both in length and location to this 5S RNA ribosomal binding site.

Previously, Schubert et al. (9), using the first 125 nucleotides at the 5' end of the negative strand of VSV RNA, presented a complex flower-like secondary structure. The complementary 3' end of the positive strand could be deduced to form similar symmetrical complex structures. Unlike the "open" polymerase binding sites proposed by our studies, the complex structure formed with 125 nucleotides places the polymerase binding site in a closed stem.

For stable secondary structures of naked RNA it has been estimated that the free energy, ΔG, should be less than -15 kilo calories per mole (10). However, it is conceivable that less stable structures may alternate in a fluctuating manner, whereby signals in a non-coding region may be turned on and off. With proteins, the RNA might remain stably in one form. Such a control mechanism based on alternating secondary structures is analogous to the control of transcription termination at the attenuator of the tryptophan operon on _Escherichia coli_ where mutually exclusive, stable base-paired structures have been identified (17, 18).

The major structural proteins that bind to VSV RNA are the RNA-binding, N protein, the large, L polymerase protein and the NS phosphoprotein. N protein is necessary for both template activity (19) and for continued genomic synthesis (20, 21). Recently, a temperature-sensitive N mutant showed that the lesion fails to affect the replication of DI RNA in the same way as standard genomic RNA (7). Therefore, both the binding to the RNA and interaction with polymerase must be considered when N protein functions are examined. A complication of such regulation of RNA synthesis is the attenuating roles of the M and the G proteins (14, 22, 23, 24).

To determine whether or not there is any validity for the structures presented here, in vitro RNA synthetic systems should be used where binding constants of the proteins and competition between synthetic oligonucleotides can be measured.

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THE EFFECT OF THE β–γ BOND OF ATP ON TRANSCRIPTION OF VESICULAR STOMATITIS VIRUS[1]

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In vitro transcription of vesicular stomatitis virus (VSV) requires much higher concentrations of ATP than of the other three nucleotides -- 500 µM compared to 22 µM for GTP and 33 µM for CTP and UTP (1). Although it is not known why so much ATP is needed, an examination of VSV transcription suggests a number of steps where the nucleotide could be involved (2,3). Transcription by the viral-encoded polymerase is sequential and polar in the gene order 3'-leader-N-NS-M-G-L-5'. Leader RNA is a 47-nucleotide-long transcript which initiates with A and contains a high proportion of A residues, but is not polyadenylated. The monocistronic mRNAs coded for by the N through L genes all initiate with an A residue which is capped and methylated, and they are polyadenylated at their 3' end. The polymerase enters at the 3' end of the genome (4), and transcription occurs by either a stop-start or processing mechanism (3). Since leader begins with the sequence (p)ppACG (5), it has been suggested that the high K_m for ATP reflects initiation with an A residue. This interpretation is based on in vitro studies where pre-incubation of virus with CTP and high concentrations of ATP allows subsequent RNA synthesis at low concentrations of ATP (1). Imido ATP, which has a non-hydrolyzable β–γ phosphate bond (6,7), inhibits in vitro transcription as assayed by incorporation of labeled nucleotides into acid-insoluble material. However, if the virus is pre-incubated with ATP and CTP, then some RNA synthesis occurs when imido ATP is substituted for ATP.

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From this it was concluded cleavage of the β-γ bond of ATP was required for initiation at the 3' end of the viral genome.

We have carried out _in vitro_ transcription experiments with imido analogs. Figure 1 compares the results of transcription products of solubilized standard and DI virions synthesized with ATP, imido ATP, or imido GTP. Transcription of standard virions with ATP generates leader RNA, the 5'-N gene oligonucleotides described previously (8), and high-molecular-weight RNA indicative of messenger synthesis. However, when imido ATP is substituted for ATP, only leader RNA and the N oligonucleotides appear to be synthesized. A longer exposure of the same gel shows the presence of larger transcripts, which could be due to leader-N readthrough (9,10) or N mRNA synthesis from polymerase already at the promoter. Transcription with imido GTP results in a normal pattern of RNA products though in reduced amounts. This indicates that although the imido analogs are not recognized efficiently by the transcriptase, inhibition of mRNA synthesis is specific for the ATP analog and is not due solely to the presence of the imido group. DI virions synthesize mainly the 46-nucleotide-long DI product when either ATP, imido ATP, or imido GTP is included, as would be expected if imido ATP preferentially affects large RNA synthesis. We also see inhibition of mRNA transcription if ATP is used in very low concentrations, i.e., 25 to 50 μM (Figure 2).

Total incorporation of [α^{32}P]-UTP into acid-precipitable RNA using imido ATP is 5% of that with ATP. This correlates with the inhibition of mRNA synthesis seen in the gel. The analog has less of an effect on DI product synthesis, about 30% of the incorporation of ATP, perhaps because only a small product is being made. Imido GTP is incorporated about five times more efficiently than imido ATP by the B virus (30% of ATP). Since DI virions use imido GTP two to three times better than B virions (60-75% of ATP), this may be a reflection on the need to cap mRNAs in the standard virus transcription reaction.

Next we directly demonstrated that imido ATP could initiate transcripts. An _in vitro_ transcription reaction lacking UTP and GTP, but containing ATP and CTP, will generate large amounts of (p)ppAC representing the 5' end of leader RNA or DI particle product (4). Calf intestinal alkaline phosphatase will remove the 5' terminal phosphates from the ATP-initiated dimer but the imido group will
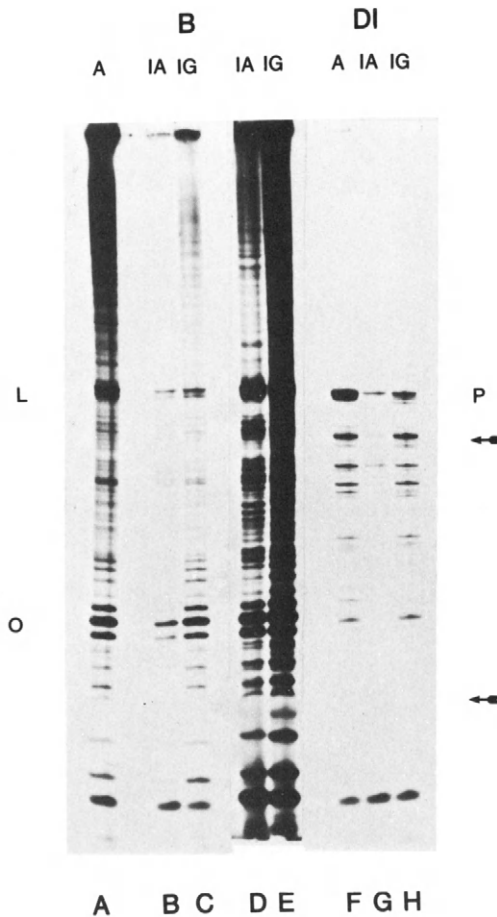
Figure 1. Autoradiograph of a 20% polyacrylamide gel with 7 M urea showing the [α^{32}P]-UTP-labeled transcripts synthesized in a standard in vitro reaction (9) by wt (lanes A–E) and DI (lanes F–H) virions. RNA synthesized for 3 h at 31°C was phenol-extracted, ethanol-precipitated, and lyophilized. Samples were resuspended in 7 M urea with xylene cyanol and bromophenol blue as dyes before electrophoresis. Lanes D and E are the same as lanes B and C, respectively, but the autoradiograph was exposed for 7 times as long. A and F (1 mM ATP); B, D, and G (1 mM imido ATP); C, E, and H (1 mM imido GTP). The location of leader (L), DI product (P), the 11- to 14-nucleotide-long 5' N-gene oligonucleotides (O), and the dyes (arrows) are noted.
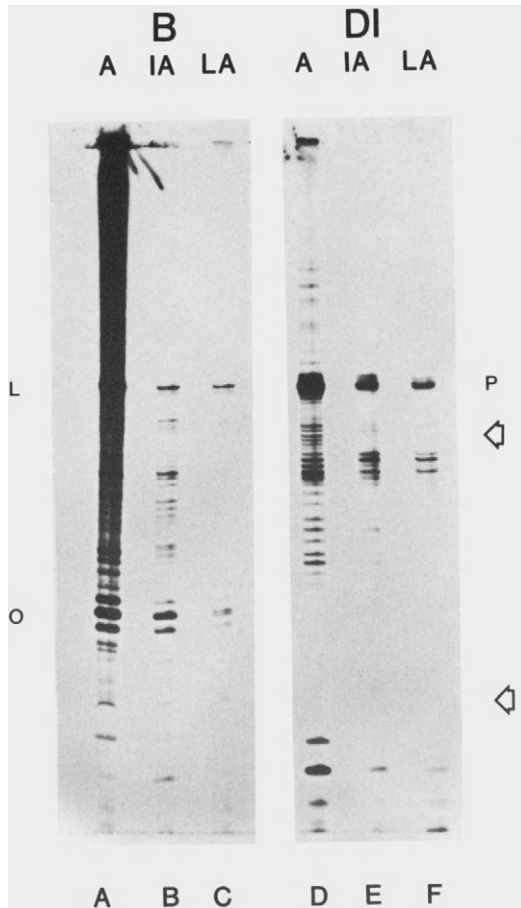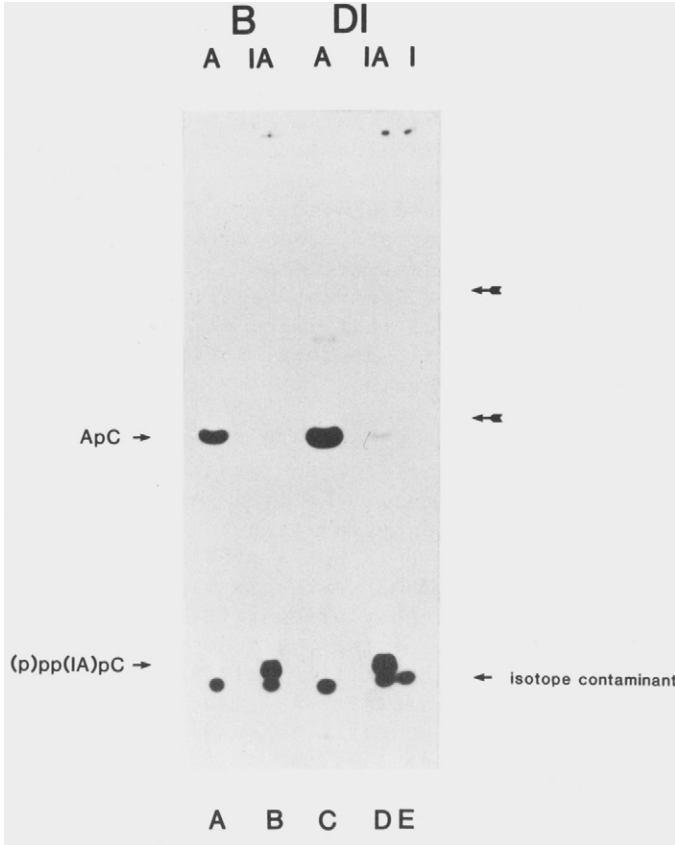
Figure 2. Autoradiograph of a 20% polyacrylamide gel with 7 M urea, showing the [α³²P]-UTP-labeled transcripts synthesized in a standard _in vitro_ reaction by wt (lanes A-C) and DI (lanes D-F) virions. RNA synthesized for 3 h at 31°C was phenol-extracted, ethanol-precipitated, and lyophilized. Samples were resuspended in 7 M urea with xylene cyanol and bromophenol blue before electrophoresis. A and D (1 mM ATP); B and E (1 mM imido ATP); C and F (33 μM ATP). Leader (L), DI product (P), 5' N-gene oligonucleotides (O), and dyes (arrows) are noted.

protect an imido-initiated transcript from phosphatase
digestion, so the two AC dimers can be distinguished by gel
electrophoresis. Figure 3 indicates that imido ATP does
initiate transcription of leader and the DI product. There
is some ATP contamination in imido ATP, but it is less than
0.1% (6). Transcripts containing all four nucleotides were
also initiated with imido ATP (data not shown).

The results presented demonstrate that both initiation
and elongation of transcripts occur with imido ATP, but
under our conditions a restricted set of transcripts is
synthesized. This is in apparent conflict with earlier
results (1), which stated that imido ATP could elongate but
not initiate transcripts. However, different methods were
used to look at the products.

In view of the high K_m for ATP, there are several
explanations for our results with the ATP and GTP analogs.
First, the polymerase protein NS is a phosphoprotein, and
its function is affected by the extent of phosphorylation
(11,12). Since imido ATP is not a substrate for protein
kinases, inhibition of mRNA synthesis may reflect a
requirement for NS phosphorylation prior to mRNA synthesis.
Alternatively, cleavage of the β–γ bond of ATP causing a
conformational change in the transcriptase may be required
for synthesis of the messengers. Transcription of the 5'-N
gene oligonucleotides indicates imido ATP can be used for
internal initiations once the transcriptase is bound to the
beginning of a gene. There are four nucleotides separating
the leader and N genes (13); if these nucleotides are not
normally transcribed, ATP hydrolysis may be needed to
translocate the transcriptase from the end of the leader
gene to the start of the N gene. A third possibility is
that because there are so many A residues as well as long
stretches of the nucleotide in leader and the mRNAs, the
polymerase has an increased chance of falling off the
template if imido ATP is substituted for ATP. Therefore it
is less likely transcription of the N mRNA would be
completed and the transcripts would not appear in large
enough numbers to be seen on gels. Polyadenylation would
probably also be inhibited, and if that is necessary before
the next gene, NS, is transcribed, incorporation would be
drastically reduced.

The results with imido GTP show that the DI particle
uses the analog more efficiently than the B particle. This
may be due to a requirement for cleavage of GTP in order to
cap the mRNAs, or to the fact that there are more G residues

Figure 3. Autoradiograph of a 20% polyacrylamide gel showing AC dimers synthesized by B (lanes A and B) and DI (lanes C and D) virions in the absence of UTP and GTP (5). The $[\alpha^{32}P]$-CTP-labeled products synthesized at 31°C for 3 h were phenol-extracted, ethanol-precipitated, and treated with calf intestinal alkaline phosphatase (0.52 U in 0.1 M Tris [pH 8.0]) for 60 min at 37°C. 7 M urea was added before electrophoresis on gels containing no urea. Bromophenol blue and xylene cyanol (arrows) were run in lanes with no samples. A and C (1 mM ATP); B and D (1 mM imido ATP); E (isotope control, no virus).

in the standard virus transcripts. We do not know. Because low ATP gives the same results as with imido ATP, it suggests high ATP concentrations are needed to transcribe the larger RNAs. But again, whether the need for ATP in large amounts is due to a specific step in the transcription process which requires cleavage of the β–γ bond, or to the presence of so many A residues in the transcripts, is not clear. It is possible that a combination of steps -- initiation, elongation, translocation, phosphorylation -- is the reason for the high K_m for ATP. At present we cannot distinguish among these possibilities.

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INTERACTIONS BETWEEN CELLULAR LA PROTEIN AND LEADER RNAs

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INTRODUCTION

Antisera from patients with systemic lupus erythematosus have been useful in the isolation and identification of cellular ribonucleoproteins.[1] In general, these autoantibodies react with determinants on cell proteins or ribonucleoproteins that are associated with small nuclear or cytoplasmic RNAs. One lupus antigen that is bound to cell RNAs of known function is the La specificity. La is a 45Kd molecular weight phosphoprotein[2] that is bound to precursor transcripts of RNA polymerase III such as transfer RNA, 4.5S, 5S, 7-2, 7S, and others.[3,4] In addition, antisera from lupus patients with the La specificity also react with small viral RNPs from adenovirus (VA) and Epstein-Barr virus (EBERs) infected cells.[5] These viral RNAs are also products of cell RNA polymerase III and tend to be more stably bound to La protein.[6] One characteristic of all of these short RNA polymerase III precursor RNAs is that they tend to have additional 3' terminal uridylate residues that may influence binding of La protein.

We have recently reported that the plus strand[7] and minus strand[8] leader RNAs of vesicular stomatitis virus also react with La antisera during the infectious cycle. The extent of La protein binding to leader RNAs was at least 50% in these studies whereas less than 1% of the RNA polymerase III precursor transcripts are normally detected in association with La protein.[9] Thus, leader RNAs of VSV differ from other La bound small RNAs in that they are not products of RNA polymerase III and that large amounts of the leader RNAs present in infected cells are bound to La protein.

Steitz and coworkers have proposed that La protein is a transcription factor for RNA polymerase III, binds to precursor RNAs and is released from the mature form of the RNA.[10] It seems unlikely that VSV uses La protein as an indispensable transcription factor per se because leader RNA synthesis is highly efficient in vitro. La protein has not been detected in VSV particles and leader RNA synthesized in vitro can not be precipitated by La antisera (unpublished observations). Although the association of leader RNAs with La protein in VSV infected cells may be fortuitous, specificity of the association is evident in that some

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shorter species of the VSV (Indiana) minus strand leader RNA
are not associated with La protein.[8] Thus, as few as 8
additional bases at the 3' end of minus strand leader RNA
result in a dramatic increase in the binding of La protein.
In this paper, we describe recent evidence that generalizes
the association of La protein with leader RNAs of VSV (New
Jersey) and rabies virus, and we propose a role for La
protein in the replicative cycle of rhabdoviruses.

RESULTS AND DISCUSSION

        Detailed studies of the association of the cellular La
protein and the VSV N protein with plus strand leader RNA of
VSV (Indiana) have indicated a transition from La to N
protein binding as the infection progresses.[7] The La protein
bound leader RNA accumulated linearly for 4 hours, plateaued
until about 6 hours, and then declined. The N protein bound
leader RNA, on the other hand, accumulated gradually at early
times in the infection but increased dramatically by 4-6
hours in an inverse relationship to La bound leader RNA. By
16 hours post infection, all of the leader RNA detected was
bound to N protein and none was detected in association with
La protein. This finding suggested a role for La protein in
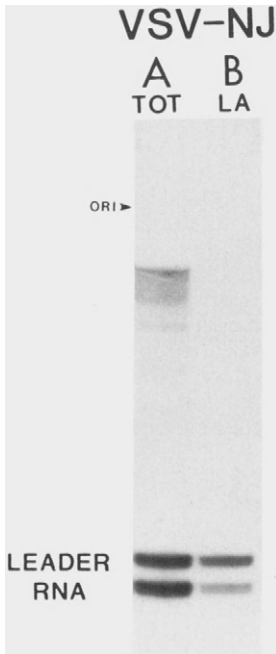VSV replication. [7]

Figure 1. Immunoprecipitation
of the leader RNA of the New
Jersey serotype of VSV with
anti-La specific serum. Lane
A, probing of the total (+)
leader RNA from extracts of
infected BHK cells; lane B,
probing of (+) leader RNA in
infected BHK cell extracts
after immunoprecipitation with
lupus antisera of the La
specificity.

We have investigated whether the New Jersey serotype of VSV shows similar interactions between the La protein and (+) leader RNA. Figure 1 shows that the plus strand leader RNA of VSV-NJ can be immunoprecipitated from infected BHK cells with La antisera.

The sequence of the VSV-NJ (+) leader RNA is similar to the Indiana serotype at the 5' end but differs significantly in the 3' half of the molecule (figure 2). We have also recently demonstrated the association of La protein with rabies virus (+) leader RNA (Kurilla, Holloway, Cabradilla, and Keene, unpublished). In these studies, a significant portion of the (+) leader RNA of rabies virus was immunoprecipitated with anti-La sera midway through the infectious cycle. Figure 2 shows a comparison of sequences in the 3' halves of the (+) leader RNAs of VSV-IND, VSV-NJ, and rabies virus. Although a consensus sequence is not readily evident, certain features are characteristic in these regions. In the 3' terminal 20 nucleotides there is a purine-rich region followed by a few terminal uridylate residues. Terminal uridylate residues have been implicated previously in the binding of La protein.[6] Probing of leader RNAs, although indirect, has shown that RNAs terminating in 3' adenylates are also bound to La protein in infected cells.[7] The presence of U residues appears to enhance binding, however.

$$VSV_{INDIANA} \quad \ldots CAUU\overset{30}{\overset{\bullet}{A}}AAAGGCUCA\overset{40}{\overset{\bullet}{G}}GAGAAACU(U\overset{50}{\overset{\bullet}{U})}\text{-OH}$$

$$VSV_{NEW\ JERSEY} \ldots AAUU\overset{30}{\overset{\bullet}{A}}UUUGGCCUA\overset{40}{\overset{\bullet}{G}}AGGGAGCU(U\overset{50}{\overset{\bullet}{U})}\text{-OH}$$

$$RABIES \quad \ldots AGCGUC\overset{40}{\overset{\bullet}{A}}AUUGCAAAG\overset{50}{\overset{\bullet}{C}}AAAAAUGU\text{-OH}$$
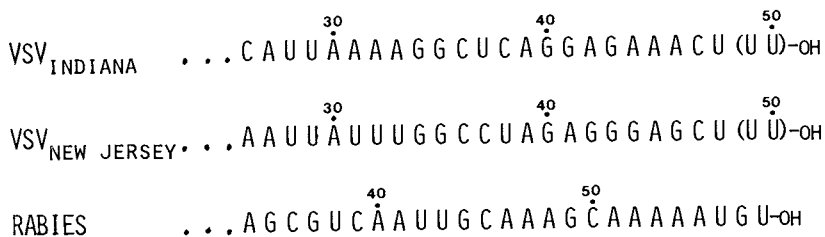
Figure 2. Comparison of the 3' ends of the (+) leader RNAs of VSV-IND, VSV-NJ, and rabies virus. Predicted sites of interaction of La protein reside in the 3' half of the leader RNAs.

Figure 3 shows nucleotide sequences at the 3' end of the minus strand leader RNAs of VSV-IND and VSV-NJ. The regions encompassing the polyadenylation site of the L mRNA and the 5' end of the genomic RNAs of these viruses are structurally different.[11] Furthermore, the nature of the interaction with La protein differs between the two VSV serotypes. The Indiana serotype synthesizes a minus strand leader RNA of about 46 nucleotides in length that is the major species in

infected cells.[8] The La bound form of the VSV-IND minus
strand leader RNA is eight nucleotides longer and is a less
abundant species in infected cells (Figure 3). The minus
strand leader RNA of VSV-NJ is about 46 nucleotides long and
this major species is bound to La protein (Wilusz and Keene,
unpublished). Thus, the Indiana serotype utilizes the entire
noncoding region of the template (up to the polyadenylylation
site of the L gene) for the production of minus strand leader
RNAs, while the New Jersey serotype appears to contain an
additional 57 nucleotides of noncoding function that is not
involved in minus strand leader RNA synthesis or La protein
binding. These findings demonstrate the specificity of La
protein binding as shown in figure 3 and argue for a general
role of La protein in the VSV replication cycle.

INDIANA   5' pppA C G A A G A C C A C A A A A C C A G A U A A A A A A A U A A A A A A C C A C A A G A G G G U C U U A A G G A U-OH

INDIANA   5' pppA C G A A G A C C A C A A A A C C A G A U A A A A A A A U A A A A A A C C A C A A G A G G G U C-OH

NEW JERSEY   5' pppA C G A A G A C A A A A A A A C C A U U C C U A A U A C A A A A G G C C A A A A G A A G G U-OH
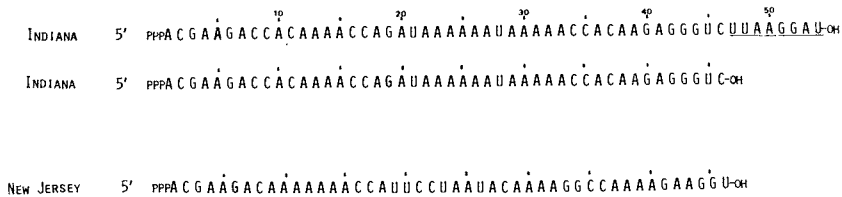
Figure 3. Minus strand leader RNAs of VSV-IND and VSV-
NJ showing the 3' region involved in binding La protein. The
La bound minus strand leader RNA of VSV-IND is 8 nucleotides
longer (underlined sequence) than the major form of minus
strand leader RNA. The minus strand leader RNA of VSV-NJ is
46 nucleotides long and fully bound to La protein.

The scheme shown in figure 4 depicts the binding of the
cellular La protein near the 3' end of the (+) leader RNA
early in the infectious cycle. As the infection proceeds,
viral nucleocapsid protein accumulates and binds at the 5'
end of the (+) leader RNA.[12] A crossover point eventually
occurs at 4 to 6 hours post infection where the accumulation
of La bound (+) leader RNA ceases but the binding of N
protein continues. We propose that La protein binds leader
RNA in the infected cell and participates in the release of
the transcript from the template. In this sense La protein
may function in the maintenance of the transcription mode _in_
vivo. When sufficient N protein is synthesized to initiate
the process of encapsidation, the N protein molecules

nucleate at the 5' end of the leader RNAs and displace or prevent binding of molecules of La protein. Thus, nucleocapsid assembly and the synthesis of full length replicative strands may be controlled and perhaps fine tuned by interplay between molecules of the cellular La and the viral N protein. This model also allows the N protein to maintain a high affinity for the VSV RNA and still raise the threshold level of N protein necessary to induce replication. Thus the feedback of N protein to inhibit its own synthesis (by inhibiting transcription of its mRNA) is delayed without the need to reduce the affinity of N protein for VSV RNA. This would allow a large pool of N protein to be available <u>in</u> <u>vivo</u>.

ABSENCE OF N PROTEIN (IN VITRO)   LOW LEVEL OF N PROTEIN   HIGH LEVEL OF N PROTEIN

▲ LA PROTEIN
O   N PROTEIN
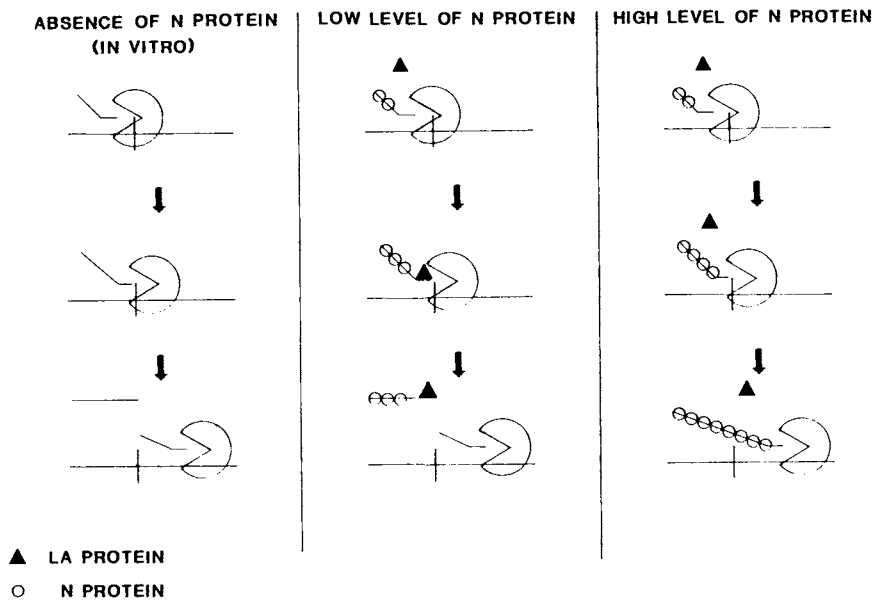
     Figure 4.   The proposed role of La protein in the replication of negative strand RNA viruses. The genomic template is indicated by the horizontal line with the leader gene/N gene junction marked with a vertical line. The large circle represents the RNA polymerase. In the absence of N protein, the polymerase will terminate at the end of the leader gene and reinitiate the N gene mRNA. In the presence of low levels of N protein, the La protein binds to the 3' end of the leader RNA transcript and prevents N protein from suppressing termination. The transcript is then released with La protein bound. In the presence of high levels of N protein, La protein is either displaced or prevented from binding to the transcript and termination is suppressed resulting in replication.

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EFFECTS OF CELL EXTRACTS ON TRANSCRIPTION
BY VIRION AND INTRACELLULAR NUCLEOCAPSIDS
OF VESICULAR STOMATITIS VIRUS

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INTRODUCTION

     Several small RNA species are synthesized in vitro by the
endogenous transcriptase of vesicular stomatitis virus. These
small RNAs can be divided into two groups. The first group
consists of RNAs that are triphosphated at their 5' termini
and show uv inactivation kinetics and times of appearance
similar to the leader RNA. These RNAs provided the basis for
the multiple-entry model of VSV transcription proposed by
Testa et al.[1] The second group consists of small capped RNA
species that have uv inactivation kinetics and time courses of
appearance similar to messenger RNA. These characteristics of
the latter species are consistent with a single-entry model
for VSV transcription.[2] Controversy remains concerning the
role of these small RNAs in VSV transcription. For various
reasons it has been difficult to detect them in vivo. As one
approach to determine whether they are synthesized in vivo, we
have prepared infected cell extracts to use in in vitro
transcription reactions. Under these conditions, we found that
the small capped and triphosphated RNAs were not synthesized.
The leader RNA was the only small RNA species detected.
Furthermore, when uninfected cell extracts were added to in
vitro reactions under conditions which normally produce large
quantities of the small RNAs, only the leader RNA was
synthesized. These results suggest that the small RNAs
represent aberrant products of in vitro transcription
reactions rather than actual precursors to mature messenger
RNAs as suggested previously.[1]

RESULTS

     Using purified viral or intracellular transcriptive
complexes (RNP cores) of VSV, we have reported previously the
identity of several small RNA species synthesized in vitro by
the genomic RNA.[2] Figure 1A shows the results of a time course
obtained from synchronized in vitro transcription reactions
utilizing purified virion RNP cores. Species 1 was identified
as the leader RNA, species 2 and 7 correspond to the 5'
terminal sequences of the NS mRNA, species 3,4,5, and 8
correspond to the 5' terminal sequences of the N mRNA and
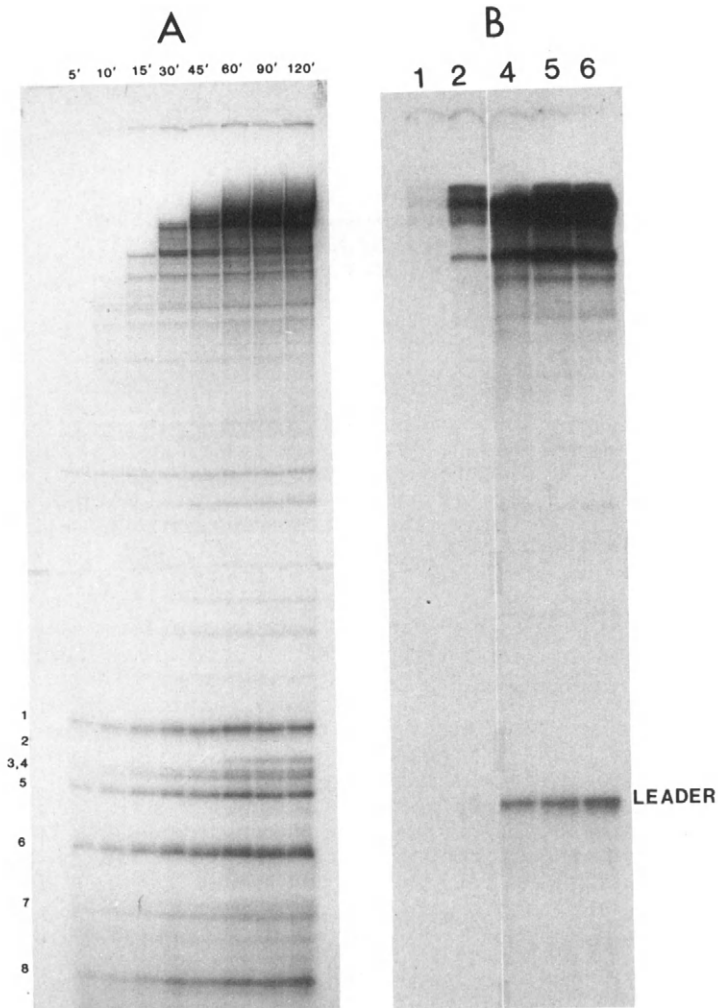species 6 originates from the leader gene.

FIGURE 1 (A) Time course of appearance of small RNA species in a synchronized in vitro transcription reaction utilizing purified virion RNP cores. Transcription reactions were synchronized by the addition of MgCl₂ or purified RNP cores. At times indicated (min. of incubation) samples were removed and anaylzed on a 5% acrylamide gel. (B) Time course of appearance of RNA species in vitro utilizing infected cell extracts. BHK spinner cells were infected with VSV at an m.o.i. of 10. At times indicated (hours post infection), cells were lysed and extracts prepared. Transcription reactions utilizing infected cell extracts were incubated for one hour at 30°C. Samples were analyzed on 5% acrylamide gels.

In order to study the synthesis of these small RNAs in
vivo, we examined transcripts synthesized in in vitro
reactions using infected cell extracts prepared at different
times after infection of BHK spinner cells with VSV. As
shown in figure 1B, the pattern of small RNAs synthesized by
infected cytoplasmic extracts is very different from that
observed when purified RNP cores were used (figure 1A). The
leader RNA is the only small RNA observed in infected cell
extracts. Messenger RNA migrating in the upper portion of
the gels is similar under both sets of transcription
conditions, however, and suggests that degradation was
minimal under these conditions.
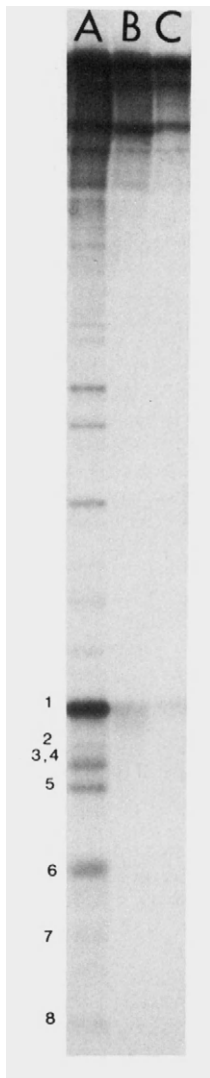
FIGURE 2. RNA transcripts
synthesized in vitro using:
purified intracellular RNP
cores in the absence of cell
extracts (A), infected cell
extracts isolated 4 hours
post infection (B), or
purified virion RNP cores in
the presence of cytoplasmic
cell extracts (C).

     In order to determine if the difference in the pattern of
small RNA synthesis was due to a structural difference between
virion and intracellular transcriptive complexes, we
substituted purified intracellular RNP cores for virion RNP
cores in in vitro transcription reactions. As shown in figure
2A, the pattern of small RNA synthesis was identical to that
seen with purified virion RNP cores (figure 1). This suggests
that the differences in small RNA synthesis are not due to
structural differences between the two templates.
     When whole cell or cytoplasmic cell extracts were added
to in vitro reactions utilizing purified virion or
intracellular RNP cores, however, the pattern of small RNA
synthesis changed (figure 2B). Although synthesized in reduced
amounts, the   leader RNA was the only small RNA detected
under these conditions. Nuclear extracts and other proteins
such as bovine serum albumin did not have this effect (data
not shown). Thus, there are factors present in the cytoplasm
of cells that influence the initiation or termination of VSV
transcripts. Whether these factors interact with the template,
the RNA products or the polymerase is unknown.
     In order to futher characterize the factors responsible
for this activity, heat inactivation studies were performed.
Cytoplasmic extracts were untreated (figure 3A), heated at 65°
C for 1 min.(figure 3C) or heated at 90°C for 1 min.(fig. 3D)
and added to in vitro transcription reactions utilizing
purified virion RNP cores. In the presence of untreated
cytoplasmic extracts the leader RNA was the only small RNA
species synthesized (figure 3B). When the extract was heated
prior to transcription, however, the ability to suppress the
synthesis of small RNAs was reduced as shown by the appearance
of species 3 to 8.
     The sensitivity of the cell factors to heat inactivation
suggests that they may be proteinaceous in nature. In order
to determine whether the   factors responsible for the
suppression of small RNA synthesis could stably associate with
transcription complexes, purified virion RNP cores were
preincubated in the presence of cytoplasmic cell extracts and
then repurified by centrifugation over discontinuous glycerol
gradients as described previously. The pattern of small RNAs
synthesized by the repurified virion cores (fig. 3E) resembled
that seen normally with purified cores in the absence of cell
extracts (fig. 3A). When intracellular cores were similarly
isolated from infected cell extracts and incubated in standard
in vitro transcription reactions in the absence of added
extract, small RNA species 1 through 8 were   synthesized
(fig. 3G). This is in contrast to the pattern seen when total
infected cell extracts were analyzed (fig. 3F). Thus, the
factors responsible for the suppression do not irreversibly
modify the template nor do they remain stably bound during the
nondenaturing isolation procedures used to prepare RNP cores.
The activity is precipitable in the presence of 80% ammonium
sulfate, however, and is stable to dialysis at 4°C overnight
(data not shown). To rule out nonspecific protein effects as
being responsible for the suppression activity, bovine serum
albumin and BHK cell nuclear extracts were added to purified
viral or RNP cores, at concentrations identical to those used

with cell extracts.   These proteins were not able to substitute for cytoplasmic extracts and the small RNAs synthesized were identical to those synthesized in the absence of cell extracts.   Thus, the effects appear to be specific for factors in the cytoplasm of host cells.
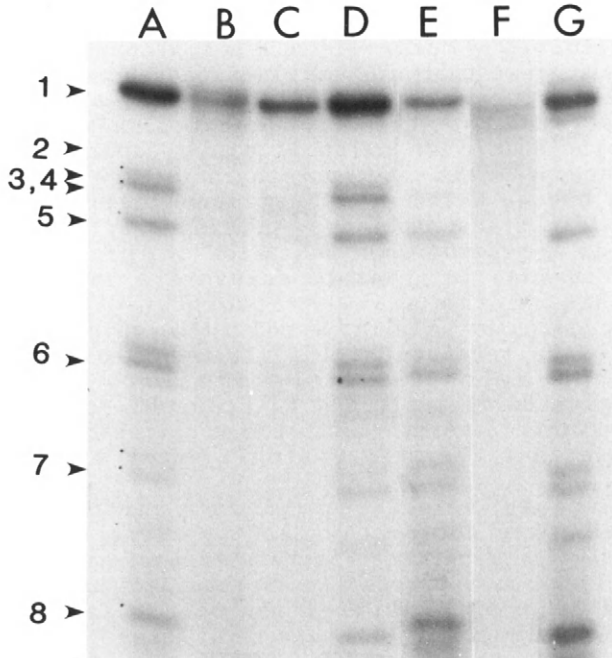

FIGURE 3. Small RNA transcripts synthesized in vitro using (A) purified virion RNP cores in the absence of cell extracts; (B) purified virion RNP cores in the presence of cell extracts; (C) purified RNP cores in the presence of cell extract heated at 60 C for 1 min; (D)purified RNP cores in the presence of cell extracts heated at 90°C for 1 min; (E) repurified virion RNP cores that were previously incubated with cell extracts at 37°C for 10 min. and reisolated by centrifugation and transcribed in the absence of cell extracts; (F) infected cell extracts prepared at 4 hr post infection; (G) intracellular RNP cores purified from extracts prepared 4 hr post infection and transcribed in the absence of cell extract.

DISCUSSION

Although it is known that VSV transcription proceeds in a linear and sequential fashion,[3,4,5] the exact mechanism of synthesis remains unclear.   One unresolved issue is whether the viral polymerase must enter and initiate transcription at precisely the 3' terminus of the template (single-entry model)[6] or whether it can also enter internally at each of the intergenic sites (multiple-entry model).[1]

The multiple-entry model was based on the finding of small triphosphated RNAs which are initiatied internally at the N and NS intergenic sites during in vitro transcription reactions. This model proposes that the short triphosphated RNAs are synthesized simultaneously at the onset of transcription and are then sequentially extended into mRNA. Whether these small triphosphated RNAs represent precursors to mature mRNA or are abortive products of the in vitro system remains unresolved. Much evidence has accumulated recently, however, to suggest that the small triphosphated RNAs may not function as precursors to mRNA. Lazzarini and coworkers,[7] have demonstrated that the triphosphated RNAs do not remain template associated, are metabolically stable and cannot be chased into capped mRNA. We and others[2,8] have shown that the 5' portions of the N and NS mRNAs accumulated sequentially during in vitro transcription suggesting that the polymerase is not solely dependent upon the prior synthesis of the short triphosphated RNAs in order to transcribe each mRNA start. The synthesis of the small triphosphated RNAs probably reflects recycling by polymerase molecules which are in excess and bound to the template at the intergenic sites during the assembly and release of the virion. Furthermore, transcription studies performed with the DI particle, LT-2,[9] and reconstitution studies[10] with the nondefective template of VSV, suggest that there is a single entry site on the VSV genome.

    In this paper, we present evidence which suggests that the small RNAs are not synthesized in extracts of infected cells. In addition, there are factors present in the cytoplasm of host cells which suppress the synthesis of these small RNA species. Whether this suppression occurs at the level of initiation or termination is unknown. Taken together, these studies provide strong evidence against the suggestion that the short triphosphated RNAs serve as mRNA precursors and are supportive of a single-entry model for VSV transcription.

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SYNTHESIS OF THE VARIOUS RNA SPECIES IN CELLS INFECTED WITH THE TEMPERATURE-SENSITIVE MUTANTS OF VSV NEW JERSEY

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TEMPERATURE-SENSITIVE MUTATIONS AFFECTING RNA SYNTHESIS OF VSV NEW JERSEY

Temperature-sensitive mutants of vesicular stomatitis virus New Jersey serotype (VSV New Jersey) were classified into six non-overlapping complementation groups (1). On the basis of the total amounts of RNA synthesised in infected BHK cells at the permissive (31°C) and restrictive (39°C) temperatures, mutants of complementation groups A, B and F were classified as "RNA negative", mutants of groups C and D as "RNA positive", whereas in the case of group E mutants two (tsE1 and tsE3) were RNA negative while the third (tsE2) was RNA positive (1).

Viral RNA synthesis in infected cells is presumed to take place in three separate stages: transcription of the mRNA species followed by synthesis of the replicative intermediate, and finally synthesis of the virion RNA (2). Activity of the virion-associated RNA transcriptase was assayed in vitro at 31°C and 39°C in order to determine at which stage RNA synthesis was inhibited in the RNA negative mutants (3). Three mutants, tsB1, tsE1 and tsF1, were found to possess a temperature-sensitive transcriptase. Since they belong to three different complementation groups, this suggests the involvement of at least three polypeptides in the transcription process.

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[1] R.G. Paterson was a recipient of a Medical Research Council grant for training in research methods.

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Furthermore, since the transcriptase activity of
other mutants of complementation groups E and F
(tsE2, tsE3 and tsF2) was not inhibited the mutated
polypeptides of these groups are presumably
multifunctional, taking part both in transcription
and replication and in the case of the "RNA
positive" mutant tsE2 possibly also in virus
maturation. Since transcription of the group A
mutants was not inhibited in the in vitro assay they
were presumed to be defective in some stage of
replication.

    High resolution DATD cross-linked
polyacrylamide gel electrophoresis and Cleveland's
partial digestion analysis established that ts
mutation affected polypeptide N of the group A
mutants (4) and polypeptide NS in the group E
mutants (5). Dissociation-reconstitution
experiments confirmed that NS is the mutated
polypeptide in group E and showed that L is the
mutated polypeptide in group B (6, 7). As yet it
has not proved possible to determine which
polypeptide of the group F mutants is affected by
the mutation.

**DETAILED ANALYSIS OF THE RNA SYNTHESISED IN INFECTED
CELLS AT THE PERMISSIVE AND RESTRICTIVE TEMPERATURES**

    To study the RNA synthesis by wild type VSV New
Jersey and its temperature-sensitive mutants BHK
cells were infected with purified virions (50 pfu
per cell) and were incubated in the presence of
10 ug/ml Actinomycin D at the permissive and
restrictive temperatures. After 6 hours of
incubation RNA species were extracted by hot-phenol,
separated by agarose-urea gel electrophoresis and
the [^{3}H]-uridine labelled RNAs were visualised by
fluorography.

    RNA Synthesis by Wild Type VSV New Jersey.   In
BHK cells infected with wild type VSV New Jersey
(Figures 1 and 3) the messenger RNAs for the five
viral polypeptides (L, G, N, NS, M) are the
predominant labelled RNA species. Besides these
there are also present four minor RNA species (a, b,
c and d). Three of these, a, c and d, are also
synthesised during in vitro RNA synthesis using
purified virus or transcribing nucleoprotein (TNP)
complexes and for this reason we believe that they
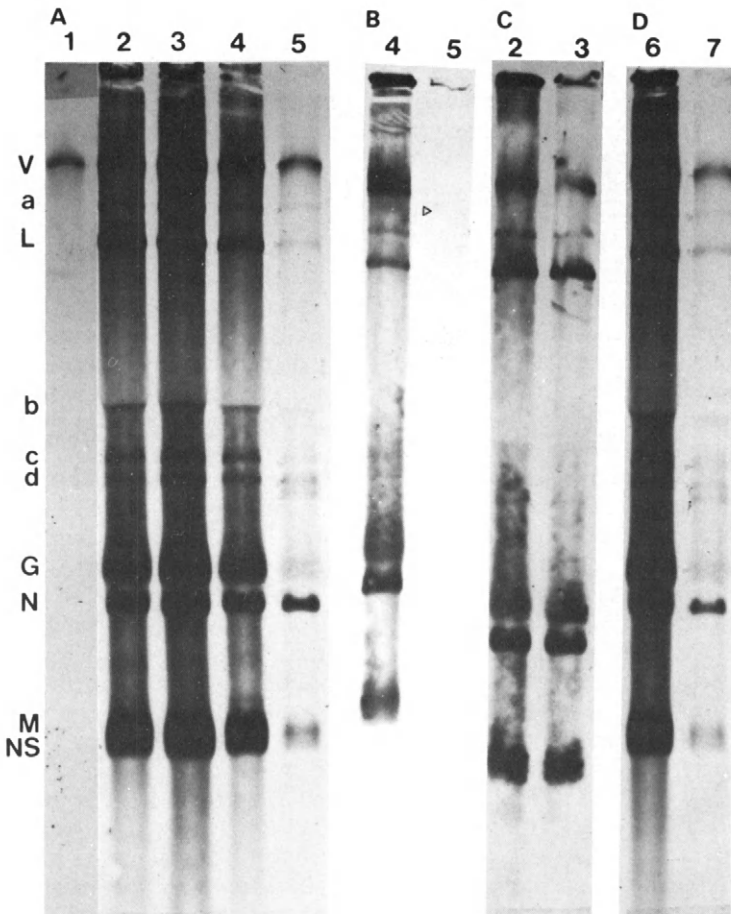
FIGURE 1. RNA species synthesised either in BHK cells infected with wild type VSV New Jersey (Panel A, tracks 2 and 3) and with the temperature-sensitive mutants tsB1 (Panel A, tracks 4 and 5), tsC1 (Panel C, tracks 2 and 3) and tsD1 (Panel D, tracks 6 and 7), or in CE cells infected with the mutant tsB1 (Panel B, tracks 4 and 5). The first track of each pair contains the RNA species synthesised at 31°C and the second track contains those synthesised at 39°C. Purified virion RNA (Panel A, track 1) was used as marker. The positions of the virion RNA (V), messenger RNAs L, G, N, NS and M (for the corresponding polypeptides) and the minor RNA species a, b, c, and d are indicated.

are also products of the transcription.
     It was not possible to analyse in detail the
various RNA species in the region of the virion RNA,
since the technique used did not resolve the high
molecular weight RNAs into discrete bands.

     RNA Synthesis by the Groups B, C and D Mutants.
Temperature-sensitive mutants of complementation
groups B, C and D (tsBl, tsCl and tsDl) synthesised
large amounts of RNA at the restrictive temperature
in BHK cells (Fig. 1, Panels A, C and D). In the
case of tsCl similar amounts of RNA were synthesised
both at 39°C and 31°C. Thus both transcription and
replication occurred at the restrictive temperature.
     Cells infected with tsBl or tsDl synthesised
the five mRNA species, the minor components and also
high molecular weight RNAs with electrophoretic
mobilities similar to the virion RNA. Although the
amounts of RNAs synthesised at 39°C were less than
at 31°C the results suggest that both mutants were
capable of transcribing and replicating their RNA at
the restrictive temperature.
     The results obtained with tsDl are in good
agreement with earlier observations but those
obtained with tsBl were unexpected since in vitro
transcription by this mutant is inhibited at 39°C.
To study this anomaly we infected chick embryo (CE)
cells with this mutant and found that RNA synthesis
was completely inhibited at 39°C (Fig. 1, Panel B).
     Thus these results confirm that mutants of
complementation groups C and D are "late" mutants.
In the case of the group B mutant they also indicate
the involvement of a host factor, present in BHK but
not in CE cells, which in some way helps polypeptide
L to overcome its heat sensitivity and to
participate in transcription and perhaps also in
replication. In this respect this mutant appears to
have some similarity to the temperature-sensitive
host range mutant tdCE3.

     RNA Synthesis by the Group A Mutants. Mutant
tsAl and tsA4 synthesised very little RNA at 39°C
(Fig. 2), indicating that transcription by the
virion-associated RNA transcriptase was restricted
in the infected cells. These results appear to
contradict the in vitro transcriptase assays where
the amounts of RNA synthesised by these mutants at
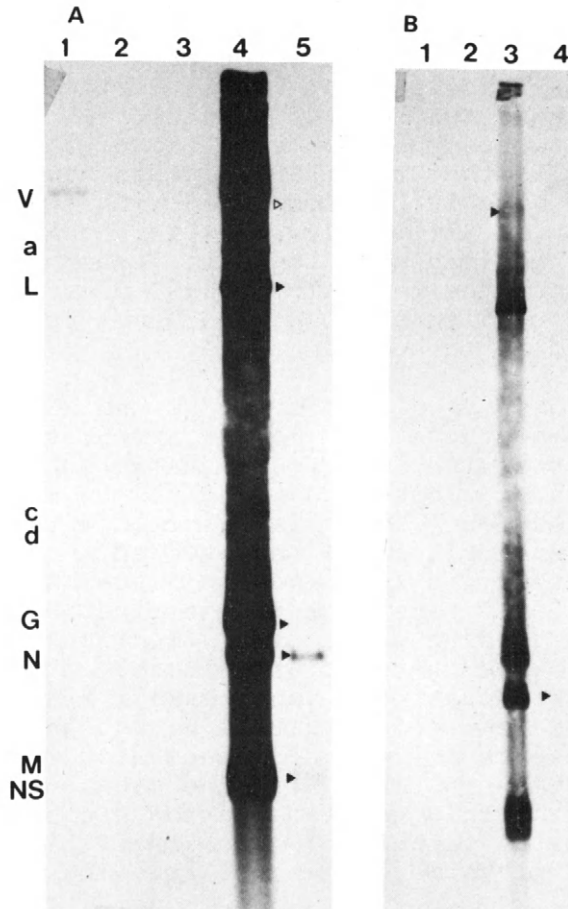39°C were similar to those synthesised by the wild

FIGURE 2. RNA species synthesised in BHK cells infected with the temperature-sensitive mutants tsA1 (Panel A, tracks 4 and 5) and tsA4 (Panel B, tracks 3 and 4). The first tracks in both pairs contain the RNAs synthesised at 31°C, and the second contain the RNA synthesised at 39°C. Mock-infected BHK cells incubated at the two temperatures (Panel A, tracks 2 and 3; Panel B, tracks 1 and 2) were used for comparison. Purified virion RNA (Panel A, track 1) was used as marker. The positions of the virion RNA (V), messenger RNAs L, G, N, NS and M (for the corresponding polypeptides) and the minor RNA species a, c, and d are indicated.

type virus (3). Furthermore, the RNA species
synthesised by these viruses in vitro at 39ºC were
similar to those synthesised by wild type virus,
suggesting that the ts mutation affected replication
rather than transcription. It is difficult to
reconcile these two sets of results. Since the ts
mutation affected the N polypeptide of these mutants
it is possible that the transcriptase synthesises
RNA at 39ºC once it has complexed with the virion
RNA-polypeptide N template. However, on reaching
the end of the template, it is no longer able to
recombine with the template at 39ºC presumably
because the configuration of the N polypeptide has
been altered at this temperature.

        RNA Synthesis of the Groups E And F Mutants.
RNA synthesis at the restrictive temperature in
cells infected with the group E mutants (Fig. 3,
Panels A and B) varied according to the mutant used
for the infection. In cells infected with tsEl no
viral RNA synthesis could be detected whereas in
cells infected with tsE2 and tsE3 viral messenger
RNA species were observed. These results are in
good agreement with the earlier findings and provide
further evidence that tsEl is probably a
transcriptase negative mutant, tsE3 a transcriptase
positive but replicase negative mutant and tsE2 a
transcriptase positive and perhaps also replicase
positive mutant. Since NS is the mutated
polypeptide in this complementation group these
results provide further evidence that this
polypeptide plays more than one role in virus
development.
        In cells infected with the group F mutants
(Fig. 3, Panel C) RNA synthesis appeared to be
inhibited in the case of tsFl but messenger RNA
synthesis was observed in the case of tsF2. These
results are also in agreement with earlier findings
and show that the mutated polypeptide in
complementation group F is also likely to be
multifunctional.

CONCLUSION

        Analysis of the RNA species synthesised in
tissue culture cells infected with the ts mutants of
VSV New Jersey provided further evidence that in
group E and F the mutated polypeptides are involved
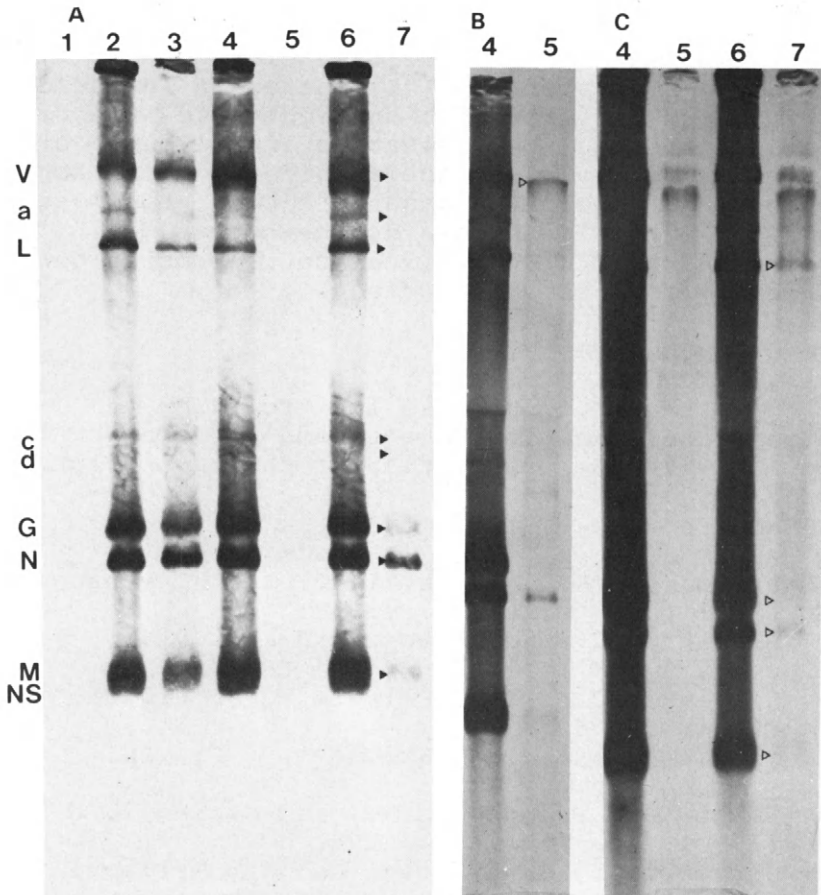
FIGURE 3. RNA species synthesised in BHK cells by the temperature-sensitive mutants of group E (Panels A and B) and group F (Panel C). The cells were infected either with wild type VSV New Jersey (Panel A, tracks 2 and 3), or with mutants tsE1 (Panel A, tracks 4 and 5), tsE2 (Panel A, tracks 6 and 7), tsE3 (Panel B, tracks 4 and 5), tsF1 (Panel C, tracks 4 and 5) and tsF2 (Panel C, tracks 6 and 7). Purified virion RNA (Panel A, track 1) was used as marker. The positions of the virion RNA (V), messenger RNAs L, G, N, NS and M (for the corresponding polypeptides) and the minor RNA species a, c, and d are indicated.

both in transcription and replication, and that
mutants in complementation groups C and D were
defective in a late function, presumably in
assembly. However, in the case of the group A
mutants analysis of the RNA species in infected
cells suggests that mutation might have affected
transcription due to a mutation in polypeptide N.
In the group B mutants the involvement of a host
factor in transciption was indicated. These new
findings may help us towards the better
understanding of VSV replication, a process only
partially understood at present.

ACKNOWLEDGEMENTS

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CHARACTERIZATION OF A MUTANT OF VESICULAR STOMATITIS
VIRUS WITH AN ABERRANT IN VITRO POLYADENYLATION ACTIVITY[1]

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The genome of the rhabdovirus vesicular stomatitis virus
(VSV) is a non-segmented, negative-sense RNA of molecular
weight 3.6-4.2 x 10^6 (1). Purified virions contain an RNA-
dependent RNA polymerase (transcriptase) (2) which, under
appropriate conditions, transcribes the genome in vitro,
producing monocistronic mRNAs which are capped, methylated
and polyadenylated (3). Three of the five viral proteins are
required for transcription: the template is genomic RNA
complexed tightly with N protein, and L and NS proteins are
also necessary for transcription (4). A fourth virally coded
protein, M, is not essential for transcription (4) but can
modulate the process (5-9). It is not known whether tran-
scription requires, or is significantly affected by, host
components which may be packaged in virions.

We have previously reported (10) that our stock of
tsG16(I), a temperature-sensitive (ts) mutant of VSV (Indiana)
(11), overproduces polyadenylic acid (poly A) in an in vitro
transcription system at all temperatures tested (27°C, 31°C
and 35°C) and that the mRNAs made in vitro by this virus
contain abnormally long tracts of poly(A). It appears that
the 5' end of tsG16(I) RNA made in vitro at 31°C is normally
modified since it is methylated to almost the same extent as
wild type (wt) product RNA, contains the same two methylated
cap structures as wt RNA in a similar ratio and is translated
as well (per pmol of UMP residues) as wt RNA in an in vitro
reticulocyte cell-free translation system (10).

     One question which arises is which component of tsG16(I)
virions is associated with this abnormal polyadenylation
activity. TsG16(I) is known to have an L protein defect which
is apparently correlated with the thermolability of tran-
scription in vitro by this virus (12). However, as mentioned
above, experiments to determine whether aberrant polyadenyla-
tion by this mutant is a temperature-dependent phenomenon
suggested that it was not. As yet, we have not been able to
isolate revertants of our stocks of tsG16 to determine
whether the abnormal polyadenylation phenotype is correlated
with the temperature-sensitivity of this mutant. The approach
used, therefore, in the studies reported here, has been to try
to identify the moiety responsible for the poly(A) phenotype
of tsG16(I) by separating mutant or wild type virions into
various components and by reconstituting the system using
homologous or heterologous components.

RECONSTITUTION EXPERIMENTS USING TEMPLATE-AND ENZYME-CONTAIN-
ING FRACTIONS

     Emerson and Wagner (13) have shown that virions treated
with Triton X-100 in the presence of 0.72 M NaCl can be frac-
tionated into a sedimentable ribonucleoprotein template frac-
tion and a non-sedimentable enzyme-containing fraction.
Individually, these fractions possess little transcriptive
activity but they exhibit considerable transcriptive activity
when recombined. To determine whether the poly(A) phenotype
of tsG16(I) was associated with the template- or enzyme-con-
taining fraction of the virus, virus was grown on BHK-21 cells
and highly purified virions (10) of either mutant or wt VSV
Indiana (San Juan strain) were treated with an equal volume
of 2X-Triton high salt solubilizer (2X-HSS; 1.44 M NaCl-18.7%
glycerol-3.74% Triton-X100-1.2 mM dithiothreitol and 10 mM
Tris-hydrochloride, pH 7.4) and separated into pellet (tem-
plate-containing) and supernatant (enzyme-containing) frac-
tions by ultracentrifugation as previously described (14).
The fractions were assayed after homologous or heterologous
reconstitution to determine their poly(A) phenotype. Virions
which were solubilized at the same time but which were not
fractionated were used as controls. Since tsG16(I) synthe-
sizes such a large excess of poly(A) compared to wt virus in
an in vitro transcription system, a convenient assay for the
aberrant polyadenylation phenotype of tsG16(I) was to use
transcription reactions (10) which contained [^{3}H]ATP,
[α-^{32}P]UTP, CTP and GTP, and to compare the molar amounts of
AMP and UMP incorporated into ice-cold trichloroacetic acid
insoluble material. The mutant and wt polyadenylation pheno-
types can readily be distinguished by this assay ((10) and
Table 1).

TABLE 1

AMP AND UMP INCORPORATION BY UNFRACTIONATED WT AND TSG16(I) VIRIONS
OR BY VIRIONS AFTER DISSOCIATION AND RECONSTITUTION FOLLOWING TREATMENT WITH 2X-HSS[a]

Virion Components	Incorporation in 120 min at 31°C[b]		Ratio AMP/UMP
	pmoles UMP	pmoles AMP	
Unfractionated wt	90.2	151.0	1.67
wtP + wt SN	52.9	75.4	1.43
Unfractionated ts	232.9	708.6	3.04
tsP + ts SN	70.6	215.1	3.05
tsP + wt SN	55.7	90.6	1.63
wtP + ts SN	76.4	270.4	3.54

[a] Purified virions were treated with an equal volume of 2X-HSS and separated into pellet (P) and supernatant (SN) fractions by ultracentrifugation as described previously (14). Solubilized, unfractionated virions and P and SN fractions (before or after recombination) were assayed in transcription reactions containing [α−^{32}P]UTP, [^{3}H]ATP, CTP, GTP and S-adenosyl methionine (10); incorporation of each isotope into ice-cold trichloroacetic acid precipitable material was determined as described previously (10).

[b] Values are not corrected for incorporation by P and SN fractions assayed separately. Neither SN fraction showed any detectable incorporation. The UMP incorporation by wtP and tsP fractions was 10.4 pmoles and 5.2 pmoles, respectively, and the incorporation of AMP was 15.9 and 23.9 pmoles, respectively.

As can be seen from Table 1, when tsG16(I) or wt virions
were separated into pellet and supernatant fractions by the
method described above and then recombined homologously, the
parental phenotype was recovered. When the pellet and super-
natant fractions were recombined heterologously, the AMP/UMP
incorporation phenotype of the ts pellet plus wt supernatant
recombination was wt whereas the phenotype for recombined wt
pellet plus ts supernatant fractions was that of the mutant.
It appeared, therefore, that the aberrant in vitro poly-
adenylation by tsG16(I) was not due to an altered polyadeny-
lation signal on the template but was due to an altered
component in the supernatant fraction.

Similar results were obtained when wt VSV Indiana
(Glasgow strain), the parental wt from which tsG16(I) was
derived (11), was used (data not shown). However, pellet frac-
tions prepared by the above method from our stocks of this wt
strain retained considerable transcriptase activity and we,
therefore, have used the San Juan strain since it has been
shown that transcriptase fractions and purified L and NS
fractions are functionally interchangeable between the Glasgow
mutants and the San Juan wild type virus (12,14).

EXPERIMENTS USING TRANSCRIBING NUCLEOCAPSIDS

The supernatant fractions prepared after treatment of the
virus with an equal volume of 2X-HSS and ultracentrifugation
contain L, NS, G and M proteins (13). To determine whether
M or G proteins could be responsible for the abnormal poly-
adenylation phenotype of tsG16(I), we prepared transcribing
nucleocapids (TNCs) which retained L and NS proteins on the
N protein-RNA template but from which M and G proteins had
been removed. Highly purified virions suspended in 15%
glycerol-10 mM Tris-hydrochloride, pH 7.4 were treated with an
equal volume of 0.5 M solubilizer (0.5 M NaCl-3.74% Triton
X-100-20% glycerol-1.2 mM dithiothreitol-4.3 mM Tris-hydro-
chloride, pH 8.0) and subjected to gel filtration on agarose
A-50m (BioRad Laboratories, Richmond, California) in 0.25 M
solubilizer (1 volume of 0.5 M solubilizer plus one volume
10 mM Tris-hydrochloride, pH 8.0). TNCs prepared by this pro-
cedure were virtually free of M and G proteins (Fig. 1), but
retained almost all of the L and NS proteins (D. M. Hunt,
unpublished data) and most of the original transcribing activ-
ity of the virus (Table 2).
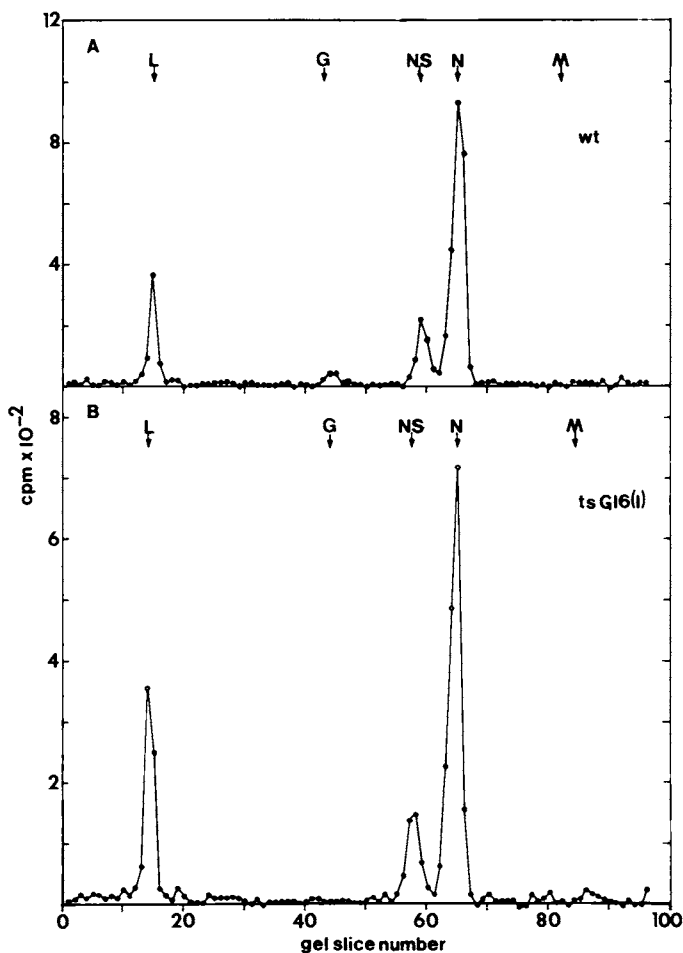
FIGURE 1.   Proteins present in transcribing nucleocapsid
(TNC) fractions.   [^{3}H] leucine labeled virions were treated
with an equal volume of 0.5 M solubilizer and subjected to
gel filtration on agarose A-50m columns equilibrated with
0.25 M solubilizer (see text).   Nucleocapsid containing frac-
tions were collected, precipitated with trichloroacetic acid
and prepared for polyacrylamide gel electrophoresis (13).
Samples were electrophoresed on 12.5% polyacrylamide-SDS slab
gels (10) with marker virion proteins in adjacent lanes.
Gels were dried, sliced, digested with NCS (Amersham Corp.):
water (9:1) for 3.5 h at 55°C and counted by liquid scintil-
lation spectrometry after addition of a xylene-based fluor.
A, wt TNCs; B, tsG16(I) TNCs.

TABLE 2

AMP AND UMP INCORPORATION BY UNFRACTIONATED WT AND TSG16(I) VIRIONS OR BY TRANSCRIBING NUCLEOCAPSIDS (TNCs) PREPARED BY GEL FILTRATION[a]

Virion Components	Ratio $\frac{\text{pmoles AMP incorporated}}{\text{pmoles UMP incorporated}}$	% recovery[b] of activity TNCs/virus
Unfractionated wt wt TNCs	2.26 2.29	91
Unfractionated ts Ts TNCs	4.24 4.42	101

[a][^{3}H]-leucine labeled purified virions were treated with an equal volume of 0.5 M solubilizer, and TNCs prepared by gel filtration on agarose A50m in 0.25 M solubilizer (see text). Fractions were assayed as described in Table 1. Incorporations were measured after 120 min at 31°C.

[b]Recoveries are calculated by comparing the UMP incorporation by virions and TNCs and correcting for the relative protein concentrations (determined by comparison of [^{3}H] leucine cpm in each fraction) and the loss of M and G proteins from the TNC (determined by polyacrylamide gel electrophoresis).

The polyadenylation phenotype of such TNCs, as measured by the ratio of pmoles AMP/UMP incorporated, corresponded to that of the parent virus (Table 2). The results in Figure 1 and Table 2 are taken from the same experiment so that a direct comparison can be made. It appeared, therefore, that M and G proteins were probably not directly responsible for the aberrant polyadenylation by tsG16(I) since virtually all of these proteins were removed (Figure 1), but almost all of the transcriptase activity and of the abnormal polyadenylation activity of tsG16(I) was recovered.

DISCUSSION

From separation-reconstitution experiments (Table 1) in which virions were treated with 0.72 M NaCl prior to fractionation, it appeared that the moiety directly responsible for abnormal polyadenylation by tsG16(I) was not the ribonucleoprotein template but, rather, a component of the supernatant fraction, which contained L, NS, G and M proteins. However, since removal of almost all of the G and M proteins by gel filtration in the presence of 0.25 M NaCl-solubilizer (Figure 1) resulted in little loss of either transcriptive activity or the aberrant polyadenylation activity (Table 2) it seemed unlikely that G or M were directly responsible for the polyadenylation phenotype of tsG16(I). Experiments are currently being performed in an attempt to determine whether the abnormal component in tsG16(I) is L protein, NS protein, or perhaps due to an abnormality in the spectrum of host proteins packaged due to a mutation in a virion component.

ACKNOWLEDGMENTS

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ROLE OF THE VIRAL LEADER RNA
IN THE INHIBITION OF TRANSCRIPTION
BY VESICULAR STOMATITIS VIRUS[1]

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Following infection of many cell types by vesicular sto-
matitis virus (VSV), a rapid decrease in cellular RNA synthe-
sis is observed (1-4). Studies with UV-inactivated virus
demonstrated that only a small portion of the 3' end of the
genome need be transcribed to obtain this inhibitory activity
(5). Because the VSV genome was shown to be transcribed
sequentially from its 3' end (6, 7), it was suggested that
the first viral transcript, the leader RNA, was the inhibitor
molecule. McGowan et al. (8) demonstrated that purified
plus-strand leader RNA could inhibit DNA-dependent transcrip-
tion in a soluble HeLa cell extract and studies by Kurilla et
al. (9) showed that the leader RNA was present in the nucleus
of infected cells.

In recent studies, we examined the ability of the VSV_{NJ}
and VSV_{Ind} serotypes to inhibit transcription and provided
additional evidence for the role of the leader RNA (10). We
also demonstrated that the VSV_{NJ} leader RNA was a better in-
hibitor of transcription than the VSV_{Ind} leader RNA. Addi-
tional studies (Grinnell and Wagner, unpublished) have de-
fined the sequence and structure of the leader RNA involved
in the inhibition of transcription. In this paper we provide
a summary of these data.

INHIBITION OF CELLULAR RNA SYNTHESIS IN VSV INFECTED CELLS AND QUANTITATION OF LEADER RNA PRESENT

The effects of VSV_{NJ} and VSV_{Ind} on cellular RNA synthe-
sis were compared by pulse labeling infected cells with tri-

Hours After Infection
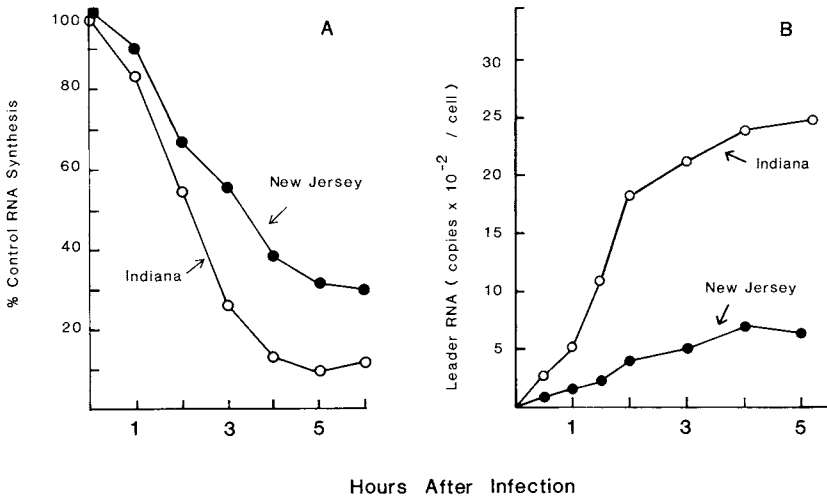
FIGURE 1.   (A) Inhibition of cellular RNA synthesis and
(B) kinetics of leader RNA production by VSV_{NJ} and VSV_{Ind} in
infected cells.   MPC-11 cells were infected with the viruses
and were pulse-labeled with [^{3}H]uridine for 10 min at hourly
intervals after infection.   The amount of cell RNA synthesis
was determined, relative to mock-infected cells, by the
amount of acid precipitable radioactivity in each sample.   To
detect the plus-strand leader RNA, cell RNA samples were
hybridized with a 42S VSV genomic RNA that had been 3'-end-
labeled with [^{32}P]pCp (11), followed by digestion with pan-
creatic and T1 ribonucleases.   Double-strand products were
separated on 10% polyacrylamide gels and the amount of leader
RNA was determined from the number of Cerenkov counts hybrid-
ized and the specific activity of the probe.

tiated uridine at various times after infection and measuring
the amount of acid precipitable incorporation.   As shown in
Figure 1A, both serotypes inhibited cellular RNA synthesis
although VSV_{Ind} did so more rapidly and to a greater extent.
This difference in inhibitory activity between the two sero-
types was independent of the multiplicity of infection or the
cell type used.
        To determine if the difference in the level of inhibi-
tion observed in Figure 1A might relate to differences in the
amount of intracellular leader RNA, we examined the kinetics
of leader RNA production.   In both VSV_{NJ} and VSV_{Ind}-infected
cells, the amount of intracellular leader RNA increased
steadily with time (Figure 1B).   The level of leader in
VSV_{Ind}-infected cells reached ~2400 copies/cell by 5h after

infection, but in VSV_{NJ}-infected cells, only reached ~625
copies/cell, or approximately four times less.

PRODUCTION OF LEADER RNA IN VIVO BY UV-INACTIVATED VIRUS

The difference in the level of cellular RNA synthesis
inhibition obtained with VSV_{NJ} and VSV_{Ind} (Figure 1A) was not
as drastic as the four-fold difference in the amount of the
leader RNAs detected in these cells (Figure 1B). While this
suggested that the VSV_{NJ} leader RNA might be a more efficient
inhibitor of transcription, such an assessment would best be
made by comparing levels of leader RNA in cells at the same
level of RNA synthesis inhibition. To this end, cells were
infected with virus that had been irradiated with increasing
UV-doses and after 5h, the levels of transcription inhibition
and intracellular leader RNA were determined. At increasing
UV doses, the ability of VSV to inhibit transcription and to
produce leader RNA decreased. For both of these functions,
the UV inactivation curves had essentially the same slope
(not shown) and even at a dose of 20,000 ergs/mm², the in-
fecting virus still inhibited transcription and produced
leader RNA. In contrast, the 37% (1/e) survival dose _in vivo_
for the N protein, whose gene is the first to be transcribed
after the leader gene, is ~400 ergs/mm² (5) and at a dose of
600 to 700 ergs/mm² is not detectable.

From the UV inactivation curves, we determined the
amount of leader RNA present at fixed levels of cellular RNA
synthesis inhibition. At all levels of inhibition, there was
approximately five times more leader RNA present in VSV_{Ind}-
infected cells than in those infected with VSV_{NJ}. Thus,
although much less VSV_{NJ} leader RNA is produced in infected
cells (Fig. 1B), this virus may effectively shut off cellular
RNA synthesis by means of a more efficient inhibitor than
that of VSV_{Ind}.

INHIBITION OF TRANSCRIPTION IN VITRO BY THE LEADER RNAS

The apparent difference in inhibitory efficiencies of
the two leader RNAs in infected cells was also observed when
we directly compared their abilities to inhibit DNA-dependent
RNA synthesis in the _in vitro_ HeLa cell transcription system
described by Manley et al. (12). In this system, we utilized
two DNA templates, pBR322 recombinants of the adenovirus-2
late promoter (LP), transcribed by RNA polymerase II, and the
adenovirus-associated (VA) gene, transcribed by RNA polymer-
ase III. The effect of increasing concentrations of purified
VSV_{NJ} and VSV_{Ind} leader RNA on the transcription of both tem-
plates is shown in Figure 2. At nearly all concentrations
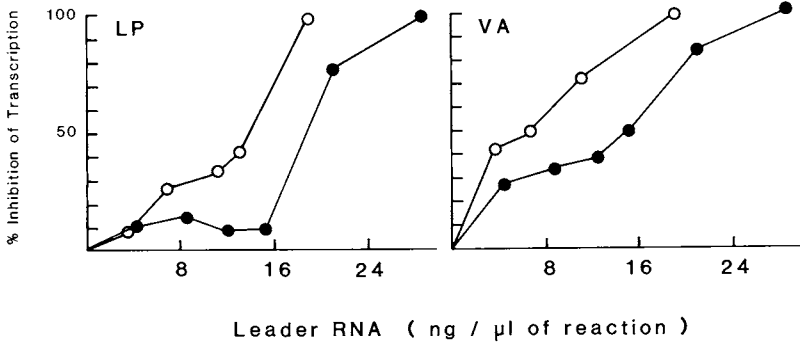tested, the New Jersey leader was more effective in inhib-

FIGURE 2. Dose response to the inhibition of transcrip-
tion by the New Jersey (O---O) and Indiana (O----O) leader
RNAs. pBR322 recombinants of the Ad2 late promoter (LP) and
associated RNA (VA) genes (37ng/µl of reaction, 9 x 10⁻⁸M)
were incubated in a soluble HeLa cell extract alone or with
increasing concentrations of purified leader RNA. The extent
of inhibition was determined by dividing the amount of tran-
scription of each template in reactions containing leader by
the amount in reactions with no leader added. The amount of
transcription was determined by Cerenkov counting of the spe-
cific transcripts sliced from gels.

iting _in vitro_ transcription of both the LP and VA genes. In
the range tested, it appeared that the minimal concentration
of Indiana leader needed to inhibit the LP gene was much
higher, approximately 16ng/µl of reaction compared to ~4ng/µl
of reaction for the New Jersey leader. However, increasing
the concentrations of VSV_{Ind} leader above 16ng/µl resulted in
rapid inhibition of transcription within a relatively narrow
range of concentration. In contrast, the inhibition of VA
gene transcription by the VSV_{Ind} leader occurred at lower
doses. Also, the VSV_{NJ} leader was more effective in inhib-
iting the VA gene than was the Indiana leader. However, both
leader RNAs appeared to be more effective in their ability to
inhibit transcription by RNA polymerase III (VA gene) than by
polymerase II (LP gene).

EFFECT OF DOUBLE-STRANDED REGIONS IN THE LEADER RNA ON THE
INHIBITION OF TRANSCRIPTION

A comparison of the RNA sequences of the VSV_{NJ} and
VSV_{Ind} leader RNAs (Figure 3) shows that they are quite
similar; the ten nucleotide differences are highlighted by
underlining. Four of the nucleotide differences are in an
AU-rich region (nucleotides 18-34) suggested to resemble the
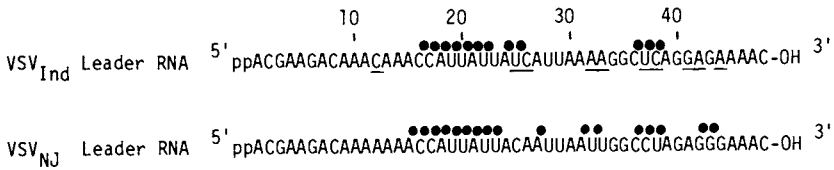
FIGURE 3. Comparison of the nucleotide sequence and sites of RNase V1 cleavage in the leader RNAs of VSV_{Ind} and VSV_{NJ}. The unique regions of VSV_{Ind} leader are highlighted by underlining. RNase V1 cleavage sites (dots) were determined by analyzing partial digests of 3' end-labeled leader RNA on RNA sequencing gels. Sequence data obtained from Colonno and Banerjee (16, 17).

TATA homology and five are in the purine-rich 3' sequences, which McGowan et al. (8) suggested to be similar to RNA polymerase recognition sequences. We determined that both leader RNAs had secondary structure, which encompassed the AU-rich region. This was accomplished by digesting end-labeled leader RNAs with the double-strand specific RNase V1 (13) and separating the partial digestion products on a 20% polyacrylamide sequencing gel (data not shown). The locations of major cleavage sites are indicated in Figure 3. Using the method of Tinoco et al. (14), we have predicted the stabilities of several stem-loop structures; the VSV_{NJ} leader RNA appears to be capable of forming a more stable secondary structure than the VSV_{Ind} leader RNA (i.e. -4.5 vs. -0.6 kcals for VSV_{NJ} and VSV_{Ind}, respectively).

We also examined the effect of eliminating the secondary structure of the leader RNAs on their ability to inhibit transcription of the VA and LP templates in vitro. The intercalating agent, proflavin, has been used to perturb secondary structure (15), and leader RNA heat denatured in the presence of 1μM proflavin contained no double-stranded regions detectable by RNase V1 cleavage. Therefore, we measured the level of transcription inhibition by the leader RNAs in the presence and absence of proflavin. At a concentration of 1μM, proflavin itself did not significantly reduce the level of VA or LP gene transcripts. In the presence of this agent, there was a slight reduction in the inhibitory activity of the VSV_{Ind} leader RNA but a significant reduction in the inhibitory activity of the VSV_{NJ} leader RNA (Table 1). After proflavin treatment, the levels of inhibition by VSV_{NJ} leader RNA approximated the levels obtained with an equimolar amount of VSV_{Ind} leader RNA. However, a significant level of inhibition remained. Thus, secondary structure may be impor-

TABLE 1
INHIBITORY EFFECT OF LEADER RNAS
ON IN VITRO TRANSCRIPTION OF THE AD 2 LATE PROMOTER (LP)
AND VA GENES IN THE PRESENCE OF PROFLAVIN

Leader RNA added[a] (amount)	Proflavin (1µM)	% inhibition of RNA transcribed on template	
		VA	LP
VSV_{Ind} (16ng/µl)	–	50	38
VSV_{Ind} (16ng/µl)	+	43	36
VSV_{NJ} (15.5ng/µl)	–	87	74
VSV_{NJ} (15.5ng/µl)	+	44	42

[a]The amount of leader used represents a molar concentra-
tion of approximately 1×10^{-6}M. The amount of each template
(37ng/µl) represents a concentration of 1×10^{-8}M for the VA
gene and 9×10^{-9}M for the LP gene.

tant in the efficiency of transcription inhibition particu-
larly by the VSV_{NJ} leader RNA.

VSV SEQUENCES INVOLVED IN IN VITRO TRANSCRIPTION INHIBITION

We examined the possibility that a recombinant cDNA of
the leader RNA might inhibit polymerase II and III-directed
transcription and would allow for a more detailed analysis of
the sequences involved. The recombinant plasmid, originally
a gift from R. Lazzarini, National Institutes of Health, con-
tained approximately the first 390 nucleotides of the 3' end
of the VSV_{Ind} genome, i.e. leader RNA sequences and part of
the N gene. The cDNA insert was isolated by Pst-1 cleavage
from the pBR322 vector (VSV sequences plus GC tails) and was
then digested with the restriction endonuclease DdeI to pro-
duce three fragments: DdeIC, containing most of the leader
RNA sequence (GC tails and leader nucleotides 1 to 36);
DdeIB, containing the remaining leader sequence and 5' end of
the N gene mRNA sequence; and DdeIA, containing only N gene
mRNA sequence and GC tails. For each fragment, we isolated
the DNA strand corresponding to the VSV plus-sense and exa-
mined its ability to inhibit both VA and LP gene transcrip-
tion. The DdeIC leader fragment exhibited significant inhib-
itory activity (Figure 4). We found no significant effect of
the DdeIB or DdeIA fragment or poly [dG-C] on transcription
at levels ten times those needed to obtain inhibition by
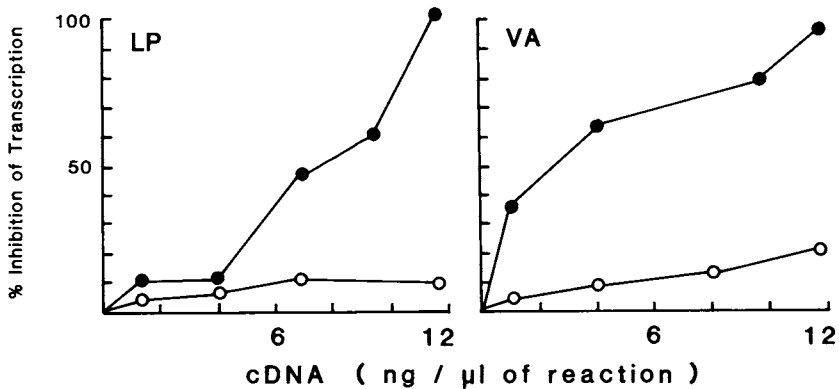
FIGURE 4. Dose response to the inhibition of transcription of the Ad2 LP and VA genes by the plus strands of the DdeIC fragment (leader sequences; O---O) and DdeIA fragment (N gene sequences; (O---O) of a cDNA clone of the 3' end of the VSV genome. The extent of transcription inhibition of each template was determined as in Figure 2.

the DdeIC fragment. In fact, less cDNA leader was required to inhibit transcription than was required for the leader RNA itself (Figure 2). This probably is because the cDNA is less sensitive to nuclease degradation during incubation in the HeLa cell extract than is the RNA. It is interesting that the dose-response patterns of the DdeIC fragment for the inhibition of both the VA and LP genes (Figure 4) were similar to those of purified leader RNA (Figure 2), i.e. inhibition of the VA gene transcription occurred at lower concentrations and reached higher levels than observed for the inhibition of LP gene transcription. This similarity in dose response patterns suggests that the cDNA leader serves as an appropriate model for the leader RNA.

The DdeIC fragment contains the AU-rich (AT) or TATA-like sequences that also appear to be important in the efficiency of transcription inhibition. To further assess the importance of these sequences, we examined the effect of two synthetic oligodeoxynucleotides on transcription of the VA and LP genes: one containing the AT-rich sequences (Oligo I: ATTATAATCATTA) and the other, sequences near the 3' end of the leader (Oligo II: AGGCUCAGGAGA). The AT-rich Oligo I molecule markedly reduced the levels of both VA and LP gene transcription. However, the Oligo II molecule exhibited little inhibitory activity even at very high concentrations (data not shown).

Leader RNA appears to play a dominant role in the inhibition of cellular transcription in VSV-infected cells.

Using a reconstituted HeLa cell transcription system, we have
determined that the AU(AT)-rich region of the leader RNA or
cDNA sequence alone is capable of inhibiting transcription
directed by both RNA polymerase II and III and that secondary
structure in this region is important. The leader RNA of VSV
may prove to be a useful model for studying regulation by
small nuclear RNAs of cellular origin.

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TEMPERATURE SENSITIVE MUTANTS OF VSV INTERFERE WITH THE
GROWTH OF WILD-TYPE VIRUS AT THE LEVEL OF RNA SYNTHESIS[1]

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Temperature-sensitive (ts) mutants of a number of viruses
are capable of interfering with the replication of wild-type
virus (1). Youngner and Quagliana (2) demonstrated that ts
mutants of VSV interfere with the replication of wild-type
VSV at both the permissive and nonpermissive temperatures.
Since the ts mutants form plaques which are much smaller than
those formed by wild-type VSV (wt-VSV), the progeny of a
mixed infection can be analyzed with respect to the relative
yields of ts and wt-VSV. By such analysis, it was found that
the growth of some ts mutants is enhanced during a mixed
infection at the nonpermissive temperature. Therefore, these
ts mutants not only interfere with the replication of wt-VSV,
but are "rescued" during a mixed infection.
     To extend these observations, we have examined in greater
detail the level at which ts mutants of VSV interfere with
the replication of wild-type virus. Mixed infections with
wt-VSV and 2 ts RNA⁻ mutants were studied.

RNA SYNTHESIS DURING MIXED INFECTION

     Cumulative RNA synthesis was measured in actinomycin D
treated BHK cells which were either singly infected with
wt-VSV, ts G 11, or ts G 41, or were doubly infected with
wt-VSV and a ts mutant. Infected cells were incubated at
$39.5°C$; virus yield and the incorporation of ^3H-uridine were
measured (Figure 1 and Table 1).
     When cells are infected with wt-VSV and ts G 11 at a
multiplicity of infection (MOI) of 1 and 10, respectively, the
yield of wt-VSV is reduced by greater than 90% relative to an
infection by wt-VSV alone. The yield of ts G 11 reflects a

TABLE 1

VIRUS PRODUCED DURING MIXED INFECTION AT 39.5°C WITH wt-VSV
AND ts MUTANTS

Cells infected with:	Ratio of yields Mixed infection/single infection	
	wt-VSV[b]	ts Mutant[b]
wt-VSV x ts G 11 (1 x 10)[a]	0.07	5800
wt-VSV x ts G 11 (10 x 10)	0.45	14000
wt-VSV x ts G 41 (1 x 10)	0.0002	1.2
wt-VSV x ts G 41 (10 x 10)	0.001	1.0

[a]Numbers in parentheses are the multiplicities of infection of wt-VSV and the ts mutant: (wt-VSV x ts virus).

[b]Culture medium was harvested at 8 hours after infection. Plaque-forming virus was assayed in chicken embryo monolayers at 34°C.

rescue of greater than 5000-fold (Table 1). However, cumulative RNA synthesis is restricted in such doubly-infected cells (Figure 1, Panel A). There is approximately an 8-fold reduction of RNA synthesis in doubly infected cells relative to cells infected with wt-VSV alone. When an MOI of 10 of each virus is used, interference and rescue are again reflected in the yields of each virus. Cumulative RNA synthesis is also reduced under these conditions, although to a lesser extent (Figure 1, Panel B).

In contrast, during a mixed infection of wt-VSV (MOI = 1 or 10) and ts G 41 (MOI = 10), the yield of wt-VSV is decreased by at least 3 logs and there is no rescue of ts G 41 (Table 1). The synthesis of viral RNA is almost completely inhibited (Figure 1, Panels C and D).

In a double infection with wt-VSV and ts G 11 the level of RNA synthesis represents a combination of the RNA synthesized by wt-VSV and "rescued" RNA synthesis by ts G 11.
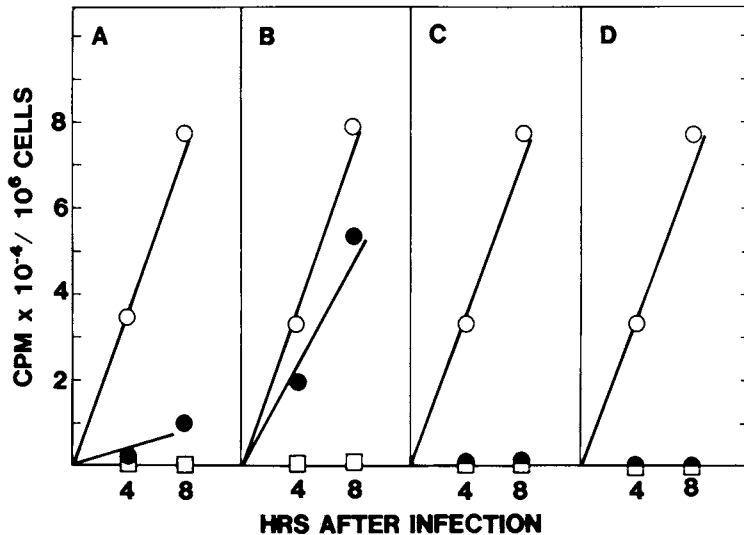
FIGURE 1.   RNA synthesis in BHK cells infected with
wt-VSV and ts mutants.   Cells were singly infected with wt-VSV
(0——0), a ts mutant (□–□), or doubly infected with wt-VSV
and a ts mutant (●——●).   Cumulative RNA synthesis at 39.5°C
was measured at 4 and 8 hours post-infection.   Panel A, wt-VSV
x ts G 11 (MOI = 1 x 10); Panel B, wt-VSV x ts G 11 (MOI 10 x
10); Panel C, wt-VSV x ts G 41 (MOI 1 x 10); Panel D, wt-VSV x
ts G 41 (MOI 10 x 10).

On the other hand, in a mixed infection with wt-VSV and ts G
41, there is neither rescue nor viral RNA synthesis.   For this
reason, ts G 41 was used in the majority of mixed infections
described in this report.
       Co-infection with wt-VSV and either ts G 11 or ts G 41
did not reduce primary transcription compared to wt-VSV alone
(data not shown).   Therefore, the inhibition of wt-VSV RNA
synthesis during a mixed infection is not due to interference
at the level of primary transcription.

WHAT SPECIES OF RNA ARE SYNTHESIZED DURING A MIXED INFECTION
WITH wt-VSV AND ts MUTANTS?

       Cells were singly infected with wt-VSV, ts G 11 or
ts G 41, or were doubly infected with wt-VSV and a ts mutant
at an MOI of 10 for each virus.   RNA was extracted after an
8 hour incubation at 39.5°C in the presence of ^{3}H-uridine and

subjected to electrophoresis in 1% agarose gels. For
analytical purposes an equal amount of TCA precipitable radio-
activity was applied to the gel for each virus combination.
Therefore, the RNA patterns presented in Figure 2 do not
represent the amount of RNA synthesized, but show the
relative amounts of the individual RNA species synthesized.
     It is clear from the fluorogram (Figure 2) that all
species of mRNA, as well as 42S virion RNA, are synthesized
during a single infection with wt-VSV and during mixed
infections with wt-VSV and either ts G 11 or ts G 41 (compare
lanes A, C, and E). This is not unexpected during
co-infection with ts G 11. In this case, although growth of
wt-VSV is reduced, the rescue of the ts mutant clearly
requires synthesis of all viral RNAs. In a mixed infection
with wt-VSV and ts G 41, on the other hand, there is no
rescue of the interfering mutant, yet all viral RNAs are
synthesized. Interference is not due to a decreased level of
synthesis of one or several species of RNA; all viral RNAs
are synthesized in near normal proportions, although at much
reduced levels.
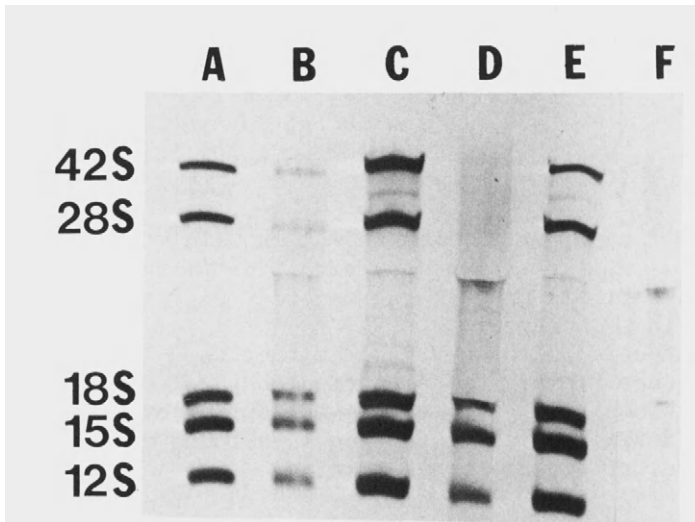
     FIGURE 2. RNA synthesis during single and mixed infec-
tion. BHK cells were infected with either wt-VSV (Lane A),
ts G 11 (Lane B), ts G 41 (Lane D), or were doubly infected
with wt-VSV and ts G 11 (Lane C) or wt-VSV and ts G 41
(Lane E). Lane F shows RNA synthesis in mock-infected cells.

EFFECT OF RELATIVE MULTIPLICITIES OF wt-VSV AND ts G 41 ON
RNA SYNTHESIS

     Cells were singly infected with ts G 41 at an MOI of 10,
wt-VSV at multiplicities of 100, 50, 10, 5, and 1, or were
doubly infected at those multiplicities (Figure 3).
Cumulative RNA synthesis was measured over a 10 hour incuba-
tion at 39.5°C. While the synthesis of RNA was inhibited in
all double infections relative to RNA synthesis by wt-VSV
alone, the degree of inhibition was dependent on the ratio of
the MOIs of wt-VSV and ts G 41. When wt-VSV was used at an
MOI of 100 and ts G 41 at an MOI of 10, cumulative RNA
synthesis was reduced to 67% of the value for cells singly
infected with wt-VSV at an MOI of 100. With decreasing
multiplicity of wild-type virus, more inhibition was observed;
when the ts virus was present in 10-fold excess, RNA synthesis
was less than 2% of the control value.
     An obvious interpretation of these results is that the
degree of inhibition of RNA synthesis is a reflection of the
proportion of cells which are actually infected by both a
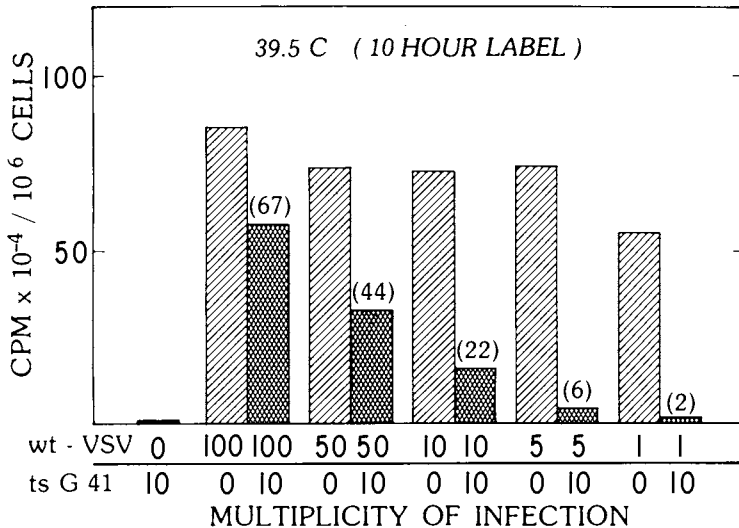
     FIGURE 3. The effect of relative multiplicities of
infection of wt-VSV and ts G 41 on RNA synthesis in mixed
infections. Cells were infected at the indicated multi-
plicities of infection. Cumulative RNA synthesis was
measured over a 10 hour incubation at 39.5°C.

wild-type and a ts virus. Only when the multiplicity of ts
virus is greater than the multiplicity of wt virus will all
cells infected by wt virus also be infected with a ts virus.
Synthesis of viral RNA would be inhibited under these
conditions of infection. The failure of Spindler and Holland
(3) to observe interference in a mixed infection with ts G 11
and wt-VSV when both viruses were used at a multiplicity of
2.5 is difficult to understand. Perhaps at those MOIs a
significant fraction of the cells may not have been doubly
infected.
      Interference was also examined under conditions where
infection with the ts mutant was at hourly intervals after
infection with wt-VSV. The ability of the ts mutant to
interfere with wt-VSV replication decreased with increasing
time between infections (data not shown). Further experiments
demonstrated that this loss of activity was due, at least in
part, to superinfection exclusion; the ts virus was unable to
infect cells which had been previously infected with wt-VSV
(4).

SENSITIVITY OF INTERFERENCE TO UV-IRRADIATION

      In order to determine if the ability of ts mutants to
interfere with wt-VSV replication is dependent on gene
expression by the mutant, an interference experiment was
carried out with UV-irradiated ts G 41. The ts mutant was
diluted in saline to yield an MOI of 10 for infection. The
diluted virus was irradiated with a UV lamp (15W) at a
distance of 50 cm for the times indicated, mixed with wt-VSV
(MOI = 1), and used to infect cells. Under these conditions
of irradiation, the titer of ts G 41 was reduced 10-fold by a
14 second exposure (Figure 4). Infected cells were labeled
with ^{3}H-uridine for 8 hours at 39.5°C. Duplicate cell
cultures were used to assess viral yield. Virus yields and
RNA synthesis are expressed as a percentage of the values
obtained during an infection with wt-VSV alone (Figure 4).
      With unirradiated ts G 41, there is a 95% inhibition of
wt-VSV replication, as reflected by yield and RNA synthesis.
With increasing times of irradiation, ts G 41 gradually loses
the ability to interfere with both yield of wt-VSV and the
synthesis of RNA. After 30 seconds of irradiation, the titer
of ts G 41 is reduced by 100-fold, and RNA synthesis and
wild-type yield are restored to approximately 20% of control
levels. By 75 seconds of irradiation, the infectivity of ts G
41 is reduced by at least 5 logs, reducing the effective MOI
to ∿ 0.001. At this point, the yield and RNA synthesis in
mixed infections are identical to those in cells infected with
wt-VSV alone. These results suggest that some gene expression

by the ts mutant is essential.

DISCUSSION

    We have examined the level at which ts mutants of VSV
interfere with the replication of wt-VSV. Ts G 41 was chosen
for study because, unlike some other interfering mutants, this
mutant is not rescued during a mixed infection at the non-
permissive temperature. The synthesis of viral RNA is
inhibited during a mixed infection with ts G 41; under the
appropriate conditions of infection, RNA synthesis is
inhibited by greater than 98%. However, primary transcription
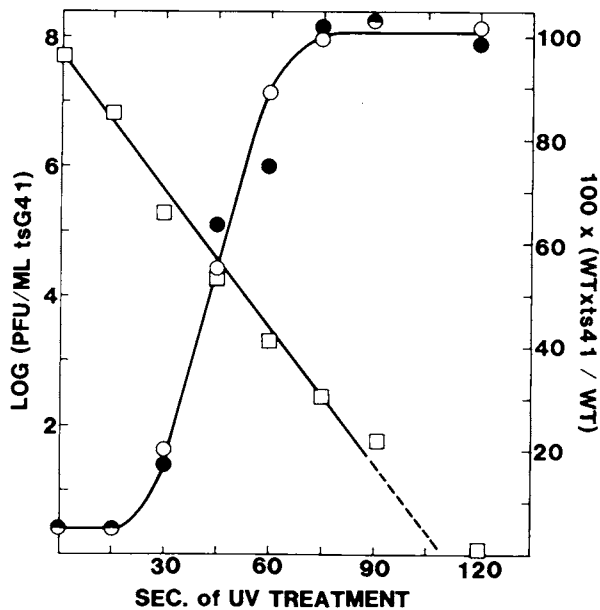
    FIGURE 4. The effect of UV-irradiation of ts G 41 on its
ability to interfere with wt-VSV. Ts G 41 was UV-irradiated
for the times shown (□-□). Cells were infected with wt-VSV
and ts G 41 which had been irradiated for increasing times.
Cumulative RNA synthesis (O——O) and yield of wt-VSV (●——●)
were measured at 8 hours after infection. Results are
expressed as percentage of the values obtained with wt-VSV
alone.

is not affected. All species of mRNA and 42S genome RNA are
synthesized during the mixed infection. UV-irradiation of the
ts mutant results in a progressive loss of its ability to
interfere with RNA synthesis by wt-VSV and the production of
infectious virus.

     The ability of RNA⁻, ts mutants of NDV to interfere with
the replication of wild-type NDV has also been reported to
involve inhibition of RNA synthesis (5). When primary chicken
embryo cells are doubly infected with wt-NDV and a ts mutant,
RNA synthesis at the non-permissive temperature is inhibited
by 50-90% relative to infection with wt-NDV alone. Inhibition
of RNA synthesis is also observed in cells doubly infected
with wild-type Semliki Forest virus and a ts mutant (6). It
is possible that a common mechanism, namely, a block of RNA
synthesis, may be responsible for interference by ts mutants
with the growth of RNA viruses.

ACKNOWLEDGEMENTS

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ROLE OF VESICULAR STOMATITIS VIRUS PROTEINS
IN RNA REPLICATION[1]

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VSV RNA REPLICATION IN VITRO

   Replication of the genome of vesicular stomatitis virus
(VSV) is dependent upon protein synthesis (1,2). It is pres-
ently unknown which viral proteins are actually required to
support this process. It is also unknown which of the VSV
proteins, if any, act to regulate RNA replication. In order
to more fully characterize VSV RNA replication in terms of
the role of the viral proteins in promoting and regulating
genome-length RNA synthesis, we have developed an in vitro
system that supports the RNA replication and nucleocapsid as-
sembly of a defective interfering (DI) particle of VSV as a
function of protein synthesis (3,4). This system, consisting
of intracellular DI nucleocapsid templates and a micrococcal
nuclease-treated rabbit reticulocyte lysate, replicates VSV
RNA when protein synthesis is programmed by the addition of
viral mRNA.
   In the experiments described here, we have used nucleo-
capsid templates of the VSV DI particle, DI-T, which contains
only the 5' 25% of the genome of wild type virus (5,6). This
was done to produce a system in which RNA replication could
be studied in the absence of any mRNA synthesis. The DI nu-
cleocapsids are not able to synthesize viral mRNAs because
they lack complete information for all the structural genes.
In the absence of protein synthesis, the DI templates synthe-
size only a 46 base long leader RNA (3,7,8). Since mRNAs are
not made by DI nucleocapsids, protein synthesis and, hence,
RNA replication in this system can be controlled by the

---

addition of exogenous mRNA. Therefore, by adding individual
VSV mRNAs to program the expression of individual viral pro-
teins, it was possible to investigate the requirement for
each of the viral proteins in VSV RNA replication. Further,
by adding varying amounts of individual VSV mRNAs to reac-
tions synthesizing genomic RNA, it was possible to examine
the ability of each of the viral proteins to regulate VSV RNA
replication.

PROTEIN REQUIREMENT FOR RNA REPLICATION

    The protein requirement for synthesis of genome-length
RNA was examined by programming the in vitro system with indi-
vidual hybrid-selected N, NS, or M mRNAs (4) to determine if
N, NS, or M proteins, when translated individually in the
presence of enzymatically active DI nucleocapsids, could pro-
mote the synthesis of genome-length RNA. Synthesis of viral
RNA and proteins was examined simultaneously in each reaction
by double-labeling with [^{3}H]-UTP and [^{35}S]-methionine. Fig.
1A shows the ^{3}H-labeled RNA products and Fig. 1B shows the
^{35}S-labeled protein products. The results demonstrated that
synthesis of N protein alone was sufficient to promote the
synthesis of both positive- and negative-strand genome-length
DI RNA. Neither NS or M protein by themselves were able to
support the synthesis of genome-length DI RNA.

EFFECT OF M AND NS PROTEINS ON RNA REPLICATION

    The results described above showed that N protein alone
was required for the synthesis of genome-length RNA in vitro.
The following experiments were designed to determine whether
the synthesis of a viral protein, specifically NS or M pro-
tein, in addition to N protein affected the level of genome-
length RNA synthesis.
    Various studies have indicated that NS and M proteins
may play a role in regulating VSV RNA synthesis. For NS pro-
tein, several different phosphorylated forms are known to
exist (9,10). It has been shown that different forms of NS
protein differ in their ability to support VSV transcription
in vitro (9). Also, several groups have shown that purified
M protein when added in large amounts to in vitro transcrip-
tion reactions will inhibit mRNA production (11,12,13,14).
Although both NS and M proteins are able to affect transcrip-
tion, the ability of these proteins to affect VSV RNA replica-
tion has not been studied.

    NS Protein Inhibits RNA Replication. To examine the
effect of NS and M proteins on VSV RNA replication, varying
amounts of hybrid-selected NS and M mRNA were added
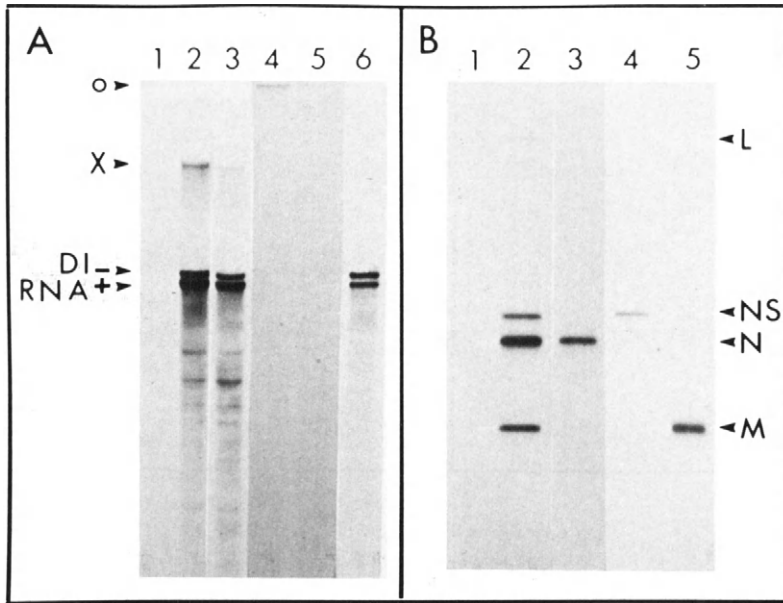
FIGURE 1. In vitro genome-length RNA and protein synthe-
sis in reactions containing individual hybrid-selected viral
mRNAs. Reaction mixtures consisted of a nuclease-treated
rabbit reticulocyte lysate, buffers, salts, nucleoside tri-
phosphates, amino acids, [^{3}H] UTP, [^{35}S] methionine, purified
intracellular DI nucleocapsids and the appropriate viral mRNA
(3). Individual mRNAs were purified by hybridization to cDNA
immobilized on nitrocellulose filters (4). Incubation of re-
action mixtures for 3 hr at 30°C was followed by electrophore-
sis of deproteinized RNA in 6M urea-agarose gels (Panel A)
and by PAGE analysis of proteins (Panel B). (1) No added
mRNA, (2) 0.74 µg of total polyadenylated VSV RNA, (3) 5 µl
of hybrid-selected N mRNA, (4) 4 µl of hybrid-selected NS
mRNA, (5) 4 µl of hybrid-selected M mRNA, (6) in vivo-labeled
DI intracellular nucleocapsid template RNA, including a
larger DI genome (X).

individually to the replication system along with a constant
amount of N mRNA and intracellular DI nucleocapsids. The RNA
and protein products are shown in Fig. 2. As above, the reac-
tion programmed with only N mRNA produced both positive- and
negative-strand genome-length RNA (Fig. 2A, lane 5). Reac-
tions programmed with an increasing amount of NS mRNA and a
constant amount of N mRNA produced an increasing amount of NS
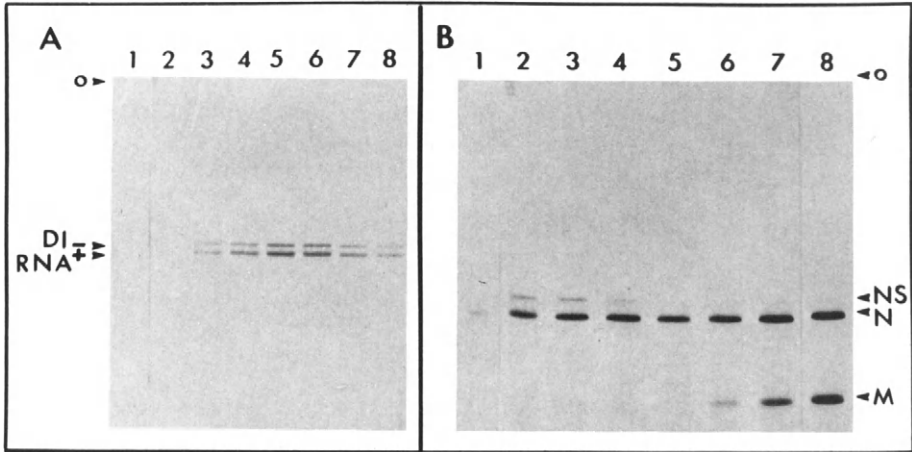
FIGURE 2.   Effect of addition of hybrid-selected NS mRNA
or M mRNA to reactions containing N mRNA.   Reaction mixtures
(see Figure 1) contained constant levels of purified intracel-
lular DI nucleocapsids and hybrid-selected N mRNA, except for
the control to which no mRNA was added.   Varying amounts of
either purified NS mRNA or purified M mRNA were added with
the N mRNA.   RNA (A) and protein (B) products were analyzed
as for Figure 1.   (1) No added mRNA, (2-8) 3 µl N mRNA plus
the following additions, (2) 3 µl NS mRNA, (3) 1.5 µl NS
mRNA, (4) 0.5 µl NS mRNA, (5) N mRNA alone, (6) 0.5 µl M
mRNA, (7) 1.5 µl M mRNA, (8) 3 µl of M mRNA.

protein relative to a constant amount of N protein (Fig. 2,
Panel B).   As the molar ratio of newly made NS:N protein
increased, the amount of DI genome-length RNA synthesized
decreased.   In this experiment, at a molar ratio of newly
made NS:N protein of 2:1, DI genome-length RNA synthesis was
completely inhibited.   The molar ratio of 2:5 gave 50% inhibi-
tion of RNA replication.

    M Protein Inhibits RNA Replication.   The ability of M
protein to influence RNA replication was measured by adding
increasing amounts of purified M mRNA to reactions containing
a constant amount of N mRNA and DI intracellular nucleocap-
sids.   The results showed that, similar to NS protein, as the
molar ratio of newly made M:N protein increased the amount of
RNA replication decreased.   However, whereas RNA replication
was blocked at a molar ratio of newly made NS:N protein of

2:1, at a molar ratio of newly made M:N protein of 2:1, repli-
cation was inhibited only by approximately 50%.

NS and M Proteins Inhibit Replication in the Presence of
All Viral Proteins. To test the possibility that increasing
amounts of NS or M protein relative to N protein may not be
inhibitory to replication when all five newly synthesized
viral proteins are present, variable amounts of NS and M
mRNAs were added to reactions containing constant amounts of
total VSV mRNA and intracellular DI nucleocapsids. The
results showed that as the amount of newly made NS or M pro-
tein relative to N protein increased, the amount of VSV DI
RNA replication decreased (data not shown). Thus, newly made
NS and M protein, whether in the presence of other newly syn-
thesized viral proteins or only newly synthesized N protein,
inhibited replication.
     In order to determine whether any mRNA when added to the
replication system in increasing amounts relative to N mRNA
would nonspecifically inhibit RNA replication, varying
amounts of rabbit globin mRNA were added to reactions contain-
ing a constant amount of total VSV mRNA and DI intracellular
nucleocapsids. No difference was observed in RNA replication
between reactions containing no globin mRNA and those contain-
ing differing amounts of this message (data not shown).
     M protein has an isoelectric point (pI) of approximately
9.0 (13). To test whether the ability of this protein to in-
hibit RNA replication was solely the result of its high pI,
cytochrome C, a protein with a pI of 9.1, was added in in-
creasing amounts to reactions programmed with total viral
mRNA and intracellular DI nucleocapsids. The addition of
cytochrome C at molar concentrations approximating those pro-
duced in vitro by M mRNA did not affect replication (data not
shown). This indicated that the high pI of M protein could
not be the only mechanism by which this protein inhibited
replication.

ROLE OF N, NS, AND M PROTEINS IN RNA REPLICATION

     We have examined the protein synthesis requirement for
VSV RNA replication and the ability of newly made NS and M
proteins to influence this process using a cell-free replica-
tion system. Our results demonstrated that only N protein
was required to promote genome-length RNA synthesis by intra-
cellular DI nucleocapsids. Newly made NS and M proteins both
inhibited VSV RNA replication in vitro. We can only suggest
purposes for these inhibitory effects. It is possible that
one function of NS protein in the virus life cycle is to modu-
late the balance between RNA replication and transcription,

thereby preventing the depletion of the N protein pool avail-
able for RNA replication. During budding, a region may be
formed at the junction of the nucleocapsid and the plasma mem-
brane which has a high M:N ratio. It is here that M protein
may act to inhibit replication to prepare the nucleocapsid
for release from the cell.

In summary, we have used an in vitro system to show that
N protein alone is sufficient to fulfill the protein synthe-
sis requirement for VSV RNA replication. In addition, we
have examined the effect of newly made NS and M proteins on
RNA replication and have found that although not required for
progeny RNA synthesis, these proteins may play important
roles in the replication of VSV RNA in the infected cell.

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VESICULAR STOMATITIS VIRUS PROTEINS REQUIRED FOR THE
IN VITRO REPLICATION OF DEFECTIVE INTERFERING
PARTICLE GENOME RNA[1]

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We have previously described a procedure which yields
extracts of vesicular stomatitis virus (VSV) infected cells
which will support the in vitro RNA replication of both
wild type VSV and its defective interfering (DI) particle,
MS-T (1). Infected cell monolayers are permeabilized by
incubation for 60 seconds with lysolecithin after which
they are scraped from the dish and disrupted by pipetting.
Following the removal of nuclei by centrifugation a
cell-free extract is obtained. We have shown that this
extract will support the replication and encapsidation of
wild type 42S RNA and DI 19S RNA in vitro for 90 minutes.
We have also shown that initiation of RNA replication
occurs in vitro in these extracts by adding purified
detergent-disrupted DI particles to extracts derived from
cells infected with wild type VSV alone and demonstrating
the synthesis and encapsidation of 19S DI genome RNA.
    Replication of viral RNA in the system described here
utilizes soluble proteins which are present in the infected
cells when extracts are made. We have shown that extracts
can be separated by centrifugation to yield one fraction
containing viral nucleocapsids and a second fraction
consisting of soluble proteins. These two fractions can be
combined to reconstitute the replication of viral RNA, and
the soluble protein fraction alone can support the
initiation of RNA replication. We have, therefore, turned
our attention to an investigation of the soluble protein

TABLE 1

IN VITRO ASSEMBLY OF MS-T NUCLEOCAPSIDS WITH
[^{3}H]LEUCINE-LABELED VSV INFECTED CELL SOLUBLE PROTEIN

Reaction Conditions[a]	Nucleocapsid Proteins (cpm)[b]		
	L	NS	N
– Replication	117	1149	1154
+ Replication	417	1405	8429

[a]VSV infected BHK cells were labeled with [^{3}H]leucine from 3.5 to 4.5 hr postinfection. A cytoplasmic cell extract was prepared in the absence of ribonucleoside triphosphates and the [^{3}H]-labeled soluble protein fraction was isolated as described previously (1). MS-T intracellular nucleocapsids were isolated from VSV + MS-T infected cells at 4.5 hr postinfection (1). Samples of MS-T nucleocapsids were incubated for 90 min at 30°C with the [^{3}H]leucine-labeled VSV soluble protein fraction in the absence or presence of ribonucleoside triphosphates and an energy regenerating system, which prevents or allows RNA synthesis, respectively.

[b]The products were treated with ribonuclease to degrade mRNAs and the nucleocapsids were purified by sucrose gradient centrifugation (2). The nucleocapsid associated [^{3}H]leucine-labeled proteins were quantitated by scintillation counting of individual proteins following their separation by SDS–polyacrylamide gel electrophoresis and detection by fluorography (3).

preparation from infected cells in order to more clearly define the proteins necessary to support RNA replication.

VIRAL   PROTEINS   ASSOCIATED   WITH   MS-T   NUCLEOCAPSIDS REPLICATING IN VITRO

To determine which viral proteins are assembled into nucleocapsids during in vitro replication, [^{3}H]leucine-labeled infected cell soluble proteins were mixed with MS-T nucleocapsids from infected cells and incubated in the presence or absence of ribonucleoside triphosphates, conditions which support or prevent in vitro replication,

respectively. The nucleocapsid products were then treated
with ribonuclease and purified on sucrose gradients.
Proteins associated with these nucleocapsids were separated
by gel electrophoresis and quantitated (Table 1).   In the
absence of RNA replication, the apparent exchange of all
three nucleocapsid proteins, including the N protein, with
the template nucleocapsid occurs.   When replication of
viral RNA is allowed to proceed, there is a 4-fold increase
in the amount of L protein associated with the
nucleocapsids, and a 7-fold increase in the amount of N
protein associated with the nucleocapsids.   The amount of
NS protein barely increases over the control level.

MONOCLONAL ANTIBODIES AS PROBES FOR VIRAL RNA REPLICATION

     Monoclonal antibodies have been used to study the
components of the soluble protein fraction of infected
cells which are necessary for in vitro RNA replication.
The addition of an anti-M monoclonal antibody to an
unfractionated in vitro replication reaction has no effect
on either transcription or replication.   Each of six
different anti-N monoclonal antibodies, however, inhibits
both replication and transcription by greater than 90% when
added to in vitro reactions (data not shown).   The anti-N
antibodies apparently bind to the N protein on the nucleo-
capsid template and inhibit all RNA synthesis.
     As an alternative approach, soluble protein prepara-
tions were depleted of N protein by incubation with anti-N
antibodies, followed by removal of antigen antibody
complexes by binding to S. aureus (Cowan strain).   The
remaining soluble protein, depleted of N protein, is unable
to support the in vitro replication of MS-T nucleocapsid
RNA (data not shown), thus providing evidence that N
protein is required for VSV RNA replication.
     These monoclonal antibodies were used to immunoprecip-
itate [3H]leucine-labeled infected cell soluble protein
preparations in the absence of detergents (Figure 1).   Each
of the six anti-N monoclonal antibodies (lanes B through G)
precipitate not only the N protein, but they also
precipitate about 10% of the NS protein present in the
preparations.   When detergents are present during the
incubation of antibody with antigen, only the N protein is
immunoprecipitated (data not shown).   These data suggest
that a detergent-sensitive complex between the N and NS
proteins exists in the soluble protein fraction of infected
cells.   This protein complex may be required for efficient
in vitro RNA replication.
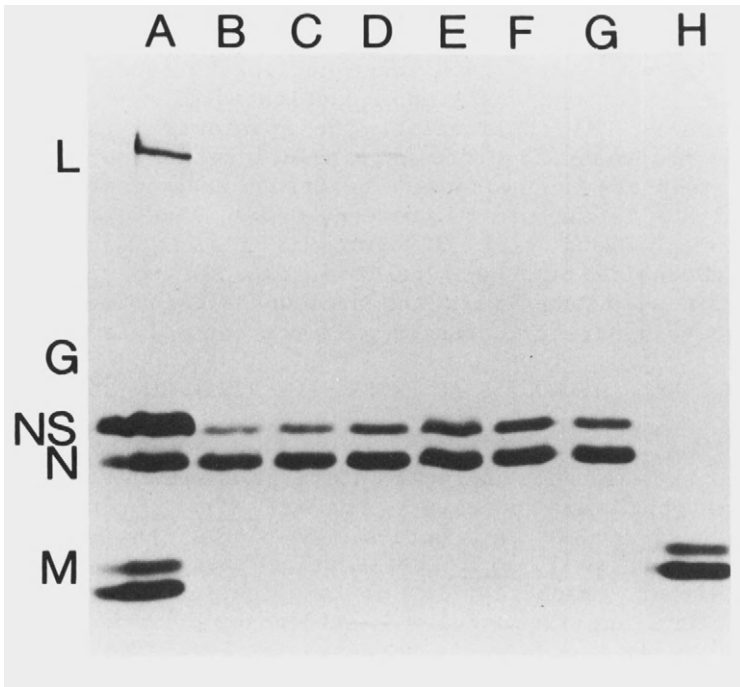
FIGURE 1.   Immunoprecipitation of proteins from the
soluble protein fraction of VSV infected cells with
monoclonal antibodies to VSV proteins.   Identical samples
of an [^{3}H]leucine-labeled soluble protein fraction from VSV
infected BHK cells prepared in a standard replication
reaction mixture in the absence of detergents were
incubated with antibodies for 2 hr at 4°C.   The monoclonal
antibodies (ascites fluid) to the VSV N and M proteins were
generously donated by Dr. D. Lyles (4).   Dr. A.K. Banerjee
provided αN-SS (5).   The antigen-antibody complexes were
collected by binding to S. aureus (Cowan strain), eluted
and analyzed by SDS polyacrylamide gel electrophoresis and
fluorography (3).   Proteins immunoprecipitated by
polyclonal αVSV serum (A); αN-SS (B), αN-A3A4 (C); αN-10G4
(D); αN-21F9 (E), αN-19B10 (F); αN-8C11F10(G); and αM-23H12
(H).

COMPONENTS OF THE INFECTED CELL SOLUBLE PROTEIN PREPARATION
ACTIVE IN MS-T RNA REPLICATION IN VITRO

     To examine possible forms of the VSV N protein
functional in in vitro RNA replication and nucleocapsid
assembly, in view of our results with the anti-N monoclonal
antibodies, we analyzed [^{3}H]leucine-labeled infected cell
soluble proteins by centrifugation on 5-20% glycerol
gradients. Fractions were collected and immunoprecipitated
with anti-VSV serum and the proteins were analyzed by
polyacrylamide gel electrophoresis (Figure 2A). The N
protein sediments in two broad peaks: as a low molecular
weight form in fractions 2, 3 and 4, representing about 60%
of the total N protein, and as a high molecular weight form
in fractions 8 through 10 with a molecular weight greater
than 300,000 based on sedimentation of the L protein
($\sim$200,000 MW) in fraction 6. Note that little if any NS
protein sediments with the high molecular weight form of
the N protein. Fractions 2, 3 and 4 from the top of the
glycerol gradient were also immunoprecipitated with an
anti-N monoclonal antibody (Figure 2B). As is seen with
unfractionated soluble protein, the anti-N monoclonal
antibody precipitates, in addition to N protein, a portion
of the NS protein present in the samples, suggesting that
an N-NS complex persists in these gradient fractions.
     Unlabeled infected cell soluble protein was analyzed
on a parallel gradient and selected fractions containing N
protein were assayed for their ability to support the
replication of MS-T nucleocapsid RNA in vitro as shown in
Figure 3. As a control, lane A shows significant synthesis
of nucleocapsid associated MS-T genome RNA after the
addition of unfractionated infected cell soluble protein to
intracellular MS-T nucleocapsids. Proteins in gradient
fraction 2 support some MS-T 19S RNA synthesis (lane B);
however, the proteins in fraction 3, which contain the peak
of the lower molecular weight N protein and of the putative
N-NS protein complex (Figure 2), show replication activity
close to control levels (lane C). By contrast, low levels
of RNA replication occur when the proteins in fractions
8-10 of the gradient are assayed (lanes D-F, respectively).
These latter fractions contain the higher molecular weight
form of the N protein, representing 40% of the total N
protein on the gradient, with only trace amounts of the NS
protein. Based on these results, we suggest that a complex
consisting of the N and NS proteins exists in VSV infected
cells, and that this complex may be the substrate for viral
RNA replication. The higher molecular species of the N
protein is a poor substrate for viral RNA replication,
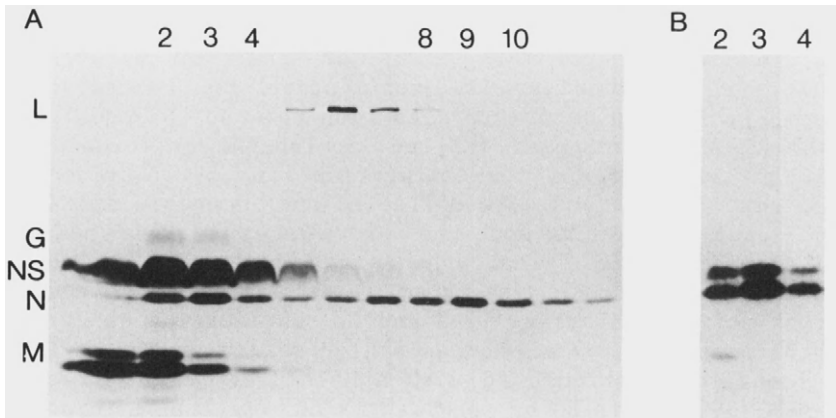
FIGURE 2. Fractionation of the soluble protein portion of VSV infected cells by glycerol gradient centrifugation. VSV infected BHK cells were labeled with [^{3}H]leucine from 3.5-4.5 hr postinfection and a cell extract was prepared in the replication reaction mixture as described (1). The extract was clarified by centrifugation in an SW65 rotor at 50,000 rpm for 75 min at 4°C. The resulting soluble protein fraction was analyzed by sedimentation on a 5-20% (w/v) glycerol gradient in buffer containing 0.2 M NH$_4$Cl, 0.1 M HEPES, pH 7.4, 7mM KCl, 1 mM DTT in an SW41 rotor at 32,000 rpm for 22 hr at 4°C. One ml fractions were collected from the top of the gradient, viral proteins in the indicated fractions were immunoprecipitated with anti-VSV serum (A) or anti-N monoclonal antibody (B) and analyzed by SDS-polyacrylamide gel electrophoresis. Sedimentation is from left to right.

perhaps due to its state of aggregation and/or the lack of the NS protein.

SUMMARY

The experiments described here utilize monoclonal antibodies to VSV proteins in conjunction with the reconstitution of components of an in vitro replication system to study the mechanism of VSV RNA replication. Based on these data we propose that the essential components for VSV replication include the nucleocapsid template containing the RNA-N complex and the L and NS proteins and, in addition, a putative N:NS protein complex.
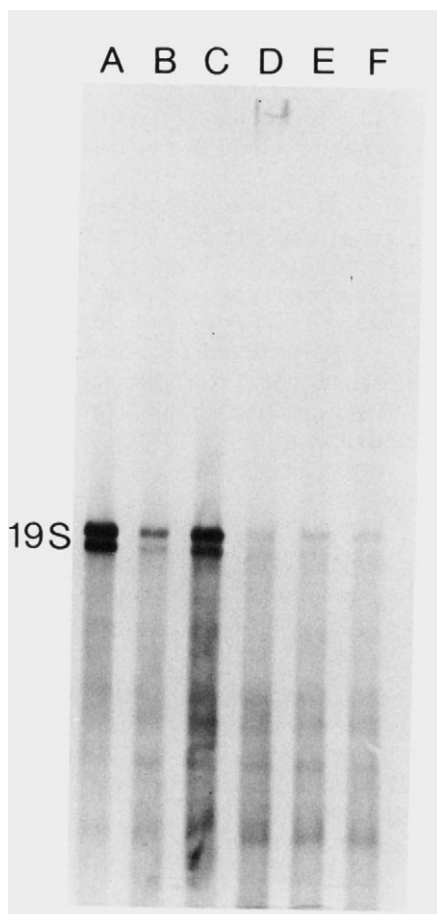
FIGURE 3.   Ability of glycerol gradient fractionated
VSV soluble protein to support the replication of MS-T RNA.
An unlabeled infected cell soluble protein fraction was
isolated and separated by glycerol gradient centrifugation
as described in Figure 2.   Samples of selected fractions
were assayed for their ability to support the in vitro
replication of MS-T nucleocapsids from infected cells by
incubation for 90 min at 30°C in the presence of [³H]UTP.
The nucleocapsid associated product RNA was purified by
banding on CsCl gradients.   The RNA was extracted from the
nucleocapsid peak and analyzed by electrophoresis on 1.5%
agarose gels in 6M urea containing 25 mM Na citrate, pH 3.5
as described previously (1).   MS-T   19S   product RNA
synthesized with the addition of: unfractioned VSV soluble
protein (A), or gradient fractions 2 (B), 3 (C), 8 (D), 9
(E), and 10 (F).

In preliminary experiments we have additional evidence from column chromatography that the N and NS proteins do form a stable complex in vivo. We suggest that it is the N:NS protein complex, rather than N protein alone, which serves as the substrate for N protein binding to nascent replicative RNA. It appears that the NS protein is dissociated upon the binding of N protein to the RNA, since we can detect no coordinate binding of NS and N proteins during in vitro RNA replication and nucleocapsid assembly (Table 1). In accord with the model of Kolakofsky and coworkers (6, 7) the N protein bound to nascent RNA then acts as an antiterminator to allow the polymerase to read through the intercistronic boundaries to synthesize and encapsidate genome RNA.

ACKNOWLEDGEMENT

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CHARACTERIZATION OF POLYCISTRONIC TRANSCRIPTS IN NEWCASTLE DISEASE VIRUS-INFECTED CELLS[1]

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Newcastle Disease Virus (NDV), a paramyxovirus, is a negative-stranded RNA virus whose genome is transcribed into six monocistronic messenger RNA's (1,2). These RNA's sediment on sucrose gradients at 18S and 35S (3,4). The 18S mRNA's encode the NDV membrane (M) protein, phosphoprotein (P), nucleocapsid protein (NP), fusion protein (F), and hemagglutinin-neuraminidase (HN) protein (5). The 35S mRNA encodes the L protein (6). Figure 1 shows total intracellular NDV RNA resolved on a formaldehyde-agarose gel. The 18S RNA which contains five species (5) resolves into four bands in this gel system. The 35S RNA contains one species (1,2).

Using the nuclease inhibitor nucleoside vanadyl complexes (7), we have detected five new species of intracellular RNA which sediment at 22S to 28S on sucrose gradients. These transcripts contain poly A and represent 15 to 25% of the total intracellular poly A-containing RNA. The molecular weights of these species range from 0.96×10^6 to 1.57×10^6 daltons (Figure 1).

The coding capacity of the NDV genome (mol. weight 5.2×10^6 - 5.7×10^6 daltons) (8) is accounted for by the six well characterized monocistronic messenger RNA's. Thus it was likely that the 22-28S RNA contained sequences present in the monocistronic RNA's (9, 10). To characterize the specific sequences present in each of the five 22S-28S RNA species, cDNA clones which contained sequences of each of the NDV genes were used as probes on Northern blots of total NDV intracellular RNA. Clones were derived by reverse transcription of NDV mRNA using standard methods. The gene from which each clone is derived was identified by hybrid selected translation (11).

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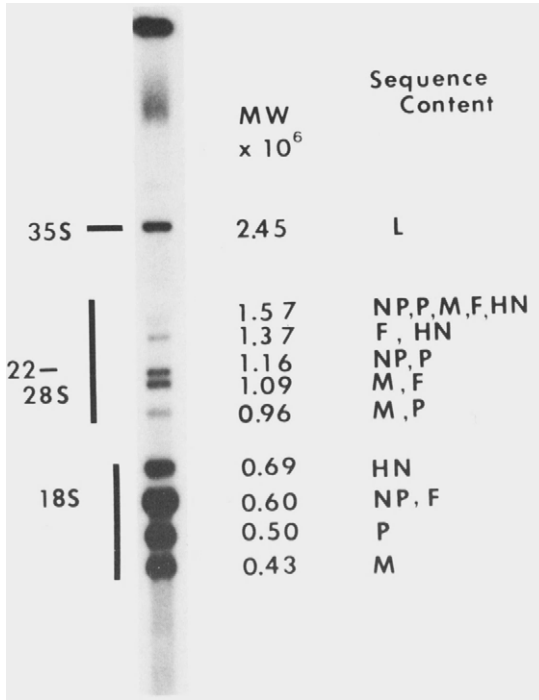
Figure 1:   Total intracellular RNA resolved on a 1% formaldehyde gel.

By Northern blot analysis, we found that the 0.96 x 10^6 dalton species contains M and P gene sequences.   The 1.09 x 10^6 dalton species contains M and F gene sequences while the 1.16 x 10^6 dalton species contains P and NP gene sequences.   The 1.37 x 10^6 dalton species contains F and HN gene sequences.   Thus these four species each contain sequences from two genes (see Figure 1). They likely represent transcription across two adjacent genes.   The fifth 22-28S species is resolved as a wide band with a molecular weight of from 1.57 to 1.61 x 10^6 daltons.   This RNA contains sequences of NP, P, M, F and HN genes.   The most likely explanation for this species is that it contains a mixture of two or three different RNA's, each of which represents transcription across three adjacent genes.   Thus we feel that the 22S-28S RNA's represent transcription across adjacent genes to generate polycistronic transcripts, either dicistronic or tricistronic.   And, in fact, the sizes of these

transcripts correspond closely to the sizes predicted by the sums of two or three monocistronic transcripts (Table I).

By ordering these species according to overlapping sequences (Figure 2), it is possible to establish a map order for the NDV genome of NP,P,M,F,HN. This order confirms the UV transcription map of Collins et al (12).

TABLE 1

SIZES OF TRANSCRIPTS GENERATED BY TRANSCRIPTION ACROSS ADJACENT GENES x10[6] DALTONS

Gene combinations	Predicted size[a]	Measured size[b]	Predicted size minus Poly A[c]	Measured size minus Poly A[d]
NP-P	1.10	1.16	1.04	1.12
P-M	0.93	0.96	0.87	0.92
M-Fo	1.03	1.09	0.97	1.03
Fo-HN	1.29	1.37	1.22	1.36
HN-L	3.14	NS		
NP-P-M	1.53	1.56	1.44	1.52
P-M-F	1.53	1.58	1.44	1.52
M-F-HN	1.72	NS		
F-HN-L	3.74	NS		
F-HN-Y[e]	–	1.61[e]	–	1.52

a Predicted size determined by adding the molecular weights of the monocistronic mRNA's shown in Figure 1.

b Sizes measured on Northern blots.

c Predicted size determined by adding the molecular weights of the monocistronic mRNA's without Poly A as determined from Figure 3.

d Sizes measured from Figure 3.

e An undefined space (Y) adjacent to HN must be postulated to account for this species.

```
                    |_____1.63_____|
              |_____1.57_____|
       |___1.57___|

       NP   P   M    F    HN   y              L
     |_.60_|.50_|.43_|.60_|_.69_|.2_|        2.45            |

     |___1.16___|
           |___.96__|
               |___1.09___|
                   |___1.37___|
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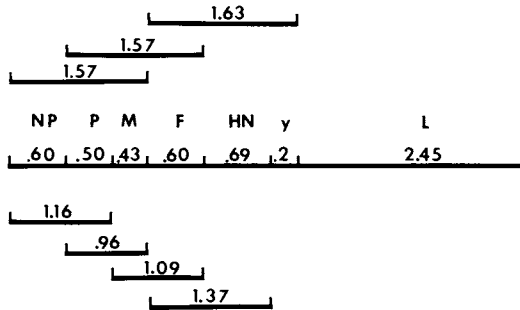
Figure 2: Map order of NDV Generated by overlapping sequences.

Polycistronic transcripts have also been found by Herman et al (13) in in vitro vesicular stomatitis virus transcription reactions, as well as in infected cells. These polycistronic transcripts contain long stretches of internal poly A at the intercistronic boundaries. We, therefore, asked if the NDV polycistronic transcripts contain such sequences. That these transcripts are slightly larger than their predicted size (Table 1), might lead one to suspect the presence of long stretches internal poly A.

In order to detect internal poly A, we hybridized NDV RNA to oligo dT and then digested the hybrid structures with RNase H. Any double-stranded structure should be susceptible to digestion. If there is internal poly A, then the 22S to 28S RNA should disappear after digestion. Such a digestion does reduce the size of the 18S RNA species slightly consistent with the removal of 3' poly A. In addition, the sizes of the 22S-28S RNA species are slightly reduced, consistent with removal of 3' poly A sequences (Figure 3). However, these species remain intact. Thus by this procedure, no internal poly A sequences are detected. Thus the mechanisms responsible for the generation of these species may reflect transcription events different from those responsible for VSV polycistronic transcripts.
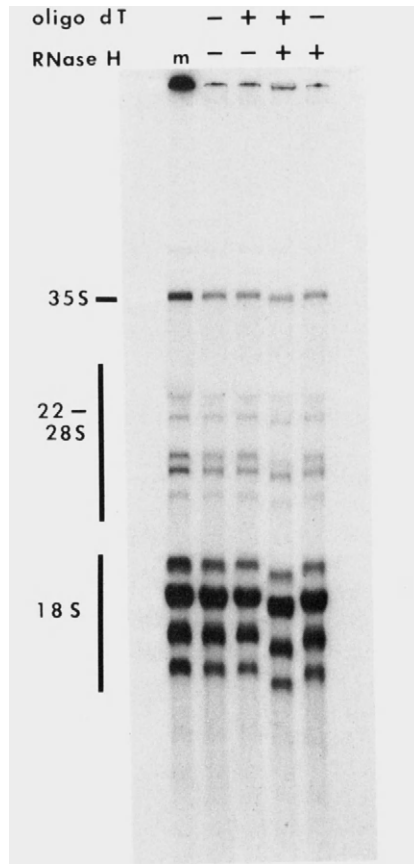
Figure 3: Digestion of NDV RNA with RNase H in the
presence of Oligo dT. RNA digested with RNase H in the
presence of Oligo dT is indicated by + control digestions
containing no RNase H or Oligo dT, or neither are
indicated at top of the figure.
m = marker RNA not subjected to reaction conditions.

The details of the transcription mechanisms of
paramyxoviruses have not been extensively explored. By
analogy with VSV, two mechanisms are possible: processing
of a long transcript or sequential start-stop synthesis of
each mRNA. The existence of polycistronic transcripts
containing no internal poly A is compatible with either
model. If a hypothetical nuclease were inefficient in
attacking cleavage sites in between cistrons, then various
combinations of polycistronic transcripts would be found.

Alternatively, if the NDV polymerase is inefficient in the termination of synthesis at the end of each cistron, then a certain percentage of transcripts would occur in a polycistronic form.

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MOLECULAR STUDIES ON CANINE DISTEMPER VIRUS REPLICATION[1]

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INTRODUCTION

The morbilliviruses, a subgroup of the paramyxoviridae which includes measles virus, canine distemper virus (CDV) and bovine rinderpest virus, cause acute febrile illness in their hosts. Measles virus and CDV are also known to establish persistent infections involving the central nervous system in some cases; measles is the cause of subacute sclerosing panencephalitis (SSPE) in man and CDV causes old dog encephalitis, but the mechanism whereby persistence is established is not understood (1). Molecular differences have been shown in the replication of viruses isolated from SSPE and old dog encephalitis brains. Sera from SSPE patients lack antibodies against the virus M protein while sera from dogs with similar demyelinating diseases contain antibodies to all canine distemper virus proteins (2). The establishment of persistence may nevertheless involve similar genetic mutations, and the induction of defective interfering (DI) RNAs in both viruses. Differential protein expression may occur in addition to this. In this communication we describe the virus-specific RNA species that can be observed in Vero cells infected lytically and persistently with CDV and provide evidence of defective RNA production in the persistently infected cells.

VIRUS-SPECIFIC RNAs PRODUCED IN LYTIC INFECTIONS

For lytic virus infections we used Vero cells infected
at a multiplicity of 0.1 p.f.u./cell with CDV (Onderstepoort
strain). RNA was labelled in vivo (in both infected and
uninfected cells) using ^{32}P-orthophosphate. Nuclear and
cytoplasmic extracts were prepared by treating the cells with
1% Nonidet p40 and 0.2% DOC (3). Purified RNA was then
fractionated into polyadenylated and nonpolyadenylated RNA
by oligo(dT) cellulose chromatography (4). Analysis of RNA
by polyacrylamide gel electrophoresis showed only one high
molecular weight virus-specific RNA band in the polyadenylated
RNA. To improve separation, the RNA was subjected to electro-
phoresis under completely denaturing conditions in 1.5%
agarose gels containing 2M formaldehyde (5). The RNA was
then blotted to nitrocellulose paper (6) and autoradiographed.
This method greatly improved the separation of the higher
molecular weight RNAs and a series of seven virus-specific
RNAs could be detected in the polyadenylated RNA. When RNA
from similar cells was labelled in the presence of actino-
mycin D (10 µg/ml) no labelled polyadenylated RNA could be
detected in the uninfected cells, while all the virus-
specific RNAs remained insensitive to the drug (Figure 1).
No virus-specific RNA could be detected in the nuclear
fractions.
The most abundant virus messenger RNA species (RNA
band 6) has been cloned in a bacterial plasmid, and found
to code for the most abundant virus structural protein, the
nucleoprotein (Barrett and Mahy, submitted). When infected
cells were labelled with ^{32}P at various times post infection
(Figure 2) no virus-specific mRNA could be detected up to
12 h.p.i. In more detailed studies on the time of appear-
ance of virus mRNAs (data not shown) virus-specific RNA was
detected from 16 h.p.i. and was maximal at 24 h.p.i. It
can be seen from Figure 2 that while all host cell poly-
adenylated RNA is sensitive to 10 µg/ml actinomycin D, some
host cell non-polyadenylated RNAs are resistant to inhibition
by the drug. These host RNAs are reduced significantly as
infection proceeds, probably due to the cytocidal effect of
the virus. However, in the absence of actinomycin D the host
cells are still capable of significant host mRNA synthesis
at 24 h.p.i. (compare tracks 3 and 4, Figure 2).

VIRUS-SPECIFIC RNAs PRODUCED IN PERSISTENT INFECTIONS

We have established a persistent CDV infection by multiple
undiluted passages of virus in Vero cells and culturing the
surviving cells. The persistently infected cells were
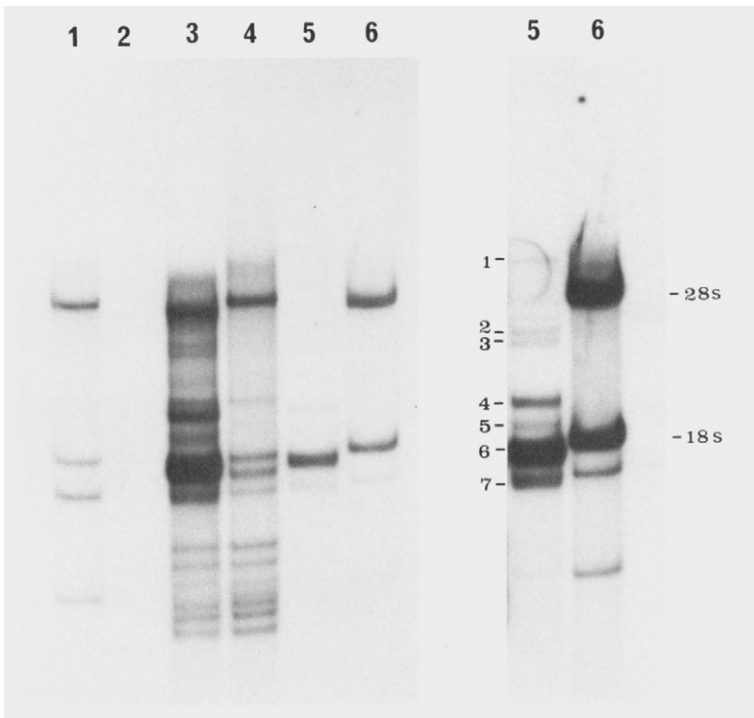
FIGURE 1.   Analysis of ^{32}P-labelled polyadenylated (A+)
and non-polyadenylated (A-) RNA from uninfected and CDV
infected Vero cells.   Cells were phosphate-starved at 24
h.p.i. for 3 hr and then labelled for 3 hr with ^{32}P-ortho-
phosphate (2 mCi/15 cm dish).   Cytoplasmic and nuclear
fractions were prepared by treating the cells with 1%
Nonidet P40 and 0.2% deoxycholate (3).   RNA was fractionated
into (A-) and (A+) species by oligo(dT) cellulose chroma-
tography (4).   RNA samples were denatured in 50% formamide,
2M formaldehyde and 1 x MOPS (20 mM MOPS, 5 mM sodium
acetate, 1 mM EDTA, pH 7.0) for 5 min at 60% and separated
by electrophoresis on a 1.5% agarose (HGT) gel containing
2M formaldehyde and 1 x MOPS at 50 mA for 2 hr (5).   The
gel was soaked in 20 x SSC and blotted to nitrocellulose
(6) before exposure to X-ray film.   Where indicated actino-
mycin D (AMD) was added (10 μg/ml) during both the phosphate
starvation and labelling periods.   A longer exposure of
tracks 5 and 6 is shown on the right.   The positions of the
AMD-insensitive RNAs (1-7) and the 28S and 18S ribosomal
RNAs are indicated.

1, Uninfected (A-) RNA; 2, uninfected (A+) RNA+ AMD; 3,
infected (A+) RNA; 4, uninfected (A+) RNA; 5, infected (A+)
RNA + AMD; 6, infected (A-) RNA.
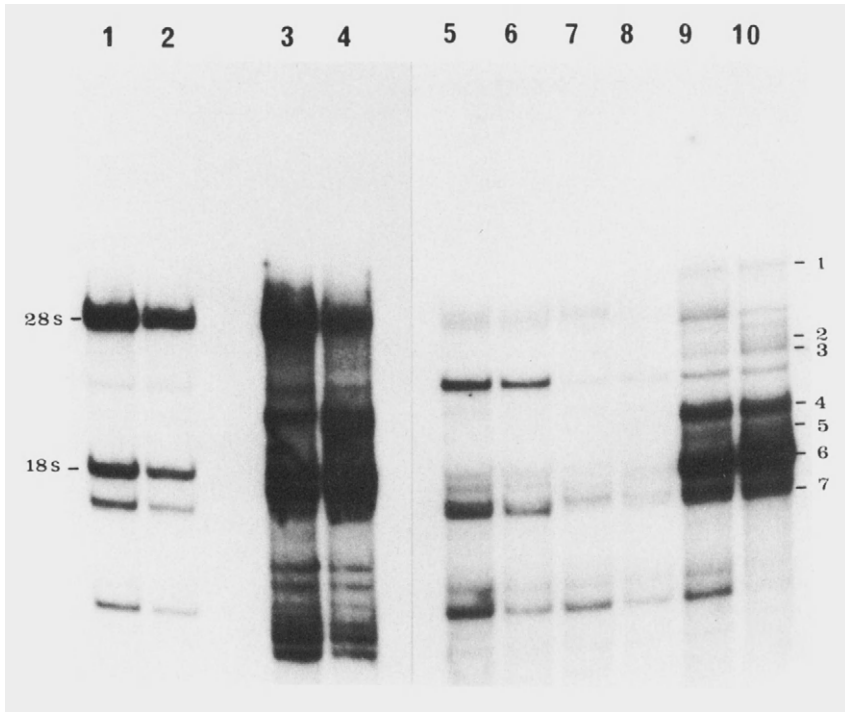
FIGURE 2. Analysis of ^{32}P-labelled RNA isolated from
the cytoplasm of uninfected and CDV infected Vero cells at
various times post infection. RNA was labelled in vivo with
^{32}P and separated by electrophoresis in 1.5% agarose-formalde-
hyde gels as detailed in Figure 1. Where indicated AMD was
present during the 3 hr phosphate starvation period and during
the 3 hr labelling period at 10 μg/ml. The time post
infection indicates the time at which phosphate starvation was
begun. The positions of the 28S and 18S ribosomal RNA
markers and the virus-specific mRNAs (1-7) are indicated at
the sides of the figure.
     Tracks 1 and 2, (A-) RNA; 3 and 4, (A+) from uninfected
and infected cells, respectively. Tracks 5-10 represent
total cytoplasmic RNA labelled in the presence of AMD.
5, uninfected cell RNA; 6-10, infected cell RNA labelled
at 4, 8, 12, 20 and 24 h.p.i., respectively.

resistant to superinfection and over 90% showed virus specific
antigens on immunofluorescence. Electronmicrographs showed
typical virus nucleocapsids in the cytoplasm of persistently
infected cells (Figure 3). Most of the infectious virus
remained cell-associated and was not released into the medium.
In contrast, lytically infected cells release most of the
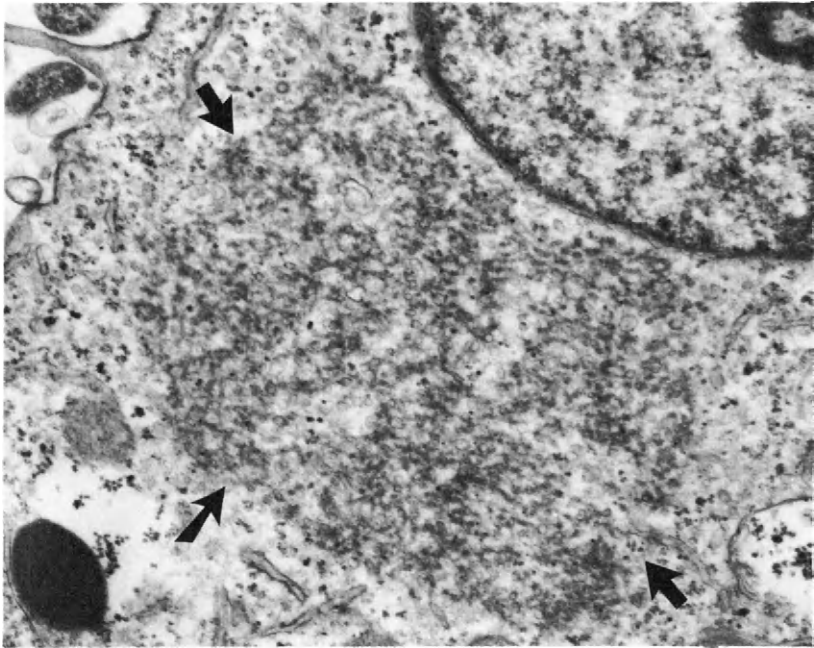
        FIGURE 3. Electronmicrograph of a persistently infected
Vero cell. Arrows show the position of virus nucleocapsids.
Part of the nucleus can be seen in the top right-hand corner.
Magnification x 21,600.

infectious virus into the medium and the cell monolayers
display gross cytopathic effects by 24 h.p.i.
        Figure 4 shows the RNA species detected in persistently
infected cells labelled in the presence of actinomycin D
(10 µg/ml). Four virus-specific bands were observed which
were resistant to DNase₁ digestion. Only one RNA (RNA 3)
corresponded in size to a virus-specific mRNA detected in
lytic infections i.e. to mRNA 6, the mRNA for the virus nucleo-
protein (Barrett and Mahy, submitted). The other three RNAs
did not correspond to any virus-specific mRNA nor to any host
cell RNA resistant to actinomycin D.
        To determine whether or not RNA 3 from persistently
infected cells was the virus nucleoprotein mRNA, unlabelled
RNA was hybridised with ³²P-labelled plasmid DNA containing
a CDV nucleoprotein-specific insert. This DNA hybridised with
two DNase-resistant bands, one of which (number 2) co-migrated
exactly with nucleocapsid mRNA from lytically infected cells
(Figure 4). The second band of higher molecular weight did
not correspond in molecular weight with any of the RNAs
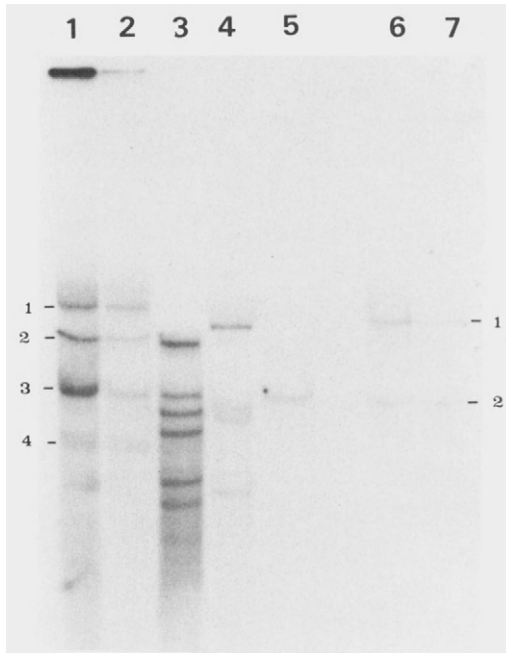
FIGURE 4. Analysis of RNA from Vero cells persistently
infected with CDV. The cells were phosphate starved for 3 hr
prior to labelling with ^{32}P orthophosphate (2 mCi/15 cm dish).
AMD (10 µg/ml) was present during the 3 hr starvation period
and during the 3 hr labelling period. Total nucleic acid was
extracted from the cells by digesting the cell monolayers with
5 ml pronase (0.5 mg/ml) in 50 mM Tris HCl, pH 7.5, 25 mM Na
Cl, 5 mM EDTA, 0.25% SDS at 37^O for 1 hr. Immediately after
addition of the pronase the viscous cell extract was passed
several times through a syringe needle to shear the DNA.
Nucleic acid was then purified by phenol : chloroform (1:1)
extraction. In some cases the DNA was digested with $DNase_1$
(50 µg) in 2.0 ml 100 mM NaCl, 15 mM $MgCl_2$, 100 mM Tris HCl,
pH 7.4, for 5 min at room temperature. RNA was then purified
by a second phenol : chloroform extraction. Unlabelled
nucleic acid from persistently infected cells was also run on
the same gel (tracks 6 and 7). These tracks were cut from
the nitrocellulose sheet and hybridised (6) with ^{32}P nick-
translated plasmid DNA containing a virus nucleoprotein
gene-specific insert (Barrett and Mahy, submitted). The most
abundant virus-specific mRNA detected in lytic infections
(track 5), which codes for the virus nucleoprotein, can be
seen to co-migrate with RNA3 from persistent infections
(tracks 1 and 2). The nucleoprotein clone (224) hybridises
with an RNA of the same size in (tracks 6 and 7)   continued.

observed in either lytically or persistently infected cells
labelled in vivo. It is not clear if this band represents
a true virus-specific RNA or is an artefact due to aggre-
gation, since a similar high molecular weight band hybridised
when the nucleoprotein clone was hybridised to (A+) RNA from
lytically infected cells. No hybridisation was detected with
(A+) RNA from uninfected cells, however. Table 1 lists the
molecular weights of the virus-specific RNAs detected in
lytic and persistent virus-infected cells.

TABLE 1

MOLECULAR WEIGHTS OF VIRUS-SPECIFIC RNAs DETECTED IN LYTIC
AND PERSISTENT INFECTION OF CANINE DISTEMPER VIRUS

RNA no.	1	2	3	4	5	6	7
Lytic	.96	.78	.76	.64	.58	.50	.43
RNA No.	1	-	2	-	-	3	4
Persistent	.84	-	.70	-	-	.50	.39

RNAs were labelled with ^{32}P-orthophosphate in the
presence of 10 µg/ml actinomycin D. Molecular weights
(x 10^{-6}) were determined from their migration in agarose-
formaldehyde gels by comparison with influenza A virus
(fowl plague) RNA markers of known nucleotide composi-
tion (7).

CONCLUSIONS

RNA transcribed in lytic and persistent infections with
CDV was analysed by electrophoresis in denaturing agarose-
formaldehyde gels. Seven virus-specific polyadenylated RNAs
(i.e. mRNAs) could be detected from 16 h.p.i. and synthesis

Figure 4 continued

Track 1, persistently infected cell RNA; 2, as 1 but
treated with DNase1; influenza A (fowl plague) RNA markers;
4, total cytoplasmic RNA from uninfected cells labelled in
the presence of AMD; 5, total cytoplasmic RNA from lytically
infected cells (24 h.p.i.) labelled in the presence of AMD;
6, unlabelled nucleic acid from persistently infected cells
hybridised with ^{32}P-clone 224 DNA; 7, as 6 but treated with
DNase1 before electrophoresis.

was maximal at 24 h.p.i. No virus-specific RNA synthesis
could be detected in the nuclei of infected cells, and it is
probable that the cytoplasm is the site of all virus RNA
synthesis. Four virus-specific RNAs could be detected in
persistently infected cells, only one of which corresponded
in molecular weight to a virus-specific RNA, the nucleo-
protein mRNA from lytic infections. The identity of this RNA
in persistent infections was confirmed using a plasmid DNA
containing a CDV nucleoprotein gene insert as a hybridisation
probe. The function of the other virus-specific RNAs in
persistently infected cells is unknown. However, aberrant
RNAs of similar size have previously been detected in Vero
cells persistently infected with SSPE (8). The generation of
these unusual subgenomic RNAs in CDV persistent infection is
under investigation.

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RNA-DEPENDENT RNA POLYMERASE ASSOCIATED
WITH RESPIRATORY SYNCYTIAL VIRUS

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Respiratory syncytial virus (RSV), initially isolated by
Chanock et al. (1) is a large pleomorphic, enveloped virus
which resembles paramyxoviruses in general morphology and
size (2-4). The absence of detectable hemagglutinating and
neuraminidase activities in RSV envelope glycoproteins has
resulted in the classification of this virus in a separate
genus, Pneumovirus, within the Paramyxoviridae family (5).
RSV contains a negative-strand RNA genome of approximately
5×10^6 daltons (6, 7) that has the capacity to code for at
least 9 distinct complementary mRNAs (6-8). The formation of
viral mRNAs in infected cells is the first biosynthetic reac-
tion in the virus replication cycle, suggesting that the virus
contains a RNA-dependent RNA polymerase (transcriptase). The
purpose of the present investigation was to demonstrate
whether a transcriptase is associated with purified RS virions
and to determine the optimal conditions for its expression
in vitro.

IN VITRO REACTION

Conditions for Activity. Human RS virus (Long strain)
was used throughout this study. Virus was grown and purified
as previously described (6) and further concentrated by sedi-
mentation onto a 60% sucrose cushion. Sucrose, an inhibitor
of transcriptase activity, was removed by chromatography of
the virus on a short column of Sephadex G-100 equilibrated
with STE (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.9, 1 mM EDTA).
Results in Table 1 indicate that a transcriptase was pre-
sent in purified preparations of RSV and define the conditions
required for its in vitro activity. The data were obtained
with [^{3}H]UTP as radioactive substrate and activity was meas-
ured as the incorporation of label into acid-precipitable pro-
duct. Optimum activity was obtained at pH 7.9 and 30°. The
reaction required both mono- and divalent cations. Sodium
or potassium ions were equally effective at concentrations of

TABLE 1

OPTIMUM CONDITIONS FOR RSV IN VITRO TRANSCRIPTION[a]

Conditions	Fresh virus[b] pmols incorp.		Aged virus[b] pmols incorp.	
Complete	12.3	(100)[c]	69.8	(100)[c]
-lysolecithin	4.7	(38)	44.6	(64)
$-MgCl_2$; $+MnCl_2$	3.2	(26)	19.5	(28)
-CTP, GTP, ATP	9.6	(78)	51.8	(74)
+DTT (4mM)	1.8	(15)	67.4	(97)
+RNase (50 μg)	1.6	(13)	10.4	(15)
+DNase (50 μg)	11.1	(90)	65.6	(94)
+Act. D (10 μg)	11.8	(96)	66.3	(95)

[a] Assays in an incubation volume of 0.25 ml contained: Tris-HCl, pH 7.9, 60 mM; GTP and CTP, 0.83 mM; ATP, 1.66 mM; NaCl, 120 mM; $MgCl_2$, 8 mM; lysolecithin, 0.04%. When substituted for $MgCl_2$, the $MnCl_2$ concentration was 2 mM. The labeled substrate, [^{3}H]UTP, was present at a concentration of 0.10 mM and contained 200 cpm per pmol. Incubations were for 60 min. at 30°.

[b] Virus concentration was 9.2 μg of viral protein assayed shortly after preparation (fresh) of after 6 weeks of storage (aged).

[c] Values in parentheses represent percentages of the complete reaction.

120 mM. Magnesium appeared to be the divalent cation of choice at an optimum concentration of 8 mM. Substitution of magnesium by manganese ions at concentrations of 2 mM or higher resulted in marked inhibition of activity. The reaction was not sensitive to DNase or actinomycin D but was greatly inhibited by the addition of RNase.

Although the conditions were in general similar to those reported for other negative-strand RNA viruses, the RSV system did show some anomalies. Dependence of the reaction on detergent was incomplete. This was most probably due to the virus purification procedure which includes a sonication step to remove contaminating cellular vesicles (6). It is likely that sonication partially disrupted some virus particles allowing transcriptase activity to be expressed in the absence of detergent. The activity was also not greatly depressed by the omission of unlabeled nucleoside triphosphates. Similar results were obtained when CTP or ATP were used as labeled substrates (data not shown). There is precedent for both lack of dependence on detergent (9, 10) and nucleotides (9, 11, 12)

in other viral systems.

A more unusual finding was made when the effect of the reducing agent, dithiothreitol (DTT), on the RSV transcriptase was tested. In contrast to results obtained with transcriptases of other negative-strand RNA viruses (10, 12, 13) which showed at least partial dependence on DTT, the activity associated with fresh preparations of RSV was inhibited 85% by 4 mM DTT. Lower concentrations of DTT (1 to 3 mM) were also found to inhibit the reaction substantially (at least 75%).

Another surprising finding was made when the activity of aged virus was tested. It was observed that transcriptase activity of virus stored at 4° for several weeks increased 5 to 6-fold (Table 1). This was a consistent finding made with all virus preparations. In contrast to fresh virus, the activity of aged virus was neither inhibited nor stimulated by DTT. It was also observed that dependence of the reaction on detergent was decreased. Dependence on nucleotides, mono- and divalent cations as well as sensitivity to RNase of both fresh and aged virus preparations were entirely similar. The reasons for the increased activity of aged virus and for the different responses to DTT and detergent are not presently understood.

Dependence of Activity on Virus Concentration and Time. Figure 1 shows that transcriptase activity of aged virus was not linearly dependent on virus concentration. Increasing viral protein from 1.84 to 3.68 μg resulted in only a 1.47-fold increase in RNA formation. A pattern of progressively lower amounts of product synthesis over the expected was observed when viral protein was increased to 51.52 μg, suggesting the presence of an inhibitor of transcription. Inhibition was most evident when fresh virus was tested. A 10-fold increase in viral protein (1.67 to 16.7 μg) resulted in only a 1.2-fold increase in product formation. Moreover, 20 to 30-fold increases in concentration resulted in inhibition of RNA synthesis (data not shown). These results suggest that the putative inhibitor was partially inactivated during storage of the virus.

Dependence of the activity of aged virus on reaction time is shown in Figure 2. The rate of the reaction was linear for the first 30 min. and then decreased. Synthesis continued at a decreasing rate for 5 hours. Beyond 5 hours the amount of acid-precipitable product slowly declined (data not shown) suggesting breakdown probably caused by RNase contamination of the system. With the exception that less product was formed, fresh virus showed essentially the same time course (data not shown).
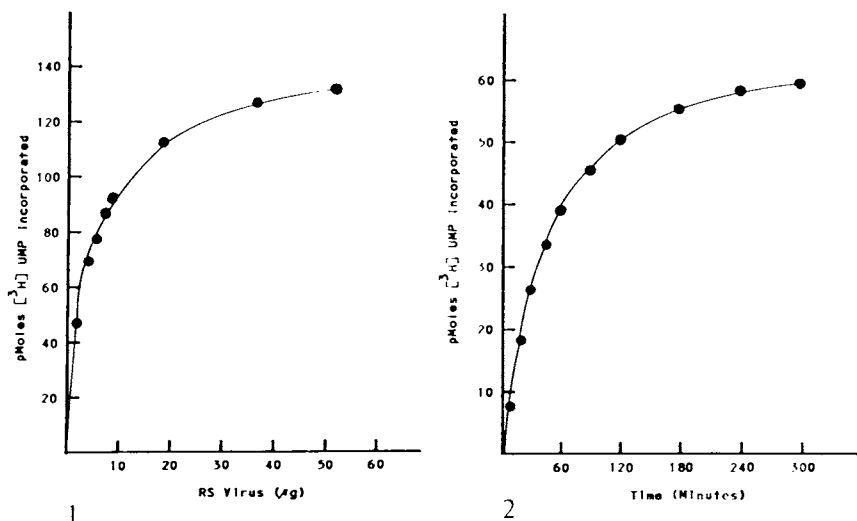
FIGURE 1 (Left). Dependence of transcriptase activity on virus concentration. Reaction volumes of 0.25 ml containing from 1.84 to 51.52 µg of viral protein were prepared (see legend to Table 1). After a 2 hour incubation, RNA was acid-precipitated, filtered and radioactivity determined.

FIGURE 2 (Right). Dependence of transcriptase activity on incubation time. Reaction volumes of 1.5 ml containing 55.2 µg of viral protein were prepared (see legend to Table 1). Reactions were started (0 time) by the addition of ^{3}H-UTP (200 cpm/pmol). At the indicated times 100 µl aliquots were withdrawn, added to 0.2 ml of stopping mixture, and RNA acid-precipitated, filtered, and radioactivity determined.

CHARACTERISTICS OF SYNTHESIZED RNA

Polyadenylation. In order to determine whether in vitro synthesized RNA had the properties expected for viral mRNAs, several characteristics of the RNA were examined. First, polyadenylation of the product was analyzed by affinity chromatography. In vitro formed RNA labeled with [^{3}H]UMP was phenol-extracted and applied to [oligo-dT] cellulose columns. In the several experiments carried out about 50% of the product was bound by the columns, indicating the presence of poly A tracts. After elution of the bound RNA and rechromatography on a second affinity column, about 97% of the RNA was again bound, confirming that 50% of the total synthesized RNA was polyadenylated (poly A+).

Sedimentation. In vitro synthesized RNA was separated into poly A+ and poly A- RNA by chromatography on [oligo-dT]

cellulose. Each species of RNA was then sedimented on linear
sucrose gradients. As shown in Figure 3A, about 40% of the
poly A+ RNA sedimented at 13-25S, approximately the size ex-
pected for some RSV mRNAs. The remaining RNA was smaller (4-
7S). All of the product was at least 99% sensitive to RNase
digestion. Poly A- RNAs gave essentially the same profile
with the exception that a small peak, representing about 10%
of the total, sedimented at 30-35S (Figure 3, Right). This
faster sedimenting RNA was relatively resistant to RNase,
suggesting the presence of transcriptive intermediates that
were not dissociated from template RNA.
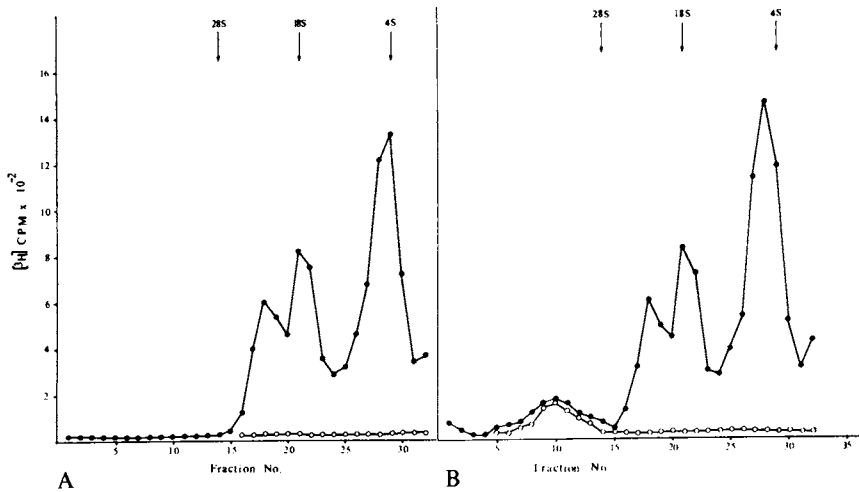
FIGURE 3. Sedimentation analysis of in vitro synthe-
sized RNA. [^{3}H]UMP-labeled product formed in a 2.5 ml reac-
tion volume (see legend to Table 1) containing 92 µg of viral
protein and incubated for 2 hours was separated into poly A+
and poly A- RNAs by [oligo-dT] cellulose chromatography.
After ethanol precipitation the RNAs were dissolved in H_2O,
heat denatured and centrifuged on 32 ml sucrose gradients (5-
20% sucrose w/v in STE, pH 4.9). Centrifugation was at
20,000 rpm, 4° for 17 hours in a Beckman SW28 rotor. Size
markers, 4, 18 and 28S RNAs, were centrifuged on a separate
gradient. Fractions of 1 ml were divided into equal aliquots.
One set was digested with RNases A and T$_1$ (1 and 10 µg/ml,
respectively) for 30 min. at 37°. Aliquots were then acid-
precipitated, filtered and radioactivity determined. A (Left):
poly A+ RNA, ●-●, no RNase, 0-0 , RNase treated; B (Right):
poly A- RNA.

TABLE 2
ANNEALING OF UNLABELED 50S VIRION RNA TO
[3]H-URIDINE LABELED IN VITRO POLY A+ RNAs

Annealing Mixture (μl)[a]		cpm[b]		
		No RNase	With RNase	% Resistant
poly A+ RNA	(5)	809	473	58
poly A+ RNA	(10)	799	479	60
poly A+ RNA	(15)	736	478	65
Self-annealing		806	16	2

[a]Varying amounts (μl in parenthesis) of unlabeled geno-
mic 50S RNA extracted from purified virions were mixed with
constant amounts of [^{3}H]UMP labeled poly A+ RNA (1800 cpm)
synthesized in vitro. RNA samples in 0.1 ml H_2O were dena-
tured by heating at 100° for 1 minute and cooled by immersing
in ice water. Samples were made 2 X SSC and 50% formamide
and annealed as described (6). Each sample was then divided
into two equal portions, one set of which was digested with a
mixture of RNases A and T_1 (1 and 10 μg/ml, respectively) at
37°C for 30 minutes. Acid precipitable radioactivity of each
sample was then determined.
[b]Values represent the average of two experiments.

Annealing. Complementarity of in vitro synthesized RNA
to RSV genomic 50S RNA was investigated by hybridization.
Constant amounts of poly A+ RNA were annealed to increasing
amounts of 50S RNA. Results in Table 2 show that self-anneal-
ing of poly A+ RNA resulted in only a 2% resistance to RNase
digestion indicating that the product was virtually free of
contaminating 50S RNA. In contrast, annealing in the presence
of RSV 50S RNA increased RNase resistance to 65%. Failure to
obtain a value closer to 100% may have been due to insuffi-
cient amounts of 50S RNA or to the presence of substantial
amounts of small RNAs in the product.

CONCLUSION

The data presented here strongly suggest that RSV con-
tains RNA-dependent and poly A polymerase activities for syn-
thesis of viral mRNAs. Definitive proof for this conclusion
depends on obtaining an in vitro system free of contaminating
RNase and a demonstration that in vitro formed poly A+ RNAs
are of the same size as in vivo synthesized RSV mRNAs and
functional in cell-free translation. Studies to obtain this
evidence are currently in progress.

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THE EFFECTS OF INTERFERON ON MEASLES VIRUS RNA SYNTHESIS

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We have been interested in the effect of interferon (IFN)
on measles RNA synthesis in several different systems.
Earlier studies examining the effect of IFN on negative strand
viruses focused on action at the translational or post-
translational level (1-5); the studies described here examine
effects at the transcriptional level.

MEASLES INFECTED LYMPHOCYTES

One of the cell-virus systems studied here was human
peripheral blood mononuclear cells infected with measles
virus. Earlier work suggested that this infection could be
induced to change from persistent to productive when IFN-alpha
was removed (6). We have confirmed those data, and have
further examined the effects of IFN-alpha on accumulation of
measles virus specific RNA in these cells.
Table 1 shows that there is a major effect on the
accumulation of 50s RNA when IFN is present, correlating with
the loss of virus production and a change to a persistent
infection. Thus, when the infection becomes productive, after
addition of phytohemagglutinin (PHA) (exp. 3) or addition of
anti-IFN-alpha, (exp. 8-10), cells show fluorescence with
measles positive serum, and 50s measles specific RNA accumu-
lates. Under these growth conditions, IFN concentration in
the cell media (columns 4 and 5) is reduced to 50 to 625 U/ml
compared to 3000 U/ml. In the presence of anti-measles serum
(exp. 4) FA is negative since virus cannot bud from cells in
the presence of antibody (Ab); however, 50s RNA does accumu-
late in the cells and IFN is reduced. The control (exp. 5),
using measles-negative serum, resembles the persistent infec-
tion (exp. 2). Moreover, the addition of exogenous IFN can
overcome the effect of PHA (exp. 6) and, to some extent,
that of anti-measles Ab (exp. 7). In contrast, there seemed

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TABLE 1

EFFECTS OF TREATMENTS WHICH AFFECT INTERFERON LEVELS
ON MEASLES VIRUS REPLICATION IN HUMAN LYMPHOCYTES[a]

Treatment	FA[b]	50s RNA	IFN	
			After Treatment	At Harvest
1. Uninfected	−	−	125	Less than 5
2. Infected	±	−	3125	1250
3. ___, + PHA[c]	+	+	625	125
4. ___, + Anti-measles Ab after 3 hours	−	+	25	625
5. ___, + Measles-neg Serum after 3 hours	−	−	1000	1000
6. ___, + PHA + 1000 U/ml IFN-beta day 4	−	−	1000	250
7. ___, + Anti-measles Ab after 3 hours + 1000 U/ml IFN-beta day 4	±	+	125	3125
8. ___, + Anti-IFN after 3 hours	+	+	250	125
9. ___, + Anti-IFN after 3 hours + PHA day 4	+	+	50	625
10. ___, + Anti-IFN + PHA both on day 4	±	+	625	125

a Exogenously added IFN was interferon-beta or interferon-alpha, which gave comparable results. Anti-interferon was directed against interferon-alpha.
    b Immunofluorescence, unfixed cells.
    c Phytohemagglutinin.

to be no qualitative effect on measles mRNA synthesis, though
its quantity was decreased following IFN treatment (not
shown).

However, because the IFN-treated lymphocytes seem to have
additional low molecular weight measles RNA instead of 50s, it
is possible that these observations reflect increased break-
down of measles 50s RNA rather than lack of synthesis. This
would be consistent with what is known of the antiviral action
of IFN, and its action in induction of a ribonuclease (7-11).
Studies using monoclonal antibodies specific for components of
the measles nucleocapsid suggested that measles nucleocapsids
also do not accumulate in these cells (11).

MEASLES INFECTED VERO CELLS

In an attempt to generalize these findings to a simpler
system, we examined the effect of IFN on measles virus RNA
synthesis in Vero cells, a cell type which produces only low
levels of endogenous IFN. Interferon-beta was used for all
Vero cell studies. Added IFN at more than 25 U/ml caused a
substantial decrease in released virus amounting to 3 to 5
logarithmic units. However, there was no decrease in 50s RNA
synthesis when IFN treatment was used (12). Thus the effect
of IFN in this system was not analogous to that in
lymphocytes.

An examination of mRNAs from Vero cells showed that there
was actually an increase in ^{3}H-uridine-labeled mRNA in the
presence of IFN especially in mRNA of 16s or less. However,
when this mRNA was used to direct in vitro translation, as
shown in Figure 1, it was not translated into measles specific
proteins at a level commensurate with its concentration,
except for a polypeptide coelectrophoresing with measles M
protein. If in fact this mRNA represents measles specific
RNA, why is it not translated?

To answer this, we first examined polysomes from infected
cells and found that after treatment with 250 U/ml of IFN the
polysome size was smaller, 151s compared to 175s. Ribosome
binding of mRNA after incubation in an in vitro translation
assay for various times and separation in high salt sucrose
gradients showed, in addition, that polysome formation, which
was complete using untreated mRNAs, was not begun in IFN-
treated RNAs after 5 minutes incubation. However, binding did
occur within a short period of additional incubation. Thus,
the kinetics of polysome formation differs in IFN-treated and
untreated cells.

TABLE 2
THE EFFECT OF INTERFERON ON CAPPING AND
TRANSLATION OF MEASLES mRNA

	mRNA	Translation	Cap
NO IFN	22s	NP, P, F_1, some M	ND^a
	16–20s	NP, P, F_1, some M	0, II
	12s	no translation	ND
+ IFN	22s	no translation	ND
	16–20s	no translation	no cap
	12s	M, some NP	I, II

^{a}ND = not done.
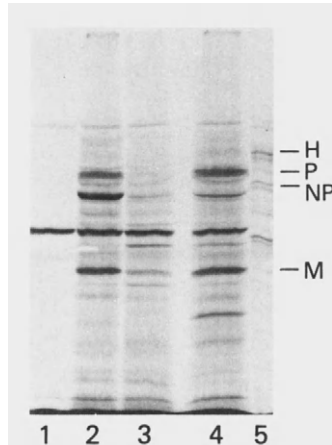
FIGURE 1. In vitro translation of measles mRNA from
untreated and IFN-treated Vero cells. Products of an in vitro
reticulocyte translation were autoradiographed after
separation on a 10% acrylamide gel run in Laemmli buffer (13).
Lane 1. Endogenous translation, no added mRNA. Lane 2.
Translation of measles mRNA from untreated Vero cells. Lane
3. Translation of measles mRNA from IFN-treated Vero cells
(250 units/ml). Lane 4. Translation of control measles mRNA.
Lane 5. Measles polypeptides labeled in Vero cell culture.

Attempts to characterize the mRNAs for differences with
or without IFN treatment were substantially negative. There
was no substantial difference in percentage of polyadenyla-
tion, or in methylation and capping. Examination of the cap
by DEAE-cellulose ion exchange column chromatography after
alkaline phosphatase treatment revealed no aberrant structures
at the 5' end as a result of IFN treatment. There was,
however, a small difference in the percentage of mRNAs having
5' terminal cap I and cap II structures. When mRNAs were
fractionated according to size and examined for the presence
of cap and for the ability to direct translation in the
reticulocyte lysate system, the smaller mRNAs existing after
IFN treatment appeared to have caps and be translatable, while
in untreated cells it was the 16-20s mRNAs which were capped
and translatable as seen in Table 2. Thus, with IFN treatment
of infected cells it appears that the functional mRNAs which
accumulate are smaller than those in untreated cells.

IN VITRO STUDIES

We next explored whether this difference was a property
of the in vitro polymerase or, alternatively, a posttrans-
criptional modification of mRNA. To examine this, we studied
in vitro RNA synthesis using purified measles virus nucleo-
capsids. We first examined total in vitro polymerase activity
in measles virus infected Vero cells as a function of
exogenously added IFN. Cells were treated with IFN 24 hours
before infection, and virus and viral nucleocapsids were
harvested from the post-nuclear supernatant, centrifuged on
isopycnic sucrose-D_2O gradients, and assayed for polymerase
activity across the gradient. As Figure 2 shows, the total
amount of polymerase activity is about the same with and
without IFN treatment; however, after IFN treatment a larger
proportion of the activity is associated with nucleocapsids
than with complete virions, a reflection of a decrease of
intact infectious virions following IFN treatment. When the
products of in vitro transcription were analyzed on sucrose
velocity gradients, no difference was seen with or without IFN
treatment. Similarly, IFN addition to the in vitro RNA
polymerase reaction has no effect on product yield or
characteristics. When 2.8 nM to 2.8 uM of oligo (2'-5') A, a
product which can induce the effect of interferon in intact
cells, was added to the in vitro transcription reaction, no
effect was seen on the size of the RNA products or the extent
of polyadenylation.
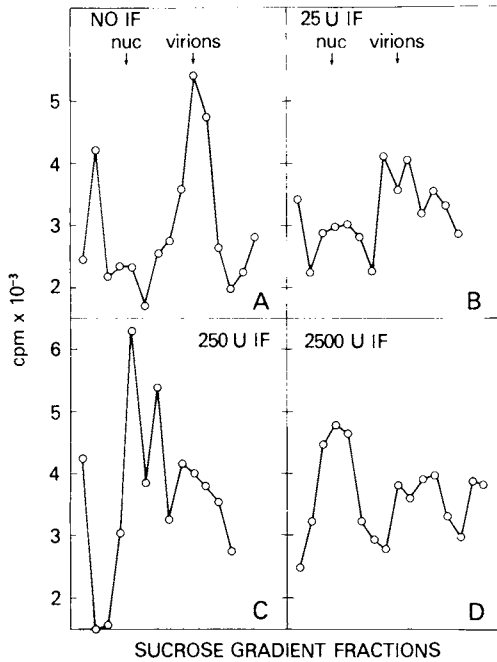
FIGURE 2. The effect of interferon on transcriptase
activity of virions. Virions harvested from measles infected
Vero cells pretreated with (A) 0, (B) 25 units/ml, (C) 250
units/ml, and (D) 2500 units/ml IFN-beta, were purified on
15-65% sucrose in D_2O gradients, and gradient fractions were
assayed for polymerase activity by incorporation of
radioactivity (14).

OVERALL EFFECTS OF INTERFERON

Thus, we conclude that the effect of IFN on measles RNA
synthesis is probably not at the level of transcription.
Instead, it appears to occur posttranscriptionally, perhaps
involving RNA stability. In IFN-treated measles infected Vero
cells, those mRNAs containing methylated caps at the 5' end
appear to be a smaller size than those in untreated infected
cells. In IFN-treated cells, the larger mRNAs, of the size of
normal measles mRNAs, appear defective in their caps, in their
ability to bind to ribosomes, and in their ability to be
translated. There is an increased amount of 12s mRNA, which
contains a cap and is translatable in vitro to a product

coelectrophoresing with measles M protein. Increased RNA
breakdown mediated by an induced endonuclease is consistent
with known IFN effects (6,7,8,9), but to our knowledge,
differential susceptibility of mRNAs to the endonuclease has
not been reported. Our data do not support the notion of
aberrant ribosome binding due to aberrant cap structure after
IFN treatment; rather, when the cap is absent, there appears
to be no ribosome binding, while when the cap is present, it
appears to be comparable to that found in a normal infection,
and ribosome binding and translation occur.

     In the lymphocyte system, on the other hand, the RNA
species which is unstable is the genomic RNA; mRNAs are made
and are translatable into measles protein in vitro and in
infected cells. It is our interpretation that the inability
to accumulate 50s genomic RNA is a reflection of inhibition of
virus budding in lymphocytes when IFN-alpha is induced, which
in turn leads to disassembly and breakdown of viral
nucleocapsids.

     We have examined RNA synthesis in three different measles
RNA synthesizing systems and we have found that in vitro,
there is no effect of interferon or of interferon induced
products, such as oligo A, suggesting that association with
cellular components may be needed to produce an effect. In
Vero cells, where little endogenous interferon is produced, we
have seen that IFN results in a preponderance of lower
molecular weight mRNAs which appear to be nonuniformly capped.
This results in a differential effect on translatability of
the message, probably due to methylation and capping. In
lymphocytes, which do not normally support measles infection,
the presence of interferon interferes with the accumulation of
50s genomic RNA.

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CONSTRUCTION AND EXPRESSION OF A CHIMERIC GENE OF GLYCOPROTEIN G AND MATRIX PROTEIN M OF VESICULAR STOMATITIS VIRUS[1]

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The information necessary for the synthesis, transport, and modifications of secretory and membrane proteins is believed to be contained in discrete regions or domains of the polypeptide which are decoded by specialized mechanisms in the cell (1). These domains, or 'topogenic sequences' (2) which can be a transient or permanent feature of the polypeptide, include the signal sequence which initiates the translocation of proteins into and across specific membranes, the stop transfer sequence which in the case of transmembrane proteins serves to interrupt the signal sequence initiated translocation and insures a proper asymmetric orientation of the polypeptide and sorting sequences which serve to target the newly synthesized polypeptide along selected subcellular pathways to its site of function. The glycoprotein G of vesicular stomatitis virus contains a transient amino terminal hydrophobic signal sequence which is involved in translocation of the polypeptide across the endoplasmic reticulum as well as a hydrophobic COOH-terminal domain which anchors the polypeptide in the envelope of the virions (3,4,5). In order to examine the basis for the specificity and function of these hydrophobic domains, we have constructed a chimeric gene containing the signal sequence coding region of G protein fused to the bulk of the coding sequence of M protein. The fusion of the signal sequence coding region of the G protein to a protein that does not normally transverse membrane bilayers and its expression in mammalian cells would indicate whether or not the signal sequence is sufficient to initiate and maintain vectorial translocation across the membrane.

The predicted amino acid sequence of M (6) reveals the presence of a sequence of the type Asn-X-Ser/Thr, which is a concensus sequence for glycosylation (7).   In M protein however, this target site is not glycosylated.   The attachment of a signal sequence to the M protein may insert it into the RER and thus could allow glycosylation in this site.
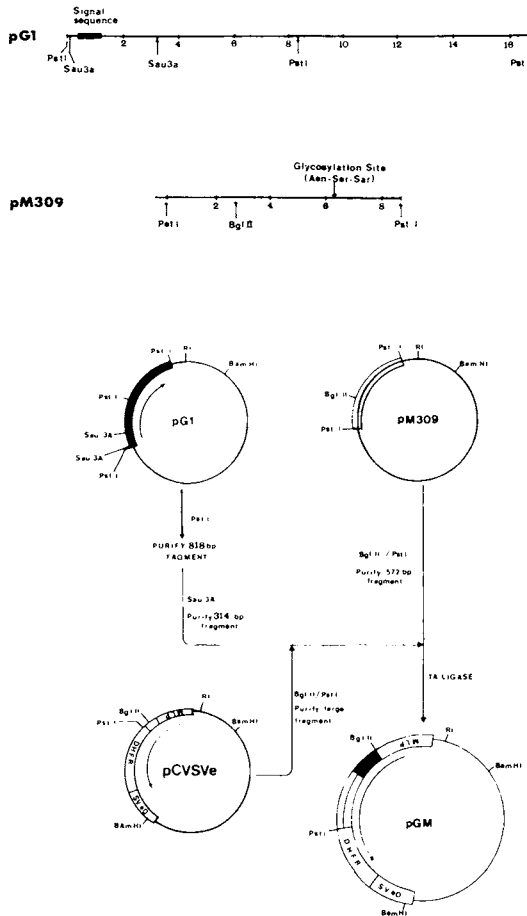
FIGURE 1.   Strategy used for the construction and cloning of a G-M chimeric gene.   The map coordinates for pG1 and pM-309 are from Rose and Gallione (6).   All DNA fragments were purified by gel electrophoresis prior to ligation.   MLP, adenovirus major late promoter, DHFR, cDNA sequence coding for the dihydrofolate reductase gene.

CONSTRUCTION OF CHIMERIC GENE

    The G/M chimeric gene was constructed from the recombin-
ant plasmids pGl and pM309 containing cDNA copies of the com-
plete coding sequences of the mRNAs specific for G and M pro-
tein, respectively (6) (kind gift of Dr. Jack Rose).
    pGl contains Sau3A sites at nucleotide positions 10 and
324. Thus this restriction fragment would contain the entire
signal sequence coding region as well as the 5' untranslated
region required for ribosome binding. This fragment would
also contain sequences coding for the first 82 amino acids of
the mature G protein. pM309 contains a unique Bgl II site
located at nucleotide position 259. Ligation of the Sau3A
pGl fragment with Bgl II cut pM309 would produce an in phase
hybrid gene containing the G specified sequences mentioned
above as well as sequences coding for the 157 carboxy
terminal amino acids and the entire 3' untranslated region of
the M gene.
    The 314 bp Sau3A fragment of pGl was obtained by digestion
with Sau3A of the 414 bp fragment generated by digesting pGl
with Pst I. The 572 bp Bgl II/Pst I fragment from pM309 was
obtained by digesting pM309 with these enzymes. These frag-
ments were ligated directly into the mammalian expression
vector pCVSVe (gift of Drs. R. Kaufman and P. Sharp). pCVSVe
contains sequences coding for dihydrofolate reductase and was
used to express this protein in CHO cells (8). This vector
also contains the major late transcription promotor of adeno-
virus, the SV40 72 base pair repeat, and the SV40 poly A site.
pCVSVe contains unique Bgl II and Pst I cleavage sites
suitable for insertion of foreign DNA.
    The fusion of the Sau3A fragment with the Bgl II/Pst I
cut pM309 fragment would generate a product having a Bgl II
cohesive end at the 5' end and a Pst I cohesive end at the 3'
end. Thus cloning the hybrid gene so constructed into Bgl II/
Pst I cut pCVSVe would place the gene in the correct orientat-
ion for transcription just downstream from the regulatory
sequences of the vector. This cloning strategy is summarized
in Fig. 1. Several clones of the expected size (approximately
900 bp larger than Bgl II/Pst cut pCVSVe) were isolated foll-
owing transformation of E. coli LE293. Several of these are
shown in Fig. 2A. Digestion of recombinants containing Bam Hl
show that pGM03, 04 and 07 contain the fused G/M gene while
pGM12 contains just the pM insert (Fig. 2A).
    Fusion of G to M in the correct orientation and ligation
into pCVSVe was determined by digestion of the pGM clones with
Bgl II/Pst I, seen in Fig. 2B. pGM 07, generates a fragment
coincident with the fragment generated from Bgl II/Pst I cut
pM309. pGM03, 04, on the other hand produced a fragment of
lower mobility. Since pGM07 was shown to contain an insert

of the size expected for the G-M hybrid, it must contain the
G specific fragment in the incorrect orientation while pGM03,
04 contain the GM hybrid in the correct orientation.

EXPRESSION OF CHIMERIC GENE IN MAMMALIAN CELLS

pGM03 was introduced into COS-1 cells by the calcium
phosphate transfection procedure (9,10). The cells were
labeled with ^{35}S methionine 40-48 hours after transfection
and cell extracts were prepared. The cell extracts were
immunoprecipitated with anti M antibody and analyzed by SDS-
polyacrylamide gel electrophoresis.
    As shown in Fig. 3, lane h, a protein having an apparent
molecular weight of approximately 36000 was precipitated by
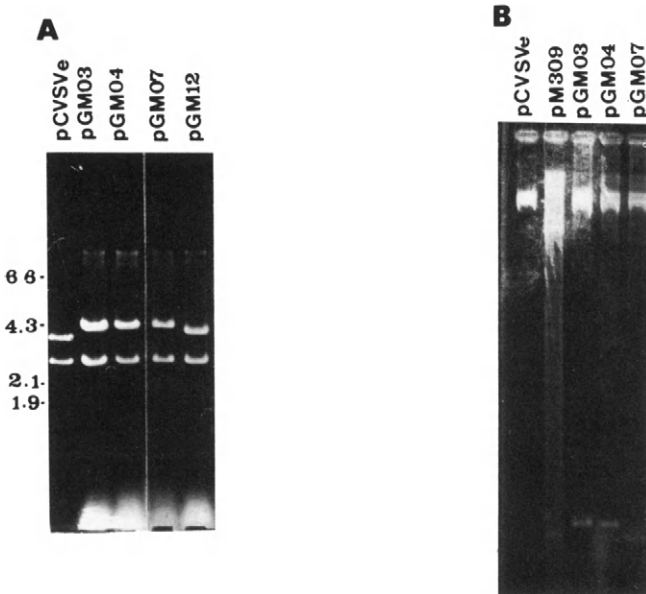
    FIGURE 2. Digestion of recombinant plasmids. Ligated
recombinant plasmids constructed as described in Fig. 1 were
used to transform E. coli LE392 to tetracycline resistance.
Positive colonies were selected at random from the plates,
grown to saturation in 5 ml of L broth containing the select-
ive antibiotic and plasmids purified and digested with Bam HI
(panel A) or with Bgl II and Pst I (panel B). The size
markers (in Kilobase pairs) are from a sample of Hind III
digested λ DNA run in a separate well.
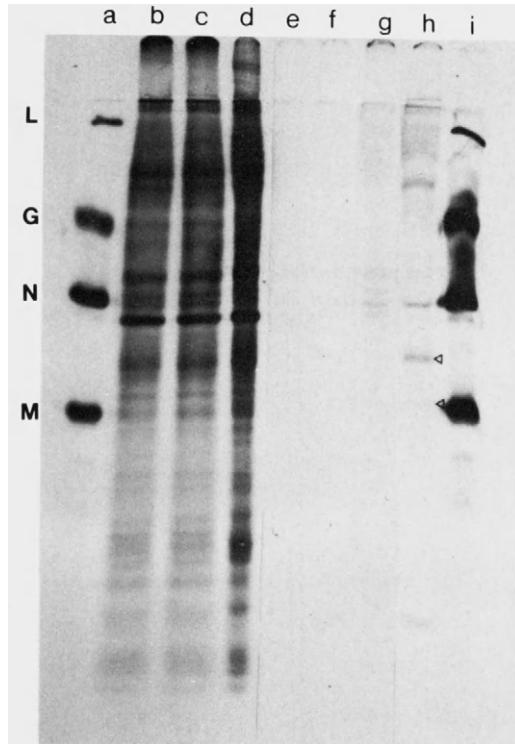
FIGURE 3. Polyacrylamide gel analysis of proteins immuno-
precipitated from COS-1 cells transfected with pCVSVe or pGM-
03. [^{35}S]–methionine labelled cell extracts prepared from COS
-1 cells transfected with pCVSVe or the recombinant plasmid
pGM03 were immunoprecipitated and analyzed on a 12% polyacry-
lamide gel. Lane a, [^{35}S]–methionine labelled VSV; lane b,
sample of whole cell extract from COS-1 cells; lanes c and d,
whole cell extract from COS-1 cells transfected with pCVSVe
and pGM03, respectively; lanes e and f, pCVSVe transfected
cells immunoprecipitated with normal antiserum and anti–M
antiserum, respectively; lanes g and h, pGM03 transfected cells
immunoprecipitated with normal antiserum and anti–M antiserum,
respectively. The open arrowheads indicate two protein
species, have apparent molecular weights of approximately 36 K
and 32 K respectively, specifically immunoprecipitated from
pGM03 transfected cells by anti–M antiserum.

anti-M from pGM03 transfected cells. This protein was not
precipitated from these cells with normal rabbit serum nor
from pCVSVe transfected cells immunoprecipitated with anti-M
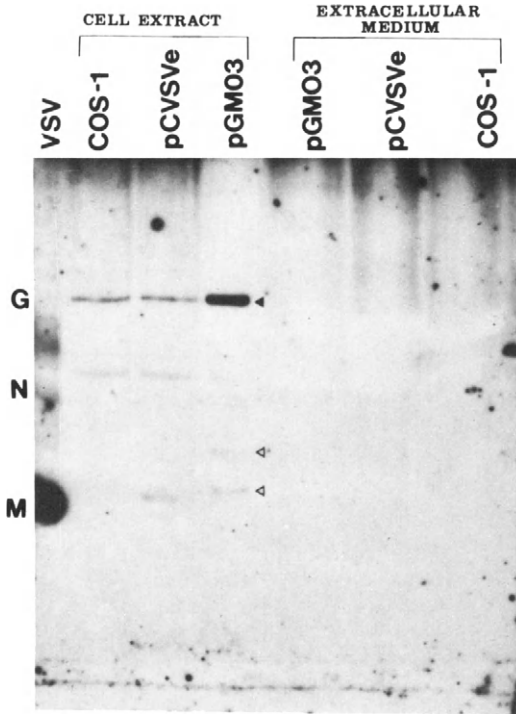
FIGURE 4. Immunoblotting of COS-1 transfected cells with
anti-M antibody. COS-1 cells, grown in 60 mm culture plates
were transfected with pCVSVe or pGM03. 48 hours post-trans-
fection, the cells were collected, washed, and lysed with
sample buffer. One half of each sample was run on a 10% poly-
acrylamide gel. For the extracellular medium, the growth
medium was collected from each plate, precipitated with ace-
tone and the precipitate was collected, washed, and solubil-
ized in sample buffer. One half of the total precipitated
material from each sample was run on the same gel. Following
electrophoresis, the proteins were transferred onto nitro-
cellulose and reacted with anti-M antibody followed by [125]I-
protein A (11). The open arrow heads indicate protein
species reactive with anti-M antibody and only present in the
pGM03 transfected cells.

or normal serum (lanes e and f). Therefore, this protein
appears to be specifically precipitated by anti-M only from
cells containing the chimeric gene. A minor protein having a
molecular weight of approximately 31-32,000 was also
immunoprecipitated by anti-M antibody.
    These results were confirmed by immunoblotting as shown
in Fig. 4, lanes b-d. In this case there were two protein
species having molecular weights of approximately 32,000 and
36,000 immunoreactive with anti M which were present in pGMO3
transfected cells but not present in cells transfected with
pCMSVe or from nontransfected cells. A 80,000D protein
present in pGMO3 transfected cells was also present in the
other cell extracts.

SECRETION OF M DERIVED PROTEINS

    The culture medium was collected from cells 40-48 hrs
following transfection and analyzed by immunoblotting with
anti M antibody. No M specific polypeptides seemed to be
secreted by cells transfected with pGMO3 (Fig. 4, lanes e-g).
    The 36,000D polypeptide immunoprecipitated by antibody
therefore may represent a hybrid protein containing NH_2-
terminal peptide of G fused onto a truncated M protein.
Experiments are in progress to determine if the signal
sequence has been processed and the hybrid protein is gly-
cosylated in vivo.

ACKNOWLEDGEMENTS

    We thank Drs. J. Rose, R. Kaufmann, P. Sharp, L. Prevec
and K. Ghosh for kind help.

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INTRACELLULAR PROCESSING OF VESICULAR STOMATITIS VIRUS AND NEWCASTLE DISEASE VIRUS GLYCOPROTEINS[1]

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Intracellular pathways have evolved to insure that newly made proteins arrive at their proper destinations (1). Plasma membrane glycoproteins follow a pathway that generally involves insertion of nascent polypeptides into the membrane of the rough endoplasmic reticulum (RER) and subsequent transport through the Golgi membranes and finally insertion into the plasma membrane (1,2,3,4).

Perhaps all plasma membrane glycoproteins follow a similar path. By virtue of possessing a signal sequence (2) which insures the proteins' insertion into the membrane of the RER, plasma membrane glycoproteins may enter a membrane flow which ultimately results in insertion into the plasma membrane. However, in polarized cells such as MDCK cells, the glycoprotein (G) of vesicular stomatitis virus (VSV) is inserted on one side of the cell, the basolateral side, while the glycoproteins of paramyxoviruses and myxoviruses are inserted on the apical side of the cell (5,6,7). Such a result argues for two pathways to the cell surface in at least these cells.

We explored the idea that plasma membrane glycoproteins are processed in different ways in non-polarized chicken embryo cells. We reasoned that different pathways may be reflected in different kinetics of transport to the cell surface. We, therefore, compared the transit times of the VSV G protein and the Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) glycoprotein to the Golgi membranes and to the cell surface of chick embryo cells. Our data show that the transit time of the HN protein to the cell surface is much longer than that of the G protein. The difference in the transit time can be accounted for by the time it takes the HN glycoprotein to reach the Golgi membranes.

We first asked how long it takes pulse-labelled
glycoprotein to reach the cell surface. Our assay for
cell surface glycoprotein utilizes antibody binding to
intact cells. Monolayers of infected cells were
pulse-labelled with [^{35}S]-methionine or pulse-labelled
and chased with cold methionine. After various lengths of
chase, washed monolayers were incubated with anti-NDV or
VSV antisera. Molecules on the outside surface of cells
will bind antibody while internal proteins will not.
Unbound antibody is washed away, the cells are lysed and
immunobeads containing goat anti-rabbit IgG used to
precipitate immune complexes. Cell surface molecules will
be precipitated while internal polypeptides including
glycoprotein which has not yet reached the cell surface
will not precipitate.
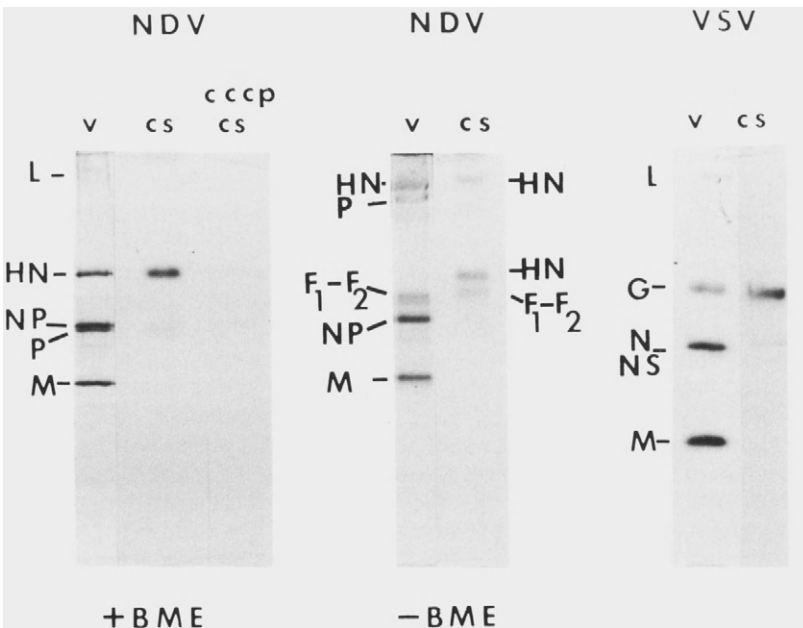
Figure 1: Antibody Binding Assay of Cell Surface
Molecules.
    v = virion proteins, cs = cell surface proteins,
    cccp = carboxyl cyanide m-chlorophenylhydrazone.

Figure 1 shows the result of such an assay. The NDV
HN protein is precipitated after such a procedure, as well
as a polypeptide with a molecular weight of 56,000
daltons. That this polypeptide is the fusion protein is
shown by the fact that it comigrates with the F_1-F_2
complex if electrophoresed under non-reducing (-BME)
conditions. The internal polypeptides, NP, P, and M
proteins, are not precipitated. It should be noted that
while virion-associated HN protein is resolved only as a
multimer seen at the top of the gel if electrophoresed
under non-reducing conditions (8), cell surface HN is seen
in both monomeric and multimeric forms.

That this protocol precipitates only glycoproteins at
the cell surface and not glycoproteins which have not yet
reached the cell surface is shown by the use of CCCP. An
inhibitor of oxidative phosphorylation, CCCP is known to
block intracellular migration of glycoproteins (9,10).
Addition of CCCP to cells just after the pulse-label
blocks the migration of the labelled protein to the cell
surface (9,10). In fact, no NDV proteins are detected by
our assay after CCCP addition.
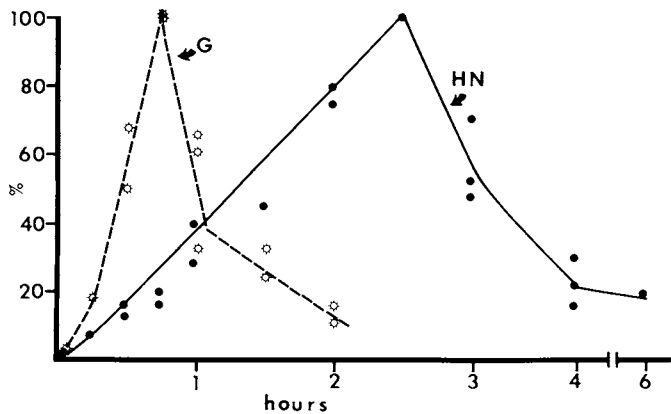
Figure 2: Appearance of Glycoproteins at the Cell
Surface.

Values are expressed as the percentage of maximal
amount precipitated after onset of chase.

     This protocol also results in the precipitation of
only the VSV G protein and not the internal proteins, N,
NS, M and L.
     Using this assay, we were able to follow the kinetics
of HN and G protein at the cell surface. After varying
lengths of chase, pulse-labelled G protein appears at the
cell surface and then disappears as the protein is
incorporated into virions. Note the VSV G enters the
plasma membrane rapidly with a half-time of approximately
27 minutes and exits rapidly as reported previously
(11,12). However, the HN protein enters the plasma
membrane more slowly, with a half-time of approximately 78
minutes, and exits quite slowly. Surface HN can be
detected by 6 hours after the onset of the chase.
     We next asked how long it takes the two molecules to
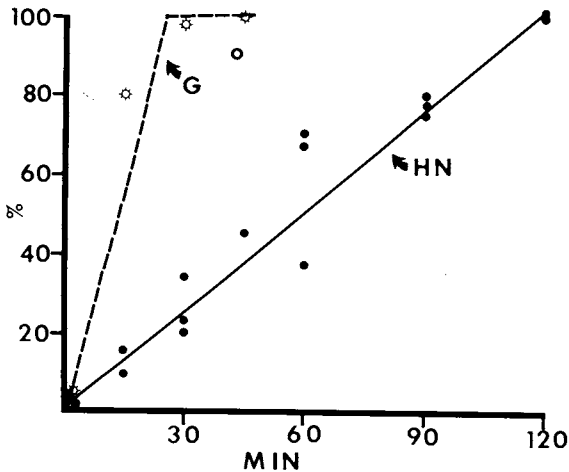
     Figure 3: Appearance of Endoglycosidase H-resistant
glycoprotein.
     Values are plotted as percent of total glycoprotein.

reach the trans-Golgi membranes. In the trans-Golgi, the carbohydrate on glycoprotein is converted to resistance to endoglycosidase H by the addition of periferal sugars (4). We, therefore, asked how long it takes the two glycoproteins to acquire endoglycosidase H resistance. Figure 2 shows a plot of the appearance of endoglycosidase H-resistant glycoprotein with time of chase.

The G acquires endoglycosidase H resistance very quickly. The NDV HN protein becomes resistant quite slowly. As reported previously, the G protein acquires endoglycosidase H resistance with a half-time of approximately 13 minutes (11,12,13). In contrast, the HN protein acquires endoglycosidase H resistance with a half-time of approximately 60 minutes.

In summary, the G protein reaches the cell surface with a half-time of approximately 27 minutes and reaches the trans-Golgi membranes with a half-time of approximately 13 minutes. Thus, the G migrates from the Golgi membranes to the cell surface with a half-time of approximately 14 minutes. By contrast, the HN reaches the cell surface with a half-time of approximately 78 minutes and the trans-Golgi membranes with a half-time of approximately 60 minutes. Thus, the HN protein migrates from the Golgi membranes to the cell surface with a half-time of approximately 18 minutes, a time not significantly different from that of the VSV G protein. Thus, the difference in the kinetics of intracellular transport of the two glycoproteins resides primarily in the transit from the RER to the trans-Golgi membranes.

If all plasma membrane glycoproteins were transported by similar mechanism, the kinetics of transport should be identical. Results presented here, therefore, argue for different pathways to the cell surface.

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RESCUE OF VSV IN PERSISTENTLY INFECTED L CELLS
BY SUPERINFECTION WITH VACCINIA[1]

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Mouse L cells persistently infected with vesicular
stomatitis virus (VSV) are resistant to superinfection with
VSV and other viruses (1). Interferon (IFN) plays a part in
the modulation of this persistent infection, since treatment
of the cells with anti-IFN serum converts the persistent
infection to a lytic infection (2). Thacore and Youngner
showed that vaccinia can rescue VSV from the inhibitory
effects of IFN in L cells (3), and recently we have reported
that this rescue occurs at the level of VSV protein synthesis
(4). We have also identified a specific kinase inhibitory
factor (SKIF) produced by vaccinia that inhibits the IFN-
induced, dsRNA-dependent protein kinase (4).
    In this study we identify the point at which the
replication of superinfecting wild-type VSV (wt-VSV) is
blocked in persistently infected L cells (L_{VSV}) and show that
vaccinia is able to reverse the block to wt-VSV growth.
Infection with vaccinia produces a slight but reproducible
stimulation of the growth of the persistent virus (VSV-PI) in
L_{VSV} cells.

VACCINIA MEDIATED RESCUE OF VSV GROWTH

    Coinfection with vaccinia (MOI = 10) has a profound
effect on growth of wt-VSV in L_{VSV} cells (Figure 1). By 24
hours after infection with wt-VSV alone (MOI = 10), little or
no replication of the wt-VSV is observed; however, L_{VSV} cells
doubly infected with wt-VSV and vaccinia produce approximately
100 times more wt-VSV than singly infected cells, a yield
similar to that seen in normal L cells. The growth of VSV-PI

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is stimulated by about 10-fold by infection with vaccinia.
This stimulation occurs whether or not L_{VSV} cells are also
superinfected with wt-VSV.

        Since both VSV infection and high doses of IFN can affect
the penetration of VSV into L cells (5,6), we compared the
ability of ^{35}S-labeled wt-VSV (MOI = 10) to enter L_{VSV} cells
and uninfected L cells. Uninfected L cells and L_{VSV} cells
take up virus at the same rate: 3.5% of the radioactive virus
enters the cells after incubation at 37°C for 1 hour.
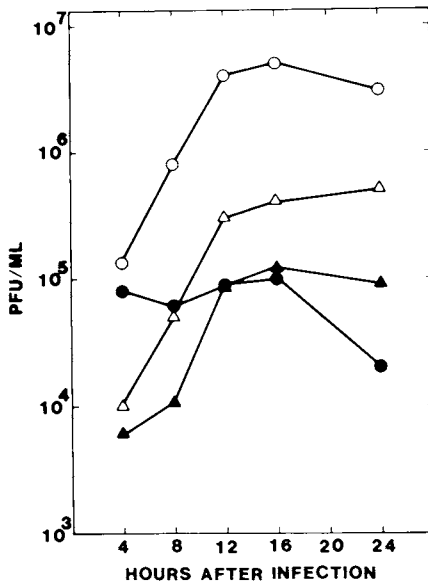
        FIGURE 1. Growth of VSV in L_{VSV} cells with and without
vaccinia. Monolayers of L_{VSV} cells in 24-well trays were
mock- or vaccinia-infected (MOI = 10). After 2 hr at 37°C the
cells were mock- or VSV-infected (MOI = 10) in the presence of
5 μg/ml of actinomycin D. The inoculum was removed after 1 hr
at 37°C, the cells were washed 2 times with medium and
recultured in medium containing actinomycin D. At the
indicated times after VSV infection, the fluid from individual
wells was harvested and infectivity assayed in primary chicken
embryo cells at 37°C. VSV-PI and wt-VSV are easily distin-
guished by plaque size. VSV-PI from mock-infected cells (▲),
VSV-PI from cells superinfected with vaccinia (Δ), wt-VSV from
cells superinfected with wt-VSV (●), and wt-VSV from cells
superinfected with wt-VSV + vaccinia (O).

THE EFFECT OF VACCINIA ON VSV GENE EXPRESSION

To determine if vaccinia has any effect on VSV RNA synthesis, primary transcription by superinfecting wt-VSV was measured in the presence or absence of coinfecting vaccinia by labeling L_{VSV} cells with ^{3}H-uridine in the presence of actinomycin D and cycloheximide. Vaccinia produces only a modest stimulation of the incorporation of ^{3}H-uridine into TCA precipitable material (Figure 2). This analysis is complicated by the fact that early RNA synthesis by vaccinia is only partially sensitive to inhibition by actinomycin D in the presence of cycloheximide (unpublished observation). Therefore, we measured primary transcription in L_{VSV} cells by isolating the RNA and testing its ability to direct the synthesis of VSV protein in an in vitro rabbit reticulocyte lysate (Figure 3). Vaccinia has little or no influence on the
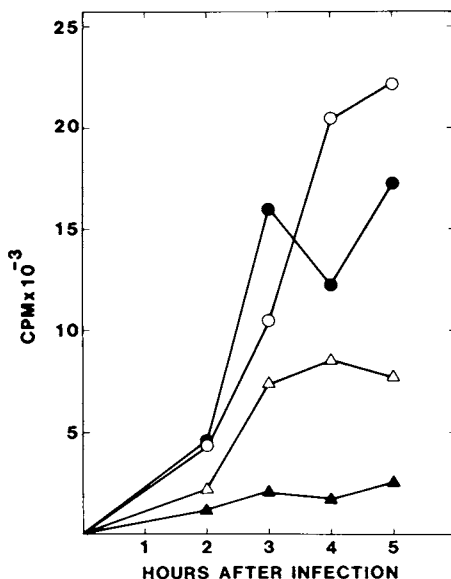
FIGURE 2. Primary transcription by VSV in L_{VSV} cells with and without vaccinia. Monolayers of L_{VSV} were infected with vaccinia as described in Figure 1. After 2 hr at 37°C, the cells were mock- or VSV-infected (MOI = 10) in the presence of actinomycin D and cycloheximide (200 µg/ml). The inoculum was removed after 1 hr at 37°C, and the cells were washed, and recultured in medium containing actinomycin D, cycloheximide, and ^{3}H-uridine (10 µCi/ml). At the indicated times after VSV infection, cultures were lysed in 1% SDS and the ^{3}H-uridine incorporated into TCA precipitable material determined. Uninfected (▲), vaccinia infected (△), wt-VSV infected (●), and wt-VSV + vaccinia infected (O).
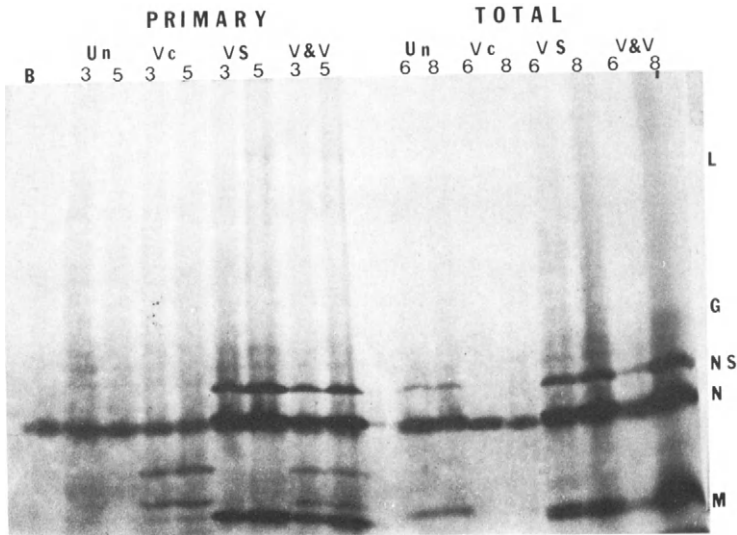
FIGURE 3. The amount of functional VSV mRNA in L_{VSV} superinfected with VSV and vaccinia. Monolayers of L_{VSV} were infected with wt-VSV and vaccinia as described in Figure 2 (to measure the level of functional primary transcripts) or as described in Figure 1 (to measure the amount of total VSV mRNA). RNA was isolated at the indicated hours after VSV infection by phenol extraction followed by ethanol precipitation. The RNA preparations were assayed for the ability to direct protein synthesis in a rabbit reticulocyte lysate translation system. An equal amount of purified RNA was added to each reaction to give a final concentration of 50 μg/ml of lysate. Products synthesized during 60 minutes of incubation at 30°C were analyzed by SDS-PAGE. B indicates the lane which received lysate to which no exogenous RNA was added. Un refers to mock-infected L_{VSV}, Vc indicates vaccinia infected L_{VSV} cells, VS refers to VSV infected cells and V&V indicates doubly infected L_{VSV} cells.

amount of functional VSV primary mRNA isolated from these cells. In addition, similar analysis of total VSV mRNA was performed using RNA isolated at 6 or 8 hours after VSV infection from singly and doubly infected L_{VSV} cells. Again, vaccinia coinfection has little effect on the amount of functional total VSV mRNA (Figure 3).

Analysis of protein synthesis in L_{VSV} cells superinfected with wt-VSV and vaccinia revealed that vaccinia produces a dramatic stimulation of the synthesis of VSV protein (Figure 4). Persistently infected cells were mock-infected, singly

infected with wt-VSV or vaccinia, or doubly infected with VSV + vaccinia; the cells were labeled with ^{35}S-methionine for 30 minutes at 2, 4, and 6 hours after infection. Examination of extracts from these cells by SDS-PAGE revealed that little or no VSV protein is synthesized in cells infected with VSV alone. However, large amounts of all five VSV proteins are produced in cells doubly infected with VSV + vaccinia.
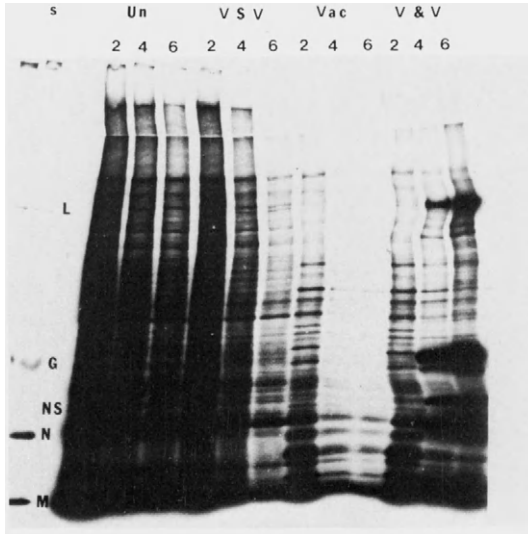
FIGURE 4. SDS-PAGE analysis of protein synthesis in L_{VSV} cells superinfected with wt-VSV and vaccinia. Monolayers of L_{VSV} cells were infected with vaccinia and VSV as described in Figure 1. At 2, 4, and 6 hr after VSV infection the cells were recultured in methionine-free medium to which ^{35}S-methionine (20 µCi/ml) had been added. After 30 minutes at 37°C, cell extracts were prepared and analyzed by SDS-PAGE as described (4). Un refers to mock-infected L_{VSV}, VSV refers to wt-VSV infected L_{VSV}, Vac to cells infected with vaccinia, and V&V to cells infected with VSV + vaccinia. The lane labeled S received a standard of ^{35}S-labeled VSV proteins. The numbers at the top of each lane indicate the hour after VSV infection that the cultures were labeled with ^{35}S-methionine.

VACCINIA-MEDIATED INHIBITION OF THE IFN-INDUCED PROTEIN KINASE

The ability of vaccinia to rescue VSV in IFN-treated cells has been correlated with the SKIF-mediated inhibition of the IFN-induced protein kinase activity (4). Mock-infected,

singly infected, and VSV + vaccinia-infected L_{VSV} cells were
assayed to determine if the IFN-induced protein kinase
activity was present and if vaccinia infection resulted in an
inhibition of the kinase activity in these cells. Kinase
activity was detected by the phosphorylation of P1, a protein
of M_r 67,000, whose phosphorylation has been correlated with
IFN-induced, dsRNA-dependent protein kinase activity (7). The
IFN-specific kinase is detectable in L_{VSV} cell extracts in the
presence of dsRNA, although in lower amounts than seen in
extracts from normal L cells treated with 100 units/ml of IFN
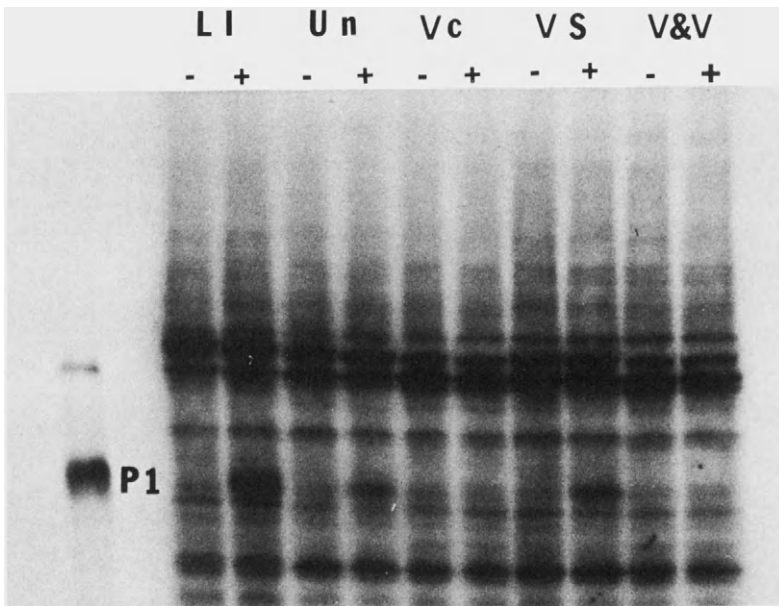
FIGURE 5.   Protein kinase activity in L_{VSV} superinfected
with VSV and vaccinia. Monolayers of L_{VSV} were mock-infected
(Un), singly infected with VSV (VS) or vaccinia (Vc), or
doubly infected with VSV + vaccinia (V&V) as described in
Figure 1. At 2 hr after VSV infection, S10 lysates were
prepared as described (8). An S10 lysate was also prepared
from uninfected normal L cells which had been treated for 24
hr with 100 units/ml of mouse IFN (LI). The extracts were
incubated at 30°C for 30 minutes in the presence of 0.4
mCi/ml γ-^{32}P-labeled ATP with (+) or without (−) the addition
of 0.4 µg/ml of poly I:C. The products of this reaction were
analyzed by SDS-PAGE. P1 marks the migration position of the
67,000 molecular weight protein phosphorylated by the IFN-
induced protein kinase (7).

(Figure 5). As in IFN-treated cells, this kinase function is inhibited in extracts from L_{VSV} cells infected with vaccinia.

DISCUSSION

We have shown that coinfection with vaccinia reverses the inhibition of the growth of superinfecting wt-VSV in L cells persistently infected with VSV. Vaccinia has little or no direct effect on wt-VSV RNA synthesis, as measured by primary transcription or total transcription, but dramatically increases the synthesis of wt-VSV protein. The mechanism of rescue of VSV by vaccinia in L_{VSV} cells appears to be the same as the mechanism of rescue of VSV in IFN-treated L cells, namely, stimulation of VSV protein synthesis. It is interesting to note that IFN-induced protein kinase activity is present in L_{VSV} cells and that vaccinia is able to inhibit this enzyme in L_{VSV} cells as it does in IFN-treated L cells.

Although vaccinia also stimulates the growth of endogenous VSV-PI in persistently infected cells, the effect is less dramatic than the effect on superinfecting wt-VSV. This difference in extent of rescue of VSV-PI may be due to the expression of genetic differences between wt-VSV and VSV-PI, e.g., the altered RNA synthesis pattern of VSV-PI reported by Frey and Youngner (9). An alternative explanation is that there are simply fewer VSV-PI genomes available in L_{VSV} cells for rescue by vaccinia.

ACKNOWLEDGMENT

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THE EFFECT OF VESICULAR STOMATITIS VIRUS
ON ADENOVIRUS TYPE 2 REPLICATION[1]

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VSV INHIBITION OF EUKARYOTIC MACROMOLECULAR SYNTHESIS

Vesicular Stomatitis Virus (VSV) is an enveloped
rhabdovirus containing an RNA-dependent RNA polymerase
(1,2). The 42 S negative stranded genome RNA is
sequentially transcribed to yield as an initial product a
plus-strand 47-nucleotide leader which is neither capped nor
polyadenylated (2,3). Transcription of the leader RNA is
required for the syntheis of the five monocistronic
messenger RNAs which are capped and polyadenylated (1,2).
The Viral Function Responsible for Inhibition.
Eukaryotic RNA,DNA and protein syntheis are drastically
altered by an infection with VSV. The general
characteristics of VSV inhibition of host cellular
macromolecular synthesis have been well characterized
(4,5,6). Experiments to determine the viral function and
the cellular target that interact to cause the phenomenon
known as shut-off have recieved considerable attention
during the last few years (5,6,7,8). Studies have used UV-
irradiation, ts mutants, and DI particles of VSV to
determine that transcription of at least leader RNA is
required for the inhibition of cellular macromolecular
synthesis (5,6,7,9). Others previously (10) used a DNA
dependent in vitro transcription system (11) made with
extracts from HeLa cells to directly test whether VSV leader

RNA is the viral function required for the inhibition of
cellular RNA. Using VSV RNAs transcribed in vitro it was
determined that the VSV leader RNA was at least 30 times
more effective in the inhibtion of the specific initiation
of RNA transcription in vitro then any other RNA tested
(10). More recent data obtained by independent
investigators confirms these previous results using two
strains of VSV (12).

    Previous studies (6,10) have tentatively identified RNA
polymerase II and III as the cellular target for the
inhibiton of transcription. However, the precise
identification of the cellular traget(s) affected by VSV
infection has not been made. Use of these in vitro systems
currently offers us our best opportunity to investigate the
cellular target in the shut-off phenomenon.

    Studies of the VSV inhibitor of cellular protein and
DNA synthesis have also implicated the WT leader sequence as
a possible inhibitor (5,9,10). The use of in vitro
translation systems are yielding additional information on
the shut-off function required by VSV to inhibit cellular
protein synthesis (15). VSV inhibition of cellular DNA
synthesis has not been studied in depth (5). The lack of an
in vitro replication system which would reflect the cellular
process of DNA synthesis has hampered further study.
However, we describe below a system which allows us to
approach the effects of VSV on DNA synthesis in vitro.

    Adenovirus Replication In Vitro: A Model for Eukaryotic
DNA Synthesis. Our previous findings that VSV drastically
inhibits cellular DNA synthesis in vivo by transcription of
at least leader RNA has promted us to investigate the
mechanism by which cellular DNA synthesis is inhibited.
Although the capacity of VSV to shut-off cellular DNA
synthesis could be secondary to its effect on RNA synthesis,
it seems unlikely based on the almost identical rates of
inhibition of both cellular RNA and DNA synthesis (5).

    The sequence of steps required for DNA synthesis in
eukaryotic cells is too complex to dissect in vivo. The
interacting components can only be analyzed in vitro under
specific conditions. Unfortunately, a system which supports
the initiation of cellular DNA synthesis does not presently
exist. A simple model for the accurate in vitro replication
of adenovirus DNA has been developed (13). Since adenovirus
relies primarily on cellular processes for its DNA
synthesis, we have chosen this model to analyze the effects

of VSV on eukaryotic DNA replication. To this end,
experiments have been designed to exploit the in vitro
replication system developed for adenovirus DNA.

    Nuclear extracts from adenovirus-infected HeLa cells
preserve the characteristics of adenovirus DNA replication
(13). The nuclear extracts require exogeneous adenovirus
DNA-terminal protein complex as a template, they replicate
strands in a 5' to 3' direction, and simultaneously displace
the complementary strand (13). The general characteristics
of adenovirus replication in vivo and in vitro are well
established (for review,14) which makes it possible to
assess the biological relevance of the synthetic reactions
in vitro.

USE OF THE AD 2 REPLICATION SYSTEM WITH VSV RNA

    The Specificity of Cell Extracts. We first determined
the nature of the DNA synthesized from the adenovirus type 2
(AD 2) DNA template using HeLa cell extracts. Extracts of
HeLa cells were obtained as described (13) and mixed with
Xba-1-cleaved AD 2 DNA. These five Xba-I-cleaved
restriction fragments were replicated in vitro in the
presence of ^{32}P-dCTP for 60' at 31^0C. The labeled products
were resolved by electrophoresis on 0·7% agarose gels and
visualized by autoradiography (data not shown). Typically
40-55% of the radioactivity was incorporated into the C and
E terminal fragments of newly synthesized viral DNA. These
terminal fragments represent less than 16% of the total
genome yet contain greater than 50% of the total activity
incorporated. Therefore, our reaction conditions were
specific and suitable for the studies below.

    Effect of VSV Plus-Strand RNA on AD 2 Replication:
Kinetics of the Reaction. Previously it was (5) reported
that VSV transcription is required to shut-off MPC-11 and L
cell DNA synthesis. To test this possibility, VSV genome RNA
was transcribed in vitro and the resulting syntheisized plus
strand RNAs were separated form the viral nucleocapsids by
ultracentrifugation (10). Phenol extracted purified in
vitro transcribed VSV RNAs were then added into the AD 2
replication system and the amount of incorporation into acid
precipitable counts determined (10).
    Figure 1 shows the kinetics of replication of the AD 2
DNA template in the presence of increasing concentrations of
VSV plus-strand RNA. The relatively low concentration (12
ng/ul) actually stimulated the incorporation of ^{32}P-dCTP
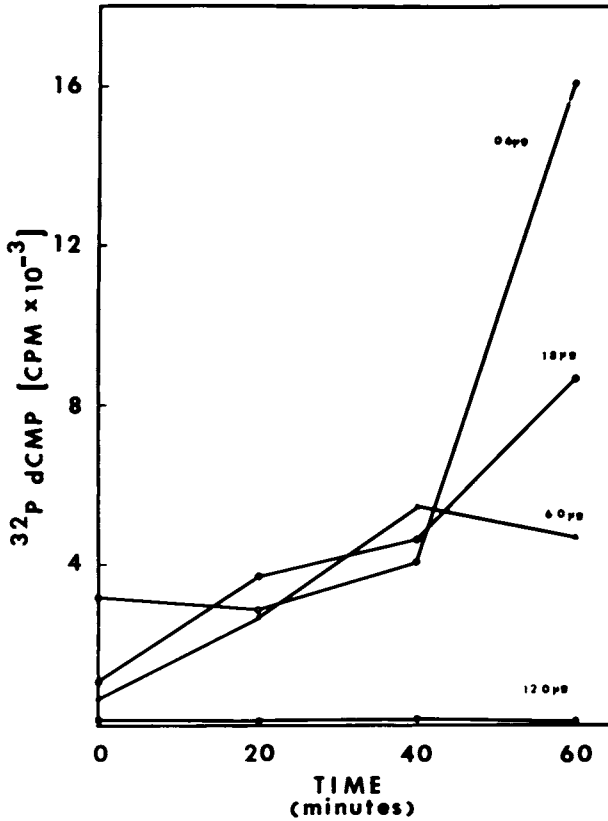
Figure 1. The effect of VSV plus-strand RNA on
adenovirus type 2 replication in vitro. Purified VSV was
transcribed in vitro (10) and added into the AD 2
replication extract prepared as described (13). The
concentrations indicatated were added into a 50 ul reaction
and at the times indicated 10 ul aliquots were removed and
the amount of radioactivity incorporated determined (10).
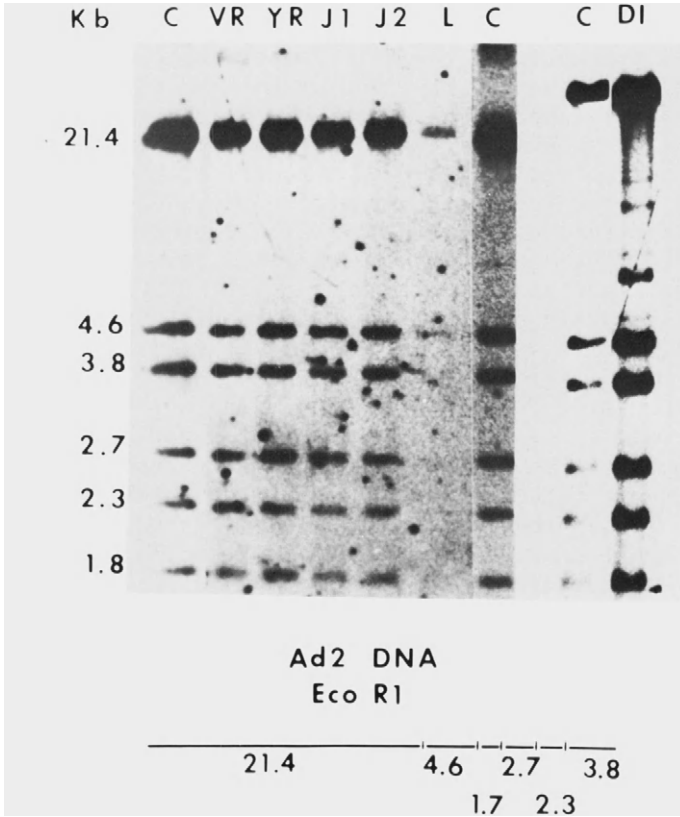
Figure 2.   Effect of various viral and non-viral RNAs
on AD 2 replication in vitro.   Total VSV RNA (excluding
leader), VR;   yeast RNA, YR;   WT leader RNA, L;   and
synthetic oligonucleotides containing portions of the WT
leader RNA, J-1, and J-2; were added into the replication
reaction at the same concentration (15 ng/ul).   The DI
leader RNA was added at a concentration of 30 ng/ul.   The
reactions were incubated at 31^0 C for 60'.   Samples were
processed as described and visualized by autoradiography
(13).

into acid preciptiable counts. Higher concentrations (36-
120 ng/ul) of VSV in vitro transcribed RNA did not
significantly inhibit the reaction.   A reduction of >95% in
total DNA synthesis was observed in the presence of the
highest concentration of VSV RNA tested (240 ng/ul).   The
kinetics of the replication reaction were quite similar for
each concentration tested despite the degree of inhibition
obtained.   In the experiments described below we test
individual species of RNA to define which VSV plus-strand
RNA(s) is capable of inhibiting the specific replication of
AD 2 DNA.

      Comparative Effect of Various Viral and Nonviral RNAs
on AD 2 DNA replication.   The effects of various viral RNA
transcripts and several nonviral RNAs were tested at the
same concentration for their capacity to inhibit AD 2
specific DNA synthesis.   In each case preparations of VSV WT
plus-strand leader RNA, DI leader, VSV incomplete and
complete mRNA excluding leader, yeast RNA, synthetic
oligonucleotides J-1 or J-2 were added to the reaction.   The
reaction mixture contained: HeLa-cell extracts, AD 2 DNA
template, the appropriate buffer and deoxynucleoside
triphosphates required for AD 2 replication in vitro.
Incorporation of ^{32}P-dCMP into AD 2 specific DNA was
determined by electrophoresis on 0.8% agarose gels, which
were analyzed by autoradiography.   The radioactivity in the
gel slices was quantitated by Cerenkov counting and scanning
the gels with a densitometer.
      A representative autoradiogram is shown in Figure 2.
The relatively low concentration of WT leader added (15
ng/ul) markedly inhibited replication of the AD 2 specific
DNA by >90%.   The only other RNA tested at this
concentration which resulted in >24% reduction of AD 2
specific DNA was J-1 which is a synthetic oligonucleotide
made from sequence between nucleotides 18 to 25 of the WT
leader.   The addittion of the DI leader in concentrations
which were 3.5 times higher than the molar amount of VSV WT
leader resulted in no loss of AD 2 specific DNA synthesis.
Indicating that a sequence unique to the WT leader is
responsible for the inhibition of AD 2 DNA synthesis.

ROLE OF VSV PLUS STRAND LEADER RNA IN HOST-SHUTOFF

      The relationship between the inhibition of cellular RNA
synthesis and cellular DNA synthesis by VSV remains poorly
defined.   The availability of techniques to study the

specific replication of AD 2 in vitro have allowed us to
begin investigations on the inhibitory action of VSV
specific RNA products in greater depth. Adenovirus
replication is dependent to a large extent on the host
cellular enzymes. Therefore, it has provided us with an
opportunity to determine whether the inhibition of
eukaryotic DNA synthesis by VSV occurs via a cascading
mechanism or by a direct effect on the cellular replication
mechanisms.

     We tested the effect of adding preformed viral RNA
products on the in vitro replication of AD 2 specific DNA.
At the concentrations tested only VSV leader RNA has
exhibited a very strong capacity to inhibit AD 2 DNA
replication (0.5 uM). Other RNAs tested (specifically DI
leader RNA) at concentrations of 3.5 - 6.0 uM higher than WT
leader did not significantly effect the reaction. Current
dogma suggests that AD 2 replication is not affected by the
addittion of purified RNASE, does not require an RNA primer,
but is dependent on the binding of an 80,000 molecular
weight protein to the 5′ termini of the DNA to initiate
synthesis (13,14). We have found when T_1 RNASE is added
into the reaction with leader that DNA synthesis is restored
to some degree. The DNA synthesis observed with WT leader
in the presence of T_1 RNASE is primarily repair polymerase
activity and not the result of the specific initiation of
replication as determined by the lack of incorporation of
isotope into the terminal fragments of AD 2 DNA. We have
subsequently found that our RNASE is contaminated with DNASE
which makes it difficult to speculate further.

     Experiments are now in progress to define the sequences
unique to the WT leader which are important in inhibition of
AD 2 replication in vitro. We will also determine if there
is a specific interaction of the WT leader with some
component of the replication extract. In addittion, studies
of VSV's effect on the replication of adenovirus in tissue
culture cells will ascertain whether the results obtained to
date are the consequences of an in vitro system or truly
representative of the events in vivo.

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INHIBITION OF ADENOVIRUS AND SV40 DNA SYNTHESIS
BY VESICULAR STOMATITIS VIRUS[1]

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Inhibition of host cell DNA synthesis by lytic viruses
is generally considered to be an indirect result of the
inhibition of host cell protein synthesis (1,2). However,
recent studies of VSV infected mouse cells indicated that the
kinetics of the inhibitions of protein and nucleic acid
synthesis are sufficiently different to suggest, that the two
events are unrelated (3). Although certain hypotheses have
been advanced about the putative action of VSV, the complex-
ity of the eukaryotic cell presents difficulties in the
design and interpretation of meaningful experiments. In the
experiments described in this communication, the problem was
approached by using DNA viruses as targets for DNA synthesis
inhibition by VSV. SV40 was selected because of the strin-
gent dependency of its DNA synthesis on the host cell cycle,
histones and DNA polymerase. This dependency suggested
similarities between the cellular and SV40 DNA replicative
mechanism, and hopefully also a correspondence in the inhibi-
tory mechanisms by VSV. Adenovirus, although it replicates
in a much more autonomous way independent of the cell cycle,
can serve as a convenient source of an in vitro DNA repli-
cating system for inhibition studies.
    VSV inhibited SV40 DNA synthesis in Vero cells to
approximately the same extent as that of cellular DNA.
Unlike in normal SV40 infections, incompletely supercoiled
SV40 DNA species accumulated in the VSV inhibited system to
the extent that they were detectable by steady state label-
ing. A similar accumulation of incomplete SV40 DNA super-
coils was also observed when protein synthesis was inhibited
with cycloheximide in the absence of VSV.
    Dana and Nathans (4) have shown that a short pulse
(10-15 min) of SV40 DNA labeling established a linear

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gradient of radioactivity in the molecules synthesized during
the pulse. The radioactive counts increased with distance
from the single origin of replication. Using the slope of
this gradient as a measure of DNA chain elongation rates we
found that this rate remained unchanged during inhibition by
VSV. The absence of any effect on the rate of DNA synthesis
and the similarity in the electrophoretic profiles of cyclo-
heximide and VSV inhibited SV40 DNA synthesis, suggested that
the major effect of VSV on SV40 DNA synthesis was through
inhibition of protein synthesis.

     Horwitz et al. (5) have demonstrated that adenovirus DNA
synthesis became independent of protein synthesis late in the
infectious cycle. If DNA synthesis inhibition by VSV were
indirectly associated with the inhibition of protein synthe-
sis, late adenovirus DNA replication would be expected to be
insensitive to VSV. We report here, however, that late
adenovirus DNA synthesis in HeLa cells, although not very
sensitive to cycloheximide, was extensively inhibited by VSV.

INHIBITION OF SV40 DNA SYNTHESIS BY VSV.

     The effect of SV40 DNA synthesis was investigated in
Vero cells synchronized by a double thymidine block. The
cells were infected with SV40 after the first release and
with VSV 4 hrs after the second release. Incorporation of
^{3}H thymidine into intracellular SV40 DNA (Hirt super-
natants) was followed at various times after infection (Table
1). The extent of incorporation into cellular DNA (Hirt
precipitates) was determined (data not shown). The extent of
SV40 DNA synthesis inhibition was very similar to that of
cellular DNA, increased as the VSV infection progressed and
reached a maximum of approximately 70%. The experiment was
not continued beyond a time when more than 20% of the cells
became permeable to trypan blue, indicating extensive cell
killing.

     Characterization of intracellular SV40 DNA synthesized
in the presence and absence of VSV or cycloheximide.
     SV40 infected cells in the presence and absence of VSV
or cycloheximde were labeled with ^{32}P, intracellular SV40
DNA was isolated from Hirt supernatants and electrophoresed
as described in the legend to Fig. 1. Lanes a and b show
profiles of newly synthesized SV40 DNA in the absence of
either VSV or cycloheximide. In lanes d and e were SV40 DNAs
isolated from cells which were superinfected with VSV for the
specified periods of time. Lane c represents SV40 DNA
obtained from VSV-free cells treated with cycloheximide 2 hrs

INCORPORATION OF ^{3}H THYMIDINE INTO SV40 DNA (HIRT SUP.) IN
SYNCHRONIZED VERO CELLS WITH AND WITHOUT VSV INFECTION

Time (hr) after release	CPM SV40 + ts31VSV	SV40
4 VSV added	--	--
5	19,150	19,280
7	13,225	26,250
9	15,030	34,200
11	11,110	33,320
13	4,510	15,830

Vero cells in 60 mm plastic petri plates were synchronized by
a double thymidine block as described by Pages et al. (10).
Immediately following the first release, the cells were
infected with 50 PFU/cell of SV40 strain 776 for 90 min at
39°C. Following the second release, at the indicated time
the cells were superinfected with 5 PFU/cell of VSV mutant
tsG31 at 39°C for 30 min. This mutant inhibited SV40 DNA
synthesis as well as the wild type but cell detachment and
death (as defined by exclusion of trypan blue) were delayed.
In this way it was possible to follow the effect of VSV
infections over a longer period of time (up to 8 hrs at least
80% of the cells were viable). One hour before the indicated
time 1.5 μCi/ml of ^{3}H thymidine (60-80 Ci/mM) were added to
the medium. Following a two hour incubation, the cells were
washed with cold saline and intracellular SV40 DNA was
isolated by the Hirt procedure (11).

before the pulse. In lanes a and b the heaviest fast moving
band corresponds to the supercoiled form of SV40 DNA and the
lighter band in the middle of the lanes represents the
circular relaxed forms. The slower moving bands near the top
of the gel probably contain the dimeric forms described by
Sundin and Varshavsky (6). In addition to these bands, lanes
c, d and e also exhibit ladder-like patterns characteristic
of partially supercoiled DNA forms with varying numbers of
twists. The similarity of the SV40 DNA profiles obtained by
inhibition of protein synthesis with cycloheximide (lane c)
to those obtained from VSV superinfeted cells (lanes d and
e), suggested that VSV may not effect DNA synthesis directly.
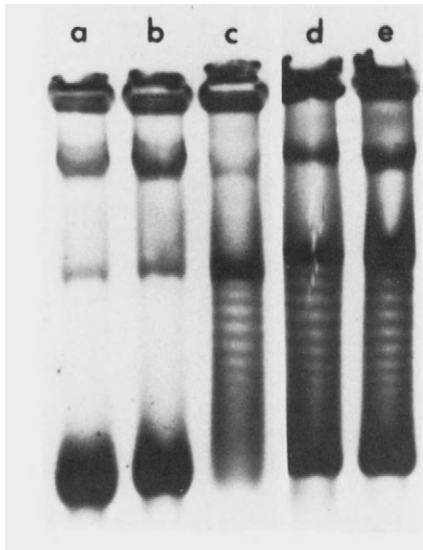
FIGURE 1.   Electrophoretic profiles of ^{32}P labeled
intracellular SV40 DNA in the presence and absence of either
VSV or cycloheximide.

     Vero cells, synchronized and infected with SV40 as
described in Table 1, were either superinfected, mock
infected of treated with 50 μg/ml of cycloheximide, 4 hrs
after release.   The cells were labeled at the intervals
indicated with 0.1 mCi/ml of ^{32}P.   Intracellular SV40 DNA
was isolated by the Hirt procedure (11) and electrophoresed
at 4 V/cm for 12 hrs in a horizontal 0.8% Agarose (Seakem)
gel, using 8.9 mM tris base, 8.9 mM boric acid and 0.25 mM
EDTA as buffer.
     Lanes a and b: SV40 DNA labeled 10-12 and 6-8 hrs after
release respectively; lane c: SV40 DNA in the presence of
cycloheximide labeled 6-8 hrs after release; lanes d and e:
SV40 DNA in the presence of VSV labeled 10-12 and 6-8 hrs
after release respectively.

     Relative SV40 DNA elongation rates in the presence and
in the absence of VSV.
     The possible existence of a direct effect on DNA
synthesis was examined by comparing SV40 DNA chain elongation

rates in the presence and absence of VSV. These rates were
measured, using a method originally applied by Dana and
Nathans (4) to locate the origin of replication in SV40 DNA.
In this procedure a linear radioactive gradient, with counts
increasing as a function of distance from the origin of
replication, was established by short (10-15 min) pulse
labeling (^{3}H thymidine) of non-synchronized nascent SV40
DNA molecules. At a given duration of the pulse, the slope
of the gradient reflects the rate of DNA chain elongation,
since this rate alone determines the proportion of molecules
whose synthesis is finished during the pulse. These slopes
were determined by measuring radioactive counts in
restriction fragments obtained with a mixture of HindII and
HindIII enzymes, suitable normalization of the counts with a
randomly labeled ^{32}P control and corrections for
thymidine content based on the known SV40 DNA nucleotide
sequence (4,7). The relative slopes obtained in the presence
and absence of VSV, respectively were 4.55 + 0.17 x 10^{-3}
and 4.63 + 0.23 x 10^{-3} per nucleotide after a 10 min
pulse and 4.20 + 0.17 x 10^{-3} and 4.0 + 0.08 x 10^{-3}
after a 15 min pulse. These results demonstrated that VSV
inhibition of SV40 DNA synthesis did not effect the chain
elongation rates. The identical elongation rates in the
presence and absence of VSV and the similarity of the
electrophoretic profiles of intracellular SV40 DNA in VSV
infected and cycloheximide treated cells, suggested that the
VSV effect was primarily indirect, through inhibition of
protein synthesis.

INHIBITION OF ADENOVIRUS DNA SYNTHESIS BY VSV.

     Horwitz et al. (5) demonstrated, that late adenovirus
DNA synthesis was independent of protein synthesis. If the
action of VSV on DNA synthesis was indirect through the
inhibition of protein synthesis, late adenovirus DNA
synthesis should be insensitive to superinfection with VSV.
Adenovirus DNA synthesis in the HeLa cells 19 hrs after
infection, was followed by incorporation of ^{3}H thymidine in
the presence or absence of either VSV or cycloheximide. High
molecular weight DNA and adenovirus DNA were separated by
high salt precipitation (Hirt procedure). Incorporation of
radioactive counts was measured in both fractions. As the
data in Table 2 indicate, incorporation of ^{3}H thymidine
into either fraction was relatively insensitive to
cycloheximide, although host protein and DNA synthesis were
strongly inhibited in control experiments under these
conditions (98% and 75% respectively). The insensitivity to
cycloheximide suggested that the radioactive counts in both

TABLE 2

INCORPORATION OF ^{3}H THYMIDINE INTO ADENOVIRUS DNA IN NON-
SYNCHRONIZED HELA CELLS 19 HRS AFTER INFECTION AND IN DOUBLY
INFECTED CELLS THUS AFTER VSV INFECTION.

Type of	CPM	
infection	Sup.	Prec.
Adeno	67,380	180,490
Adeno + VSV	33,430	66,040
Adeno + Cyclohex.	61,240	131,510

Subconfluent cultures of HeLa cells on 100 mm diameter
plastic tissue culture plates (approximately 1 x 10^7 cells)
were mock infected or infected with approximately 50 PFU/cell
of adenovirus type 2. Twelve hours later, the cells were
either infected with VSV tsG33 5 PFU/cell at 39°C or mock
infected. When required, two hours prior to pulsing cyclo-
heximide was added to a final concentration of 50 µg/ml to
the media covering the cells. The cells were pulsed at 19
hours post adenovirus infection with media containing 1
µCi/ml ^{3}H thymidine (77.2 Ci/mmol) for 30 min. After the
pulse, the cells were rinsed and assayed for ^{3}H thymidine
incorporation by the Hirt procedure (11). The precipitates
in solution were sonicated with three 10 second pulses with a
Heat Systems-Ultrasonics Inc. Sonifier Cell Disruptor Model
185 setting 4.5. An aliquot of the supernatant and of the
sonicated precipitates was precipitated with 10% trichloro-
acetic acid (TCA) in the presence of 200 µg bovine serum
albunim (BSA) as carrier and counted in a toluene based
scintillation fluid.

fractions represent adenovirus DNA synthesis. Extensive
integration of adenovirus-like sequences into host cell high
molecular weight DNA has been reported in the literature
(8,9). At 36 hrs after infection, the adeno-genome
equivalents incorporated into host cell DNA were estimated at
500-1000 copies (8). The association of the radioactive
counts with adenovirus DNA sequences in the high molecular
weight DNA fraction (Table 2) was further demonstrated by
annealing experiments. After sonication, the DNA was
annealed to cellulose nitrate filters containing varying
quantities of denatured non-radioactive adenovirus DNA. With
excess cold DNA, approximately 82% of the radioactive counts
were retained by the filters. DNAs labeled in the presence
and absence of cycloheximide followed similar hybridization
kinetics (Fig. 2).

   Adenovirus DNA synthesis was inhibited by VSV in doubly
infected cells. Seven hours after VSV infection the extent
of inhibition in the Hirt supernatant was 51% (Table 2). In
the Hirt precipitate the apparent inhibition was 64%, but not
all the counts incorporated into this fraction in the
presence of VSV were in adeno-DNA sequences. Annealing
experiments of the sonicated high molecular weight DNA
fraction indicated that only 24% of the radioactive counts
were retained by the filters, suggesting that 76% of the
counts might be associated with cellular DNA sequences (Fig.
2). A suitable correction of the data in Table 2, based on
the annealing data, resulted in 90% inhibition of adenovirus
DNA sequences in the high molecular weight DNA fraction.
Unlike in the absence of VSV, extensive synthesis of cellular
DNA seems to have taken place. A quantitative comparision
with parallel uninfected controls indicated that cellular DNA
synthesis, as measured by ^{3}H thymidine incorporation, was
completely restored in the mixed infection.
   The data presented here suggested that the primary cause
of SV40 DNA synthesis inhibition by VSV was the inhibition of
protein synthesis. Because of extensive similarities between
SV40 and host cell DNA synthesis it is likely that the
inhibition of protein synthesis, on which both types of DNA
synthesis are stringently dependent, is the primary factor in
their inhibition by VSV. On the other hand, the more
autonomous synthesis of adenovirus DNA might be inhibited by
a different mechanism and could involve steps unique to DNA
synthesis of this virus.
   The observation that in adenovirus infected cells,
superinfection with VSV restored cellular DNA synthesis, is
difficult to explain. Since either virus alone inhibited
cellular DNA synthesis, it appeared that their ability to do
so was compromised in the mixed infection. At present it is
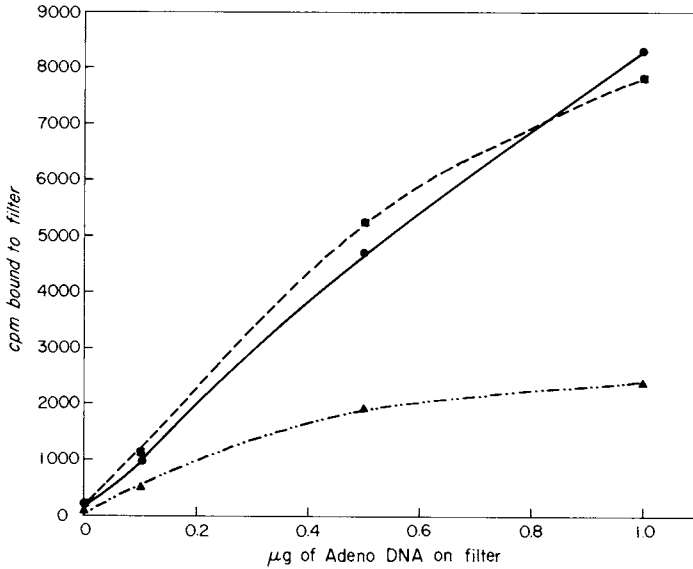
FIG. 2. HeLa cells were infected as in Table 2 except that the
Hirt precipitates were not sonicated, but were dialyzed
against 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10 mM EDTA.
The DNA was extracted by the procedure of Maki et al. (12).

Adenovirus DNA purified from virions was sonicated and
denatured with NaOH at a final concentration of 0.2 M for one
hour. To neutralize the solution 2 M Tris-HCl was added.
The DNA was passed through a filter made of Gene Screen (New
England Nuclear). The filter was washed with 1 x SSC (0.15 M
NaCl, 0.015 M sodium citrate, pH 7.0) and air dryed. The DNA
was baked onto the filter for 2-4 hours at 80-100°C. Smaller
filters were punched out of this filter and placed into
siliconized conical eppendorf tubes.

The hybridization was carried out esentially as
described in the Gene Screen manufacters instruction booklet.
The procedure entailed prehybridizing the filters with a
hybridization solution of 50% formamide (deionized), 2 x SSC,
0.05 M Na_2HPO_4/NaH_2PO_4, pH 6.5, 0.02% polyvinyl-
pyrrolidone (M.W. 40,000), 0.02% BSA, 0.02% ficoll (M.W.
400,000) and denatured salmon sperm DNA (100 μg/ml) overnight
at 42°C. That was followed by hybridization with sonicated
and denatured DNA purified from the Hirt precipitates (10,000
cpm/filter) in the hybridization solution for 22 hrs at 42°C.
The filters were then washed twice with a 0.3 M NaCl, 0.06 M
Tris-HCl, pH 8.0, 0.002 M EDTA solution for 5 min at room
temperature, twice with a 0.3 M NaCl, 0.06 M Tris-HCl, pH

not clear whether cellular DNA synthesis is completely
restored in the sense that all nucleotide sequences are
synthesized in the proper temporal order. If that were the
case, it is not clear how the two viruses effect each others
infectious cycle so as to negate their influence on these
cellular events. Experiments are in progress to provide
answers to these questions.

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8.0, 0.002 M EDTA, 0.5% SDS solution for 30 min at 60°C, and
twice with a 0.003 M Tris base solution for 30 min at room
temperature. The filters were dried and counted in a toluene
based scintillation fluid. DNA from adenovirus infected
cells in the presence ■----■ and absence ●——● of cyclo-
heximide. DNA from cells doubly infected with adenovirus and
VSV ▲-··-▲.

EXPRESSION OF MEASLES VIRUS RNA IN BRAIN TISSUE[1]

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Measles virus is a human pathogen which causes both acute and subacute infections, and has also been implicated in multiple sclerosis. Following acute measles, the individual develops a life-long immunity to reinfection. However, in some cases, the virus establishes a persistent infection in the central nervous system (CNS) which gives rise to the slowly progressing disease subacute sclerosing panencephalitis (SSPE). Current evidence suggests that virus persistence in SSPE is associated with a specific lesion in the production of virus matrix (M) protein, although all other polypeptides are produced. The lack of this major structural polypeptide is thought to disrupt the virus maturation process and lead to a strictly cell-associated phenotype, thus accounting for the slowly progressing nature of the disease. In order to assess the involvement of measles virus in SSPE, we decided to clone the virus genomic RNA, so producing specific probes with which to examine virus expression within diseased tissue.

CLONING PROCEDURE

Genomic RNA was isolated from intracellular nucleocapsids produced in lytically infected MA160 cells (1). The cloning procedure was straight forward and used standard methods. In brief, genomic RNA was polyadenylated at its 3' end (2) and then used as template for oligo-dT primed cDNA synthesis by reverse transcriptase. Single stranded cDNAs were converted to double strands, tailed and joined to plasmid pBR322 at the Pst1 site. E. coli cells were transfected with this DNA and recombinant clones were screened by in situ hybridization using a probe obtained by reverse transcription

of polyadenylated 50 S MV RNA. 15 clones were selected with
inserts ranging from 500 to 1000 bp in size. These were
further characterized by Northern blotting. Three reacted with
uninfected cell RNA, the other 12 reacted only with measles
virus infected cell RNA. These clones could be divided into
four groups, according to their hybridization behaviour, as
shown in Figure 1.
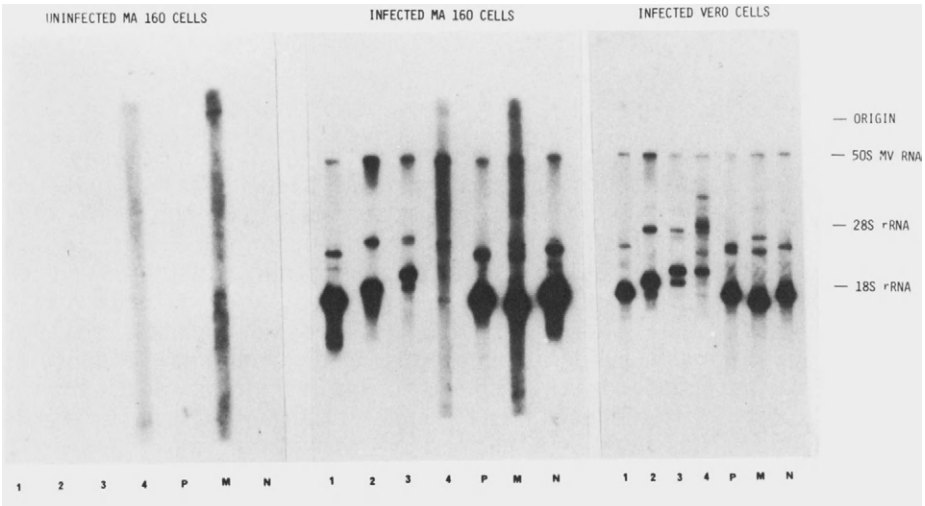
Figure 1. Identification of MV-specific RNA species from lyti-
cally infected MA 160 and Vero cells.
Cytoplasmic RNAs were electropohoresed in a vertical 1 % aga-
rose slab gel containing 6 mM methylmercury hydroxide, and
blotted directly to nitrocellulose. Filters were baked, cut
into small strips and hybridized with individual nick-trans-
lated 32P labeled cDNA clones. Clones 1-4 are derived from
virus genomic RNA and habe been assigned tentatively as: 1,
Nucleocapsid; 2, Haemagglutinnin; 3, Fusion; 4, L. clones P,
M, N are derived from virus m RNA (3).

Three size classes of virus- specific RNA were detected. The
largest comprises 50S genome-size molecules, and the smallest,
most abundant species constitutes the virus mRNA. In addition
all clones also recognised RNA species of intermediate size
(is-RNA). Clones of group 1 showed an identical hybridzation
pattern to that obtained using cloned copies of sequences con-
tained in the mRNA for nucleocapsid protein (kindly provided
by Dr. S. Rozenblatt) (3). These clones also cross-hybridized

to the nucleocapsid specific DNA itself. The sequence of the
first several hundred nucleotides has been determined (Fig. 2)
and the start of N-mRNA was located by S1 mapping.

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                                                      ↓                        100
ACCAAACAAAGTTGGGTAAGGATAGTTCAAATCAATGATCATCTTCTAGTGCACTTAGGATTCAAGATCCTATTATCAGGGACAAGAGCAGGATTAGGGA


                      ALU                                           BAM        200
TATCCGAGATGGCCACACTTTTAAGGAGCTTAGCATTGTTCAAAAGAAACAAGGACAAACCACCCATTACATCAGGATCCGGTGGAGCCATCAGAGGAAT
       MET ALA THR LEU LEU ARG SER LEU ALA LEU PHE LYS ARG ASN LYS ASP LYS PRO PRO ILE THR SER GLY SER GLY GLY ALA ILE ARG GLY ILE


                                                                               300
CAAACACATTATTATAGTACCAATCCCTGGAGATTCCTCAATTACCACTCGATCCAGACTTCTGGACCGGTTGGTCAGGTTAATTGGAAACCCGGATGTG
  LYS HIS ILE ILE ILE VAL PRO ILE PRO GLY ASP SER SER ILE THR THR ARG SER ARG LEU LEU ASP ARG LEU VAL ARG LEU ILE GLY ASN PRO ASP VAL


                                                                               400
AGCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTATTTGTGGAGTCTCCAGGTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTA
  SER GLY PRO LYS LEU THR GLY ALA LEU ILE GLY ILE LEU SER LEU PHE VAL GLU SER PRO GLY VAL GLN LEU ILE GLN ARG ILE THR ASP ASP PRO ASP VAL


                                                  KPN            500
GCATAAGGCTGTTAGAGGTTGTCCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAGGTACCAACATGGAGGATGAGGCGGACCAATACTT
  SER ILE SER LEU LEU GLU VAL VAL GLN SER ASP GLN SER GLN SER GLY LEU THR PHE ALA SER ARG GLY THR ASN MET GLU ASP GLU ALA ASP GLN TYR PHE


                                                                               600
TTCACATGATGATCCAATTAGTAGTGATCAATCCAGGTTCGGATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACCCTGAGGGATTCAACATGAT
  SER HIS ASP ASP PRO ILE SER SER ASP GLN SER ARG PHE GLY TRP PHE GLU ASN LYS GLU ILE SER ASP ILE GLU VAL GLN ASP PRO GLU GLU GLY PHE ASN MET
```

Figure 2. Sequences of the 5' terminal region of MV positive
strand RNA and of the region encoding the N-terminal of the
nucleocapsid protein. The point where N mRNA starts is marked
by an arrow.

        The sequence of the first 9 nucleotides of the cDNA
matched perfectly the 3' sequence of genomic RNA. This proved
that group 1 cDNAs are derived from the exact 3' end of genomic
measles virus RNA. Group 2 cDNAs hybridized to a mRNA of
approximately 2100 nucleotides (Fig. 3) which was not detect-
ed using any of the clones derived from measles virus mRNAs
(P,N,M) provided by Dr. Rozenblatt. Group 3 clones hybridzed
to the same mRNA as group 2, but also recognized an additional
mRNA of about 2650 nucleotides in size. Experiments are in
progress to identify the proteins encoded by these mRNAs which
we believe are the haemagglutinin (H) and fusion protein (F)
for groups 2 and 3 respectively. Group 4 is most probably
derived from an area towards the 5' end of the genome. This
clone hybridizes to several RNAs in the A(-) fraction which
are probably defective RNAs. No hybridization is detectable to
the A(+) fraction. This clone could represent part of the L
gene. Transcripts of this gene are known to be present only in

low amounts in the A(+) fraction. The nature of the is-RNA is
not yet fully understood. Some of these molecules are poly-
adenylated and are found in the A(+) fraction (Fig.3). These
bands are of positive polarity, as intermediate-sized RNA
hybridizes to a recloned M13 probe of negative sense, derived
from group 1 inserted sequences. This suggests that these RNAs
are polycistronic mRNAs or polymerase read-throughs, which are
also known to occur in other negative strand viruses (4,5).
The existence of these measles virus polycistronic RNAs per-
mits the determination of the order in which proteins are
encoded on the virus genome. This we have tentatively deter-
mined as: L-2(H?)-3(F?)-M-P-N. This includes the reassignment
of an H clone as P (6).
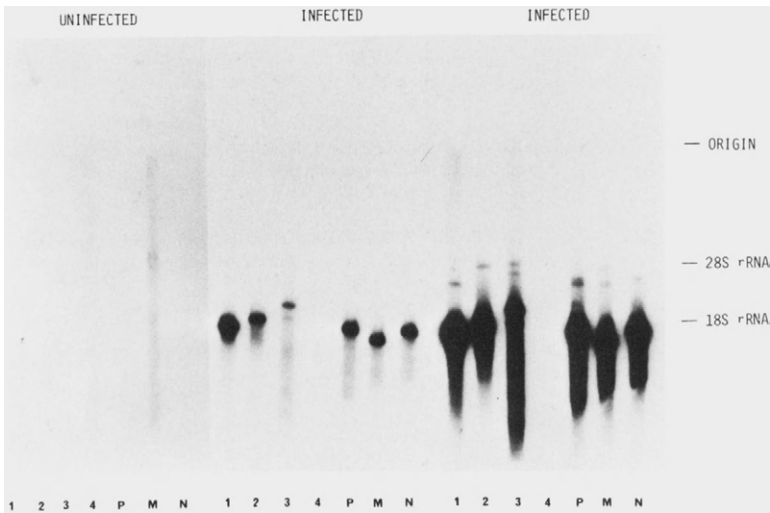
Figure 3. Northern blot of oligo-dT selected A(+) cytoplasmic
RNAs from Vero cells, hybridized to nick-translated, cloned
cDNAs as in Fig. 1.

EXAMINATION OF BRAIN RNA

       The available cDNAs were used to characterize virus RNA
in measles virus infected rat brains. In these studies suck-
ling rats were infected with the neurotropic measles virus
strain (CAM) which induced an acute encephalitis after an in-
cubation period of 10-15 days. Infectious virus could be iso-
lated from diseased brain. In addition, another group of suck-
ling rats were intracerebrally inoculated with a lytic measles
virus rescued from an SSPE cell line (MF). This persistently

infected cell line produced M protein and released infectious
virus following physiological trauma (7). These animals also
developed an acute encephalitis after an incubation period of
approximately two weeks.

RNA extracted by the guanidium isothiocyanate technique
(8) was examined by the method of Northern blotting using the
cloned measles virus DNAs. The pattern of virus-specific RNAs
expressed in the CAM-infected brains was similar to that ob-
served in lytically infected cells in vitro. All three size
classes of virus-specific RNA (with the exception of L
message) could be detected with each probe. However, the re-
sults were different using RNA from SSPE-MF infected rat brain
(Fig. 4). All mRNAs except P and L could be detected, although
the amount of matrix protein mRNA was greatly reduced and
there was a high concentration of the intermediate-sized P-M
transcription read-through. This indicates that expression of
M-mRNA by this SSPE virus may be decreased in CNS tissue.
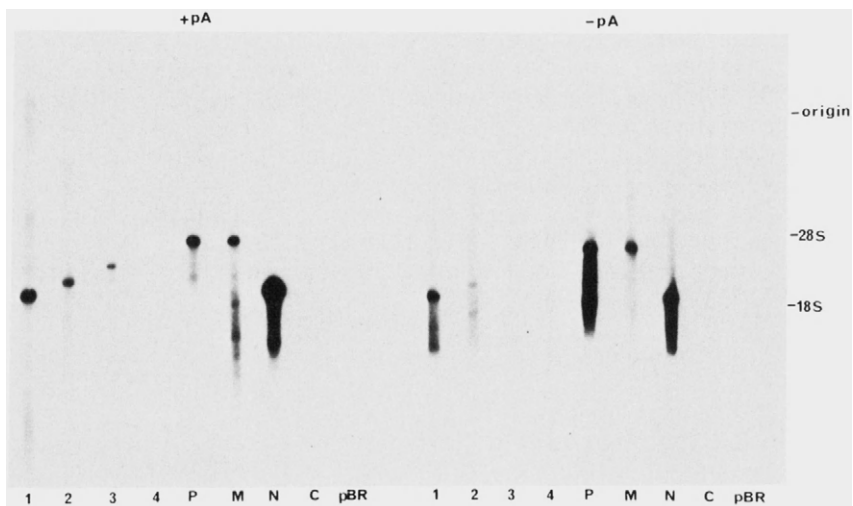
Figure 4. Northern blot of oligo-dT selected RNAs from SSPE-MF
infected rat brain hybridized to nick-translated, cloned cDNAs
as in Fig. 1 and 2. Control cDNAs C = cDNA of corona mRNA 7
(kindly provided by Dr. Siddell), pBR = pBR322 without insert.

The presence of M protein specific mRNA does not imply
that functional matrix protein is necessarily synthesised
within the infected brain. One SSPE cell line is known in which
M protein mRNA is not utilized correctly in translation re-
actions (9). Further experiments are in progress to investi-

gate the activity of these mRNAs in in vitro protein synthe-
sising reactions. This study demonstrates the usefulness of
these techniques in analysing the state of measles virus gene
expression in diseased tissue.

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TEMPORAL CHANGES OF MEASLES VIRAL PROTEINS IN HELA CELLS
ACUTELY AND PERSISTENTLY INFECTED WITH MEASLES VIRUS[1]

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Measles virus, a paramyxovirus, normally causes an
acute infection with accompanying classic symptoms of rash
and fever. Occasionally, however, the virus can generate a
persistent infection. The neurological disorder subacute
sclerosing panencephalitis (or SSPE) is one clinical
manifestation of persistent measles virus infection. SSPE
is the best studied example in man of a persistent viral
disease caused by a virus that normally causes an acute
infection (1). As such, measles virus persistence offers an
excellent system for the study of viral persistence in man.
     The study of persistent infections has been greatly
aided by the establishment of persistently infected tissue
culture cell lines in the laboratory. By comparing
persistently and acutely infected cell lines, one may glean
some information about the possible cause and/or consequence
of persistent infections. This report presents some
preliminary results of one such study in progress in our
laboratory.
     The purpose of this study was to examine the fate, with
time, of newly synthesized viral proteins during persistent
and acute infections. Acutely and persistently infected
HeLa cells were subjected to a one-hour pulse of radioactive
labeling, then chased for varying lengths of time with
medium containing no radioactive label. The resultant
labeled intracellular viral proteins were isolated by immune
precipitation and examined by SDS-PAGE.
     Acute infection was accomplished by infecting HeLa
cells with wild type measles virus   at a multiplicity of
infection of one. Two HeLa cell lines persistently infected
with measles virus (2, 3, 4) were studied. The first

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persistently infected cell line, K11, produces extracellular
virus at a low level (a productive persistent infection).
The second line, K11A, was derived from K11 after extended
culture in the presence of measles specific antisera.
Unlike K11, K11A produces no detectable extracellular virus
(a non-productive persistent infection). However, virus can
occassionally be recovered from K11A cells following
extensive co-cultivation with suitable permissive cells (5).
In this regard K11A closely resembles the brain cells of
patients with SSPE, which also produce no directly
detectable infectious virus yet from which SSPE virus has
occassionally been isolated by co-cultivation techniques
(1).   We have previously reported differences, detected by
pulse labeling followed by SDS-PAGE, between the M (matrix),
H (hemagglutinin), and NP (nucleocapsid) proteins of these
persistently infected cells compared to acutely infected
cells (6).   Differences were also seen between the two
persistently infected cell lines.

PULSE-CHASE EXPERIMENTS

     Pulse-chase experiments have previously been done with
cells acutely infected with wild type measles virus (7, 8).
In the study reported here, the results for the acutely
infected cells were identical to those of the productive
persistently infected cell line K11.   Therefore, due to
space limitations,   gels of the acutely infected cells are
not shown.

     Stability of viral proteins with time. The major viral
proteins from acutely infected HeLa cells and K11 cells
appeared to be stable throughout the chase period (Figure
1).   On the other hand, the viral M (matrix) protein band
from K11A cells diminished in intensity and eventually
disappeared during the chase (Figure 2).   At the end of the
labeling period (lane 0), the M protein formed a prominent
band on the gel.   Two hours into the chase, the intensity of
the M protein band was greatly diminished.   The band was
barely visible after a chase of four hours and by the end of
the six-hour chase, it had all but disappeared.   Therefore,
it appears that the M protein in K11A is less stable than
that in both acutely infected HeLa cells and the other
persistently infected line.
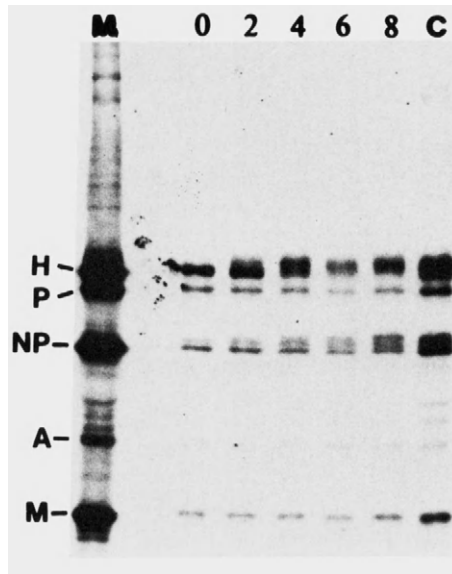
Figure 1.   Fluorogram of a pulse-chase experiment in K11 cells.   Cell monolayers were labeled with [^{35}S]methionine for one hour as previously described (7, 9). Cells subjected to chase periods were washed three times with 1 ml of medium supplemented with a large excess of cold methionine (150 mg/L) and 10% fetal calf serum and then reincubated in the same medium. After either a pulse labeling period alone, or a pulse followed by a chase, the monolayers were harvested, immune precipitated with anti-measles antiserum (9), run on SDS-PAGE (10) and processed for fluorography.   Viral proteins: H, hemagglutination protein [80,000 daltons (80K)]; P, nucleocapsid associated phosphoprotein (70K); NP, nucleocapsid protein (60K); M, matrix protein (37K); A, cellular actin (43K). L, involved in transcription (200K) and F_1 (41K) and F_2 (20K), the 2 components of the fusion protein are not consistently detected and were not analyzed.   Gel   lanes: M, measles proteins as marker; 0, no chase; 2, 2 hr chase; 4, 4 hr chase; 6, 6 hr chase; 8, 8 hr chase; C, continuous labeling for 8 hours.
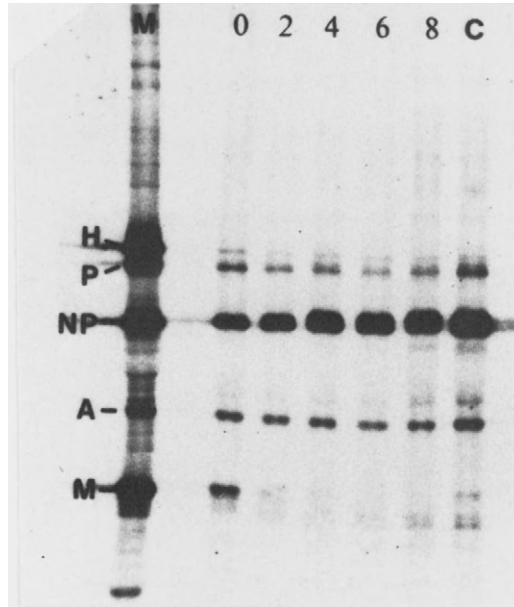
Figure 2.   Fluorogram of pulse-chase labeled K11A cells.   Conditions were identical to those of Figure 1, but with K11A cells.

Processing of viral proteins. Two viral proteins, the major nucleocapsid protein (NP) and viral hemaglutinin (H), in acutely infected cells and K11 were processed over a period of time (Figure 1). In the course of the chase, the initial band of each protein gradually disappeared with the concommitant appearance and subsequent increase in intensity of a new band of slightly higher molecular weight just above the original band. The NP and H proteins are phosphorylated (11) and glycosylated (7, 12), respectively. The new protein bands are probably the more highly phosphorylated and glycosylated forms of their respective precursor proteins (6, 13).

    The situation in K11A was somewhat different. The processing of NP appeared to proceed normally (Figure 2). (This is seen more clearly in a shorter exposure of this gel, shown in Figure 3). On the other hand, there was no detectable processing of the H protein during the chase period. As late as eight hours into the chase, no new protein band appeared above the original H protein band.
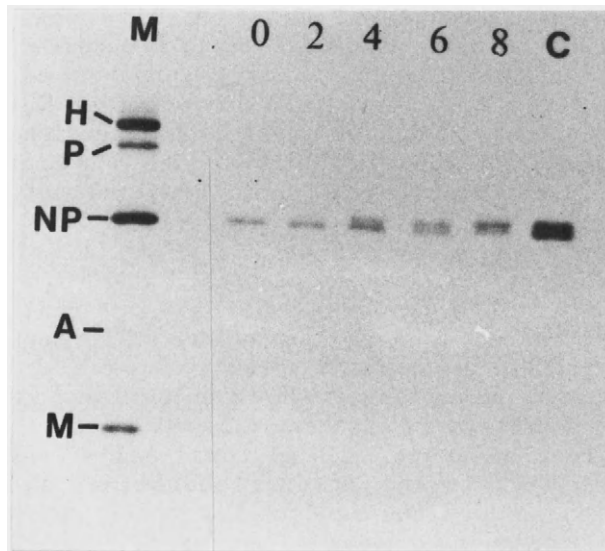
Figure 3. A shorter exposure of the gel in Figure 2.

Therefore, the H protein is either not processed or is
processed very slowly. In addition, there also appears to
be less H protein present in the K11A cells. It is not known
whether the failure of the H protein to be processed (or its
reduced quantity) in K11A cells is related to the
instability of the M protein in these cells.

DISCUSSION

     We have previously reported alterations in the
electrophoretic mobilities or quantities of the M, H, and NP
proteins in the persistently infected cell line K11A
compared to cells acutely infected with wild type measles
virus (6, 13). Using pulse-chase experiments, we have
reported here two additional significant differences between
the viral proteins of these non-productive, persistently
infected K11A cells compared to the viral proteins of cells
acutely infected with measles virus. The viral M protein in
K11A cells was unstable. In addition, the H protein in K11A
cells did not undergo the processing observed during acute
measles virus infection.

A second persistently infected cell line, K11, that does produce some infectious extracellular virus, had a stable M protein and normal processing of H protein. Since the K11A cell line was derived from K11 cells, it is possible that the alterations in M and H detected here may be involved in the biological difference between K11 and K11A, i.e., the change from a productive persistent infection to a non-productive persistent infection.

The cause of the instability of the K11A M protein is not known. The protein may be inherently unstable, it may be unstable due to temperature sensitivity (the pulse-chase experiments were performed at $37^{\circ}C$), or it may be more susceptible to proteolytic degradation. It is also possible that the K11A cell M protein is normal, but that the K11A cell itself has changed in such a way as to degrade that particular protein faster than normal.

The lack of processing of the H protein and the reduced quantity of H protein in K11A cells appeared to be reflected in the biological properties of these cells. K11A cells have reduced surface viral immunofluorescence and reduced hemadsorption ability compared to K11 cells and HeLa cells acutely infected with measles virus (2, 3, 6). These differences are consistent with either reduced amounts of the viral hemagglutination (H) protein in the cell membrane or abnormal processing of this protein.

The lack of processing of the H protein and the instability of the M protein in K11A cells may be coincidental or they may both be linked to the mechanism of non-productive persistence. The instability of the M protein could also be involved in the lack of processing and reduced quantity of H. Since the M protein may be involved in anchoring H protein in the cell membrane, an unstable M protein could result in H protein being lost from the membrane. This would not only reduce the total amount of H present, but would eliminate processing events that occur while H is in the cell membrane.

Reduced H protein and lack of its processing could be involved in the mechanism of non-productive viral persistence. K11A was derived from K11 through extensive culture in the presence of antisera directed against measles virus antigens. This process may have selected in favor of persistently infected cells which produce little or no cell surface viral antigens (as suggested by the antigenic modulation model for viral persistence (14)). Should presence of viral proteins on the cell surface be necessary for alignment of viral nucleocapsids and subsequent viral budding, selection in favor of cells which lack viral proteins on the cell surface would also be selection in

favor of cells which cannot produce extracellular viruses.

K11A cells are of special interest because of the close similarity of the non-productive viral persistent state to that in the brain cells of patients with SSPE. In both systems, no detectable infectious virus is produced, and virus can be recovered only following extensive co-cultivation with cells permissive for measles virus. The instability of the K11A cell M protein also appears similar to that of the M protein in SSPE infected brain cells. A great deal of evidence supports the theory that in SSPE the viral M protein is present in greatly reduced amounts (1), and that this may be due to synthesis of an unstable M protein (1, 15, 16). Thus, we have both an in vivo and an in vitro persistent measles infection with similar biology and an apparently similar molecular defect, instability of the M protein.

It is tempting to speculate that mutations in the M protein are involved in the mechanism of establishment of non-productive persistent infections. However, it is also possible that persistent measles virus infection leads to alterations in the measles virus M protein. If this is the case, then instability of the M protein may be involved in the maintanence of non-productive measles virus persistent infections.

More than one mechanism probably exists for the generation of non-productive persistently infected cells. We have found that late passages of K11 cells produce fewer extracellular virus particles than earlier passages (as much as a 40-fold reduction in 150 passages). Yet these cells contain M proteins of normal stability and their H proteins are processed in an apparently normal manner. As this dimunition of virus yield occurs as a result of extensive serial passage in the absence of antisera, the possibility of an alternate pathway to the non-productive state cannot be ruled out. Experiments are in progress to test out the various hypotheses.

ACKNOWLEDGMENTS

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MODULATION OF MEASLES VIRUS-SPECIFIC PROTEIN SYNTHESIS BY
CYCLIC NUCLEOTIDES AND AN INDUCER OF ADENYLATE CYCLASE[1]

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    Measles virus (MV) replication in mammalian cells is
inhibited by a variety of biological and chemical agents
including specific antibody (1,2), interferon (3-5), cytocha-
lasin B (6,7), anaesthetics (8,9), prostaglandins (10), and
cyclic nucleotides (10-12). The mechanisms by which these
substances inhibit MV replication is unknown or poorly under-
stood. However, certain virus replication processes are
affected similarly by many of these inhibitors. These
processes include (1) assembly of nucleocapsids and virions,
(2) intracellular nucleocapsid distribution, (3) fusion of
cells, and (4) virus-specific protein synthesis.
    The results of other studies indicate that the establish-
ment and/or maintenance of persistent MV infections involves
tissue- or cell-specific restriction of virus replication
(13-16). Interestingly, the virus replication processes
affected by the inhibitors mentioned above also appear to be
affected in persistent infections. It has been suggested that
a common bio-molecular mechanism may be involved in the inhibi-
tion or restriction of MV replication observed in these
studies (11,12).
    Previous workers have shown that the synthesis of certain
MV structural proteins is restricted in tissues from the
central nervous system (CNS) of patients with subacute sclero-
sing panencephalitis or SSPE (17,18). Similar restriction of
MV-specific protein synthesis has also been described in virus-
carrying cell lines derived from biopsied SSPE tissue (19).
While the synthesis of a number of MV-specific proteins was
shown to be decreased in these studies, the virus M protein
was most significantly affected.
    In a separate study, the synthesis of the M protein was

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shown to be inhibited by treatment of acutely infected human
amnion cells (AV3) with adenosine 3',5' monophosphate (cyclic
AMP)(11). A subsequent study showed that treatment of acutely
infected human neural cells with an inhibitor of phosphodies-
terase (papaverine) also inhibited the synthesis of the virus
M protein (12). These findings suggested that elevated intra-
cellular concentrations of cyclic AMP in CNS tissues may be
involved in restricting the synthesis of the M protein in
SSPE.

We have been interested in the mechanism by which cyclic
AMP inhibits the production of MV structural proteins and what
role this may play in the evolution of persistent MV infec-
tions. In this paper, we describe the effects of cyclic AMP
and an inducer of adenylate cyclase (dopamine) on MV-specific
protein synthesis in persistently infected AV3 cells (16).

In our initial experiments, we examined the synthesis of
uninfected AV3 cell proteins following exposure to various
concentrations of cyclic AMP or dopamine. Concurrently, we
examined the synthesis of MV-specific proteins in acutely and
persistently infected cells which were also treated with the
chemicals. Figure 1 shows the relationship between the syn-
thesis of cellular and virus-specified proteins and the cyclic
nucleotide concentration used to treat the cells. Similar
results were obtained for dopamine although the concentrations
used were approximately 10 fold lower (data not shown). While
significant inhibition of cellular protein synthesis was not
seen when concentrations of up to 10 mM cyclic AMP were used
to treat uninfected cells, the synthesis of viral proteins was
clearly inhibited in both acutely and persistently infected
cells. The inhibition was more pronounced in acutely infected
cells and paralleled the decreased production of infectious
particles reported earlier (11).

While the synthesis of the virus M protein was markedly
affected in cyclic AMP-treated acutely infected cells (data
not shown), there was only a generalized inhibition of all
virus structural proteins in persistently infected cells
(see Figure 2). At cyclic AMP concentrations greater than or
equal to 500 μM, the HA, P, and F_1 viral proteins were not
readily detected by immunoprecipitation and SDS-PAGE. However,
since these proteins were not produced at high levels in
untreated cells, it was difficult to evaluate the actual
extent of their inhibition.

The parameters used for the treatment of the persistently
infected cells with cyclic AMP and dopamine in these experi-
ments were based on those used in earlier studies for the
treatment of acutely infected cells, i.e. 24 hours of exposure
followed by radio-labelling and immunoprecipitation (11). The
production of the M protein in the persistently infected cells
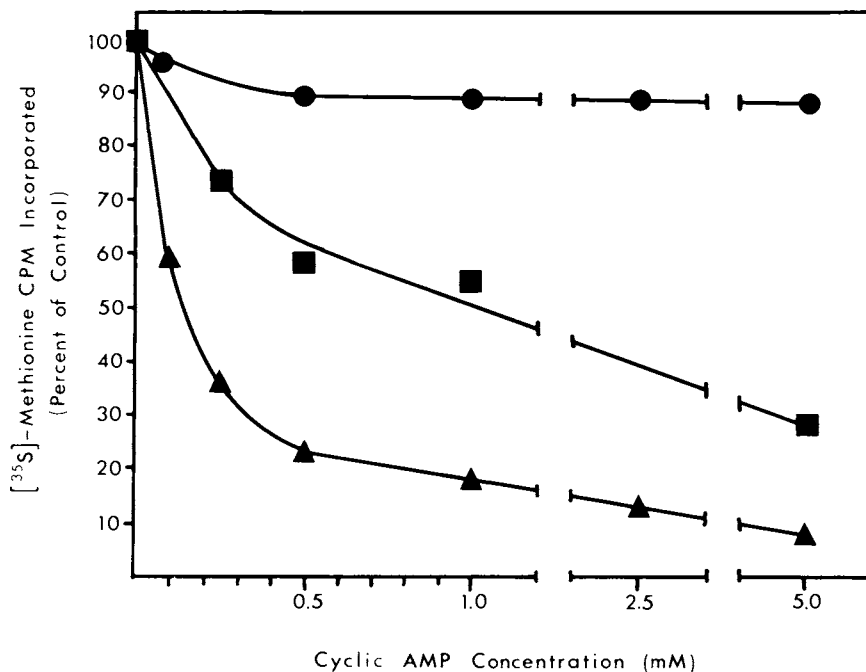
Figure 1. Measles virus- and cell-specific protein
synthesis in uninfected (●), acutely infected (▲), and
persistently infected (■) human amnion cells (AV3) following
exposure of cells to different concentrations of cyclic AMP
for 24 hours. All cells were labelled with [^{35}S]methionine
at 40 µCi/ml for 4 hours in the presence of cyclic AMP. Cell-
specific incorporation of radio-isotope was determined by
counting TCA-precipitable materials in a beta-scintillation
counter; virus-specific isotope incorporation was determined
by similarly counting immunoprecipitable materials from
acutely and persistently infected cells.

suggested that the treatment protocol was not sufficient to
inhibit the protein's synthesis or that the biosynthetic step
involved was no longer susceptible to the inhibitory effects
of the chemical. Although treatment of the cells with cyclic
AMP and/or dopamine for up to one week further inhibited the
overall synthesis of the viral proteins, it did not appear to
specifically block the synthesis of any particular protein
(data not shown). This seemed to indicate that the biosynthe-
tic step involved in the inhibition of virus M protein
production in acutely infected cells was not susceptible to
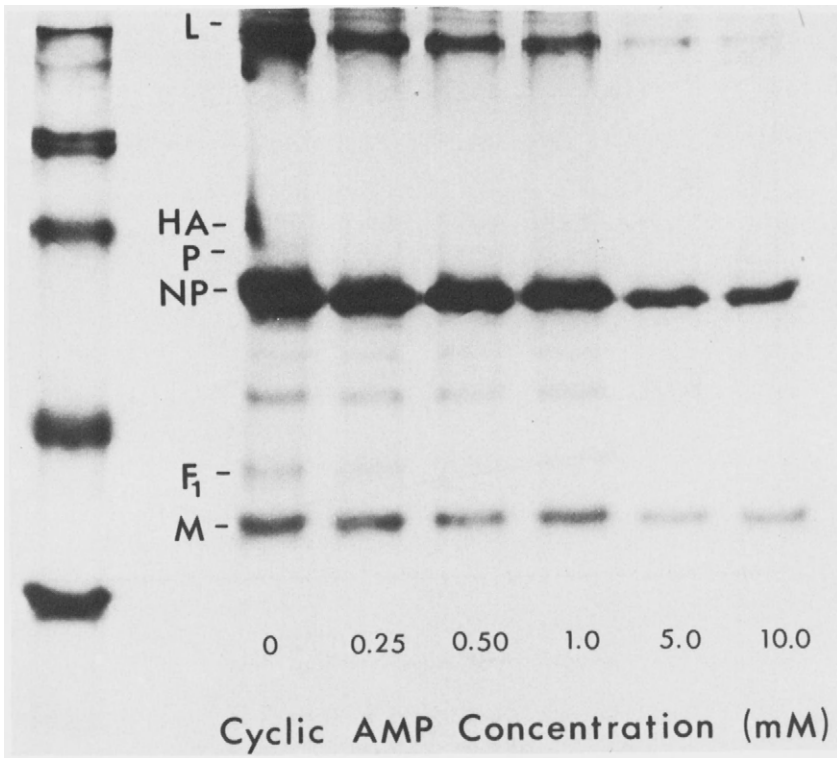
Figure 2.   Immunoprecipitates of [^{35}S]methionine-labelled cyclic AMP-treated persistently infected AV3 cells as analyzed by SDS-PAGE and fluorography.   Radio-labelled molecular weight marker proteins are shown in the left of the figure.   Immunoprecipitation was carried out as previously described (11) using uniform conditions for each sample.

inhibition in persistently infected cells.   Although this result seems paradoxical, it may indicate that initiation of M protein production is the step affected by cyclic AMP and that once initiated (transcribed? translated?) it is no longer as susceptible to the inhibitory effects of the chemical. Nevertheless, there does seem to be a generalized inhibitory effect of cyclic AMP on MV-specific protein production.
    Interestingly, chronic treatment of the AV3/MV cells with cyclic AMP and/or dopamine also resulted in a pronounced redistribution of intracellular virus antigens.   As shown in Figure 3, substantial numbers of MV inclusion bodies in cell nuclei were observed in such chemical-treated cells.   With prolonged treatment, dissapation of cytoplasmic inclusion
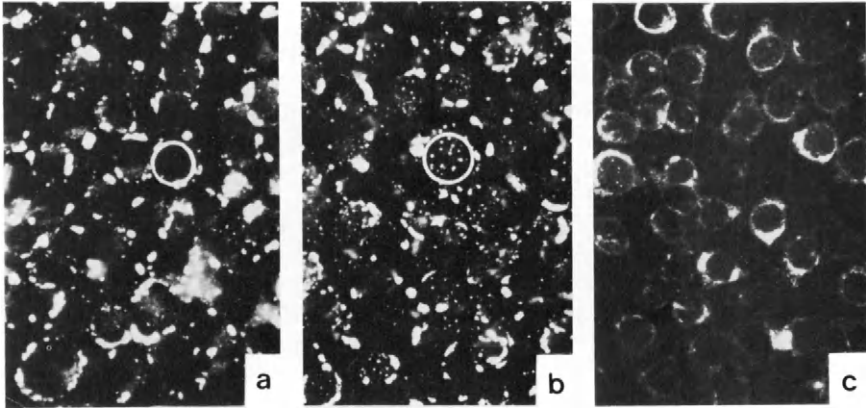
Figure 3. Expression of MV antigens in persistently
infected AV3/MV cells as detected by immunofluorescence (a)
and after treatment of 1 mM cyclic AMP for 4 days (b) or
250 μM cyclic AMP for 6 weeks (c). The encircled areas in (a)
and (b) encompass single cell nuclei showing the relative
levels of MV antigens.

bodies also occurred. The significance of these observations
is unclear. It is perhaps worth noting that intranuclear
MV inclusion bodies occur frequently in SSPE CNS tissues and
have previously been proposed as diagnostic markers of the
disease (20).
    Although the exact mechanism by which cyclic AMP inhibits
MV replication was not determined in the present study, the
modulatory effect of the chemical on MV-specific protein
synthesis and intracellular antigen distribution in persis-
tently infected cells seems to support the hypothesis that
cyclic nucleotide levels may play regulatory roles in such
infections.

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FINE STRUCTURAL ANALYSIS AND PHOSPHORYLATION SITE
DETERMINATION IN VSV NS PROTEIN[1]

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NS protein is a phosphoprotein with a molecular weight of
25,000 (1) and it has been suggested that phosphorylation of
this small enzyme subunit may be involved in regulation of the
VSV RNA polymerase enzyme function by mediating the switch
from transcription to replication (2,3,4,5). We decided that
the only way to precisely relate the changes in enzyme
function to phosphorylation changes was to determine the sites
of phosphorylation in NS protein for active and inactive
enzyme with the ultimate goal of determining if changes in
sites of phosphorylation correlated with changes in enzyme
function.

ANALYSIS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

First, we have studied how the highly acidic nature of NS
protein, predicted from the DNA sequence (1), affects its
behavior in polyacrylamide gels. NS protein migrates
anomalously on sodium dodecyl sulfate (SDS) polyacrylamide
gels (6,7). NS protein has also been shown to exhibit at
least two molecular weight forms in different gel systems
which contain urea (2,3,5). Under no conditions has NS
protein been shown to migrate as its true molecular weight of
25K, as determined by DNA sequence data (1). The reason for
this aberrant migration is unclear but it has been suggested
that phosphorylation might cause the abberant migration of NS
protein either by changing the charge of the protein (2, 5);
through the formation of phosphodiester bonds which leads to
dimers of NS protein (8); or because of it bound SDS poorly

due to its highly acidic nature as indicated by DNA sequence
data (1). A schematic representation of the amino acid
sequence of NS protein deduced from the cDNA sequence is shown
in Fig. 1.
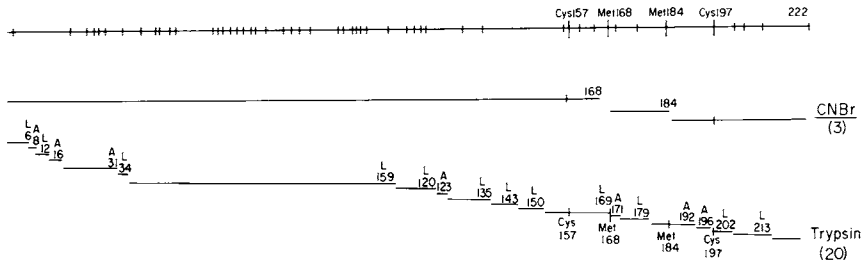
FIGURE 1. NS protein amino acid sequence (1). On the
first line the short vertical lines represent aspartate and
glutamate residues. Second line shows the 3 CNBr peptides.
Third line shows tryptic peptides, ending with arginine (A) or
lysine (L).

In order to establish what the basis for the aberrant
electrophoretic behavior of NS protein was, we analyzed VSV
proteins on polyacrylamide gels using the detergent
cetyltrimethylammonium bromide (CTAB) instead of SDS (9).
CTAB is a cationic detergent and might be expected to bind to
the acidic residues of NS protein and perhaps result in a
migration which more closely corresponded to its true
molecular weight. Figure 2 shows polyacrylamide gel analysis
of VSV proteins in three different buffer systems. Figure
2A,B shows that NS protein migrates as a 48K protein in our
standard Tris-glycine buffered gel system. When the buffer
was changed to an acidic buffer (Fig.2C,D) NS protein migrated
with an apparent molecular weight of 35K. When the gels were
made with the acidic buffer and the detergent in the samples
and the gels was changed to CTAB, NS migrated with an apparent
molecular weight of 32K (Fig.2E,F,G). Positive identification
of NS protein was made by using VSV intracellular RNPs,
labeled with [^{32}P], in which NS is the only radiolabeled
protein (Fig.2B,D,G). In the CTAB system M protein migrates
slightly slower than its true molecular weight of 26K so in
Figure 2E M and NS proteins were not resolved.There is also no
evidence of more than one form of NS protein on either acid-
SDS gels or CTAB gels (data not shown). Since in the CTAB

gels NS protein migrated very close to its true molecular
weight the hypothesis that it migrates anomalously on SDS gels
because it binds SDS poorly appears to be substantiated by
these data, and the suggestion that it might be a dimer
appears unlikely.
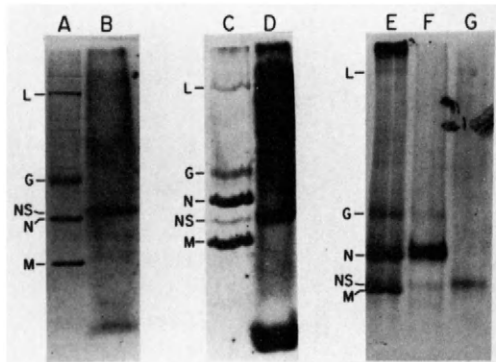
FIGURE 2.   PAGE analysis of NS protein. Intracellular
RNPs radiolabelled with [35S]methionine (lanes F) or [32P]
orthophosphate (lanes B,D,G); or virions radiolabeled with
[35S]methionine (lanes A,C,E) were analyzed by SDS Tris-
glycine PAGE (lanes A,B)(10), SDS β alanine PAGE (lanes
C,D)(9) or CTAB β alanine PAGE (lanes E,F,G)(9).

Analysis of CNBr Cleavage Products. The deduced amino acid
sequence of NS protein showed that most of the acidic groups
were located on the amino-terminal half of the protein (1).
Therefore we analyzed this domain of NS protein further by
analyzing the CNBr cleavage products for the amount of
phosphorylation of these CNBr peptides and for their migration
on SDS polyacrylamide gels. The sequence predicts cleavage at
methionines 168 and 184 creating a large peptide of a
molecular weight of 18,028, containing the N-terminus of the
protein, and 2 small peptides (MW, 4943 and 2139).
     The sequence of NS protein predicts that it will contain
a total of 75 aspartate and glutamate residues, 90% of which
are in the large CNBr peptide. This large N-terminal fragment
should also contain [35S]cysteine. Therefore, in order to
identify the large acidic terminal peptide, NS protein was
isolated from intracellular RNPs (which contain only one form
of NS protein, (2)), radiolabeled with [3H]aspartate and
[3H]glutamate, [35S]cysteine, or [32P] orthophosphate, cleaved

with CNBr and analyzed by PAGE (12). Figure 3, lane 1, shows
that residual uncleaved NS protein migrated with an apparent
molecular weight of 45Kd. The large major CNBr peptide
migrated with a molecular weight of 32Kd. This large peptide
contains the majority of the [^{35}S]cysteine (Fig. 3,lane 1);
the acidic residues (Fig. 2,lane 2); and the phosphoamino
groups (Fig 3., lane 3), as predicted by the sequence.
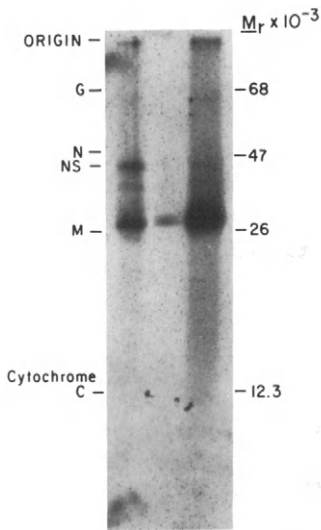
FIGURE 3. PAGE analysis of
NS CNBr peptides. NS protein
from intracellular RNPs (11) was
isolated from SDS Tris -glycine
polyacrylamide slab gels,
cleaved 18hr with 5mg CNBr,
dried and analyzed by SDS-urea
phosphate PAGE (12). NS was
radiolabeled with either
[^{35}S]cysteine (lane 1),
[^{3}H]aspartate and [^{3}H]glutamate
(lane 2), or [^{32}P]
orthophosphate (lane 3).

   The sequence also predicts the existence of a smaller
[^{35}S]cysteine-containing peptide. This peptide is shown in
Fig.4A, migrating right above cytochrome C. This peptide
contains no detectible [^{32}P], [^{3}H]aspartate or [^{3}H]glutamate
(Fig. 3). Therefore our analysis showed that NS protein is
highly acidic and contains the majority of its acidic residues
and phosphoamino groups on its large N-terminal CNBr
peptide. Furthermore, this large CNBr peptide migrates with
an apparent molecular weight of 32Kd rather than 18Kd as the
sequence would predict.
Analysis of Peptides on CTAB Gels. In order to obtain a more
accurate estimate of the molecular weight of the CNBr peptides
we analyzed the peptides using the detergent CTAB instead of
SDS for PAGE. NS protein, radiolabeled with [^{35}S]cysteine,
was cleaved with CNBr and analyzed by SDS-urea PAGE (12)(Fig.
4A). One can see that there were two major peptides migrating
with apparent molecular weights of 33Kd and 15Kd. The 33Kd
peptide peptide was then isolated from the SDS gel and

analyzed by CTAB PAGE (Fig. 4B). Under these conditions it
migrated with an apparent molecular weight of 17Kd, very close
to the molecular weight (18Kd) predicted by the DNA sequence
(1). This provided further evidence that this is the acidic
N-terminal peptide predicted by the sequence. Furthermore it
shares with NS protein the property of aberrant migration on
SDS PAGE, probably because of its highly acidic nature.
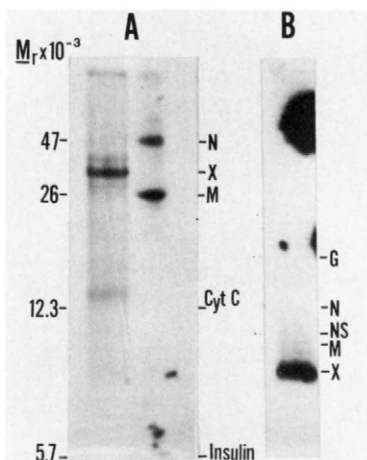
FIGURE 4. CTAB PAGE
analysis of NS CNBr peptides.
NS protein from intracellular
RNPs, radiolabeled with
[^{35}S]cysteine were analyzed by
SDS-urea PAGE (A,lane 1), the
large band (X) was cut out of
lane 1, electroeluted,
concentrated and analyzed by
CTAB PAGE (B). Lane A1,
[^{35}S]cysteine RNPs. Lane A2,
[^{35}S]methionine virions. B,
peptide X from A.

ANALYSIS OF NS PROTEIN TRYPTIC PEPTIDES

After we had localized the sites of phosphorylation of NS
protein to the amino terminal CNBr peptide we did a more
detailed analysis in order to more precisely localize the
sites. For this purpose we chose tryptic peptide analysis by
high performance liquid chromatography (HPLC)(13). By using
the amino acid sequence of NS protein deduced from the DNA
sequence (1), the nature of the tryptic peptides could be
predicted, and one could determine which specific amino acids
these peptides contained. Once the peptides were identified
their specific sites of phosphorylation could be determined.
From the sequence, 20 tryptic peptides were predicted; three
of the tryptic peptides could be positively identified by
labeling with [^{35}S]methionine, [^{35}S]cysteine and [^{3}H]lysine.
The sequence predicts that peptide 13 should have all the

above labels, peptide 16 should have only [^{35}S]methionine label and peptide 18 should have [^{35}S]cysteine and [^{3}H]lysine.

Purified NS protein, radiolabeled with either [^{35}S]methionine or [^{3}H]lysine, was digested with trypsin and the peptide samples were combined and analyzed by HPLC (13), as shown in Figure 5. The data show that NS protein radiolabeled with [^{35}S]methionine contained two tryptic peptides (fractions 22,30). The first peak (faction 6) contained a highly positive charged residue, and was shown not to be a peptide by further analysis (data not shown). Two methionine-containing tryptic peptides were consistent with the prediction made from the DNA sequence. However, Figure 5 also shows that both peptides radiolabeled with [^{35}S]methionine also contained [^{3}H]lysine instead of only one as predicted by the sequence.
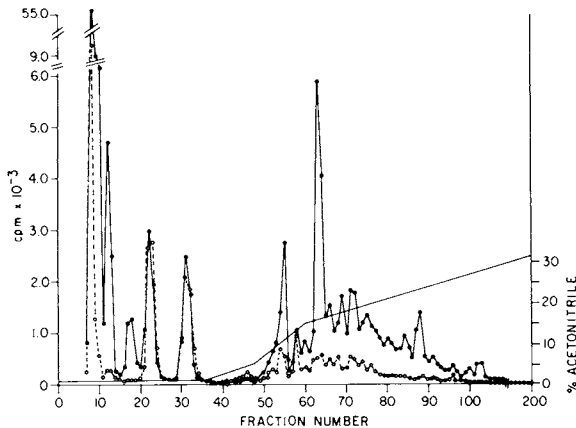
FIGURE 5. HPLC analysis of NS protein. NS protein was purified from intracellular RNPs by SDS PAGE, performic acid treated and digested with trypsin (20mg/ml,4hr,20C).Peptide samples, radiolabeled with either [^{35}S]methionine (0---0) or [^{3}H]lysine (0---0), were combined and analyzed by reversed phase HPLC. Peptides were eluted from a Whatman C18 column in a gradient of acetonitrile in 0.1% phosphoric acid.

To further analyze the tryptic peptides, NS protein was labeled with [^{35}S]cysteine and Figure 6A shows the HPLC profile of the tryptic peptides. One can see that instead of two peptides labeled with [^{35}S]cysteine as predicted, that there are at least three major and two minor peptides. In addition, peptide 13 was predicted to contain both [^{35}S]cysteine and [^{35}S]methionine, but there were no peptides observed that contained both amino acids (Fig. 5 and 6).

It was possible that the three or more peaks of [^{35}S]cysteine seen by HPLC analysis resulted from the additon of one or more phosphate residues to one or both of the cysteine peptides predicted by the cDNA sequence. The addition of a phosphate residue(s) to a tryptic peptide might be expected to change the behavior of this peptide on HPLC, leading to two or more new peaks eluting from the column. To test this possibility, we treated [^{35}S]cysteine labeled NS tryptic peptides with bacterial alkaline phosphatase before HPLC analysis. This treatment had been shown to remove about 50% of the label from [^{32}P]-radiolabeled NS protein (data not shown). The HPLC profile of the phosphatase-treated NS peptides (Fig.6B) shows no change from the untreated sample (Fig.6A). Therefore, the presence of phosphate groups on the cysteine-containing peptides did not appear to affect the elution of these peptides from HPLC suggesting that there were more that two cysteine-containing tryptic peptides in NS protein.
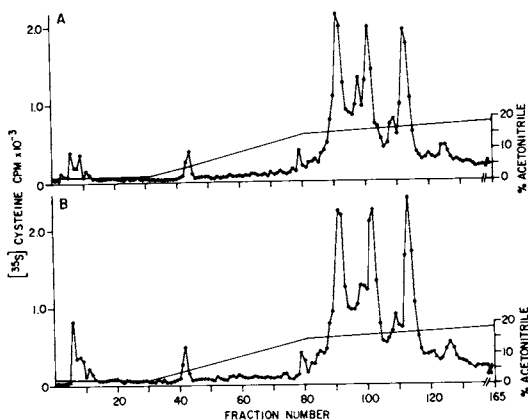
FIGURE 6. HPLC analysis of NS protein. A: NS protein, purified and analyzed as in Fig. 5 except radiolabeled with [^{35}S]cysteine. B: same as A except that after trypsin treatment the peptides were treated with bacterial alkaline phosphatase.

We have also attempted to identify which tryptic peptides of NS protein were phosphorylated. Tryptic peptides, radiolabeled with either [^{32}P] orthophosphate or [^{3}H]aspartate and [^{3}H]glutamate, were analyzed together by HPLC (Fig. 7). One can see that there were at least 11 peptides containing apartate and glutamate, confirming the highly acidic nature of NS protein, predicted by the sequence

(1). We attempted to identify the large 9.9Kb acidic tryptic
peptide using several different types of size exclusion
chromatography in buffers of pH 1.5, 3.0, 7.4 and in 67%
acidic acid, but in each case the peptides aggregated and were
eluted in the void volume. Fig. 7 also shows that there were
4 major phosphopeptides (peaks at fractions 110-111, 117, 123
and the shoulder at fraction 120), two of which contained
acidic residues (peak 110-111, shoulder 120). Additional
analysis (data not shown) has shown that neither of the
[^{35}S]methionine-containing peptides coelute with the
phosphopeptides. However the first phosphopeptide (Fig 7,
peak 110-111) coelutes with the third major [^{35}S]cysteine-
containing peptide (data not shown). More specific
identification of these peptides and the localization of the
phosphate residues in the sequence will have to wait until the
descrepencies between the published cDNA sequence (1) and the
results of our analyses of the tryptic peptides are resolved.
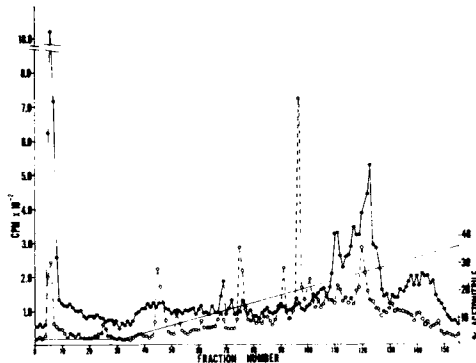
     FIGURE 7. HPLC analysis of NS protein. NS protein was
purified and analyzed as in Fig. 5 except radiolabeling was
done with [^{32}P] orthophosphate (O---O) or [^{3}H]aspartate and
[^{3}H]glutamate (0---0).

     Although the sequence predicts that only one of the two
methionine peptides should contain a lysine, our analysis
showed that both contained lysines. This could have been
caused by incomplete digestion, however other studies have
shown that digestion was complete by 2hr (data not shown)
whereas in all the data shown digestion was for at least twice
that long. The possibility does exist that the phosphate
groups interfered with the specificity of the trypsin,
preventing cleavage at lys179.

Our data also showed that neither methionine peptide contained a cysteine residue. Incomplete digestion would have increased the probability that they were in the same peptide so that cannot be the cause of the discrepency. The hetergeneity of the cysteine peptides was probably not caused by phosphates disturbing the elution patterns since treatment of the peptides with alkaline phosphatase did not change the elution patterns. Incomplete cleavage could account for the extra cysteine peptides but not for the fact that methionine and cysteine do not coelute.

Strain differences between the strain sequenced and the one analyzed for tryptic peptides was not the reason for the discrepencies, since analysis of the tryptic peptides of the sequenced strain also showed the same differnces (data not shown).

In summary, we have shown that specific tryptic peptides, predicted from the published sequence (1), cannot be identified by specific amino acid radiolabeling and analysis by HPLC. Work is in progress to clone the NS gene in order to sequence this VSV gene and resolve the descrepencies shown above. We have also confirmed the highly acidic nature of NS protein and shown that this is very likely the cause of its aberrant migration on SDS PAGE. In addition, we have shown the phosphates residues are located on the large CNBr N-terminal peptide. Further work is in progress to isolate the large CNBr peptide and cleave it with trypsin in order to further pinpoint the positions of the phosphoamino acids. In addition, differences between virion, cytoplasmic soluble and cytoplasmic-RNP NS protein phosphorylation sites will be studied.

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MAPPING PHOSPHATE RESIDUES REQUIRED FOR VSV
TRANSCRIPTION ON THE NS PROTEIN MOLECULE[1]

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NS PHOSPHORYLATION AND VSV TRANSCRIPTION

Vesicular stomatitis virus (VSV) transcription seems to
be activated by phosphorylation of a minor component of the
virus nucleocapsid, the NS protein (1,2). Nucleocapsids that
transcribe rapidly contain a highly phosphorylated form of
NS, designated NS2, which migrates ahead of the more abun-
dant, less heavily phosphorylated form, NS1, in some electro-
phoretic conditions (1-3). An extra cluster of chymotryptic
phosphopeptides, denoting specific sites of phosphorylation,
also distinguishes NS2 from NS1 molecules (2). Partial enzy-
matic dephosphorylation of NS2 converts it to NS1, removes
these extra phosphates, and reduces transcriptase activity
about 80% (2). In this work, we have located phosphorylation
sites common to NS1 and NS2 in the primary structure of the
protein and we have obtained some indications of where NS2-
specific phosphates reside.

CLEAVAGE AT METHIONINE

The NS protein has a molecular weight of about 22K (K =
1,000), according to the base sequence of its gene (4).
Based on the predicted amino acid sequence (4), we expected
three cyanogen bromide (CNBr) fragments (Fig. 2). When
$^{32}PO_4$-labeled NS was treated with CNBr, a large peptide with
a molecular weight of about 17K, and a smaller, 4K peptide,
were seen, corresponding to the expected N-terminal and C-
terminal fragments, respectively (Figs. 1A and 2). The
third, internal fragment that should have been generated may
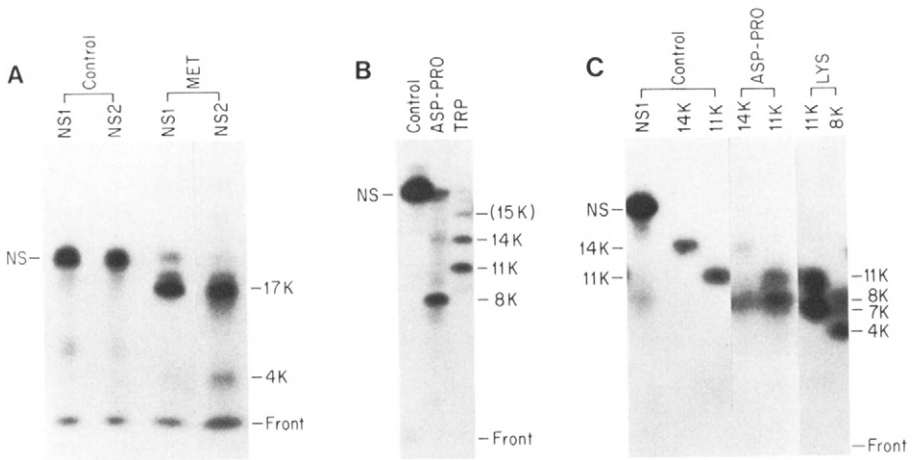
FIGURE 1. Polyacrylamide gel electrophoresis of [^{32}P]phosphate-labeled NS (2) and its cleavage products. In all cases, wet gels were exposed to X-ray film without fixing or staining, to obviate losses of low molecular weight peptides. Ovalbumin (45K), trypsin (24K), lysozyme (14K), Aprotinin (6.5K) and insulin B chain (3.5K) were used as unlabeled molecular weight markers. A. NS1 and NS2 from virions were isolated separately by gel electrophoresis and cleaved at MET with CNBr in 70% formic acid for 2 hr at 24°C in the dark, before electrophoresis in a 10% polyacrylamide gel. B. NS1 from virions was cleaved at ASP-PRO in 70% formic acid at 50°C for 16 hr (6), or at TRP with 3-bromo-3-methyl-2-[(2-nitrophenyl)thio]-^{3}H-indole (BNPS-skatole) in 70% acetic acid, for 16 hr at 24°C in the dark (7) and fragments were separated by electrophoresis in a 10% to 18% polyacrylamide gradient gel. C. The 14K and 11K products of BNPS-skatole treatment were extracted from gels and cleaved at ASP-PRO as in panel B; the same 11K peptide and the 8K product of ASP-PRO cleavage were cleaved at LYS$_{35}$ with trypsin (20 μg/ml in 0.01 M Tris-HCl, pH 8.0, 37°C, 16 hr.). Products were separated as in panel B.

have migrated in the radioactive band at the electrophoretic buffer front, since it is expected to have a low molecular weight (about 1.6K). The 4K fragment from NS2 molecules contained more radioactive phosphate than its counterpart from NS1 molecules, suggesting that this fragment contains some sites that are specific to NS2. It also appears that the 17K fragment is the main repository of phosphorylated sites common to both NS1 and NS2 molecules (2).

CLEAVAGE AT TRYPTOPHAN

To further localize phosphorylated residues in the 17K CNBr fragment, ^{32}P-labeled NS molecules were cleaved at TRP. Two major peptides with apparent molecular weights of 14K and 11K were obtained in addition to a larger peptide, tentatively assigned a molecular weight of 15K (Fig. 1B). The 14K and 15K peptides appear to be products of incomplete cleavage at the three TRPs in NS (Fig. 2). (The expected 7K, 3K and 1K fragments located at the C-terminal side of NS were seen after longer autoradiographic exposures; evidently, they contain considerably less radiophosphate than the 11K peptide; data not shown.)

CLEAVAGE AT ASPARTATE-PROLINE

Formic acid treatment of either the 11K or 14K fragment, under conditions relatively specific for the single ASP-PRO linkage in NS, yielded one main ^{32}P-labeled product, an 8K peptide (Fig. 1C). The same result was obtained after ASP-PRO cleavage of intact NS (Fig. 1B), indicating that most of the phosphate residues in NS and in the 11K and 14K fragments reside in this 8K region. The 8K fragment appears to be located at the N terminus of the NS molecule, because it was also obtained when the 17K CNBr fragment was cleaved at the ASP-PRO site (data not shown).

CLEAVAGE WITH TRYPSIN

To locate the phosphorylated sites in the 11K peptide and its 8K subfragment more precisely, each was digested separately with trypsin. The only trypsin-sensitive bond in either peptide is at LYS_{35}, since LYS_{50} is followed by a PRO, rendering it resistant to tryptic digestion (5). The 11K peptide yielded a ^{32}P-labeled fragment about 7K in molecular weight, whereas the 8K piece yielded a 4K subfragment (Fig. 1C). Based on the site specificities of the cleavage reactions that yielded these 7K and 4K subfragments, they appear to reside between residues 35 and 78 (Fig. 2). Comparing these findings with our previous chymotryptic peptide maps of NS1 and NS2 and with the predicted phosphoaminoacid compositions of these chymotryptic peptides, we note that there are five peptide bonds between residues 35 and 78 which are potentially chymotrypsin-sensitive (at LEU_{39}, PHE_{40}, TYR_{53}, PHE_{54} and TYR_{75}) and that the two predicted products (peptides 41-53 and 55-75) contain both phosphoserine and phosphothreonine, characteristic of the two groups of consistently phosphorylated chymotryptic peptides found in
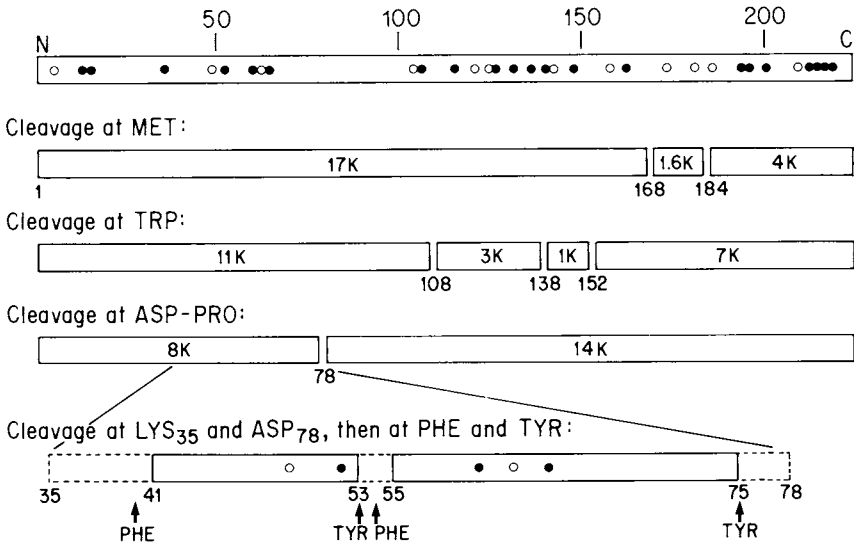
FIGURE 2. Diagram of the primary structure of the NS molecule and its expected cleavage products, based on the data of Gallione et al. (4). In the top band, N is the amino terminus and C is the carboxyl terminus. Open circles are the positions of THR residues and filled circles designate SER residues. The peptides expected from specific cleavages are shown below. Numbers below gaps represent amino acid residue positions at sites of cleavage.

both forms of NS (2). In addition to having an additional SER, peptide 55-75 is twice as rich in acidic amino acids as peptide 41-53, so we hypothesize that the former represents the previously designated electronegative chymotryptic peptide 1 and the latter represents the less electronegative chymotryptic peptide 2. Each of these peptides had been further resolved into subspecies that probably reflect variable phosphorylation or chymotryptic cleavage (2).

CONSTITUTIVE AND VARIABLE PHOSPHORYLATION SITES

Fig. 2 summarizes the deductions we have made from these cleavage studies. We believe that most of the sites in the NS molecule that are constitutively phosphorylated reside between residues 41 and 75, buried in the tertiary structure of the molecule and thereby protected from enzymatic dephosphorylation. Similar findings have been made by J.C. Bell

(Ph.D. Thesis, McMaster University) and L. Prevec (personal communication). The secondary sites of phosphorylation that are specific for NS2 molecules and which fully activate the viral transcriptase evidently reside elsewhere. The 4K C-terminal CNBr peptide may contain such sites. This peptide was more heavily phosphorylated in NS2 molecules (Fig. 1A), and it exhibits a high local concentration of SER residues (4), which may be exposed to the environment and the protein kinases that reside there.

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MATRIX (M) PROTEIN REQUIREMENT FOR THE BINDING
OF VESICULAR STOMATITIS VIRUS RIBONUCLEOCAPSID
TO SONICATED PHOSPHOLIPID VESICLES[1]

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The vesicular stomatitis virus (VSV) matrix (M) protein
is intimately associated with both the ribonucleocapsid core
and the envelope of the virus. It is, however, not exposed
at the exterior surface of the intact virion, since it is
refractory to protease digestion and ^{125}I-labeled lactoper-
oxidase iodination (1, 2). The use of lipophilic monofunc-
tional and bifunctional cross-linking reagents in intact
virions demonstrates that the M protein is in close proximity
to the inner surface of the envelope bilayer but penetrates
little, if at all, into the envelope (3). Moreover, in vitro
binding studies with lipid vesicles have shown the M protein
to be a peripheral membrane protein, which reconstitutes only
with vesicles containing acidic phospholipid components (4,
5).
    Earlier studies have shown that the major structural
proteins (N, G, and M) of VSV take independent pathways to
the site of virus assembly at the plasma membrane of the
infected cell (6, 7, 8). Appearance of M protein at the cell
membrane of an infected cell causes decreased mobility of the
already inserted glycoprotein (G) (9). The M protein is
believed to function in virus maturation as a bridge between
cytoplasmic viral ribonucleocapsids and regions of the cellu-
lar membrane into which the G protein has been inserted (10).
This process may occur through the interaction of the highly
positively charged M protein (11) with the phosphatidylserine
(PS) polar headgroups located in the inner leaflet of the
plasma membrane. Analysis of the phospholipid composition of
the VSV envelope revealed that PS comprises 18% of the total

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phospholipid and is asymmetrically distributed in the bilayer
with 85% located in the inner leaflet (12).

We have developed an in vitro model system to study the
role of M protein in the binding of VSV nucleocapsid core
structures to membrane bilayers. Nucleocapsid cores, with or
without M protein, are allowed to bind to sonicated unilamel-
lar vesicles (SUV) composed of acidic and/or neutral phospho-
lipids. Core structures are also allowed to react with nega-
tively charged SUV previously saturated with M protein. Our
results indicate that M protein is required for the optimal
binding of lipid vesicles to ribonucleocapsid cores. We have
also noted that the best binding occurs when M protein is
reconstituted with the negatively charged vesicle first, but
this may be only a steric effect.

PREPARATION OF VSV RIBONUCLEOCAPSID CORE STRUCTURES

Solubilization of VS virions in solutions containing
nonionic detergents and varying ionic strengths can selec-
tively remove the viral envelope and specific viral proteins
from the ribonucleocapsid core (13, 14). Ribonucleocapsid
cores with M protein attached (RNP/M) were prepared by sus-
pending whole VS virions (Indiana serotype) in 10 mM Tricine
(pH 7.5) containing 30 mM octylglucoside. The insoluble
cores were removed from the envelope components and soluble G
protein by ultracentrifugation. Analysis by SDS-polyacryla-
mide gel electrophoresis revealed that only the N, NS, L, and
M proteins were associated with the RNP/M complex. Electron
microscopic studies showed the RNP/M complex to be a con-
densed structure, slightly thinner and longer than an intact
virion. It has a density of about 1.30 g/cm^3.

Ribonucleocapsid core structures without M protein (RNP)
were prepared by solubilizing whole VS virion in 10 mM
Tricine (pH 8.0) containing 1% Triton X-100, 0.25 M NaCl and
dithiothreitol (0.2 mg/ml). Ultracentrifugation was able to
separate the core structures from the soluble envelope, G
protein and M protein. Analysis by SDS-PAGE revealed that
only the N, NS, and L proteins remain associated with the
RNP. Electron microscopic examination revealed the RNP to be
a long, strung out structure several times longer and thinner
than an intact virion. The density of RNP is about 1.26
g/cm^3. Core structures were stored in calcium-free and mag-
nesium-free phosphate buffered saline (PBS) at 4°C until
used.

PREPARATION OF SONICATED UNILAMELLAR PHOSPHOLIPID VESICLES (SUV)

Small sonicated unilamellar phospholipid vesicles (SUV) were prepared and purified to homogeneity by the method of Barenholz et al. (15). Lipid mixtures of either 100 mol% 1,2-dimyristoyl-3-sn-phosphatidylcholine (DMPC) or 50 mol% 1,2-dipalmitoyl-3-sn-phosphatidylcholine (DPPC) plus 50 mol% bovine brain phosphatidylserine (BPS), both containing trace amounts of [14C]DPPC as a marker, were dried and lyophilized overnight. The lipid preparations were resuspended in PBS at 35°C; each sample was sonicated for 3 min and then centrifuged for 185 min at 165,000 x g and 31°C. The upper two-thirds of the suspension, containing the SUV, was collected and used as the source of phospholipid vesicles. Vesicles range in size from 300-500Å.

RECONSTITUTION OF RIBONUCLEOCAPSIDS WITH NEUTRAL OR ACIDIC PHOSPHOLIPID VESICLES

Purified ribonucleocapsid cores without (RNP) or with (RNP/M) matrix protein were mixed with SUV of 100 mol% DMPC. The reaction mixtures were incubated for 6h at 37°C, then layered on top of a 0-66% sucrose gradient in PBS and ultracentrifuged to equilibrium. Figures 1A and 1B illustrate the density gradient analysis of the reaction mixtures. In both cases only a low level of binding, accompanied by a moderate density shift, occurred. The presence of M protein on the ribonucleocapsid core had no effect on the level of binding of neutral phospholipid vesicles to ribonucleocapsid core structures (Table 1).
Either RNP or RNP/M structures were incubated for 6h at 37°C with mixed SUV containing 50 mol% DPPC and 50 mol% BPS and then centrifuged to equilibrium in a 0-66% sucrose gradient. Figures 1C and 1D represent the analysis of these gradients. RNP structures bound the same low level of negatively charged vesicles as they had neutral vesicles (Table 1). The RNP/M structures, on the other hand, were able to bind much greater amounts of negatively charged vesicles (Fig. 1D and Table 1) than neutral vesicles (Fig. 1B). The presence of the M protein on the ribonucleocapsid also greatly increased the capability of VSV ribonucleocapsids to bind negatively charged vesicles (Fig. 1D); a large density change accompanied the increased binding. The concentration of salt had no effect on the binding by RNP/M of DPPC/BPS-SUV, indicating that the reaction was more specific than that which took place without M protein (data not shown).
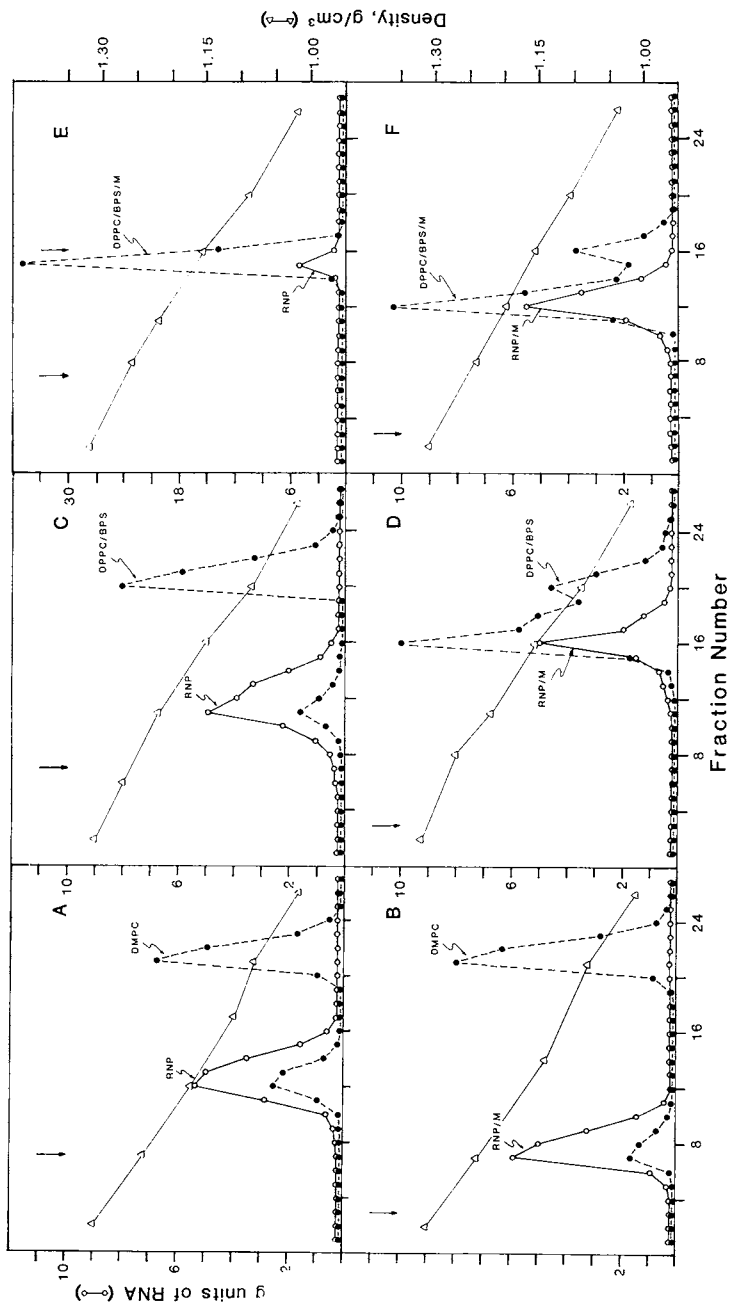
FIGURE 1. Sucrose density gradient analysis of the reconstitution mixtures of (A) RNP and DMPC-SUV, (B) RNP/M and DMPC-SUV, (C) RNP and DPPC/BPS(1:1)-SUV, (D) RNP/M and DPPC/BPS (1:1)-SUV, (E) RNP and DPPC/BPS(1:1)-SUV previously saturated with M protein, and (F) RNP/M and DPPC/BPS(1:1)-SUV previously saturated with M protein. The vertical arrows indicate where lipid-free RNP or RNP/M core structures would band in the gradient.

TABLE 1
DENSITIES AND BINDING CAPACITIES OF VSV
RIBONUCLEOCAPSIDS AND PHOSPHOLIPID VESICLES

Sample	Density (g/cm³)	Picomole of lipid bound/ μg unit of RNA[a]
VSV	1.17	
RNP/M[b]	1.30	
RNP[c]	1.26	
DMPC-SUV[d]	1.08	
DPPC/BPS-SUV[e]	1.09	
DPPC/BPS/M-SUV[f]	1.15	
RNP + DMPC	1.17	417
RNP/M + DMPC	1.23	307
RNP + DPPC/BPS	1.22	313
RNP/M + DPPC/BPS	1.15	1,988
RNP + DPPC/BPS/M	1.16	6,203
RNP/M + DPPC/BPS/M	1.19	1,892

[a] Length of RNA found in one μg of whole virion.
[b] Nucleocapsid containing the N, NS, L, and M proteins.
[c] Nucleocapsid containing the N, NS, and L proteins.
[d] Sonicated unilamellar vesicles of DMPC.
[e] Sonicated unilamellar vesicles of 50 mol% DPPC and 50 mol% BPS.
[f] DPPC/BPS-SUV reconstituted with M protein (mole ratio 100:1).

RECONSTITUTION OF RIBONUCLEOCAPSIDS WITH NEGATIVELY CHARGED
PHOSPHOLIPID VESICLES PREVIOUSLY SATURATED WITH M PROTEIN

     Matrix protein, purified by the method of Zakowski et
al. (5), was reconstituted with DPPC/BPS-SUV at a mole ratio
of 1:100; this is a ratio which will ensure saturation of the
vesicles with M protein (4). The reconstituted vesicles were
then purified by sucrose density ultracentrifugation. After
dialysis against PBS, the vesicles were stored at 31°C under
N_2 and in the dark.
     RNP or RNP/M structures were mixed with negatively
charged phospholipid vesicles previously saturated with M
protein (DPPC/BPS/M). The mixtures were incubated for 6h at

37°C and then centrifuged for 18h in a 0-66% sucrose gradient
in PBS. Figures 1E and 1F illustrate the density gradient
analysis of the two binding reactions. The presence of the M
protein on the vesicles greatly enhanced the capability for
RNP structures to bind negatively charged phospholipid ves-
icles (compare Fig. 1C and 1E). The RNP structures were also
able to bind three times as much phospholipid (Fig. 1E) as
the RNP/M structures (Fig. 1F). This may be due to the
increased area for binding afforded by the strung out struc-
ture of the RNP, rather than to any inherent tendency for
more efficient binding.

     Figures 1D and 1F illustrate that the RNP/M condensed
structure has a maximum binding capacity and that the addi-
tion of M protein to the vesicle before binding does not
increase this capacity. Whether M-M interactions are also
involved in Figure 1F could not be determined. As before, a
large density change accompanied the binding of phospholipid
vesicles by RNP and RNP/M.

     Table 1 summarizes the results of the various binding
experiments. The mixture of RNP structures with neutral
(DMPC) or negatively (DPPC/BPS) charged vesicles resulted in
only a low level of binding, which in the case of the
DPPC/BPS vesicles was easily reversed by the removal of salt
from the reaction mixture (data not shown). The reaction of
RNP/M structures with the neutrally charged DMPC vesicles
also did not lead to a high level of binding. Only when the
combination of both M protein and negatively charged vesicles
occurred was a high level of binding achieved. The combina-
tion of RNP/M and DPPC/BPS vesicles achieved six-fold greater
binding than did the reaction of DPPC/BPS vesicles with RNP;
the absence of the M protein reduced the binding efficiency
considerably.

     When M protein is on both the ribonucleocapsid (RNP/M)
and the vesicle (DPPC/BPS/M), there is no significant
increase in binding when compared to RNP/M and DPPC/BPS
vesicles without M protein. This suggests that the condensed
structure of the RNP/M complex allows for only a limited
number of vesicles to bind and that the addition of M protein
to the vesicles does not alter this limit. Possibly, the
M-protein binding sites of RNPs are saturated.

     Whereas RNP structures were inefficient at binding nega-
tively charged (DPPC/BPS) vesicles, the previous addition of
M protein to the vesicles greatly enhanced the binding capa-
bilities (more than eighteen-fold). It would appear that
the RNP can bind DPPC/BPS/M vesicles better than the RNP/M
structures, but the binding efficiency may be more a conse-
quence of the increased binding area offered by the strung
out RNP than to any inherent tendency for more efficient
binding.

Our results indicate that M protein is essential to achieve optimal binding between VSV ribonucleocapsids and sonicated phospholipid vesicles. This suggests that the M protein may interact with the negatively charged phosphatidylserine headgroups of the inner leaflet of the VS viral envelope. This interaction may be in addition to any association with the transmembrane G protein and may be a stabilizing force to help the virion maintain its shape.

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COMPARATIVE NUCLEOTIDE SEQUENCE ANALYSIS OF THE
GLYCOPROTEIN GENE OF ANTIGENICALLY ALTERED RABIES VIRUSES[1]

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The glycoprotein (G) of rabies virus is the major viral
antigen responsible for the induction and binding of virus-
neutralizing (VN) antibodies. Antigenic differences in G
among laboratory strains of rabies virus and street virus
strains detectable by the use of monoclonal antibodies
(1, 2) suggest that rabies virus can escape anti-viral
immunity by gradually changing the antigenicity of G. A
detailed analysis of the antigenic structure of rabies virus
G is therefore necessary to understand the relationship of
one virus strain to another in terms of antigenic cross-
reactivity and cross-protective immunity.
     Recently, an operational map of rabies virus G of the
CVS-11 strain was described which delineated three func-
tionally independent antigenic sites based on the grouping
of G variant rabies viruses which were resistant to neutra-
lization by one or more of a panel of anti-G monoclonal
antibodies (3). Our present goal is to physically map the
functionally independent antigenic sites on rabies virus G.
In this report, we present an expanded operational map of G
from the ERA strain of rabies virus which delineates five
functionally distinct antigenic sites, and identify
nucleotide changes in the G gene which code for amino acid
substitutions corresponding to site III epitopes in the
operational map.

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CONSTRUCTION OF THE OPERATIONAL ANTIGENIC MAP OF ERA STRAIN VIRUS GLYCOPROTEIN

Twenty-one monoclonal antibodies specific for rabies virus G and capable of neutralizing ERA strain virus were obtained from hybridomas as previously described (3, 4). These were used to isolate 52 antigenically unique mutant viruses from parental stock ERA virus as previously described (3, 5) except that a plaquing system was used in which BHK-S13 cells were suspended in agarose (6). The 52 neutralization-resistant variant viruses were arranged into groups as shown in Figure 1 by their pattern of reactivity in cross-neutralization tests with a panel of 25 monoclonal antibodies. Variants that lost reactivity to a number of

Variant strains	Number	509-6	231-22	101-1	162-3	116-1	1111-2	1121-5	613-2	1117-8	1110-3	120-6	194-2	248-8	507-1	1105-3	1113-1	127-5	1122-3	193-9	176-2	1108-1	1119-8	110-3	1120-10	1118-6	Antigenic site
RV 509-6	2	■																									I
RV 231-22	1		■																								II
RV 101-1 A	2			■	■	■	■																				
B	1			■	■	■																					
RV 162-3 A	1			■	■	■	■																				
B	2			■	■	■																					
RV 1117-8	1									■																	
RV 1103-4	3										■																III
RV 120-6	3											■															
RV 194-2	6												■														
RV 248-8 A	2												■	■													
B	8													■													
RV 718-3	2														■												
RV 507-1 A	1														■	■											
B	1															■											
C	1															■	■										
D	2															■	■										
E	1																■										
RV 127-5	2																	■	■								
RV 193-2	1																		■	■							
RV 176-2	2																				■	■					
RV 1108-1	1																					■	■				
RV 110-3	3																							■			IV
RV 1120-10	2																								■		V
	1																									■	
ERA parent																											

FIGURE 1. Operational antigenic map of ERA G. Neutralization-resistant variants (RV) were selected using monoclonal antibodies, and then tested for susceptibility (□) or resistance (■) to neutralization by antibodies in the panel. RV viruses are identified by the number of the monoclonal antibody used for the selection.

antibodies simultaneously were considered operationally
linked by the epitopes recognized by those antibodies.
Conversely, variants which were resistant to only one anti-
body revealed an epitope which was considered operationally
independent of any other epitopes recognized by the avail-
able monoclonal antibodies. The antigenic map of ERA virus
G confirms antigenic sites I, II and III previously
described for CVS virus (3). Two additional antigenic sites
(designated IV and V) were defined by monoclonal antibodies
110-3 and 1120-10 which neutralized ERA virus, but not CVS
virus.

NUCLEOTIDE SEQUENCE ANALYSIS OF VARIANT VIRUS GLYCOPROTEIN GENES

        Virion RNAs were isolated from mutant viruses repre-
senting the five operationally defined antigenic sites of
ERA strain virus G and transcribed in dideoxy-chain ter-
mination sequencing reactions (7). Transcripts were primed
with synthetic cDNA oligomers corresponding to the cloned
nucleotide sequence of mRNA specific for ERA strain G (8) or
CVS sequences which we have determined by direct cDNA
sequencing using strain common primers (unpublished data).
The cDNA products of the four base-specific reaction mix-
tures (A, C, G, and T) for each cDNA primer-virion RNA
hybrid were screened in groups of A's, C's, G's, and T's in
polyacrylamide gels. Our analysis has shown that mutations
resulting in amino acid residue changes were infrequent and
that a single residue change may be responsible for an
altered structure on the antigen directly complementary to
the combining site for a given VN antibody.

LOCATION OF ANTIGENIC SITE III IN THE RABIES VIRUS GLYCO-PROTEIN

        A single base change in the nucleotide sequence of RV
194-2, RV 248-8, RV 507-1, and RV 120-6 at nucleotide posi-
tions 1062 (G→T), 1062 (G→T), 1072 (T→G), and 1133 (G→A),
respectively, leads to an altered amino acid in the variant
G. Three of these mutations are shown in Figure 2. The
base change at position 1133 for RV 120-6 is not shown. A
single base change (G→A) at position 1062 has also been de-
tected for the RV 194-2 variant of CVS virus (data not
shown). This change in the second base of the codon C G G
for arginine-333 of the parental CVS G sequence to C A $\overline{G}$ in
CVS RV 194-2 confirmed the glutamine substitution in the
tryptic peptide replacing arginine-333 as previously
reported (9).
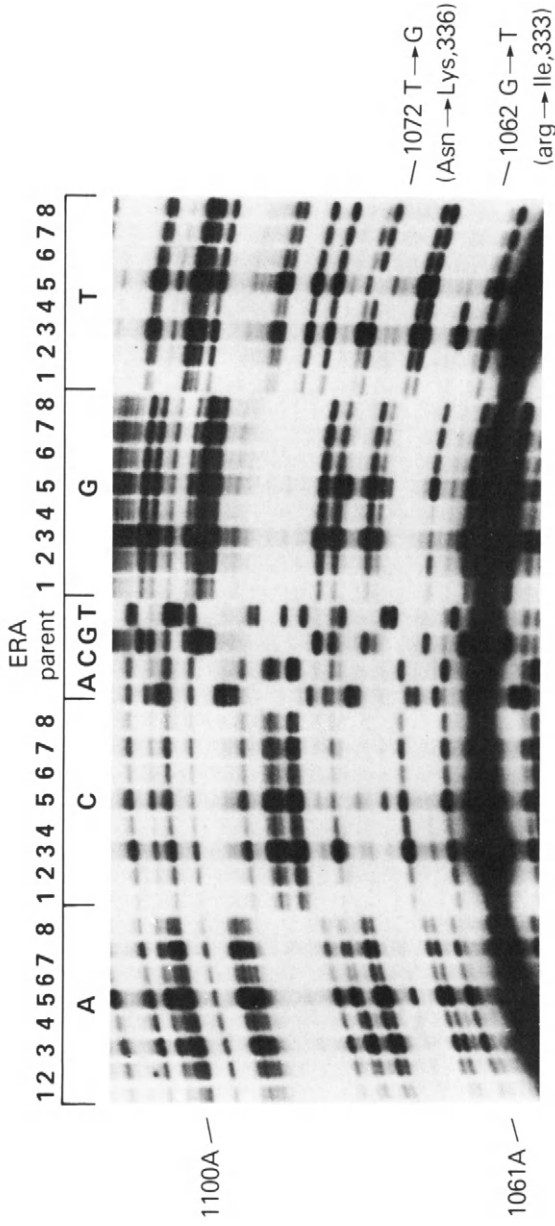
FIGURE 2. Comparative nucleotide sequence analysis of the G gene of anti-genic variant viruses. Virion RNAs from RV 509-6 (1), RV 101-1 (2), RV 194-2 (3), RV 248-8 (4), RV 507-1 (5), RV 120-6 (6), RV 110-3 (7), RV 1120-10 (8), and ERA strain parent virus were incubated in sequencing reactions with cDNA primer 1001-1018. Products of the A, C, G, and T sequence-specific reactions were separated in 8% polyacrylamide gels. The oligonucleotide primer and transcripts are numbered to correspond with the nucleotide sequences of the cloned G sequences (ref. 9).

The location of unique amino acid changes in the pri-
mary sequence of G is schematically shown in Figure 3.
Variant viruses that have been assigned to site III of the
operational epitope map (Figure 1 and ref. 3), show
clustering of mutations in the nucleotide sequence
suggesting that residues involved in the binding of VN anti-
bodies which delineate antigenic site III occupy positions
close to each other in the G molecule.

   An additional amino acid change which has been des-
cribed (10) is indicated in Figure 3 and represents the
substitution of glutamic acid in CVS variant RV 231-22
replacing lysine-198 in the parental G.

   The mapping of mutations in G of rabies virus will per-
mit us to locate functional epitopes within antigenic sites.
This study is a preparation for interpreting relationships
between rabies and rabies-related viruses and between rabies
viruses that exhibit antigenic changes as a result of
passage in different animal species.
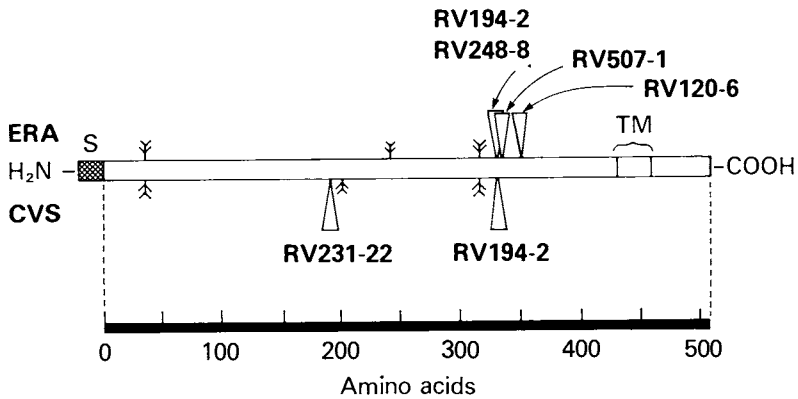
FIGURE 3. Map locations of altered amino acids in the
glycoprotein sequence of rabies variant (RV) viruses. The
diagram shows the location of mutations (darts) of RV
viruses and carbohydrate attachment sites (Ψ) in ERA and
CVS strains above and below the open bar, respectively. The
signal peptide (S) and transmembrane (TM) segments are also
shown.

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VARIATION IN GLYCOSYLATION PATTERN OF G PROTEINS AMONG ANTIGENIC VARIANTS OF THE CVS STRAIN OF RABIES VIRUS[1]

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Glycoproteins from different fixed rabies virus strains vary greatly in their electrophoretic mobilities (1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the structural proteins from virions of ERA and Flury HEP strains has revealed single bands of glycoproteins which differ by approximately 5,000 daltons. Analysis of virion proteins of CVS and PM strains has shown that two forms of the glycoprotein exist which are designated GI and GII. The slower migrating GI of CVS corresponded in electrophoretic mobility to the glycoprotein of ERA and the faster migrating GII of CVS corresponded to the glycoprotein of Flury HEP virus. Comparative tryptic peptide analysis of CVS GI and GII has demonstrated that all peptides derived by trypsin digestion of GI comigrated with those of GII (1). Thus, GI and GII appear to differ in their extent of glycosylation.

We have recently determined the structural relationship between GI and GII. In this paper, we report on the investigation of two CVS variant viruses with antigenically altered glycoproteins affecting glycosylation.

CHARACTERIZATION OF CVS VARIANT VIRUS GLYCOPROTEINS

Two antigenic variants of the CVS-11 parent virus which resisted neutralization by monoclonal antibody 231-22 and 194-2 as previously described (2) contained glycoproteins with uniquely altered electrophoretic mobilities in SDS-PAGE compared with parent virus glycoprotein (Fig. 1). Two glycoprotein species, GI and GII with molecular weights of 67,000 and 62,000, respectively, can be observed in CVS

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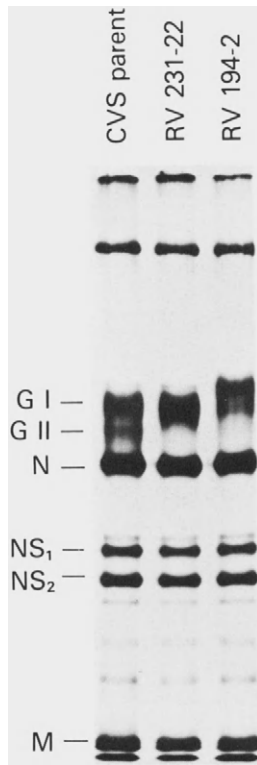
FIGURE 1.   Electrophoretic analysis of structural pro-
teins of CVS-11 parent and variant viruses.   CVS-11 parent
virus and the antigenic variants RV231-22 and RV194-2 were
labeled with [^{35}S]methionine and the purified virions were
subjected to SDS-PAGE as described (1).

parent.   Glycoprotein GII is absent in RV231-22 virus.   Both
species are present in RV194-2 virus, but they exhibit
slower electrophoretic mobilities compared with GI and GII
of parent virus.
     In order to confirm previous results comparing tryptic
peptides of CVS GI and GII (1), the virus-specific glycopro-
teins were isolated from tunicamycin-treated cells individ-
ually infected with CVS-11, RV231-22, and RV194-2 virus
(data not shown).   In every case, immunoprecipitated virus-
specific glycoproteins migrated in SDS-polyacrylamide gels
faster than virion-associated glycoprotein.   Furthermore,
only one glycoprotein species with the same molecular weight
for all three viruses could be isolated from these cells.

This suggests that the differences in the electrophoretic
pattern of virion-associated G proteins represent variation
in sugar content of these proteins.

ANALYSIS OF TRYPTIC GLYCOPEPTIDES

     Glycoproteins from CVS-11, RV231-22, and RV194-2 were
labeled with $[^3H]$glucosamine or $[^{14}C]$glucosamine, purified
by SDS-PAGE, carboxymethylated, and exhaustively digested
with trypsin to determine the chemical relationship between
GI and GII in the various viruses. The tryptic glycopep-
tides were resolved by reverse phase high-pressure liquid
chromatography (HPLC). Figure 2a shows that two major peaks
of glucosamine-labeled tryptic glycopeptides are present in
GI of CVS-11 (#1, #4) and one minor peak (#2), but only one
major peak could be resolved from GII of CVS-11. Two addi-
tional minor peaks (#3, #5) regularly observed as shoulders
to peak #4 probably represent a heterogeneity of glycopep-
tides related to #4 (or were not resolved). However, the
retention time of the single GII peak was identical to peak
#4 of GI. The pattern of tryptic glycopeptides obtained
from the single glycoprotein of RV231-22 virus (Fig. 2b) is
similar to that of GI of CVS parent. In Figure 2c, an addi-
tional glycopeptide peak (#6) was obtained from the gly-
coprotein (GI plus GII) of RV194-2 virus.
     To localize the various glycopeptides within the
deduced amino acid sequence of the CVS-11 glycoprotein (3),
the isolated peptides were subjected to automated Edman
degradation. The amino acid sequence for glycopeptide #1
(Table 1) was the same for all three viruses. Only residues
1, 2 and 4 of glycopeptide #1, which matched the amino acid
sequence of CVS glycoprotein at positions 200, 201, and 203,
were obtained. However, this partial sequence was suf-
ficient to map the glycopeptide to the predicted glycosyla-
tion site II at asparagine 204 of the CVS glycoprotein. The
first two residues of glycopeptide #2 were shown to match
the start of the sequence of glycopeptide #1. Therefore, it
is believed that peptide #2 is an incompletely cleaved pep-
tide of glycopeptide #1. With the exception of residue 6,
the entire amino acid sequence of peptide #4 was obtained.
This sequence matches residues 314 to 320 of the CVS-11 gly-
coprotein and contains the predicted glycosylation site III
at asparagine 319. Regarding glycopeptide #6 of RV194-2,
the first five residues and residues 8 and 9 were obtained.
These amino acids match residues 153, 154, 155, 156, 157,
160, and 161 of the CVS-11 glycoprotein. Since this amino
acid sequence in the parent glycoprotein does not contain a
glycosylation signal, it was suspected that an undetectable

(2a)
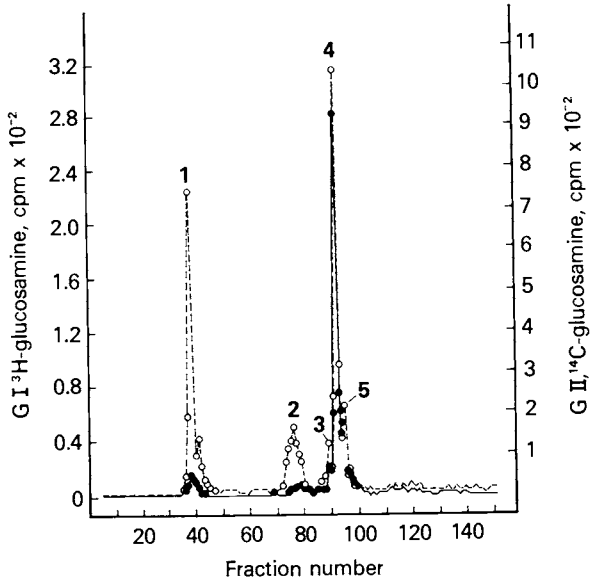
(2b)
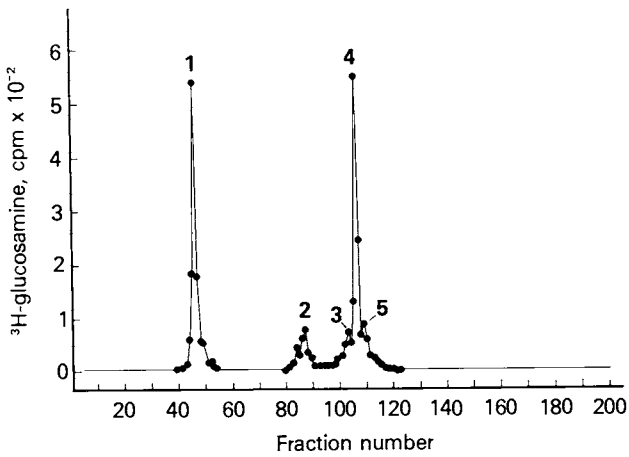
(2c)
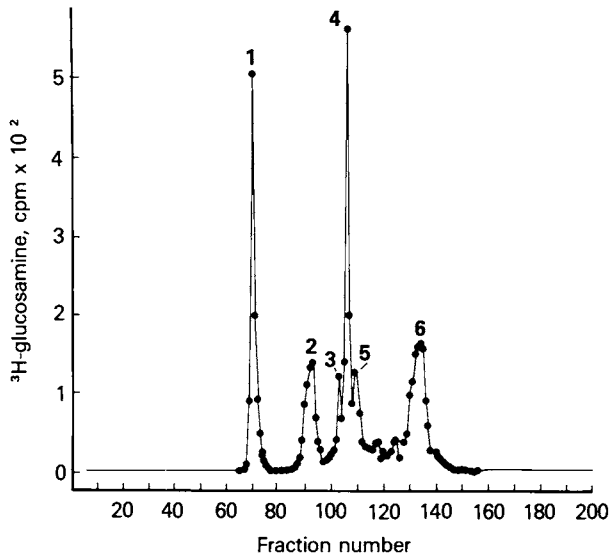
FIGURE 2. HPLC analysis of glucosamine containing tryptic peptides of glycoproteins of CVS-11 (2a), RV231-22 (2b) and RV194-2 (2c). Glycoprotein species, GI and GII, were separated by SDS-PAGE. GI labeled with ^{3}H (●——●) glucosamine and GII labeled with ^{14}C (o---o) glucosamine were mixed, carboxymethylated and exhaustively digested with trypsin. Tryptic peptides were separated by HPLC using a Beckman Ultrasphere ODS column. HPLC conditions were as follows: Solvent A was .1M NaClO$_4$, Solvent B was 70% acetonitrile, 30% H$_2$O, .1% H$_3$PO$_4$; a gradient of 0 to 90% B was run for 50 min at a flow rate of 1.5 ml/min at ambient temperature. Glycopeptides from RV231-22 and RV194-2 were prepared in a similar way as described for CVS, except that GI and GII of RV194-2 were not separated.

amino acid substitution occurred at residue 158 of the RV194-2 virus glycoprotein.

NUCLEOTIDE SEQUENCES ENCODING PREDICTED GLYCOSYLATION SITES

Overall, this direct amino acid sequencing data did not precisely identify the glycosylation sites in the different glycopeptides because asparagine, the initial amino acid which signals such a site was never obtained. Therefore, we

TABLE 1
AMINO ACID SEQUENCES OF GLYCOPEPTIDES OBTAINED BY EDMAN
DEGRADATION

Glycopeptide no.	Virus	Sequence
1	CVS, RV231-22, RV194-2	200 AS-G
2	CVS, RV231-22, RV194-2	AS
4	CVS, RV231-22, RV194-2	314 AYTIF-K
6	RV194-2	153 VFPGG--SG

determined the nucleotide sequences of regions in the paren-
tal and variant glycoprotein genes which encode the glycosy-
lation signals asn-x-ser or asn-x-thr. We employed the
dideoxy sequencing method (4) and used synthetic DNA primers
as described elsewhere (5) matching nucleotides 8-24,
553-569, and 1001-1018 of the cloned glycoprotein gene to
determine nucleotide sequences containing the predicted
glycosylation sites I, II, and III, respectively.
Furthermore, to confirm the putative extra glycosylation
site of RV194-2 glycoprotein, a synthetic DNA primer
corresponding to nucleotides 377-393 was also used.
     The nucleotide sequence of the CVS-11 glycoprotein gene
in the region of the predicted glycosylation site I (data
not shown) revealed the presence of a glycosylation signal
(asn-leu-ser) at asparagine 37 in each of CVS-11 parent,
RV231-22, and RV194-2 virion RNAs. However, since none of
the isolated glycopeptides from the glycoproteins of CVS-11
parent, RV231-22, and RV194-2 viruses possess the amino acid
sequence of site I, we believe the first glycosylation
signal is not utilized for carbohydrate attachment.
     Regarding glycosylation site II of CVS-11 glycoprotein,
nucleotide sequencing confirmed the location of the pre-
dicted glycosylation signal at asparagine 204 (3).
Moreover, since the glycosylation signal sequence was unam-
biguous it is unlikely that the two glycoprotein species in
CVS parent virus are derived from two separate gene

transcripts. Rather it is likely that the two different
glycoprotein forms reflect different conformations as pre-
viously suggested (6). Direct nucleotide sequence analysis
of this region in the glycoprotein gene of CVS-11 parent,
RV194-2 and RV231-22 viruses revealed a A→G base change in
the codon for residue 198 of RV231-22 as shown in Figure 3.
The amino acid change from lysine to glutamic acid is
located five residues from asparagine 204 in CVS glycopro-
tein. As a result of this mutation, it appears that car-
bohydrate attachment occurs at asparagine 204 in the GII
species of RV231-22 glycoprotein. This site is apparently
not utilized in the GII form of parental CVS glycoprotein.
One reason for this may be that the conformation of GII
conceals residue 204 making it inaccessible for carbohydrate
attachment. Rules established by Chou and Fasman (7) pre-
dict that this single amino acid change in the deduced pri-
mary sequence alters the secondary structure of the
glycoprotein in this region and presumably allows glycosyla-
tion site II to be utilized in the GII species.
     The predicted glycosylation signal at asparagine 319
for the glycoproteins of CVS, RV231-22 and RV194-2 viruses
was confirmed by nucleotide sequencing of the glycosylation
site 3 region of the glycoprotein gene (data not shown).
This glycosylation site is highly conserved and utilized in
all rabies virus strains examined and is also present at the
same location in vesicular stomatitis virus (VSV) G (8).
     Nucleotide sequencing of the region coding for extra
putative glycosylation site in RV194-2 virus glycoprotein
revealed the expected amino acid change at residue 158
resulting in a substitution of lysine with asparagine
(AAG→AAT). This change in the primary sequence creates a
new glycosylation site and confirms the presence of such a
site predicted by the analysis of peptide #6 from RV194-2
virus glycoprotein.
     The data presented here have shown that 1) all glycosy-
lation signals may not be utilized and 2) glycosylation may
be dependent on the secondary structure of the molecule.
The glycosylation site which is always glycosylated in
rabies virus strains and in VSV is highly conserved. At
this point, we do not know whether the variation in glycosy-
lation among antigenic variants will impair biological func-
tions of the protein.
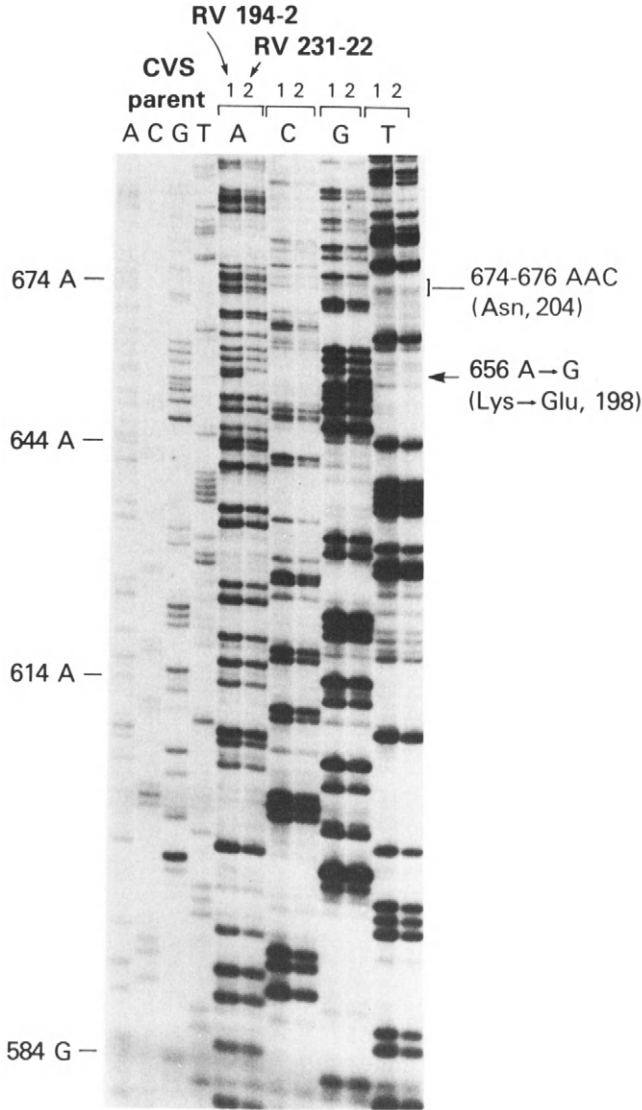
FIGURE 3. Comparative nucleotide sequence of the region encoding glycosylation site II. Virion RNAs from CVS-11 parent virus and antigenic variants RV194-2 and RV231-22 were incubated with DNA primer complementary to bases 553 to 569 in dideoxy-chain terminating sequencing reactions. Products of the A, C, G, and T sequence-specific reactions were separated in 8% polyacrylamide sequencing gel. The nucleotides of primer and transcripts are numbered to correspond with the nucleotide sequence of the cloned glycoprotein gene.

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CHANGE IN PATHOGENICITY AND AMINO ACID SUBSTITUTION
IN THE GLYCOPROTEIN OF SEVERAL SPONTANEOUS
AND INDUCED MUTANTS OF THE CVS STRAIN OF RABIES VIRUS[1]

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We have previously demonstrated that 90 % of spontaneous
or 5 Fu induced mutants of the CVS strain of rabies virus se-
lected for the resistance to neutralization by monoclonal anti-
bodies n° 194-2 and 248-8 (from the Wistar collection) were a-
virulent or attenuated in adult mice (1, 2). Those two monoclo-
nal antibodies recognize site IIIa of the CVS glycoprotein (3).
Using the same technique, Dietzschold et al (4) isolated 2 spon
taneous non pathogenic mutants of the ERA and CVS strains of
rabies virus. They found that the mutant glycoproteins diffe-
red from the wild-type by one amino acid substitution at posi-
tion 333, an arginine being replaced by a glutamine (CVS) or by
an isoleucine (ERA). Because we possess a collection of mutants
which differ in their attenuation, we decided to investigate
if they were mutated in the same position.

The nucleotide sequence of the 3' terminal region of the
glycoprotein gene was determined for 4 avirulent (AvO_1, AvO_4,
AvO_{10} and AvO_{11}) and two attenuated strains (AtO_1 and AtO_2).
AvO_1, AvO_4, AtO_1 and AtO_2 are 5 Fu induced mutants (2) while
AvO_{10} and AvO_{11} are spontaneous. The CVS parental strain and
VO_{67} (V = Virulent, O = Orsay), a 5 Fu induced mutant resis-
tant to neutralization by monoclonal antibodies n° 194-2 and
248-8 which retains its virulence, were also included in this
study.

In order to precise the difference between attenuated and
avirulent phenotypes, the pathogenic power of the mutants was
also investigated, by intramuscular or intracerebral inocula-
tion of 3 weeks-old mice with increasing doses of the virus.
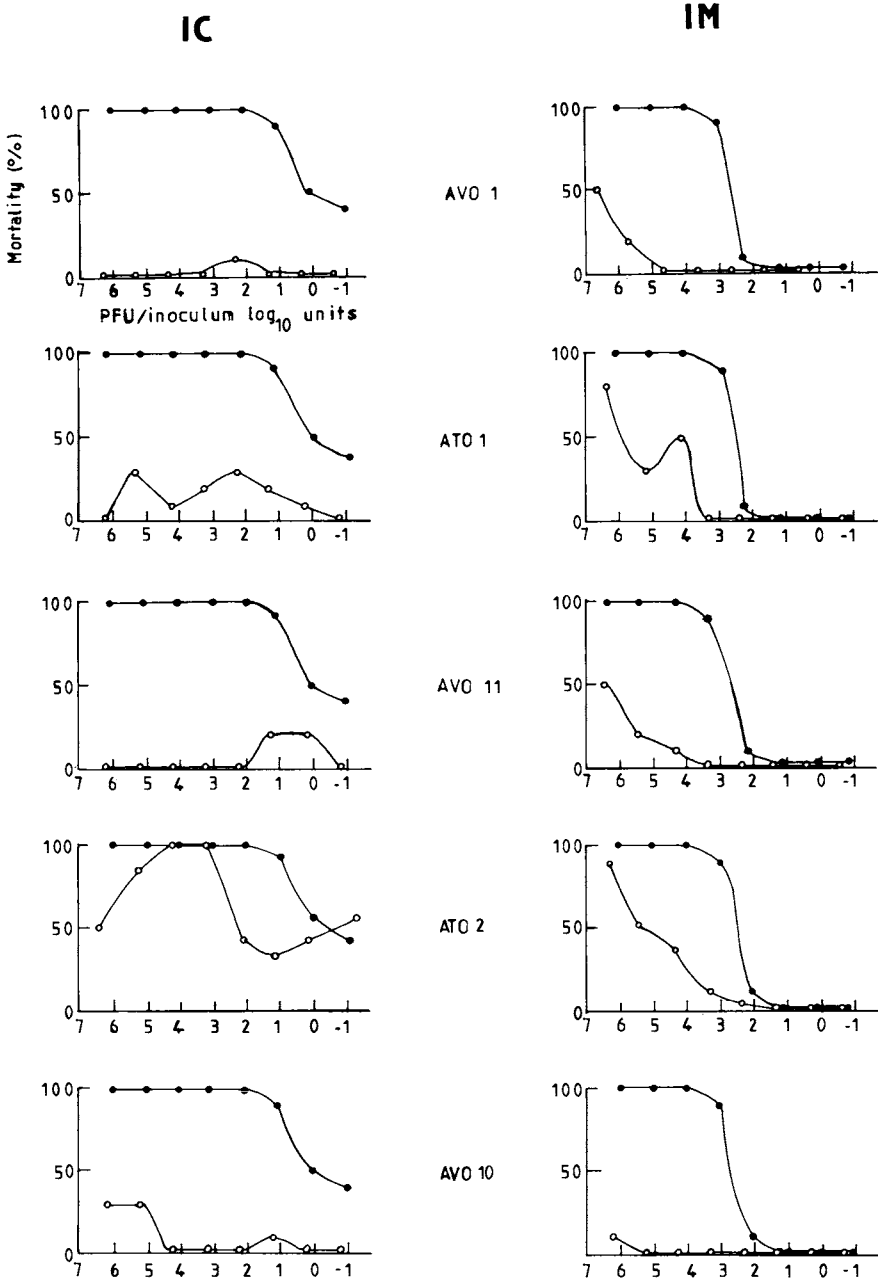
FIGURE 1.   Residual pathogenicity of AvO and AtO mutants for young mice.

Series of ten 3 weeks-old mice were inoculated intracere-
brally (IC) or intramuscularly (IM) with 30 μl (IC) or 50 μl
(IM) of increasing dilutions of CVS (●—●) or mutant strain
(O—O).

```
                                    ▼
    KFPIYTIPDKLGPWSPIDIHHLRCPNNLVVEDEGCTNLSGFSYMELKVGY    50

    ISAIKVNGFTCTGVVTEAETYTNFVGYVTTTFKRKHFRPTPDACRAAYNW

    KMAGDPRYEESLQNPYPDYHWVRTVRTTKESLIIISPSVTDLDPYDKSLH   150

    SRVFPSGKCSGITVSSTYCSTNHDYTIWMPENPRPGTPCDIFTNSRGKRA

    ▼
    SNGNKTCGFVDERGLYKSLKGACRLKLCGVLGLRLMDGTWVAMQTSDETK   250

    WCSPDQLVNLHDFRSDEIEHLVVEELVKKREECLDTLESIMTTKSVSFRR
Gln                       ▼                  Q
Arg LSHLRKLVPGFGKAYTIFNKTLMEADAHYKSVRTWNEIIPSKGCLKVGGR   350
Gly                                          G
    CHPHVNGVFFNGIILGPDDRVLIPEMQSSLLRQHMELLESSVIPLMHPLA

    DPSTVFKEGDEAEDFVEVHLPDVYKQISGVDLGLPNWGKYVLMTAGAMIG   450

    LVLIFSLMTWCRRANRPESKQRSFGGTGGNVSVTSQSGKVIPSWESYKSG

    GEIRL                                               505
```

FIGURE 2.   Aminoacid sequence of the glycoprotein of
mutant and parental CVS strains.
Complete amino acid sequence is drawn according to Yelver-
ton et al (6). The location of the primer is indicated by a
thick line. The nucleotide sequence of AvO_1 and AtO_1 was deter-
mined throughout the underlined region. The brackets enclose
the membrane-anchoring region. The arrows refer to the poten-
tial glycosylation sites. The amino acid exchange in mutants
AvO_1, AvO_4, AvO_{11}, AtO_1 and AtO_2 is presented above the wild-
type arginine 333 (R = arginine ——➤ Q = glutamine) ; the ami-
no acid exchange in mutant AvO_{10} is presented under the wild-
type (R = arginine ——➤ G = glycine).

Fig. 1 shows that none of the mutants was completely avirulent
for young mice, although the difference with the fully patho-
genic CVS strain was always evident. This was not surprising
in view of our previous finding that non pathogenic mutants
AvO_1 and AvO_2 retain their virulence for suckling mice (1).
When older mice were inoculated, the difference between atte-
nuated and avirulent mutants became more evident : animals in-
jected with increasing doses of avirulent mutants showed no
symptoms and always survived the inoculation while mice inocu-
lated with attenuated viruses had comportmental and paralytic
symptoms leading eventually to the death of the animals.
        A synthetic oligonucleotide complementary to the region
of the gene corresponding to amino acids 308-312 was used to
prime the transcription of cDNA from a mutant or wild type RNA
template, by the dideoxy chain termination procedure (5).
Around 25 % of the glycoprotein gene could be sequenced by this
method (Fig. 2). The amino acid sequence of the Orsay CVS
strain, deduced from the base sequence, was identical to that
published by Yelverton et al (6). The six avirulent and atte-
nuated mutant differ from the wild type in the triplet coding
for amino acid 333 : in mutants AvO_1, AvO_4, AvO_{11}, AtO_1 and
AtO_2, a G   A transition at base 1062 was found, the arginine
being replaced by a glutamine ; in mutant AvO_{10}, a C   G tran-
version at base 1061 was observed, the arginine being replaced
by a glycine (Fig. 3). No other difference between wild-type
and mutants was evidenced.
        No difference, whatsoever, could be detected between VO_{67}
(also mutated in site III but still virulent) and CVS. The
difference is therefore probably located before amino acid 319,
an observation which suggests that site III would correspond to
a folding of the molecule.
        Our results confirm the crucial role of arginine 333 in
the pathogenicity of rabies virus. They do not explain why our
mutants differ in their attenuation. Our hypothesis is that at-
tenuated viruses, which are less frequent than avirulent mu-
tants among the resistants to neutralization by antibodies n°
194-2 and 248-8, would have a second mutation, partially cor-
recting the loss of the arginine. Another explanation is that
some mutant stocks would be a mixture of non pathogenic viru-
ses and fully pathogenic revertants, giving an intermediate
phenotype when injected to animals. The fact that no contamina-
ting sequence could be detected in AtO_1 or AtO_2 in the CAG
triplet (Fig. 3) indicate that either the amount of revertant
virus was too low to be visible or that the reverse mutation
was in another region of the gene. Experiments are in progress
to clarify this point.
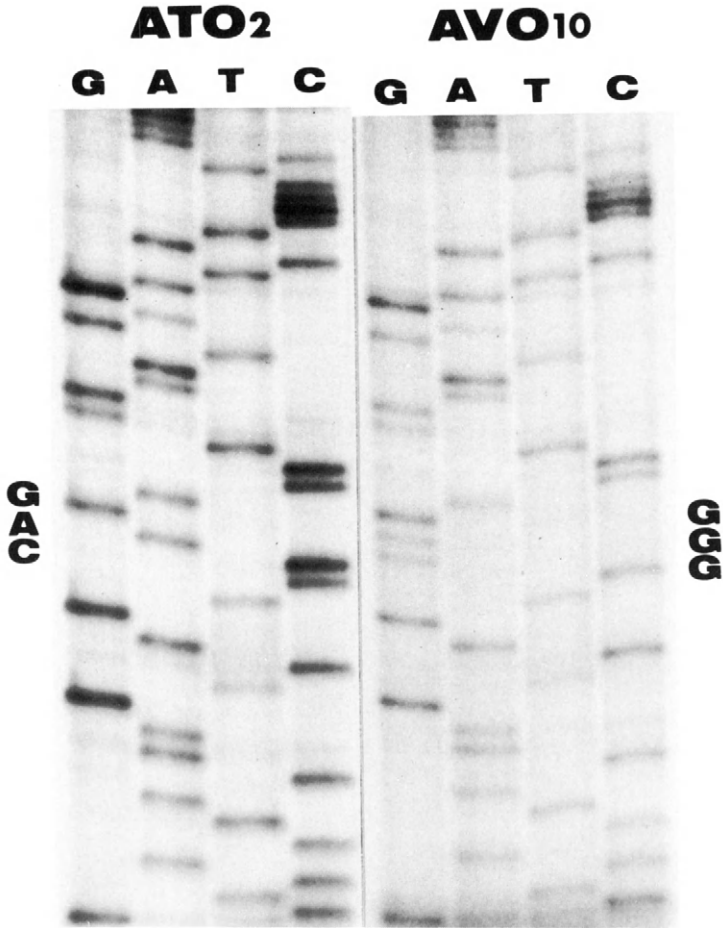
FIGURE 3.   Identification of the nucleotide changes lea-
ding to loss of pathogenicity.
    The synthetic oligonucleotide 5'-d (GTCCCAGGGTTTGGAAA)3'
was used to prime the transcription of RNA extracted from AvO_{10}
or AtO_2 purified virions, in the presence of dideoxynucleoti-
des. The CGG sequence found in the CVS parental strain is re-
placed by CAG (AtO_2) or GGG (AvO_{10}).

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HOW MANY FORMS OF THE NEWCASTLE DISEASE VIRUS
P PROTEIN ARE THERE?[1]

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PROPERTIES OF P

The P proteins of Newcastle disease virus (NDV)
are the second most abundant proteins synthesized
during infection and the P gene is second in the
transcriptional order (1). There is biochemical
(2,3) and genetic (4,5) evidence that P is invol-
ved in viral RNA synthesis, but the molecular de-
tails of its involvement are unknown. P is gener-
ally described as a nucleocapsid-associated phos-
phoprotein. However, this descriptive veneer
glosses over a rich variety of molecular forms.
Virions contain four variants of P with different
isoelectric points, only the two most acidic forms
of which are phosphorylated (6). P proteins form
disulfide-linked multimers large enough to be tri-
mers and several small nonstructural proteins which
appear to be related to P have been identified (7,8).

FORMS OF P IN INFECTED CELLS

Secondary cultures of chicken embryo (CE)
cells were either mock-infected (Figure 1A,D) or
infected with strain AV at 5 PFU/cell (Figure 1B,C)

and exposed to [^{32}P]orthophosphate (Figure 1C,D)
from 3 to 6 hours post-infection (p.i.) or to
[^{35}S]methionine (Figure 1A,B) from 5 to 6 hours
p.i. Radioactive proteins were extracted and ana-
lyzed on two dimensional polyacrylamide gels using
isoelectric focusing in the horizontal dimension
followed by SDS-PAGE in the vertical dimension
after the methods of O'Farrell (9). Only the gel
regions from pH 5.8 to 6.2 and 53 kilodaltons to
56 kilodaltons are shown in Figure 1 and in sub-
sequent figures. Five forms of P were identified
among the [^{35}S]methionine-labeled proteins from
infected cells (Figure 1B). Spots 1-4 occupied
the same positions on gels as the four P variants
from virions. The fifth form of P, identified by
partial digest peptide mapping, sometimes streaked
and sometimes focused into a well-formed spot in-
termediate in mobility between spots 3 and 4, and
more acidic than either. Form 5 was phosphory-
lated (Figure 1C) as were forms 3 and 4; however,
unlike the latter two variants, form 5 was detected
in extracts of infected cells but not virions.
Much larger amounts of form 2 relative to the other
forms of P were obtained from virions than from
infected cells (6). See Figure 3A for patterns of
P isolated from virions. The dark horizontal
streak across Figure 1B was the nucleocapsid pro-
tein, which did not focus under these conditions.
     At present, we can only speculate on the bio-
logic roles of the various forms of P in virions
and infected cells. Based on its relatively high
abundance, we suggest that form 1 may have primar-
ily a structural role in nucleocapsids. Form 2,
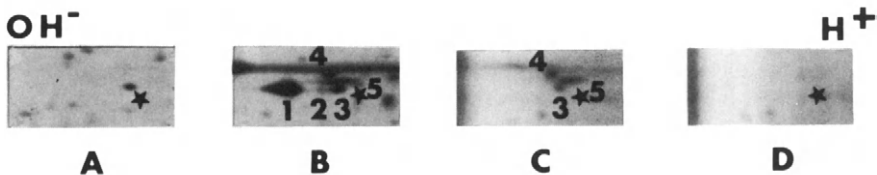
     Figure 1. Forms of [^{35}S]methionine-labeled
and ^{32}P-labeled P extracted from infected CE cells
and analyzed on two dimensional gels. The stars
mark the same position in each fluorogram (^{35}S) or
autoradiogram (^{32}P).

which is relatively enriched in virions, may be in-
volved in folding of the nucleocapsid during virion
assembly. Form 5, found in infected cells but not
virions, may be involved in replication whereas
forms 3 and 4 may be required for both transcrip-
tion and replication.

FORMS OF P MADE IN CELL-FREE PROTEIN SYNTHESIZING
SYSTEMS

     mRNA was extracted from mock-infected (Figure
2A), virulent strain AV-infected (Figure 2B), and
avirulent strain B1-infected CE cells at 9 hours
p.i. and purified on oligo-dT cellulose columns
as described previously (10). The mRNA was used
to program nuclease-treated reticulocyte lysates.
Two dimensional gel analyses of the [^{35}S]methio-
nine labeled P protein synthesized in these lysates
are shown in Figure 2. The overall pattern of P
proteins of strain AV made in vitro was remarkably
similar to the pattern from infected cells (Figure
1B), indicating that the mechanisms that generated
the P variants operated in the reticulocyte lysate
as well as in infected cells. In particular, the
P proteins made in vitro included a fifth form
similar in pI and mobility to form 5 from infected
cells, and the in vitro products were relatively
deficient in form 2. The similarities between in
vivo and in vitro P patterns of strain AV suggest
that mechanisms at the level of translation or per-
haps mRNA heterogeneity as well as post-transla-
tional modifications may be responsible for P
variants.
     Since strain-specific differences in the
patterns of P from infected cells have been reported
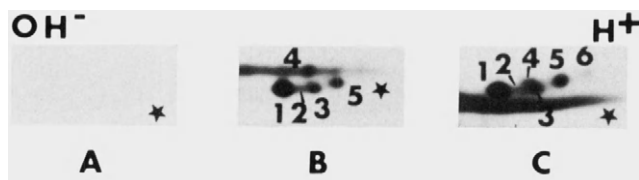
     Figure 2. Forms of the [^{35}S]methionine-
labeled P proteins synthesized in vitro and ana-
lyzed on two dimensional gels.

(11), we compared the P proteins synthesized in
reticulocyte lysates programmed with mRNA from
strain Bl (Figure 2C) to those of strain AV.
Although strain Bl has a slightly smaller nucleo-
capsid protein than strain AV and slightly larger
P proteins than strain AV (12), there are strong
similarities in the overall patterns of P variants
made in vitro. For both strains, form 1 was the
most abundant and a fifth form was produced. The
triangular cluster of forms 2, 3, and 4 of strain
AV also appeared to be present among the products
of strain Bl mRNA; however, the cluster was much
tighter and these three forms were only partially
resolved. Strain Bl produced a sixth spot which
may be an additional form of P, but it has not been
mapped. There was a hint of a sixth spot in the
in vitro pattern from strain AV (Figure 2B) as well,
but it was partially hidden in the streak of nucleo-
capsid protein.

COMPOSITION OF P MULTIMERS FROM VIRIONS

        We used a modification of the standard O'Far-
rell protocol for two dimensional gel electrophor-
esis to determine the composition of the disulfide-
linked trimers of P from purified strain AV viri-
ons. The strategy was to omit mercaptoethanol from
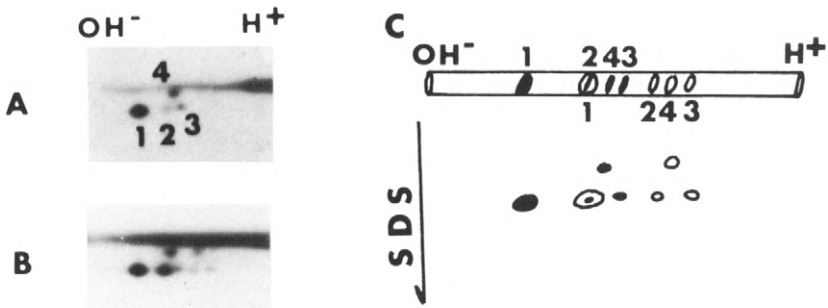
        Figure 3. Analysis of P multimers from [^{35}S]
methionine-labeled AV virions using isoelectric
focusing under either reducing (A) or nonreducing
(B) conditions in the horizontal dimension followed
by reducing SDS-PAGE in the vertical dimension.
Panel C shows a diagrammatic sketch of Panel B.

the isoelectric focusing dimension to allow P pro-
teins to focus as disulfide-linked molecules.
Under these conditions, the net charge of the P
multimers might be different enough from P monomers
to separate them. As shown in Figure 3, this ex-
pectation was fulfilled. The pattern of four P
variants from [^{35}S]methionine-labeled purified
virions analyzed under normal reducing conditions
is shown in Figure 3A. The same preparation sepa-
rated under nonreducing conditions during isoelec-
tric focusing is shown in Figure 3B. Under these
conditions, both P monomers and disulfide-linked
multimers are expected since only a portion of vir-
ion P can be extracted as multimers (6). The char-
acteristic pattern of P monomers and four additional
spots which appeared to be a duplicate of the mono-
mer pattern but shifted to the acidic side were ob-
tained. Our interpretation of this pattern is that
the shifted P variants represent disulfide-linked
homomultimers of P. Other data suggest that these
are at least homotrimers (6). Since each spot ex-
perienced the same magnitude of acidic shift, we
suggest that the subunits in all four of the homo-
trimers associate in a similar fashion. These data
are consistent with the findings of Chinchar and
Portner (2) that the disulfide-linked portions of
P are in a highly basic, protease-resistant part
of the molecule. Neutralization or shielding of
some of the basic amino acid residues in the di-
sulfide-linked multimers could have produced the
acidic shift observed by us. Further, our results
imply that modified forms of P such as phosphory-
lated forms 3 and 4 either associate only with the
same modified forms or that all subunits in a P
multimer are modified in the same way after multi-
mer formation. The strategy shown in Figure 3
should be generally applicable to the analysis of
complex disulfide-linked multimers.

A GLIMPSE OF THE MOLECULAR ANATOMY OF P

     Virus-specific proteins with apparent molecular
weights of 36,000 and 33,000 which appear to be P-
related have been detected in NDV-infected cells
and in cell free systems. Similar proteins have
been detected in mumps virus-infected cells (13).
The two forms made in vitro can be labeled with
fmet-tRNA, and they share several tryptic peptides

Protease-Resistant   Protease-Sensitive
Domain   Domain

Basic	Acidic
S-S	Met Poor
RNA Binding Site	Modified

SP1   HOOC··─────────────┤   30K
SP2   HOOC··───────────┤   24K
                    ├────────────────NH$_2$ 33K
                  ├──────────────────NH$_2$ 36K

P   HOOC├──────────────────────NH$_2$ 53K
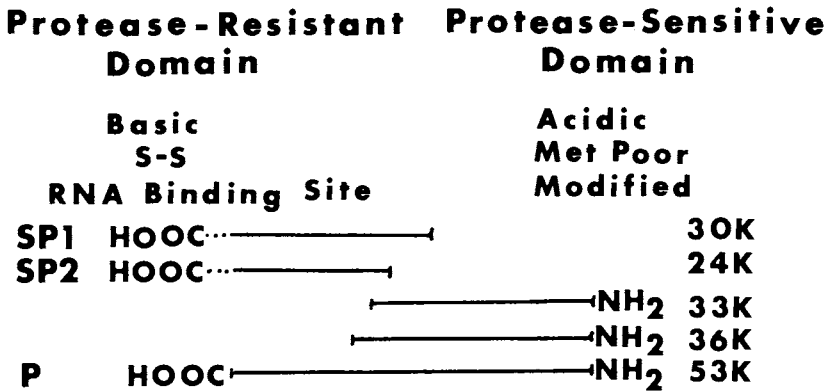
Figure 4.   Molecular anatomy of the P protein.
The relative sizes based on electrophoretic mobil-
ities of the 53 kilodalton P proteins and smaller
forms are drawn to scale.   The placement of SP1 and
SP2 at the exact C-terminus is speculative.   The
33 and 36 kilodalton *in vitro* products are shown.

which are also common to P protein (7).   Therefore,
they are thought to be overlapping fragments from
the N-terminus of P.   These fragments are more
acidic than P as determined by isoelectric focusing
and are relatively poor in methionine, containing
only 2 of the 10 methionyl tryptic peptides of full-
size P.   Additional comparisons of these fragments
and P using radioactive amino acids other than
methionine for peptide mapping would be useful.
The 36 kilodalton proteins made *in vivo* and *in
vitro* have very similar behavior on two dimen-
sional gels; however, we are less certain about the
identity of the 33 kilodalton *in vivo* and *in vitro*
products.   The 33 kilodalton protein made in infec-
ted cells has been reported to be highly basic (8)
whereas the 33 kilodalton *in vitro* product is
more acidic than full-size P.   The possibility that
the similarity in electrophoretic mobilities of
these proteins is coincidental needs further evalu-
ation.   The 36 kilodalton fragment is phosphorylated
in vivo (11) and thus contains the site(s) for at
least one type of P modification.
     Our data on P-related fragments are summarized
in Figure 4 along with data obtained by Chinchar

and Portner (2). Taken together, these results
begin to reveal the molecular anatomy of P.
Chinchar and Portner treated nucleocapsid-associated
P with S. aureus protease V8 and obtained two
highly basic fragments SP1 and SP2 which contained
the cysteine residues involved in disulfide bonding
of P multimers. These fragments remained associated
with cores and presumably also contained a RNA-
binding site of P (14). SPI and SP2 have different
partial digest peptide maps than the N-terminal
fragments made in vitro and thus represent the part
of P close to and possibly including the carboxyl
terminus.
      The P protein in its many forms is likely to
hold keys to the assembly and biological activities
of the nucleocapsid of paramyxoviruses. We are
just beginning to appreciate the complexity of this
protein.

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FOUR FUNCTIONAL DOMAINS ON THE HN GLYCOPROTEIN OF NEWCASTLE DISEASE VIRUS[1]

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COMPETITION ANTIBODY BINDING RADIOIMMUNOASSAYS

A panel of nine monoclonal antibodies specific for the hemagglutinin-neuraminidase (HN) glycoprotein of Newcastle disease virus have been prepared by fusion of SP2 myeloma cells with spleen lymphocytes from a BALB/c mouse immunized with intact, UV-inactivated virus (1). These antibodies have been used in competition antibody binding radioimmuno-assays in which each monoclonal antibody was assayed for its ability to compete for the binding of ^{125}I-labeled anti-bodies to microtiter wells coated with intact virions. Anti-bodies that compete for the binding of the labeled antibody with an affinity similar to that of the homologous antibody are assumed to be recognizing overlapping antigenic determi-nants. As shown in Table 1, these studies delineate four epitopes on the surface of the HN molecule with epitopes 2 and 3 being partially overlapping in a topological sense. This is remarkably similar to studies with monoclonal anti-bodies directed against the HN molecules of Sendai (2) and parainfluenza viruses (3) which have also identified four antigenic determinants (including two which partially overlap) on the surface of the molecule.

FUNCTIONAL INHIBITION STUDIES

The functional relevance of each of the four epitopes on the surface of the HN molecule has been determined by as-saying each antibody's neutralization of infectivity, inhi-

TABLE 1
EPITOPE ASSIGNMENTS OF ANTI-HN MONOCLONAL ANTIBODIES

Competing Antibody

125I-Antibody	HN1a	HN1b	HN1c	HN2a	HN2b	HN3a	HN4a	HN4b	HN4c	Epitope
HN1a	+	+	+	-	-	-	-	-	-	1
HN1b	+	+	+	-	-	-	-	-	-	1
HN1c	+	+	+	-	-	-	-	-	-	1
HN2a	-	-	-	+	+	±	-	-	-	2
HN2b	-	-	-	+	+	±	-	-	-	2
HN3a	-	-	-	±	±	+	-	-	-	3
HN4a	-	-	-	-	-	-	+	+	+	4
HN4b	-	-	-	-	-	-	+	+	+	4
HN4c	-	-	-	-	-	-	+	+	+	4

+ > 60% competition; $\pm$ 50-60% competition;   - < 50% competition

hition of neuraminidase, with both fetuin and
neuraminlactose as substrate, and inhibition of
hemagglutination. These data are summarized in Table 2.
     The neutralizing activity of each of the nine anti-HN
monoclonal antibodies was assayed by the commonly-used
end-point dilution method in which 100 pfu were treated with
serial two-fold dilutions of antibody from an initial
concentration of 100 ug/ml. Comparison of the specific
neutralizing activity of these antibodies can be very
misleading because of the limitation imposed by the use of a
50% neutralization point. For example, antibody $HN1_c$,
despite its relatively low specific neutralizing activity,
is the most potent neutralizer of infectivity as evidenced
by its 0.9% persistent fraction of nonneutralized virus.
Conversely, antibody $HN4_c$, although having the highest
specific neutralizing activity, is the weakest neutralizer
on the basis of its extremely high persistent fraction.
Moreover, although antibodies to all four epitopes are
capable of neutralizing viral infectivity, they do so only
to an apparently epitope-specific extent, with antibodies to
epitope 1 the strongest, those to epitopes 2 and 3 inter-
mediate and those to epitope 4 the weakest neutralizers.
     With the goal of determining the role of each of the
four epitopes in the neuraminidase (NA) activity of the HN
molecule, two-fold serial dilutions of each antibody were
assayed in NA inhibition assays with the large molecular
weight molecule, fetuin, as substrate. As in the case of
neutralization of infectivity, the specific inhibiting
activity is misleading; the percent activity remaining
following treatment with a saturating amount of antibody is
much more indicative of the importance of that epitope to NA
activity. The data also suggests a correlation between
inhibition of NA and neutralization of infectivity.
     In an effort to make a finer antigenic analysis of the
role of the HN epitopes in the NA activity of the molecule,
NA inhibition assays were also performed with the much
smaller substrate neuraminlactose. These efforts were
complicated by the apparent nonspecific enhancement of NA
activity by ascites fluids, an effect which has also been
seen (with both substrates) with a parainfluenza virus (3).
Thus, Table 2 shows the percent activity remaining following
treatment of virus with a saturating amount of an
affinity-purified preparation of each antibody. Only
antibodies to epitope 2 are capable of inhibiting NA with
the smaller substrate. As a control, the affinity-purified
preparations were shown to inhibit NA with fetuin to an
extent similar to that obtained with the ascites
preparations (data not shown).

TABLE 2

FUNCTIONAL INHIBITION STUDIES

Antibody activity	spec. neut.[a] activity	% persistent fraction	spec. NAb inh. titer (Fetuin)	% NA activity remaining (Fetuin)	% NA activity remaining (Neuraminlactose)	specific[c] HI-activity
HN1a	1280	1.6+0.9	65	3.7	130.2	8200
HN1b	640	2.7+0.8	16	15.1	105.8	3000
HN1c	640	0.9+0.6	48	1.8	127.4	6100
HN2a	640	5.3+2.0	39	14.8	41.5	2000
HN2b	640	3.4+1.2	38	11.3	29.7	900
HN3a	2560	6.4+2.1	48	31.9	92.0	12
HN4a	2560	13.8+4.7	2	42.6	97.5	8
HN4b	2560	7.6+2.9	< 2	67.8	109.3	17
HN4c	5120	16.9+2.7	< 2	60.2	116.2	4

[a]The reciprocal of the highest dilution of antibody giving a 50% reduction in plaque number relative to an anti-NP control.
[b]The reciprocal of the highest dilution of ascites fluid, on a per mg of antibody per ml basis inhibiting 50% of the neuraminidase activity.
[c]The reciprocal of the highest dilution of ascites fluid, on a per mg of antibody per ml basis inhibiting four HA units of virus.

An ascites fluid preparation of each of the antibodies was next assayed for its ability to inhibit the agglutination of chicken erythrocytes by 4 HA units of virus. Antibodies to epitope 1 had the highest specific HI activities while antibodies to epitope 2, although also inhibitory of HA, have at least a three-fold lower activity. Antibodies to epitopes 3 and 4 have specific HI activities of less than 20, comparable to those obtained with control antibodies specific for the nucleocapsid protein (NP).

Table 3 presents a summary of the functional properties of the four epitopes on the surface of the HN glycoprotein of NDV. On the basis of this, we have constructed a tentative linear map of their relative positions with respect to the HA and NA sites (Figure 1). Epitope 1 is placed closest to the HA site because antibodies to it have the highest HI titers. Epitope 2 is closest to the NA site because antibodies to it are the only ones that inhibit NA with the smaller substrate, but is placed between the two sites because antibodies to it can inhibit HA as well. Antibodies to epitopes 3 and 4 are only able to inhibit NA with the larger substrate and do not inhibit HA. Thus, they are placed the most distal to the HA site but with epitope 3 closest to the NA site as it topologically overlaps with the second epitope. We are using this map as a working model of the antigenic structure of the HN glycoprotein of NDV.

EVIDENCE FOR SEPARATE SITES FOR HA AND NA

Recent evidence from mutant and neuraminic acid analog analyses suggests that the NA and HA sites are located on different active sites on the HN molecule of paramyxoviruses (4-7). This study provides further antigenic evidence in support of this view. Although antibodies to each of the epitopes are capable of inhibiting NA with the larger substrate, antibodies to epitopes 3 and 4 are not able to inhibit the HA activity of the virus. Thus, this is evidence of an antigenic distinction between the HA and NA activities on the HN molecule. Even more convincing, however, is the failure of antibodies to epitope 1 to inhibit NA with neuraminlactose as substrate despite their extremely high HI titers. This is evidence for an antigenic separation between the two sites on the surface of the HN glycoprotein of NDV and is similar to that found with Sendai virus (2).

TABLE 3
SUMMARY OF FUNCTIONAL INHIBITION PROPERTIES OF HN EPITOPES

| HN | | Neuraminidase | | |
Epitope	Infectivity	Fetuin	Neuraminlactose	Hemagglutination
1	+++	+++	-	+++
2	++	++	++	++
3	++	++	-	-
4	+	+	-	-

FIGURE 1
LINEAR MAP OF HN EPITOPES
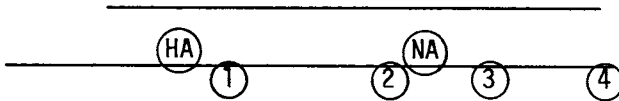
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MAPPING MUTANT AND WILD-TYPE M PROTEINS OF NEWCASTLE
DISEASE VIRUS (NDV) BY REPEATED PARTIAL PROTEOLYSIS[1]

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Partial proteolysis in SDS-polyacrylamide gels by the procedure of Cleveland et al (1) is a valuable tool in comparing proteins. However, information about the nature of the partial proteolysis fragments and the section of the molecule represented by them is not provided by this procedure. We have expanded upon this procedure by subjecting the membrane protein (M) of NDV to partial digestion with S. aureus V8 protease, and then isolating the products, redigesting them, and analyzing the resulting fragments. This has allowed us to construct a map of wild-type (AV-WT) M, and by comparison of this map with that of a ts mutant M, we have begun to locate the alteration in the mutant protein.

MAPPING AV-WT M PROTEIN

Figure 1 shows partial proteolysis of AV-WT M using increasing concentrations of protease. In addition to the undigested 41 K (kilodalton) M, 11 major fragments were generated. Each fragment was then cut from the gel and redigested with a higher protease concentration. Figure 2 shows that redigestion of band 1 (uncut M) on the left, again yields all 11 fragments; fragments 8 and 9 are difficult to separate upon redigestion. Redigestion of fragment 2 yields fragment 4 (not 3), 8/9, and 12. Fragment 3 yields fragments 4, 8/9 and 11. Fragment 4 yields fragments 8/9 and 11. At the protease concentrations used here, fragment 9 yields no new fragments on redigestion, but digestion at higher protease concentrations yields fragment 11 which is a final digestion product (data not shown).
Comparing fragments generated by redigesting each initial fragment, we generated the map of M in Figure 3;

four S. aureus V8 sites are designated A,B,C and D. An ex-
ample of the way the map was constructed follows. 2 is gen-
erated from M (fragment 1) by the loss of 2K; it has arbi-
trarily been placed at the right end. Further digestion of 2
generates 4, but not 3; thus 4, but not 3, is contained with-
in 2. Therefore, 3 must come from the other end, the left
end of the molecule. A fragment the size of 4 is generated
from both 2 and 3 and can be explained as two fragments of
similar molecular weight resulting from approximately equi-
valent fragments lost from 2 and 3: the left end to protease
site A (2K), and the fragment between sites C and D (2K).
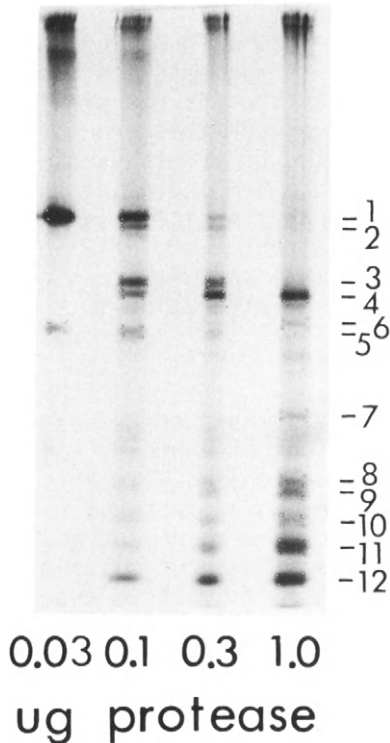
FIGURE 1.   Partial proteolysis pattern of the M of
AV-WT.   A ^{35}S-methionine labeled cell lysate was displayed
on a 10% SDS-polyacrylamide gel (2) and autoradiographed to
locate M.   Gel slices containing M were rehydrated, placed
on top of a 15% SDS-polyacrylamide gel with different
concentrations of S. aureus V8 protease, and digested (1).
Major fragments are identified, including the uncut M, #1.

Fragments on the map in figure 3 are shown in their most
likely configuration, although a few ambiguities (due in
part to our inability to separate redigested fragments 8/9)
exist at the internal sites. Fragment 7 and several minor
fragments have not been placed on the map.

LOCATING THE CHANGE IN THE M PROTEIN OF TS MUTANT D1

Virions of group D ts mutants are less infectious and
less hemolytic than AV-WT (3) and cells infected by them
package less fusion protein (F_{1+2}) into virions (4). In
addition, mutant D1's M migrates in SDS-polyacrylamide gels
with an apparent molecular weight of 40K compared to 41K for
the AV-WT M, regardless of temperature of synthesis (Figure
4 and reference 5). Revertant analysis has revealed that
altered M migration and decreased packaging of F_{1+2} are
clearly related to D1's originally selected ts phenotype (6).
Since D1's complete M migrates more rapidly, it seemed
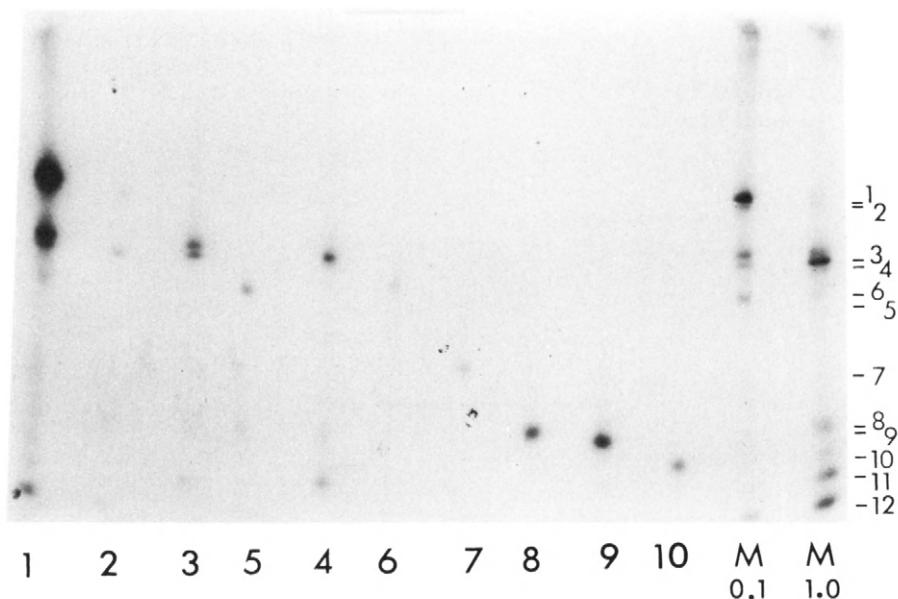
FIGURE 2.   Redigestion of partial proteolysis products
of AV-WT's M. Major fragments from the digests shown in
Fig. 1 were isolated from the track where they were present
in greatest abundance. They were redigested by the
procedures described for Fig. 1, but using the next higher
protease concentration. Products of the digestion of M with
0.1 and 1.0 ug of protease provide markers (on the right).
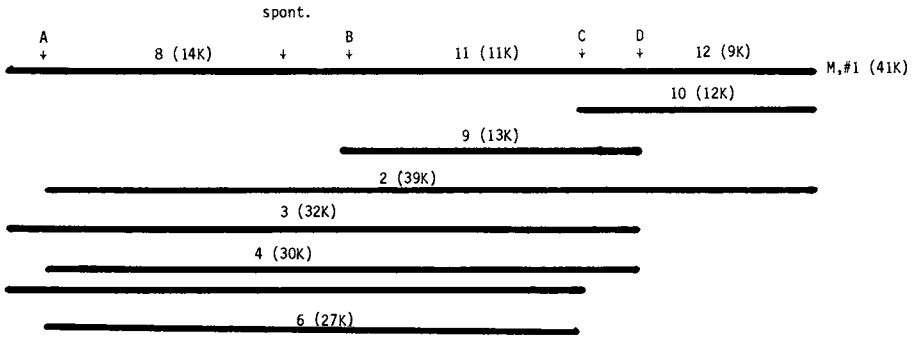
FIGURE 3. S. aureus V8 protease map of the M protein of
AV-WT. Protease sites were located by determining which
large fragments contained smaller fragments, and then
aligning overlapping fragments. The smallest fragments
which could not be digested further were placed on the full
map, and the four protease sites they define identified as
A,B,C and D. A spontaneous cleavage site whose products are
often found in the absence of protease is also shown. Two
approximately 2K fragments between the left end and A, and
between C and D, have not been isolated but are presumed to
exist because of size differences in fragments 1 and 2, and
also 10 and 12.
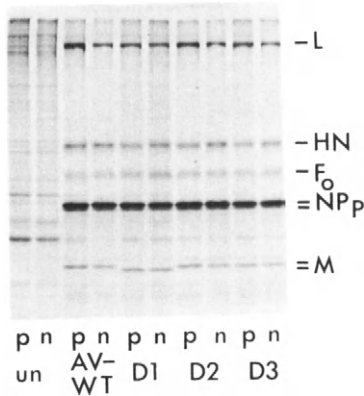
FIGURE 4. AV-WT and mutant proteins in chicken embryo
cells. Cells incubated at permissive (p) or nonpermissive
(n) temperature for 6 hours were labeled for 1 hour with 20
uCi/ml ^{35}S-methionine in medium lacking methionine, lysed
in sample buffer, and displayed on a 10% SDS-polyacrylamide
gel (2). Uninfected (un) cells, and cells infected with
AV-WT, or ts mutants D1, D2, or D3 are presented.

likely that some of its partial fragments might do so, as
well. Figure 5 shows that, in addition to the uncut M
(fragment 1), four D1 fragments, 2, 5, 10 and 12, migrate
faster. In the map of AV-WT's M in Figure 3, fragments 2,
10 and 12 are a nested set of fragments; each contains the
9K fragment 12 located at an end of the molecule. This
conclusion has recently been confirmed by digesting M from
AV-WT and D1 with S. aureus V8 in solution; under these
conditions D1's fragment 12 is smaller than that of AV-WT,
while fragment 3 from both sources is identical (data not
shown). Thus the lesion in the M protein of D1 clearly
seems to lie at an end of the molecule.
     Fragment 5 also reflects the increased migration rate of
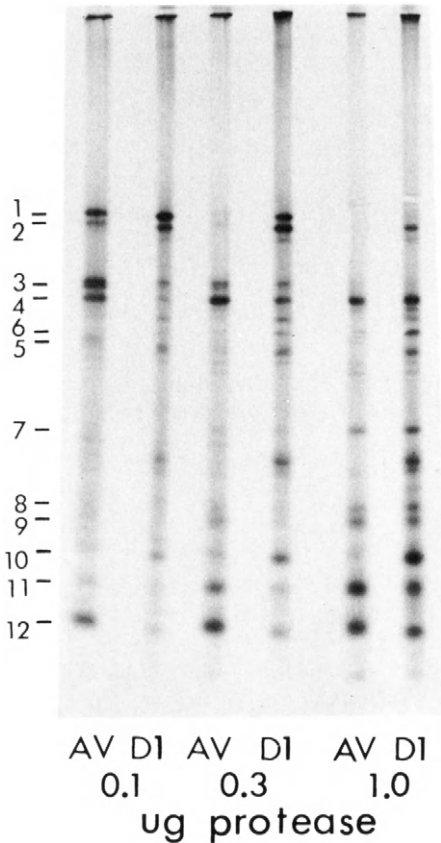
FIGURE 5. Partial proteolysis of the M protein of AV-WT
and mutant D1. M was isolated and digested as in Fig. 1.
The 12 major fragments are indicated along the side.

D1's M. It is generated spontaneously in the absence of
protease as a result of fixing the gel in acetic acid and
drying with heat (data not shown). Fragment 5 must include
the altered region of D1's M and must therefore include the
right end of the molecule. The spontaneous breakage site
must be approximately in the position shown in Figure 3.

By repeated partial proteolysis of the M of AV-WT with S.
aureus V8, we have deduced a map of this protein. By com-
paring the M of AV-WT and mutant D1 by partial proteolysis,
we have located the altered portion of D1's M to a terminal
region of the molecule. This region of M may either directly
or indirectly interact with the F glycoprotein which is re-
quired for hemolysis and infectivity (7). Such an
interaction is indicated by studies with the group D mutants
and their revertants altered in the packaging of F and its
functioning in virions (4, 6).

We do not yet know which end of M is involved in this
mutation and whether the alteration is located at the
extreme or within the terminal fragment. However, it is
tempting to speculate that the mutation is in the carboxy
terminus of M and represents a nonsense mutation causing
premature termination during translation. Analysis of the
carboxy terminus of AV-WT, D1, and its three revertants
whose M's migrate like that of AV-WT (6) will be interesting.

ACKNOWLEDGEMENTS

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STRUCTURAL CHARACTERIZATION OF HUMAN PARAINFLUENZA VIRUS 3

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Human parainfluenza virus type 3 (HPIV3) infections have great clinical significance because they occur early in life, are readily transmissible, and are likely to recur [1,2,3,]. Despite its medical importance, little is known about the molecular biology of this virus.

The most thoroughly studied parainfluenza virus is Sendai virus, a murine parainfluenza type 1 virus. Sendai virus has been shown to have 9 major structural proteins with molecular weights ranging from 145 K to 34.7 K when grown in cultured cells [4,5,6]. The biological functions and structural locations have been assigned to the major polypetides. Sendai virus contains either two or three glycoproteins depending on the host cell of origin. The largest glycoprotein, molecular weight of 69K, has both haemagglutinin and neuraminidase activities and is designated HN [5,7]. The small glycoprotein, molecular weight of 53K is involved in virus induced haemolysis, cell fusion and initiation of infection [7]. This glycoprotein is designated F. Sendai virions grown in cultured cells, have an intermediate size glycoprotein of 65K molecular weight, which has been identified as F_0, the inactive precursor of the fusion protein [7]. Three nonglycosylated proteins have been associated with the nucleocapsid and are involved in RNA polymerase activity [8]. These proteins are L protein a high molecular weight protein, P, the polymerase protein, and the NP the nucleocapsid subunit protein. It is of interest to note that the three nucleocapsid proteins are all phosphorylated [6,9]. The smallest structural protein with a molecular weight of 34.7 K is the matrix protein M. This protein is responsible for maintaining the structural integrity of the virus [5].

Preliminary works on bovine, ovine, and human parainfluenza virus type 3 [10,11,12,13] have indicated that there are similarities in the structural proteins to those of Sendai virus and the other paramyxoviruses. The characteristic which distinguishes the parainfluenza type 3 viruses from Sendai virus is that parainfluenza type 3 viruses do not need trypsin in the medium in order to form plaques [10]. This observation indicates that only the cleaved fusion protein is incorporated into the virion. Indeed, for all strains of parainfluenza type 3 viruses tested, only 2 glycoproteins are associated with the virion [10,11,12,13].

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Growth and Purification of HPIV3. One difficulty when working with HPIV3 is the low virus yields when grown in cultured cells. In order to overcome this problem HPIV3 strain C243 (provided by the Laboratory Center for Disease Control, Ottawa, Canada) has been grown in monolayer cultures of LLCMK$_2$ cells. The growth curve of the virus is shown in Figure 1. A titre of 9 X 10^7 plaque forming units/ml of culture medium was achieved within 24 hours and maintained for at least 50 hours post infection. The formation of polykaryocytes appeared at 12 hours post infection. Cell destruction became prominent 50-60 hours post infection.
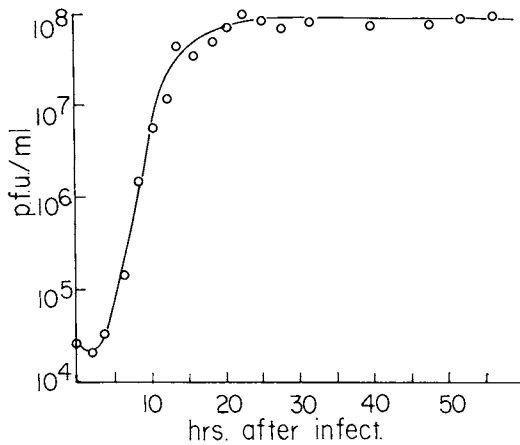
FIGURE 1. Growth curve of HPIV3 in LLC MK$_2$ cells. Cell cultures (7 X 10^6 cells) were infected at a multiplicity of infection of 3. Samples were assayed for infectivity by plaque assay using LLC MK$_2$ cells.

PROTEINS OF HPIV3

The structural proteins of the virion were labeled with radioactive precursors starting immediately after the adsorption period of one hour. The virus was grown in the presence of ^{14}C-glucosamine to label the glycosylated proteins, ^{32}P-orthophosphate to label the phosphorylated proteins and ^{35}S-methionine or ^{14}C-amino acids mixture to label all the structural proteins. The culture fluid was harvested 48 hours after infection. To remove the cellular debris the culture fluid was clarified by low speed centrifugation. The labeled virus was then pelleted by high speed centrifugation, resuspended in sample buffer and then analyzed by SDS polyacrylamide gel electrophoresis.

Identification and Molecular Weight Determination of the
structural Proteins of HPIV3.   Bovine, ovine and human para-
influenza type 3 viruses have been reported to contain 5, 6, and 8
structural proteins respectively [10,12,13]. These proteins range in
molecular weight from 17K to 125K. The variation amongst the
parainfluenza viral proteins made it necessary to identify the
structural proteins of HPIV3 and determine the molecular weights of
the proteins. HPIV3 was labeled with ^{14}C-amino acids mixture or
[35]S-methionine for 48 hours. The radioactively labeled HPIV3 was
purified and the structural proteins were analyzed in a 15% polya-
crylamide gel with a 3% stacking gel containing 0.1% SDS. Proteins
of known molecular weights have been used as references. These
include myosin (H-chain) 200K, phosphorylase B 92.5K , bovine
serum albumin 68K, ovalbumin 43K, chymotrypsinogen 25.7K, and B-
lactoglobulin 18.4K.
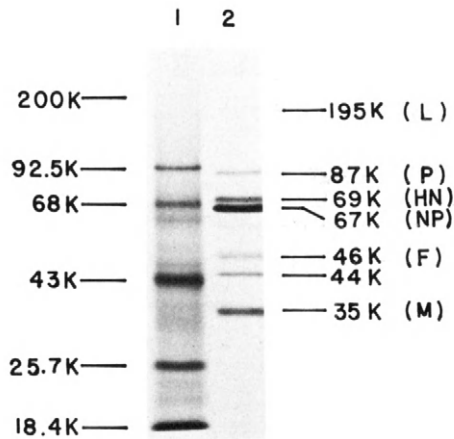
FIGURE 2.   Polyacrylamide gel analysis of marker proteins
and of HPIV3 proteins. ^{14}C-amino acids labeled myosin (200K),
phosphorylase B (92.5K), bovine serum albumin (68K), ovalbumin
(43K), chymotrypsinogen (25.7K) and B-lactoglobulin (18.4K) (1) and
[35]S-methionine labeled HPIV3 (2) were analyzed by electrophoresis
in 15% polyacrylamide gel containing 0.1% SDS.

HPIV3 contains 7 major and 2 minor structural proteins. The
major proteins have molecular weights of 195K, 87K , 69K, 67K,
46K, 44K,and 35K, for L, P, HN, NP, F, an unknown protein, and M
respectively. The minor proteins have molecular weights of 60K and
20K (Figure 2). The major structural proteins were assigned on the
basis of similarities to Sendai virus structural proteins and on this
study (see below).

To determine whether ^{35}S-methionine labeling represents the total proteins in correct proportions, a comparision was made between the viral proteins labeled with ^{35}S-methionine, or with a ^{14}C-amino acids mixture, and when the viral proteins where stained with Coomassie Brilliant Blue. The three methods represent similar molar quantities of the proteins as were found for the proteins of Sendai virus [6,14]. Thus, most experiments were carried out with ^{35}S-methionine.

The Phosphorylated Proteins of HPIV3. Phosphorylated proteins are often found in the enveloped negative strand RNA viruses. These include orthomyxo, paramyxo and rhabdoviruses. The P, NP and M proteins of Sendai and Newcastle disease virus are the predominantly phosphorylated proteins [9,15,16]. The HN and F_0 of Sendai virus are also phosphorylated [9,16].
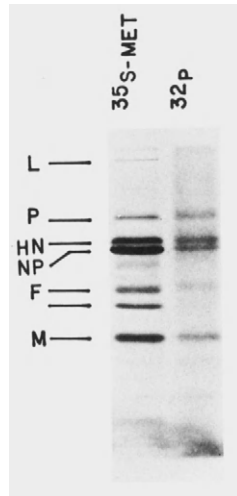
FIGURE 3. Analysis of the phosphorylated proteins of HPIV3. ^{32}P-orthophosphate labeled HPIV3 proteins were analyzed in 15% polyacrylamide gel containing 0.1% SDS. ^{35}S-methionine labeled HPIV3 proteins were coelectrophoresed for identification.

When HPIV3 was grown in the presence of ^{32}P-orthophosphate, five of the 7 major virion proteins P, HN, NP, F and M were phosphorylated (Figure 3). Of these proteins the P and M proteins were the prominently labeled. Phosphorylated proteins have been implicated as playing a role in the replication and assembly of the paramyxoviruses [9,15,16]. It therefore is not surprising to find that the nucleocapsid and envelope proteins of HPIV3 are phosphorylated. Futher study of these proteins will be important in the understanding of viral replication strategy.

Glycoproteins of HPIV3. Paramyxoviruses characteristically have at least two glycoproteins associated with their virions, the HN and F proteins. The parainfluenza type 3 viruses are no exception. Ovine, bovine and human parainfluenza type 3 viruses all have 2 glycoproteins associated with their virions [10,12,13]. The larger glycoprotein has a molecular weight of 69K-73K. The smaller glycoprotein has a molecular weight of 51K-55K depending on the virus.

The glycoproteins of HPIV3 were identified by ^{14}C-glucosamine labeling. Two proteins of molecular weights 69K and 46K were predominantly labeled with the radioactive precursor (Figure 4). These correspond to the HN and F proteins respectively. Another small protein of approximately 20K molecular weight also had some incorportion of the ^{14}C-glucosamine (unpublished observation).
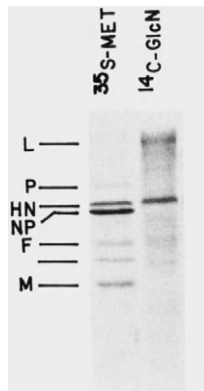
FIGURE 4. Identification of HPIV3 glycoproteins. ^{14}C-glucosamine labeled HPIV3 proteins were analyzed in 15% polyacrylamide gel containing 0.1% SDS. ^{35}S-methionine labeled HPIV3 proteins were coelectrophoresed for the reference of each protein.

The envelopes of the paramyxoviruses are associated with several enzyme activities including neuraminidase, haemagglutinin, haemolysin and cell fusion activities. These activities are attributed to the glycoproteins present on the virion surface [5,6,7]. The larger glycoprotein of Sendai, SV5 and Newcastle disease viruses is associated with the hemagglutinin and neuraminidase activities, and the smaller glycoprotein is responsible for the fusion and haemolysing activities. The major glycoprotein of HPIV3 corresponds to the HN protein of Sendai and SV5 in size and in the molar quantity incorporated into the virion. Thus, the 69K molecular weight

protein has been designated HN. Paramyxovirus F_0 protein has been shown to be cleaved post translationally into two products, designated F_1 and F_2, which are held together by disulfide bonds [18]. Under reducing conditions, cleavage of the disulfide bonds is expected, and 2 proteins are seen when the cleaved product is analyzed by SDS-PAGE. The samples in figure 4 were reduced with mercaptoethanol prior to electrophoresis. Therefore, the 46K molecular weight glycoprotein corresponds to the F_1 of other paramyxoviruses. A minor protein with an estimated molecular weight of 20K was constantly found in samples of HPIV3. This protein was labeled when the virion was grown in the presence of ^{14}C-glucosamine (unpublished observation). This may indicate that the 20K molecular weight protein is the F_2 protein These findings suggest that the putative F_0 protein of HPIV3 may have a molecular weight of about 66K.
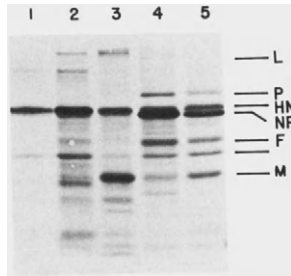
FIGURE 5. Location of HPIV3 proteins in the virion. ^{35}S-methionine labeled HPIV3 proteins were analyzed in 15% polyacrylamide gel containing 0.1% SDS after various treatments: (lane 5) total virion proteins; (lane 4) pelleted nucleocapsid protiens after triton X-100 and 1 M NaCl treatment; (lane 3) soluble envelope components after 2% triton X-100 and 1M NaCl treatment; (lane 2) envelope components after removal of M protein by dialysis to remove salt and centrifugation; (lane 1) HN protein recovered from the supernatant of high speed centrifugation to remove remaining envelope components.

Location of the Virion Proteins. The envelope proteins of SV5 and Sendai virus have been separated from the nucleocapsids by disruption with Triton X-100 in the presence of 1M KCl [6,17]. Dialysis was done to remove the KCL, resulting in the aggregation of the M protein and the aggregated protein was separated from other envelope components by centrifugation. In order to determine the location of each protein in the virion of HPIV3, a similar procedure was used. Figure 5, lane 5 represents the standard proteins of HPIV3 labeled with ^{35}S-methionine. The virions were

disrupted by 2% Trition X-100 and 1M NaCl, which solubilized the HN, F and M proteins and leaves the nucleocapsids in the pellet after ultracentrifugation. Figure 5, lane 4 represents the proteins associated with the pelletable nucleocapsids. These proteins closely resemble the proteins found in the standard virus except there is a reduction in the amount of HN and M proteins. The envelope proteins are represented in lane 3. It should be noted that the L, P, and NP proteins are not present in lane 3. Thus, the partial disruption of the virions indicates that the L, P and NP proteins are associated with the nucleocapsid, and the HN and M proteins are associated with the envelope of the virion. Track 2 represents the proteins remaining in the supernatant after dialysis to remove the salt and centrifugation of the envelope components to pellet the M protein. The M protein is virtually eliminated from this sample. The HN protein can be further separated from the envelope proteins by sucrose velocity sedimentation (lane 1). The high molecular weight proteins in lanes 1, 2, and 3 may represent polymers of the HN proteins. Similar polymers have been reported for mumps virus (19,20). It is of interest to note that the 20K molecular weight protein remains associated with the envelope proteins, and is not present in the lane with the pelleted nucleocapsid proteins. Thus, it is possible that 20K protein may represent a subunit of F_0. The F protein and the 44K protein are present in all samples shown in figure 5 with the exception of the purified HN sample. In this sample only a small amount of the 44K protein is present. The 44K protein is also associated with Sendai virus and a previous report suggested that it is a cellular polypeptide incorporated into the virion (8). The Sendai virus polypeptide is solubilized with 2% Triton X-100 and so remains associated with the envelope proteins. The origin and function of the 44K HPIV3 protein remains to be determined. The F protein of HPIV3 has not yet been isolated in pure form, because it is very tightly associated with other envelope components of the virion.

Summary. The structural proteins of human parainfluenza type 3 were studied. These proteins were designated on the basis of the work reported here, and by comparison with the properties of other paramyxovirus structural proteins. The L protein was designated on the basis of its high molecular weight and its association with the nucleocapsid. The P and the NP proteins have been assigned on the basis of their phosphorylation and by the partial disruption of the virion, which showed that they were associated with the nucleocapsids. The HN and F proteins were identified as glycoproteins on the basis of their incorporation of ^{14}C-glucosamine and HN was shown to be an envelope protein. The M protein was identified by its phosphorylation, association with the virion envelope and aggregation under low salt conditions. We suggest that the 20K molecular weight protein is a subunit of fusion protein. Further work is necessary to assign the identity and function of the 44K molecular weight protein.

ACKNOWLEDGMENTS

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CHARACTERIZATION OF STRUCTURAL PROTEINS OF PARAINFLUENZA VIRUS 3 AND mRNAs FROM INFECTED CELLS

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The human parainfluenza viruses, members of the paramyxovirus family, are second only to respiratory syncytial virus as causes of lower respiratory tract disease in young children. The most characteristic condition these agents produce in infants and young children is croup, i.e. swelling of the larynx leading to obstructed breathing and a hoarse cough. The epidemiological patterns and clinical manifestations of parainfluenza infections, in adults as well as children, emphasize the protective effect of neutralizing antibodies as well as lack of complete or long-lasting immunity. In order to gain insight into the molecular basis of pathogenicity of these viruses, we have initiated a project to study in detail the structure and function of the genes of parainfluenza virus type 3 (PI-3). Some recent progress has been made on the characterization of PI-3 in several laboratories (1-3).

VIRUS STRUCTURAL PROTEINS

PI-3, HA-1, strain 47885 (kindly provided by Dr. Robert Chanock, National Institutes of Health, Bethesda, MD) was plaque purified in BSC-1 cells (kindly provided by Dr. Robert Simpson, Waksman Institute, Rutgers University, New Brunswick, NJ). The virus was grown in BSC-1 cells (m.o.i. 2 Pfu/cell) in Dulbecco's modified Eagles medium from GIBCO (H21) containing 10% fetal calf serum. After 48 hrs, the virus from the supernatant was purified (4). For labeling with ^{35}S-methionine or ^{3}H-glucosamine, the medium contained one-fourth the usual level of unlabeled methionine and lacked glucose, respectively.

Fig. 1 shows analyses of PI-3 structural proteins by polyacrylamide gel electrophoresis. As shown in lane B, at least six distinct polypeptides (1 to 6) with some minor bands (shown by arrows) were visualized by autoradiography. Virus labeled with ^{3}H-glucosamine (lane C) specifically labeled polypeptides Nos. 3 and 5, indicating that these represent the viral glycoproteins. The nature of the band migrating near the origin has not been explored. By comparison with published data (1,3), these polypeptides probably represent the HN and F_1, respectively. Two additional bands (shown

by arrows) may represent unreduced glycoproteins HN (top) or F (bottom). The polypeptide No. 4 appeared to be the viral nucleocapsid protein (NP), since, as shown in lane E, purified nucleocapsid prepared from Triton-disrupted virions migrated at the same rate as polypeptide No. 4. The estimated molecular weights and possible identities are shown in Table 1. From these results, it seems that the molecular composition of PI-3 bears close resemblance to other members of the paramyxovirus family (5).
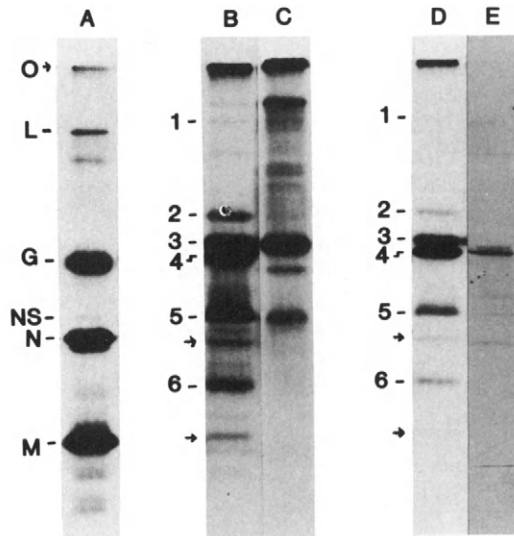
Fig. 1. Polyacrylamide gel electrophoresis of PI-3 structural proteins. (A) marker ^{3}H-methionine VSV; (B) ^{35}S- methionine PI-3; (C) ^{3}H-glucosamine PI-3; (D) ^{35}S-methionine PI-3; (E) purified RNP prepared from ^{35}S-methionine PI-3. Fluorography was carried out following electrophoresis. Nos. 1 to 6 represent PI-3 structural proteins. L,G,NS,N,M represent VSV structural proteins. Arrows indicate unidentified proteins.

TABLE 1

CHARACTERIZATION OF PI-3 STRUCTURAL PROTEINS

Protein No.	1	2	3	4	5	6
Estimated Mol.Wt. (x 10^{-3})	220	86	72	70	55	36
Glycoprotein	no	no	yes	no	yes	no
Protein Assignment	L	P	HN	NP	F_1*	M

*F_o, 68,000 (see lane C, bottom arrow, Fig. 1)

VIRUS–SPECIFIC mRNAs

To isolate virus-specific mRNAs, cells were infected with PI-3; 15 hrs and 20 hrs post-infection actinomycin D (5 µg/ml) and ^{3}H-uridine (20 µc/ml) were added, respectively. Cells were harvested at 24 hrs post-infection and total mRNA from infected cells was isolated according to the method of Glisin et al. (6). Poly(A)$^+$ and poly(A)$^-$ fractions were separated by oligo(dT)–cellulose chromatography. RNA fractions were then analyzed by electrophoresis in 1.2% agarose containing methyl-mercury as the denaturing agent (7).
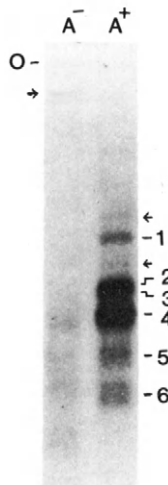
Fig. 2. Analysis of mRNAs isolated from PI-3 infected cells. ^{3}H-uridine-labeled total RNA was isolated from PI-3-infected cells and separated into poly(A)$^+$ and poly(A)$^-$ fraction by oligo(dT)-cellulose chromatography. Electrophoresis of each fraction was carried out in 1.2% agarose gel containing methyl mercury. Fluorography was carried out following electrophoresis. Nos. 1 to 6 represent PI-3 mRNAs. Arrows indicate unidentified RNA species.

As shown in Fig. 2, RNA lacking poly(A) (A$^-$ lane) contained a large RNA species (shown by arrow) with an estimated molecular weight of 5.4 x 10^6. This RNA species probably represents the full-length genome RNA of PI-3. The poly(A)-containing mRNA (A$^+$ lane) contains at least six mRNA species. The second prominent band contains two distinct mRNA species (Nos. 2 & 3) when seen at lower exposure. Two additional RNA species (shown by arrows) can also be seen. Using HeLa cell 28S and 18S ribosomal RNA markers in the same gel, the estimated molecular weights of each of the RNA species were calculated and are shown in Table 2.

TABLE 2

CHARACTERIZATION OF PI-3 mRNAs FROM INFECTED CELLS

mRNA No.	1	2	3	4	5	6
Estimated Mol.Wt.	1.65×10^6	1.0×10^6	0.92×10^6	0.76×10^6	0.5×10^6	0.3×10^6

TABLE 3

HYBRIDIZATION OF PI-3 GENOME RNA WITH ^{3}H-mRNA

Tube No.	^{3}H-mRNA μl	Genome RNA (0.01 μg/μl)	Treatment	Acid Precipitable (cpm)	RNase Resistance %
1	5	-	None	13,620	-
2	5	-	70°C, 2 hr + RNase	1,863	13.6
3	5	5	"	13,840	100

Hybridization was carried out in 1 x SSC, as described earlier (8).

Thus, it seems that possibly six monocistronic mRNAs were present in the infected cells. In vitro translation of each of the mRNA species should reveal the identity of the individual mRNAs. Finally, to demonstrate that the mRNA species were indeed virus-specific, the poly(A)$^+$-mRNAs were hybridized with excess of PI-3 genome RNA. As shown in Table 3, the entire ^{3}H-labeled mRNA entered into duplex as determined by resistance to digestion with RNase A & T$_1$. However, a small proportion (14%) of radioactivity self-annealed indicating the presence of possible high degree of secondary structure in the mRNAs.

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CHARACTERISATION OF MUMPS VIRUS PROTEINS AND RNA[1]

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PROTEINS IN MUMPS VIRUS INFECTED CELLS

Mumps is a human paramyxovirus. We have studied the polypeptides induced after virus infection by labelling with ^{35}S-methionine, ^{35}S-cysteine, ^{14}C-amino acids, ^{32}P-orthophosphate and ^{3}H-glucosamine/mannose in order to elucidate the number, types and approximate molecular weights of the virus induced proteins. We have found in the present study (Fig. 1) and earlier (1) that the virus induces in Vero cells a large 'L' protein (mol. wt. 160K); the nucleocapsid protein N (70K), which is phosphorylated and appears susceptible to proteolysis so that it is often found as an 57-60K phosphoprotein in purified nucleocapsids; the poly-merase 'P' protein (45-47K), which frequently migrates as a double band in SDS-PAGE; the matrix protein M (40K) which at least in one strain migrates as a double band (we have not yet conclusively demonstrated that the P and M proteins are phosphorylated in our virus/host system); the glycoproteins HN (80K) and FO (69K) which appears to be processed by a trypsin like enzyme to F1 (61K) and F2 (10-14K), both of which are glycosylated. These are the structural proteins of mumps virus (2,3). The unique nature of the L, HN, N, M and P proteins has been demonstrated by limited proteolysis (4,5) carried out as described by Cleveland (6), We have carried out the classical method of limited proteolysis with V8 protease as well as the strip method described in Fig. 2 which has the advantage that all the induced proteins may be analysed in one gel. Furthermore, the uniqueness of these proteins has been demonstrated by two dimensional tryptic digest peptide mapping (data not shown). These analyses have

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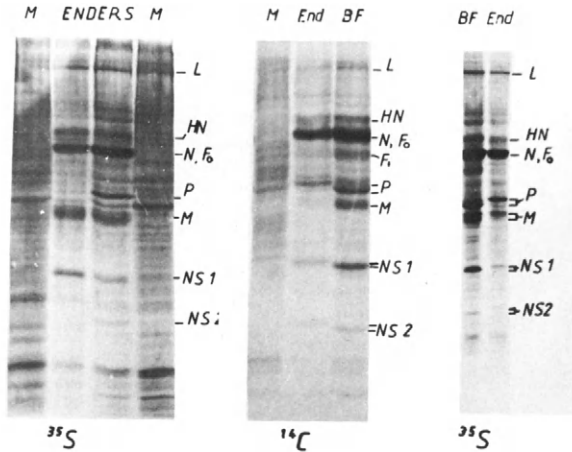
Figure 1. SDS-PAGE (8-15%) analysis of infected and mock infected (M) cell lysates radiolabelled as indicated

not been applied to the FO protein which comigrates with N, or to the F1 protein as this appears to be very poorly labelled with ^{35}S-methionine or cysteine even in pulse-chase experiments. Limited proteolysis of ^{3}H-glucosamine/mannose labelled proteins has demonstrated the distinct nature of the HN and FO and the relationship between FO and F1 (data not shown).

NON-STRUCTURAL PROTEINS

In addition to the structural proteins we have also detected two non structural proteins (1) confirmed by others (4,5) which we now propose to call NS1 and NS2. These are easily detected (Fig. 1), especially when ^{35}S-cysteine or ^{14}C-amino acids are used for NS1 labelling and ^{35}S-methionine for NS2 labelling. Limited proteolysis analysis of the whole of a first dimension gel strip (Fig. 2) indicated, besides the fact that the N protein contaminates positions above as well as below its own major band, that there is an apparent relationship between the P, and the NS1 and NS2 proteins. This has also been reported by Herrler and Compans (5). We have confirmed these data with conventional limited proteo-lysis for both the Enders and Belfast (BF) strains of mumps virus and it is of interest to note that the P as well as the NS1 and NS2 proteins show migration differences between these two strains and a comparison of the limited proteolysis
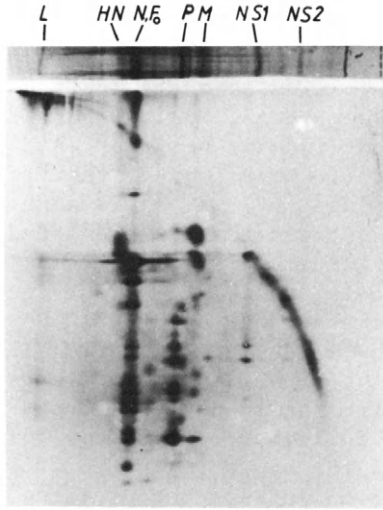
Figure 2. Limited proteolysis of whole first dimension gel strip digested with V8 protease analysed in 15% gel.
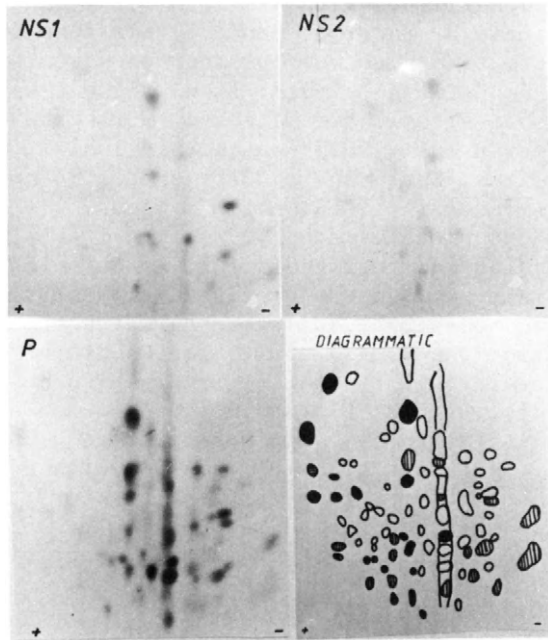
Figure 3. 2 D tryptic peptide maps of P, NS1 and NS2 plus diagram of shared spots in P, NS1 and NS2 (●); in P and NS1 (◑), in P only (O) and in P and NS2 (⊖)

patterns of the P proteins of these strains shows a difference
in one band obtained after digestion with V8 protease (data
not shown). It appeared more difficult to corroborate the
relationship between the P, NS1 and NS2 proteins after
labelling with ^{35}S-cysteine because P and NS2 label poorly
with this precursor. We employed, therefore, 2D tryptic
digest peptide mapping (7) to obtain a more direct proof of
the relationship between the P protein and the non-structural
proteins. ^{35}S-methionine labelled P, NS1 and NS2 proteins
were cut out of gels and subjected to digestion, electro-
phoresis and chromatography and the 2D peptide maps were
prepared for each of the three proteins (Fig. 3).
Combinations of the P, NS1 and NS2 digests were also analysed
in order to confirm the identity of spots with similar
migrations. The following conclusions were drawn from the
peptide maps: all the spots in the NS2 digest were present
in the P protein; all except one of these were also present
in the NS1 protein. All the spots in the NS1 protein were
also found in the P protein digests. It thus appears that
the P, NS1 and NS2 proteins contain similar tryptic peptides
with NS2 peptides being found in both the larger proteins.
The question is how the NS proteins are derived from P.
Pulse-chase experiments with ^{35}S-methionine (data not shown)
did not indicate a precursor-product relationship between
P, NS and NS2. The P as well as the non-structural proteins
appeared to be detectable after labelling with short pulses
and were stable after chase periods. Furthermore, pretreat-
ment of infected cells with proteolysis inhibitors such as
PMSF, TPCK, TLCK, ZnCl$_2$ and iodoacetamide did not lead to
the disappearance of the NS1 and NS2 bands. It seems there-
fore that these proteins are not derived from P by proteo-
lytic processing and that their relationship to P may be
similar to that between the P protein of Newcastle disease
virus and two non-structural proteins which appear to be
translated from the P mRNA and to represent the N termini
of the P protein (8). Recently, it has also been shown
that the non-structural C protein found in Sendai virus
infected cells, which has been described as a unique gene
product (7), is translated from an mRNA which comigrates
with the RNA coding for the P protein of Sendai virus (9).
Similarly, we have identified a non-structural 37K protein
related to the P protein in measles virus infected cells (10).

MESSENGER RNA AND IN VITRO TRANSLATION

     PolyA containing RNA labelled with 5-^{3}H-uridine in the
presence of actinomycin D has been extracted from infected
Vero cells by the method of Glisin (11) modified as
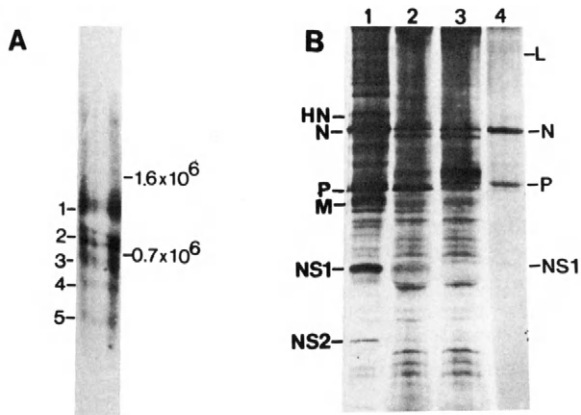
Figure 4A. Methylmercury agarose gel of ^{3}H-urd labelled polyA$^+$ RNA from mumps virus infected cells.
Figure 4B. SDS-PAGE (8-15%) of in vivo (1,4) and in vitro (2,3) synthesised proteins labelled with ^{35}S-met. Lanes 1, 2: BF-inf. cells; Lanes 3,4: Enders inf. cells.

described earlier (12). The length estimates of such RNA preparations have been made after methylmercuryhydroxide agarose gel electrophoresis by comparison to ribosomal RNA samples. Fig. 4 shows the banding pattern obtained from mumps virus infected cells. The length of the molecular species in bands 1 to 5 are approximately 3000, 2100, 1750, 1400 and 1000 nucleotides. The band of 2100 nucleotides length may actually consist of two species as it appears as a doublet in some gels. This material is the starting material for cloning experiments underway and it has also been used in in vitro translation experiments (Fig. 4B). The products directed by mRNA from infected cells in rabbit reticulocyte lysates are identifiable as the mumps N, P M and NS1 proteins. An extra band migrating ahead of the N protein may represent the unglycosylated form of the HN or FO protein. The identity of the P and N proteins has been demonstrated by limited proteolysis of the proteins synthesized in vivo and in vitro. The identity of the P and NS1 bands is further indicated by the mobility difference of these proteins in the BF and Enders strains which has also been observed in vitro.

STOECHIOMETRIC ANALYSIS

     In conclusion, it appears that the NS1 and NS2 proteins are related to P but not derived from it by limited proteolysis. Provided that all proteins are stable, as they were

shown to be in pulse-chase experiments, a scan of the ^{14}C
labelled proteins analysed by densitometry of fluorograms on
preflashed films will give an indication of the molar ratios
in which various proteins are synthesised in the infected
cell. When we use the rate of synthesis of the N protein
as reference (1.0) the rates of synthesis of the P, NS1 and
NS2 proteins in Enders infected Vero cells were 0.38, 0.38
and 0.27, respectively. In BF infected cells the figures
for P, NS1 and NS2 were 0.36, 0.65 and 0.19, respectively.
It is clear from these data that the NS1 and NS2 proteins are
major products in the infected cell and are very prevalent.
In the BF strain NS1 is made in greater copy numbers than P
and the observation that the NS proteins are not derived from
P by proteolysis and the fact that they appear to by synthe-
sized in vitro allows for interesting speculation about the
mechanisms that control the synthesis of different proteins
from possibly only one cytoplasmic mRNA.

ACKNOWLEDGEMENTS

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ANALYSIS OF THE ANTIGENIC STRUCTURE AND FUNCTION OF SENDAI VIRUS PROTEIN NP[1]

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TOPOGRAPHICAL ANALYSIS OF SENDAI VIRUS PROTEIN NP

Sendai virus contains a single-stranded RNA genome of negative polarity, which is coated by viral proteins NP, P and L to form the nucleocapsid structure (1). The functions of these proteins have yet to be assigned; however, by analogy to vesicular stomatitis virus (VSV) (2) and Newcastle disease virus (3,4), NP, the major protein species, appears to be a structural protein, while accessory proteins P and L appear to function together as the viral RNA polymerase.

The antigenic structure of NP was probed by using twelve anti-NP monoclonal antibodies in competitive-binding ELISA's (Table 1). Three antigenic sites were delineated. Sites II and III were nonoverlapping and competition was reciprocal with respect to antibodies delineating each site. Site III contains only one antibody. Antibodies to site I did not compete with antibodies to sites II and III, however, in a few instances site II and III antibodies competed nonreciprocally with antibodies to site I. These results suggest that either site I physically overlaps with sites II and III, or more likely, that the binding of antibodies to sites II or III allosterically inhibit the binding of antibodies to site I. The nonreciprocal competitions within site I may have resulted from either epitope modification after binding of the competing antibody, or from

TABLE 1

ANTIGENIC SITE DETERMINATIONS OF SENDAI VIRUS PROTEIN NP USING PROTEIN A-PURIFIED MONOCLONAL ANTIBODIES

Antigenic Site	Antibody Isotype	Competing Antibody	Competition Reaction with Peroxidase-labeled Antibody											
			M4	M6	M10	M13	M17	M18	M19	WS16	M5	M7	S11	M8
I	IgG[a]	M4	+[b]	+	+	-[c]	-	+	-	-	-	-	-	-
	IgG	M6	+	+	+	+	+	+	+	-	-	-	-	-
	IgG	M10	+	+	+	-	+	+	-	-	-	-	-	-
	IgG	M13	+	-	+	+	+	+	+	+	-	-	-	-
	IgM	M17	+	+	+	+	+	+	+	+	-	-	-	-
	IgM	M18	+	+	+	-	+	+	-	-	-	-	-	-
	IgG	M19	+	+	+	+	+	+	+	+	-	-	-	-
	IgG	WS16	+	+	+	+	+	+	+	+	-	-	-	-
II	IgG	M5	-	-	-	-	-	-	-	-	+	+	+	-
	IgG	M7	+	-	-	-	-	-	-	-	+	+	+	-
	IgG	S11	-	-	-	-	-	+	-	-	+	+	+	-
III	IgG	M8	+	-	-	-	+	+	-	-	-	-	-	+

Note: Labeled antibodies, used at concentrations which were 50-75% saturating, were incubated with duplicate serial dilutions of competitor in the competitive-binding ELISA (5). Hybridoma anti-NP antibodies were produced by fusing SP2/0-Ag14 plasmacytoma cells with splenocytes from BALB/c mice previously immunized with the Enders strain of Sendai virus disrupted in 1.5% n-Octyl-β-d-glycopyranoside.

[a]All the monoclonal antibodies were of the K light chain class.

[b]+, indicates ≥50% competition at a concentration which caused 100% competition for its homologous labeled antibody.

[c]-, indicates <50% competition.

the location of an epitope within a groove on NP.   Since
site I contains the largest number of epitopes, it is
probable that this part of NP is most accessable to antibody
binding, and it also may be the most antigenic.

EFFECTS OF ANTI-NP ANTIBODIES ON SENDAI VIRUS TRANSCRIPTION

    It is not understood how NP participates in Sendai
virus transcription, therefore, we tested the effect of the
anti-NP antibodies on in vitro transcription (Table 2).   The
following antibodies inhibited transcription by at least
50%: M4, M19, and WS16 (site I), S11 (site II) and M8 (site
III).
    In surveying the transcription inhibition caused by
each antibody, the extent of transcription inhibition did
not always correlate with antibody concentration and titer.
In general, low antibody concentrations and titers [M10,
M13, M17, 18 (site I) and M7 (site II)] caused negligible
(10-30%) transcription inhibition.   However, with antibody
concentrations of approximately 1 mg/ml [M6, M19 (site I),
and M5, S11 (site II)] transcription inhibition varied from
16-60%.   This shows that a high concentration of antibody
did not always inhibit transcription.   The antibodies appear
to have similar affinities for NP (from comparing plots of
antibody dilution versus ELISA activity, data not shown),
therefore it is likely that only specific epitopes on NP are
important in inhibiting transcription.   This concept is
supported by studies with monoclonal antibodies to influenza
virus protein NP (5) and VSV protein N (6).

KINETICS OF TRANSCRIPTION INHIBITION BY ANTI-NP ANTIBODIES

    To learn more about how the anti-NP antibodies affect
Sendai virus transcription, M4, M8 and S11 were examined for
time dependent effects on in vitro transcription (Fig. 3).
When the antibodies were added to transcribing nucleocapsids
(Fig. 3B and D), complete transcription inhibition occurred
in all cases.   This effect was similar to that observed with
antibodies to influenza virus protein NP (5) and VSV protein
N (7,8), and indicates that elongation of nascent RNA was
inhibited.   Ribonuclease contamination of the antibodies was
excluded as a possible cause for loss of transcriptase
activity since labeled product RNA was stable when incubated
with each of the three antibody preparations (data not
shown).
    In contrast, when nucleocapsids were incubated with the
antibodies before substrates were added (Fig. 3A and C), M4
and M8 caused almost complete transcription inhibition,

TABLE 2

SENDAI VIRUS TRANSCRIPTION BY PURIFIED NUCLEOCAPSIDS WITH ASCITIES

FLUIDS CONTAINING ANTI-NP MONOCLONAL ANTIBODIES[a]

Antigenic Site	Monoclonal Antibody	Antibody Titer[b]	Antibody Concentration in Ascites Fluid[c] (mg/ml)	Transcription Inhibition (%)[a]
I	M4	437,400	3.6	78
	M6	145,800	1.0	22
	M10	48,600	0.2	10
	M13	48,600	0.2	10
	M17	145,800	0.6	32
	M18	16,200	0.1	6
	M19	145,800	0.8	60
	WS16	437,400	5.2	58
II	M5	145,800	1.2	16
	M7	48,600	0.2	22
	S11	145,800	0.9	55
III	M8	1,312,200	7.5	75

[a]5 µl ascities fluid was reacted with 50 µg nucleocapsid in 50 µl at 30° for 5 min before addition of 50 µl substrates (9). Reaction mixtures were incubated for 2.5 hrs at 30°. Greater concentrations of ascites fluids were avoided because they caused nonspecific transcription inhibition. Similar results were obtained using protein A-purified antibodies (data not shown), indicating that any decrease in transcription was not due to nucleases in the ascites fluids.

[b]Antibody titers were determined by indirect ELISA (5).

[c]Monoclonal antibody concentrations were measured by ELISA (10).

while S11 inhibited transcription by only 50%. This result was repeated in three separate experiments and contrasts with that of Fig. 3D. Therefore, we suggest that the nucleocapsid structure undergoes a conformational change after exposure to substrates which increases susceptibility to inhibition by S11. Such a conformational change would indicate a role for changes in NP configuration in the control of Sendai virus gene expression.

Since M4 and M8 inhibited transcription to a similar extent, it is possible that the binding of each antibody affects the same site on NP. Since M8 competed nonreciprocally with M4 in the competitive-binding studies, it is possible that epitopes M4 and M8 are proximal to each other, however, due to the nonreciprocal nature of the competition we propose that M8 binding leads to transcription inhibition after causing a conformational change in the M4 epitope. Since epitope S11 (site II) was distinct from
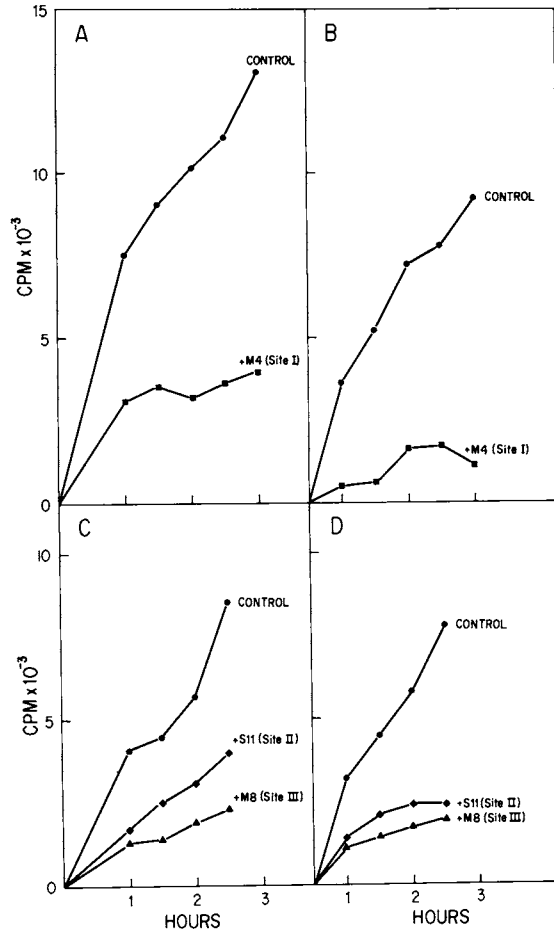
FIGURE 1. Kinetics of transcription inhibition by anti-NP antibodies in ascites fluid. (A) 18.0 µg M4 (■) were incubated with 50 µg nucleocapsids at 30° 5 min prior to the addition of substrates. (•) designates untreated control for all panels. (B) 18.0 µg M4 were added to reactions containing 50 µg nucleocapsids 25 min after transcription was initiated. ^{3}H-UTP (at 1/100 the concentration of unlabeled UTP) was added 5 min after antibody addition to measure RNA made only after antibody addition. (C) 21.0 µg M8 (▲) or 4.7 µg S11 (◆) were incubated with 30 µg nucleocapsids under the conditions of panel A. (D) 21.0 µg M8 or 4.7 µg S11 were incubated with 30 µg nucleocapsids under the conditions of Panel B. Incorporation of ^{3}H-UTP into TCA precipitable counts was determined from duplicate samples at transcription initiation and at the times indicated. Ascites fluid containing antibodies to influenza virus was used in each experiment as a negative control, and found to have no effect on transcription (data not shown).

epitopes M4 (site I) and M8 (site III) and correspondingly
inhibited transcription to a lesser extent, it is possible
that S11 affects transcription by a mechanism different from
that caused by the binding of M4 and M8.

     Possible mechanisms to explain how the anti-NP mono-
clonal antibodies inhibit transcription include: steric
hinderance of transcriptase phassage along the nucleocapsid
structure; distortion of the nucleocapsid structure by anti-
body interaction with the NP molecule; and hindrance of
possible NP movement required for exposing the RNA template
to the transcriptase.   Since all monoclonal antibodies
specific for NP did not affect transcription in the same
manner, we may infer that epitopes differ with respect to
their roles in nucleocapsid structure and/or function.

ACKNOWLEDGMENTS

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Note added to proof: We recently have found, through West-
ern blot analysis, that antibodies M5, M7, and S11 are
specific for nucleocapsid protein P, and not NP.

MONOCLONAL ANTIBODIES AS PROBES OF THE ANTIGENIC STRUCTURE AND FUNCTIONS OF SENDAI VIRUS GLYCOPROTEINS.[1]

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Paramyxoviruses initiate infection by the action of two envelope glycoproteins which cover the surface of the virion. The hemagglutinin-neuraminidase (HN), the larger of the two, has cell-binding, hemagglutinating and neuraminidase activities (1), while the other virus glycoprotein F is involved in virus penetration and has hemolysis and cell fusion activities (2). Antibodies to HN and F inhibit the biological activities associated with these molecules and appear to play an important role in preventing and limiting virus infection (3,4). Recent studies have defined the action of antibodies on HN (5-8) and F (8) more precisely by showing that hybridoma monoclonal antibodies differ in their ability to suppress viral functions. In the present study, we have used the interaction of monoclonal antibodies specific for HN and F of Sendai virus to investigate the role of individual epitopes in the expression of biological activity.

FUNCTIONAL ANALYSIS OF HN WITH MONOCLONAL ANTIBODIES; BIOLOGICAL ACTIVITIES OF HN MAP AT DIFFERENT EPITOPES.

We analyzed the antigenic structure of HN by competitive binding assays (ELISA) in which purified mouse hybridoma monoclonal antibodies, labeled with peroxidase, were competed with unlabeled antibodies for virus immobilized on polystyrene wells. Using ten anti-HN monoclonal antibodies, the competition studies delineated four non-overlapping antigenic sites on the HN molecule (data not shown). Antibodies used in our analysis bound to their respective epitopes in enzyme immunoassays (EIA) with similar titers

($\approx 10^{-5}$) and binding curves, suggesting that antibody concen-
trations and functional affinities of different monoclonal
antibody preparations were about the same.

To relate the biological activities of HN to the anti-
genic structure, and thus, physically distinct regions on
the HN molecule, anti-HN antibodies were analyzed for their
reactivity in neuraminidase (NI), hemagglutination (HI) and
hemolysis inhibition (HLI) tests (Table 1). In these tests,
antibodies binding to topologically distinct sites exhibited
unique patterns of reactivity. Thus, antibodies binding to
HN antigenic site I inhibited hemagglutination and neura-
minidase activities, whereas antibodies to site II inhibited
neither activity. Antigenic sites III and IV had different
roles in hemagglutinating and neuraminidase activities:
antibodies recognizing site III inhibited hemagglutination
but were devoid of NI activity, while antibodies reacting
with antigenic site IV displayed the reverse pattern,
inhibiting neuraminidase activity but not hemagglutination.
The observed differences between the effects of the diffe-
rent antibodies clearly depends on the region of the HN
molecule to which the antibody binds.

Although hemolysis activity (a measure of fusion) is an
F glycoprotein function, anti-HN antibodies may indirectly
inhibit hemolysis by inhibiting virus adsorption which is a
prerequisite for hemolysis. Recent reports (7,8), however,
indicate that anti-HN antibodies may inhibit hemolysis by
other mechanisms. Therefore, we tested anti-HN antibodies
to antigenic sites II and IV which lack HI activity to
determine whether hemolysis inhibition occurs with antibody-
treated virions not blocked in absorption to erythrocytes.
Hemolysis inhibition was observed by anti-HN antibodies
reacting with antigenic site II but not with site IV. This
indicates that antibodies binding specifically to HN site II
can interfere with F-mediated fusion, and thus prevent virus
penetration. As expected, antibodies to antigenic sites I
and III inhibit adsorption and therefore inhibit hemolysis.

To determine whether the antigenic structure of HN was
different on the surfaces of infected cells and virions, we
tested the binding of anti-HN antibody by EIA and immuno-
fluorescence (IF). In both cases, anti-HN binding was iden-
tical, indicating that the antigenic structure of HN was
unaltered by the budding process (Table 1). However, as we
will show, this is not the case with the F protein.

ANALYSIS OF F GLYCOPROTEIN FUNCTION AND STRUCTURE WITH
MONOCLONAL ANTIBODIES.

Competitive binding assays delineated three antigenic
sites on the F molecule. Sites I and II were non-over-

TABLE 1.

REACTIVITY OF ANTI-HN MONOCLONAL ANTIBODIES WITH SENDAI VIRIONS
AND INFECTED CELLS[a]

| Antigenic | Virions | | | | Infected Cells | | |
| | | | | | | Fluorescence | |
Site	EIA[e]	HI[b]	NI[c]	HLI[d]	EIA[e]	Surface	Cyto
I	+	+	+	+	+	+	+
II	+	-	-	+	+	+	+
III	+	+	-	+	+	+	+
IV	+	-	+	-	+	+	+

[a]Assays were performed using a 1/100 dilution of hybridoma ascites fluid for the HI and NI and 1/200 for HLI.

[b]+, antibody completely blocked hemagglutination; -, antibody had no effect on hemagglutination.

[c]+, antibody inhibited viral neuraminidase activity by >75% (O.D. 549 nm); -, neuraminidase activity was the same as samples lacking antibody.

[d]+, complete inhibition of hemolysis activity; -, hemolysis activity was the same as virus samples without antibody.

[e]+, virions EIA titer $>10^5$; +, infected cells O.D. 3-fold above cells without antibody; -, the same as cells not treated with antibody.

TABLE 2.

ACTIVITY OF F MONOCLONAL ANTIBODIES WITH VIRIONS AND INFECTED CELLS

| Antigenic | Antibody | Virions | | | | Infected Cells | | |
| | | | | | | Fluorescence | |
Site	Clone	EIA	HI	NI	HLI	EIA	Surface	Cyto
I	16	+	-	-	+	+	+	+
	28	+	-	-	-	-	-	+
	31	+	-	-	-	-	-	+
II	33	+	-	-	-	-	-	+
III	38	+	-	-	-	-	-	+

Antibody dilutions and +, - designations the same as Table 3.

lapping whereas site III was blocked non-reciprocally by site I antibodies (data not shown). As expected, hemagglutination and neuraminidase activities were not blocked by anti-F antibodies, since these functions are associated with HN (Table 2). To determine whether specific epitopes on the F molecule are involved in fusion activity, anti-F anti-

bodies were incubated with Sendai virions and tested for inhibition of hemolysis. Only a single antibody, M16, binding to antigenic site I, inhibited hemolysis. This is a surprising result, since two other anti-F antibodies mapping at the same antigenic site failed to inhibit hemolysis. The results indicate that the M16 epitope may be directly involved in hemolysis activity or the binding of antibody M16 induces a conformational change in F that alters the hemolysis active site. The results also suggest that the hemolysis active site, and thus, the fusion site, may be restricted to a distinct region on an F molecule.

To compare the antigenic structure of F in infected cells and virions, we measured the binding of anti-F antibodies to virions by EIA and to infected cells by immunofluorescence and EIA (Table 2). A striking result was that only M16 binding was detected on the surface of infected cells, whereas all of our anti-F antibodies bound to whole or disrupted virions. The results suggest that some F epitopes were sequestered on the surface of the infected cell and became uncovered by the budding process.

THE RELATIONSHIP BETWEEN ANTIBODY INHIBITORY ACTIVITY AND NEUTRALIZATION OF INFECTIVITY.

We have found that monoclonal antibodies of defined epitope specificity selectively inhibit HN and F functions. To determine the relationship between these properties and the ability of anti-HN and F antibodies to inhibit virus growth, we measured virus yields (Table 3). Virus was mixed with antibodies before adsorption or added to culture fluids after virus penetration and HA titers measured at 48 h after infection. Anti-HN antibodies to antigenic sites I, II and III inhibited virus production whether added before or after virus penetration. Since antibodies to sites I and III inhibited hemagglutination, it is likely that they block virus adsorption. Antibodies to HN site II failed to inhibit hemagglutination and neuraminidase, but inhibited hemolysis, and thus, may prevent virus penetration. An interesting finding was that anti-HN antibodies to site IV did not inhibit virus adsorption but did inhibit virus production after the penetration step. The fact that neuraminidase was the only other activity inhibited by these antibodies suggests that the viral enzyme is involved in virus release.

Of five anti-F antibodies to three different antigenic sites, the only antibody that inhibited hemolysis, M16, was also the only antibody that inhibited virus growth. The results are consistent with the known function of F in virus penetration (4) and supports the concept that fusion

TABLE 3

THE EFFECT OF ANTI-HN AND F MONOCLONAL ANTIBODIES ON THE GROWTH OF
SENDAI VIRUS

| Antibody designation | Group | Virus Yield (HA/ml) When Antibody Added[a] | | Protein specified by antibody |
		Before Adsorption[b]	After Penetration[c]	
S2	I	<3	<3	HN
16		<3	<3	HN
M2	II	<3	<3	HN
11		<3	2-3	HN
M9	III	<3	<3	HN
M12	IV	41	<3	HN
14		41	<3	HN
15		41	<3	HN
20		41	<3	HN
21		41	<3	HN
M16	I	<3	≈5	F
28		54	41	F
31		41	41	F
M33	II	54	41	F
M38	III	54	27	F
M1		41	41	M
MAF		41	41	

[a]HA titers at 48 h after infection.

[b]Before adsorption to CEL cell virus inoculum (0.5 HA in 0.1 ml)
was incubated for 30 min with 10 μl of undiluted antibody in ascitic
fluid.

[c]CEL cells were infected for 4 h to allow time for virus
adsorption and penetration. Cells were then washed 3 x with PBS, and
3.0 ml of GM added containing 30 μl of undiluted ascitic fluid.

activity may be limited to a distinct region on the F
molecule (9).

These studies indicate that anti-HN and F monoclonal
antibodies bind to distinct regions on the HN and F
proteins, and this binding specificity results in selective
inhibition of different HN and F functions. It is
noteworthy that anti-HN antibodies, besides blocking virus

absorption, may interfere with infectivity by blocking virus fusion or inhibit virus release by blocking neuraminidase activity. The inhibition of fusion may be a steric phenomenon in which antibodies to HN site II interfere with the function F. Another possibility is that HN undergoes a conformational change before F can be expressed and antibodies to HN site II prevent this change. The biological importance of HN neuraminidase activity is unknown. These studies indicate that it is required for virus release. With regard to the F protein, hemolysis and infectivity are inhibited by anti-F antibodies that bind to a distinct epitope. This may be the fusion active site. An interesting finding was that the antigenic structure of F is apparently different on the surface of infected cells and virions. This result implies that the F protein undergoes a conformational change during or after virus release.

ACKNOWLEDGEMENTS

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VARIATIONS IN ANTIGENIC DETERMINANTS OF DIFFERENT
STRAINS OF MEASLES VIRUS

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Measles is a serologically stable monotypic virus. Poly-
valent sera do not distinguish surface antigens nor internal
structural proteins of different measles virus strains in
acute nor in persistent infections. We have recently pre-
pared monoclonal antibodies against five of the six virus-
coded structural proteins of measles, the exception being the
L protein (1, 2) and used them to characterize nine strains
of measles virus giving lytic infections in vitro (3). In
order to obtain a broader view of the degree of antigenic
variation of the individual viral components, these monoclo-
nal antibodies were used to epitopically characterize five
persistent measles virus infections and additional SSPE-de-
rived lytic strains not included in the previous study (3).
    All the hybridomas with the exception of seven were
raised against the LEC strain (referred to as LEC-KI) which
has received numerous passages in our laboratory for over 10
years. The remaining seven (3 anti-hemagglutinin and 4 anti-P
protein) although raised against the Edmonston (EDM) strain
cross-reacted strongly with the LEC-KI strain. The per-
sistently infected cell lines studied were: HEpPi (4) and
Lu106 carrier (5), established in HEp-2 and Lu 106 cells
respectively with the EDM strain; MaSSPE (6) and MaPi,
established in different laboratories with the Mantooth (MAN)
strain; and HNT (7), established in Vero cells with a
hampster neurotropic variant of Philadelphia 26 strain.HNT is
nonproductive; the remaining four being semi-productive i.e.
produce moderate to low levels of plaquable virus. The lytic
measles virus strains: Woodfolk (WDF), MVO, MVP, Hu2,
Schwartz (SCH), Moraten (MOR), EDM-dilute passaged (EDM/DP)
and MAN; have been described previously (3). The five addi-

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tional SSPE-derived lytic strains used in studies on the
matrix protein were: Dean (DN), Zister (ZIS), Halle (HAL),
McClellan (MAC) and passage 5 of the original LEC isolate
(LEC-WIS) (8).

    The SSPE lytic strains were all examined in their
5-10th passage after isolation. Conditions of radioimmune
precipitation (RIPA), immunofluorescence (IF) and other
tests were as described previously (1-3).

VARIATION IN EPITOPES ON THE SURFACE PROTEINS

The nine lytic strains studied previously (3) had shown only
limited variations in their hemagglutinin (H) and no varia-
tions in their fusion (F) proteins. Limitation of variation
within these proteins probably arises from functional
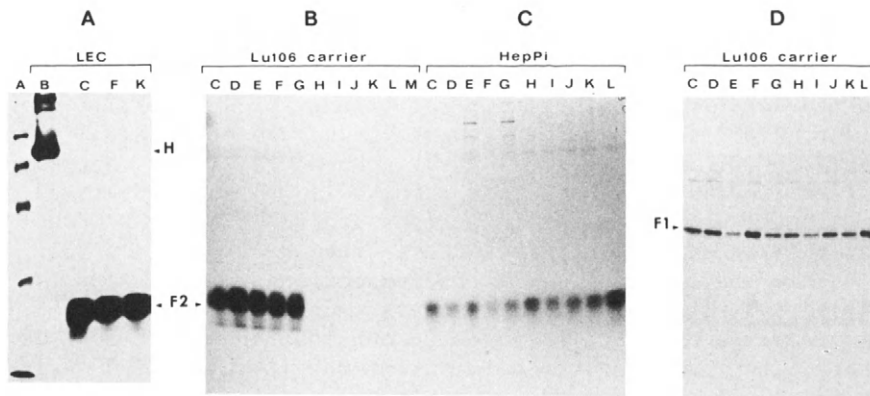
    FIGURE 1. Analysis of F protein from LEC virus infected
cells (A), Lu106 carrier (B and D) and HEpPi (C) persistent
infections by RIPA. Infected cells were labelled either with
[35]S-methionine (D) or with a mixture of 1,6-^{3}H-glucosamine
hydrochloride and 2-^{3}H-mannose (A, B and C). Immunoprecipi-
tates were analyzed on 12.5% polyacrylamide gels. Anti-F
monoclonal antibodies used were: lane C, 9-DB10; 16-AE7;
E, 16-AG5; F, 16-DC9; G, 16-EE8; H, 19-BG4; I, 19-FF4; J,
19-FF10; K, 19-GD6; L, 19-HHB4; and M, 19-HC4. An anti-H
monoclonal antibody, I-44 (lane B) and mol.wt. marker pro-
teins (lane A) are also included. The concomitantly immuno-
precipitated F1 is not seen in A, B and C as it is non-gly-
cosylated; similarly F2 is to weakly labelled by [35]S-
methionine to be seen in D.

TABLE 1
REACTIVITY OF TEN ANTI-H MONOCLONAL ANTIBODIES WITH DIFFERENT MEASLES VIRUS STRAINS IN RIPA AND IF[a]

Hybridoma Clone No.	Lytic				Persistent	
	LEC-KI	EDM/DP	WDF, MAN	SCH, MOR, MVO, MVP, Hu2	MaSSPE, MaPi, HEpPi, Lu106 carrier	HNT
7-AG11	+	-	-	-	-	-
16-CD11	+	+[w]	+	+	+	+
16-DG3	+	+	-[b]	+	-[b]	-
Others[c]	+	+	+	+	+	d

[a]Grading of reactivity: +, distinct; +[w], weak; and -, none

[b]+ in IF

[c]Hybridoma no.: I-12, I-29, I-41, I-44, 80III B2-D, 79V V17-D and 79XI C2-D

[d]+ with all except 80III B2-D and 79V V17-D in both RIPA and IF (9)

constraints. Accumulation of mutations in the surface anti-
gens in persistent infections however, could occur due to
reduction of selective pressure to keep the protein functio-
nal in such systems. This appears to hold true in some
instances such as: the H protein of HNT (Table 1; pattern of
reactivity of the lytic strains (3)are included for compa-
rison), the most defective of the five persistent infections
studied; and the F protein of Lul06 carrier. In the latter
case, two forms of the F protein were detected: a weakly
glycosylated form, which reacted with all 11 anti-F monoclo-
nal antibodies; and a strongly glycosylated form, which
reacted with 5 of the 11 anti-F monoclonal antibodies
(Figure 1). The differential reactivity of the two forms of
the Lul06 carrier F protein with the anti-F monoclonal anti-
bodies was confirmed by sequential RIPA wherein an antigen
batch was repeatedly immunoprecipitated with one anti-F
antibody until no further reactivity was observed and the
resulting depleted antigen examined with another of the
anti-F antibodies (data not shown). Such distinctly different
forms of F were not detected in MaSSPE, MaPi and HEpPi
(radioactive sugar labelled HNT F protein could not be
studied).

VARIATION IN EPITOPES ON THE INTERNAL PROTEINS

    Nucleocapsid (NP) and P Proteins. By analogy with other
negative strand viruses, the L, NP and P proteins of measles

TABLE 2
REACTIVITY OF MEASLES VIRUS PERSISTENTLY INFECTED CELL LINES
WITH ANTI-NP MONOCLONAL ANTIBODIES IN RIPA AND IF[a]

	Persistent Infection		
Hybridoma	MaPi	HNT	MaSSPE, HEpPi, Lul06 carrier
16–AC5	+	+	+
16–BB8	–[b]	–	+
16–BF2	+	+	+
16–CF7	+	–	+
16–DC3	–[b]	+	+
16–EE9	+	+	+

    [a] Grading of reactivity: +, distinct; +[w], weak; and
–, none
    [b] + in IF

virus are thought to compose the transcriptase/replicase
complex. Consequently alterations within them would be ex-
pected to be limited in lytic systems. However, mutations
within these proteins may be of importance in evolution of
persistence. Although the biological significance of the
variations detected are unknown, it is of interest that in
contrast to the nine lytic strains previously examined
wherein no major variations were detected by the six anti-NP
and six anti-P monoclonal antibodies, epitopic variations
were found in two of the persistent infections. By RIPA and
IF it was shown that the anti-P monoclonal antibodies de-
tected two major variations in the HNT P protein; while the
anti-NP monoclonal antibodies detected variations in the
NP of HNT and of MaPi (Table 2).

    Matrix (M) Protein. It has previously been shown that in
contrast to the surface and the internal NP and P proteins,
extensive epitopic variations are found in the M protein of
measles strains giving lytic infections in vitro (3) (RIPA
data included for comparison in Table 3). The persistent
infections also showed variations in M epitopes (Table 3).
Also shown in Table 3 are the M epitopic patterns of five
additional SSPE-derived strains (the previous study examined
only the MAN SSPE strain). Features of interest which emerged
from this study include : 1) There was a lack of consensus of
M epitopic patterns between the persistent infections.
2) A high consensus existed in the lytic SSPE M epitopic
patterns as 5 out of 6 strains had the same pattern. However the
fresh isolates MVO and MVP also had a similar pattern of
reactivity. 3) Lytic SSPE LEC-WIS differed markedly from
its derivative LEC-KI. The two strains also differed in their
H epitopes (data not shown). As the passage history of
LEC-KI is unclear it is not possible to assess whether the
variations arose due to extensive lab-adaptation or to conta-
mination with another strain. 4) MaSSPE and MaPi persistent
infections showed the same M epitopic patterns as lytic SSPE
MAN strain, the strain from which they were derived; indica-
ting stability in M epitopic patterns. Indications of stabi-
lity were also found in another study where the M epitopic
patterns of LEC-KI, EDM/DP and Hu2 were seen to remain
unchanged after 30 consecutive dilute passages in vitro.
Furthermore lytic SSPE MAN strains from three different la-
boratories (two in US and one in Germany) have all been found
to have the same M epitopic pattern.

    The results of this study indicate that in contrast to
lytic systems, the H, F, NP and P proteins of measles virus
accumulate mutations in persistent infections. Furthermore
the apparent conservation of M epitopic patterns in different

TABLE 3

REACTIVITY OF DIFFERENT MEASLES VIRUS STRAINS WITH ANTI-M MONOCLONAL ANTIBODIES IN RIPA[a]

Hybridoma		Vaccine		Lab	Fresh Isolates			SSPE		Persistent		
Group No.	Clone No.	LEC-KI	SCH, MOR	EDM/DP	WDF	Hu2	MVO, MVP	LEC-WIS	DN, ZIS, HAL, MAN, MAC	MaSSPE, MaPi	Lu106 carrier, HEpPi	HNT[b]
1	10-EF10	+	+	+	+	+	+	+	+	+	+	−
2	16-BB2	+	+	+	+	+	+	+	+	+	+	−
3	19-AG10	+	+	+	+	+	+	−	+	+	+	+
4	19-CG6	+	+	−	+	+	w	+	w	w	+	w
	19-GF6						+		+	+		+
5	19DC5	+	+	−	+	+	−	+	−	−	+	+
6	19-DF10	+	+	+	+	−	+	−	+	+	+	+
	19-HC5											
	19-HF6											

[a] Grading of reactivity: +, distinct; w, and −, none

[b] Data are preliminary due to difficulties in reproducibly radiolabelling HNT M protein

systems indicates that with the aid of a larger bank of mono-
clonal antibodies the M epitopic patterns could be used as a
marker for particular measles virus strains. Whether a cor-
relation with particular biological properties of groups of
strains will also emerge remains unknown.

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POSITIVE IDENTIFICATION AND MOLECULAR CLONING OF THE
PHOSPHOPROTEIN (P) OF MEASLES VIRUS

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IDENTIFICATION OF A P GENE cDNA CLONE

Measles virus, a clinically relevant infectious agent of
man, contains approximately 16 Kb of genetic information
within a single strand, negative polarity, RNA genome. The
virus readily gives rise to persistent infections and is
believed to be the causative agent for the progressive neuro-
logical disease, subacute sclerosing panencephalitis (1).
Because of a longstanding interest in the molecular
mechanisms of persistence, we have undertaken the molecular
cloning of measles virus.

Rozenblatt et al. (2) have identified several cDNA
clones of measles virus derived from oligo dT primed mRNA.
One clone, C1-G, appeared by Northern blot analysis to
hybridize to a single species of mRNA, but upon in vitro
translation, this selected message resulted in the synthesis
of two polypeptides. One of these proteins was immuno-
precipitated by an anti-HA (hemagglutinin) monoclonal
antibody. Thus, the clone was assigned on this basis as an
HA clone.

Clone C1-G was obtained and was sequenced by the method
of Maxam and Gilbert (3). The complete sequence of C1-G
indicated that this clone contained 400 nucleotides of
measles specific information and terminated in a
polyadenylated region followed by GC tails. Thus, it
appeared that C1-G represented the precise 3' end of the mRNA
that was reverse transcribed during the cloning event. The
deduced protein sequence in all three phases indicated only a

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NIH, Bethesda, Maryland 20205.
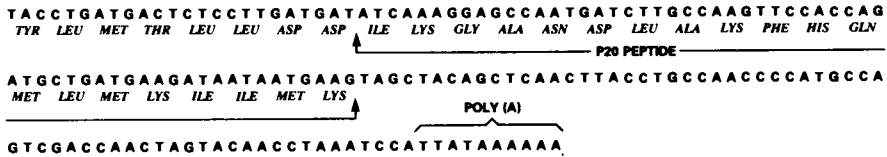
FIGURE 1. Nucleotide and peptide sequence of Cl-G used for synthetic peptide synthesis.

single open reading frame. Upon analysis, this protein sequence did not appear to contain a hydrophobic region that was expected to be present near the carboxy terminus of transmembrane glycoproteins. To positively identify the gene product of Cl-G, a 20 amino acid peptide was synthesized from the sequence deduced for the carboxy terminal region (Figure 1). The peptide (P-20) was purified by HPLC, linked to keyhole limpet hemocyanin and used as an immunogen in rabbits. Rabbit antisera were purified by affinity chromatography using the P-20 peptide coupled to a solid support resin. The affinity purified anti-P-20 was compared with two anti-measles sera and several monoclonal antibodies of determined specificity in immunoprecipitation assays. Cell lysates of measles infected Vero cells were prepared as antigen following radioactive labeling with ^{35}S-methionine (4). Analysis of the immune precipitates by SDS-polyacrylamide gel electrophoresis indicated that the affinity purified P-20 antibody precipitated a single polypeptide that comigrated with the protein precipitated by the monoclonal anti-P of measles virus (Figure 2). The P protein was also precipitated by both polyvalent anti-measles sera and was clearly distinguishable from the two forms of the hemagglutinin (HA) precipitated by these sera as well as the monoclonal anti-HA.

To determine whether the protein recognized by the P-20 antibody was a phosphoprotein, immunoprecipitation of phosphorous-32 labeled measles infected cell lysates was performed. Polyacrylamide gel analysis of the immuno-precipitates of anti-P-20 serum, affinity purified P-20 antibody and monoclonal anti-P resulted in the precipitation of the identical ^{32}P-labeled protein (Figure 2). In addition, immunofluorescent studies with anti-P-20 indicated that the antibody reacted with an internal antigen of measles infected cells. The antibody stained nucleocapsid inclusions and colocalized with the antigen recognized by anti-P monoclonal antibody.
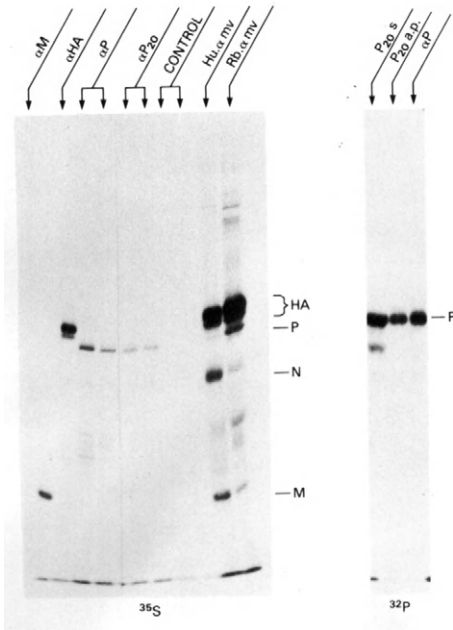
FIGURE 2. Autoradiograph of SDS-PAGE analysis of immunoprecipitated proteins from either ^{35}S-methionine labeled measles infected cell lysates (left panel) or ^{32}P-labeled measles infected cell lysates (right panel). Antisera include α M (anti-matrix protein), α HA (anti-hemagglutinin), α P (anti-phosphoprotein), α P$_{20}$ (anti-synthetic peptide, affinity purified), control (antibody not bound to affinity column from P$_{20}$ antiserum), Hu α mv (human antibody to measles virus following atypical measles), Rb α mv (rabbit anti-measles virus), P$_{20}$ s (anti-synthetic peptide serum) and P$_{20}$ a.p. (anti-synthetic peptide, affinity purified).

    Clone Cl-G and a related clone, 3A-8, identified by probing the measles cDNA library derived from 50s genomic RNA with Cl-G, were used in hybrid arrest studies (Figure 3). Poly-A selected mRNA from measles infected Vero cells was translated in vitro using rabbit reticulocyte lysates. The assignment of the P and N (nucleocapsid) proteins was based on immunoprecipitation studies (data not shown). Increasing DNA concentrations of either Cl-G or 3A-8 resulted in essentially total arrest of the translation of the P protein compared with the mock arrested mRNA preparations. The immunoprecipitation data coupled with that of hybrid arrest and immunofluorescent localization firmly establish that the nucleotide sequence contained within Cl-G is measles specific and represents the coding region for the carboxy terminus of the P protein.
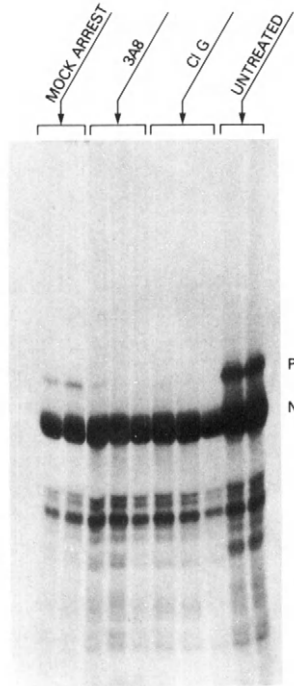
FIGURE 3. Autoradiograph of SDS-PAGE analysis of hybrid arrest and translation. Untreated, poly A selected mRNA (3 µg) was translated <u>in</u> <u>vitro</u> and the resultant peptides immunoprecipitated with rabbit anti-measles serum, mock arrest, same as untreated except taken through arrest conditions (5) with no competing DNA, 3A-8, poly A selected mRNA arrested with 1, 2 and 10 µg of 3A-8 DNA, C1-G poly A selected mRNA arrested with 5, 10, and 20 µg of C1-G DNA.

CLONING AND CHARACTERIZATION OF THE P GENE

Clones C1-G and 3A-8 were employed as probes to select a number of related clones from the 50s genomic cDNA library. Subsequent probing studies of the library have resulted in a number of cDNA clones which have been sequenced, ordered and encompass the entire P gene (Figure 4). The entire sequence of the P gene will be published elsewhere. The P cistron is comprised of 1653 nucleotides with a coding region of 506 amino acids, if the first methionine is used. Suggestive of a phosphoprotein the protein contains 15% serine plus

threonine residues. This value is in good agreement with that
of the P protein of Sendai virus (Kolakofsky, personal
communication).

Clone 9D-8 was found to contain the 3' end of the P gene
as well as to hybridize to the nucleocapsid clone, C1-15 (6).
Clone 3A-8 contains the 5' end of the P cistron and extends
approximately 350 nucleotides into the adjacent gene.
Preliminary evidence from immunoprecipitation studies with
anti-peptide serum to a region of this adjacent gene
indicates that it is the M cistron (data not shown).
Nucleotide sequence determination indicated an exact homology
for 17 nucleotides in these boundary regions with remarkable
similarity with that known for Sendai virus (7, 8,
Kolakofsky, personal communication) (Table 1). By comparison
with Sendai, the polyadenylation region for measles appears
to be ...AAUAUUUUUUU... and the tri-nucleotide ...GAA..., the
intercistronic region. Thus, it would appear that these
boundary regions are highly conserved for these
paramyxoviruses.
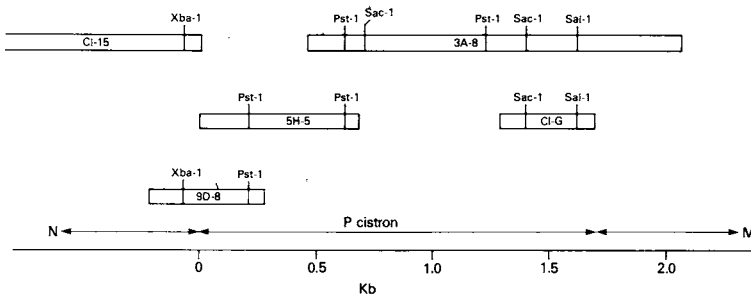
FIGURE 4. Schematic representation of cDNA clones
covering the entirety of the P cistron.

TABLE 1

POLYADENYLATION AND INTERCISTRONIC BOUNDARIES
OF TWO PARAMYXOVIRUSES

Virus	Sequence
Measles	3' AAUAUUUUUU GAA UCCU..... 5'
Sendai	3' AUUCUUUUU GAA UCCC..... 5'

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IDENTIFICATION OF A NEW ENVELOPE-ASSOCIATED PROTEIN[1]
OF HUMAN RESPIRATORY SYNCYTIAL VIRUS

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Human respiratory syncytial (RS) virus is classified in the genus pneumovirus of the paramyxovirus family. The genome of RS virus is a single negative strand RNA of at least 5.6×10^6 daltons. Ten RS viral mRNAs and their encoded proteins were identified recently (1,2,3, Collins et al, this volume). Here we demonstrate that four unique viral proteins are associated with the envelope of purified RS virions as compared to three for other paramyxoviruses.

The 24 K protein is a virion structural protein. RS virus grown in the presence of (^{3}H) leucine, was purified from the medium of virus-infected HEp-2 cells by two cycles of sedimentation through sucrose gradients. The virion proteins were analyzed by SDS-PAGE. Eight polypeptides of 200 (L), 84 (G), 68 (F_0), 47 (F_1), 42 (N), 34 (P), 26 (M) and 24 kilodaltons (K) were resolved (Fig. 1A). The 42, 34 and 26 K proteins have been identified previously as the major nucleocapsid protein, nucleocapsid phosphoprotein and matrix protein, respectively (1,4,5). The 200 K protein is a component of purified nucleocapsids (4,8 this paper) and is designated the L protein by analogy to other paramyxoviruses. The 84 K species is an envelope glycoprotein (4,5,6,7,8) and is designated G. The 68 K species has been identified as the F_0 glycoprotein (9, 10 and our unpublished data), which by analogy with other paramyxoviruses is cleaved proteolytically to generate the disulfide-linked F_1 (47 K) and F_2 (20 K) subunits of the F glycoprotein. The F_2 subunit is not visible in Fig. 1A and B, but was readily detected by coelectrophoresis of (^{3}H) glucosamine-labeled virions (data not shown). Purified virions also contained a 24 K protein (Fig. 1A); a virion protein of similar electrophoretic mobility was reported previously (4,6, 7,11) but its significance was unknown.

Comparison of (^3H) leucine-labeled and (^3H) glucosamine-labeled virions by SDS–PAGE under reducing and non–reducing conditions (data not shown) showed that the 24 K protein is unglycosylated and is distinct from the 20 K (F_2) subunit of the F glycoprotein. In addition, the 24 K protein was detected in extracts of RS virus–infected cells (Fig. 1B, lane C) and among the in vitro translation products synthesized in a wheat germ extract translation system programmed with poly (A)$^+$ mRNA from RS virus–infected cells (Fig. 1B, lane b). Peptide mapping confirmed that the 24 K proteins from virions, infected cell extracts, and the in vitro translation system were the same (Fig. 1 C & D).
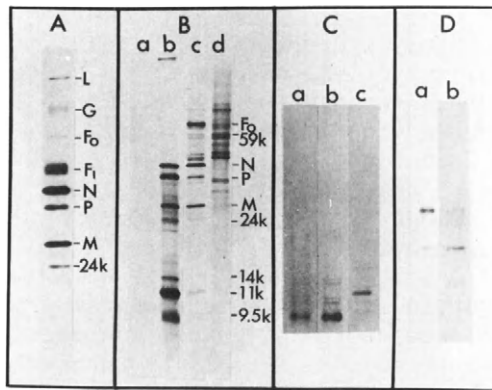
Fig. 1. Identification of the 24 K proteins. A. Proteins of purified RS virions labeled with (^3H) leucine were analyzed by 15% SDS–PAGE. B. Wheat germ in vitro translation system programmed with poly (A)–selected mRNA from (a) RS virus–uninfected or (b) infected, actinomycin D treated HEp–2 cells. (^3H) leucine–labeled products were analyzed by 15% SDS–PAGE in parallel with (^3H) labeled proteins extracted from (c) virus–infected and (d) uninfected act D–treated cells. C. Limited digest peptide mapping of the (^{35}S) cysteine–labeled 24 K protein. The 24 K protein (a) synthesized in vitro and (b) from infected cells, and (c) a comigrating cellular polypeptide were exposed to 5 ug/ml of V8 protease and analyzed by 16% SDS–PAGE. D. Limited digest peptide mapping of the (^3H) leucine–labeled 24 K protein. The 24 K proteins (a) extracted from infected cells and (b) from purified virions were digested with 5 ug/ml of V8 protease and analyzed by 15% SDS–PAGE.

The 24 K protein is unrelated to the other viral proteins. The 24 K, N, P, and M proteins were compared by tryptic pep-tide mapping. The profile of (^3H) leucine–labeled tryptic peptides separated by HPLC was different for each protein,

showing that these proteins were unrelated and that the 24 K
protein was not a fragment of one of these larger proteins,
(data not shown). In support of this conclusion, in vitro
translation of individual viral mRNAs purified by hybrid-
selection with cDNA clones demonstrated that the 24 K protein
was encoded by a unique mRNA (Collins et al, this volume).

Location of the 24 K protein in the virion. A Triton
detergent and salt dissociation method was used to distinguish
between proteins associated with the nucleocapsid and the en-
velope. (^{3}H) leucine-labeled, partially purified virus was
prepared in 4 aliquots and pelleted. The samples were treated,
processed and analyzed as described in the figure legend. As
shown in Fig. 2A, 2% Triton X-100 in 0.15M NaCl completely
solubilized the G & F proteins. Triton X-100 in 0.4 M NaCl
dissolved both glycoproteins as well as the majority of the M
and 24 K proteins. The N, P and L proteins remained associ-
ated with the insoluble (nucleocapsid) fraction.
    As a second approach to determine the virion location of
the 24 K protein, nucleocapsids were purified from infected
cell cytoplasmic extracts by gel filtration chromatography.
The extracts were adjusted to 1% Triton and immediately loaded
onto a Biogel A 15 M column (Biorad). The nucleocapsids were
found to elute with the void volume and were pelleted, dis-
solved directly in polyacrylamide gel sample buffer and analy-
zed by SDS-PAGE (Fig. 2B). These results showed that three
viral proteins, the N, P, and L proteins, were detected in
purified nucleocapsids.
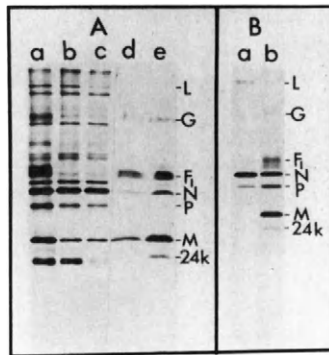
Fig. 2. Location of the 24 K protein in the virion. A. Ali-
quots of partially purified (^{3}H) leucine-labeled virions were
exposed to (b,d) 0.15 M NaCl containing 2% Triton and (c,e)
0.4 M NaCl containing 2% Triton. The (b,c) insoluble and (d,
e) soluble fraction were separated by differential centri-
fugation and analyzed in parallel with (a) untreated virions

by 15% SDS-PAGE. B. Comparison by SDS-PAGE of (a) (^{3}H)
leucine-labeled intracellular RS viral nucleocapsids isolated
from infected cell lysates by Biorad-Biogel A 15M column
chromatography and (b) (^{3}H) leucine-labeled purified RS virions.

These experiments defined three classes of virion proteins:
(i) the N, P, and L proteins which were stably associated with
nucleocapsids, (ii) the F and G glycoproteins which were
envelope-associated and quantitatively solubilized with Triton,
and (iii) the M and 24 K proteins which were almost completely
solubilized with Triton and 0.4 M NaCl and, like the two
glycoproteins, were not detected in column-purified nucleo-
capsids. Previously, a virion protein that probably corresponds
to the 24 K protein was suggested to be nucleocapsid associ-
ated (4). However, the results shown here indicate that, like
the other viral envelope proteins, the 24 K protein was solu-
bilized with 2% Triton and 0.4 M NaCl.

SUMMARY

     A 24 K protein, unrelated to the other viral proteins,
was identified in purified virions, extracts of infected cells,
and among viral polypeptides synthesized in vitro. Two methods
determined that the 24 K protein was envelope-associated.

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CHARACTERIZATION OF THE GLYCOPROTEINS OF RESPIRATORY SYNCYTIAL VIRUS

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Human respiratory syncytial (RS) virus is the most important etiologic agent of lower respiratory illness in infants (1). RS virus has been tentatively placed in the genus Pneumovirus of the paramyxovirus family (2). However, RS virus differs from the paramyxoviruses in several important respects as it has no hemagglutinating or neuraminidase activity (3) and its nucleocapsid is smaller in diameter than that of paramyxoviruses (4). Surface glycoproteins of enveloped viruses are intimately involved in the adsorption and penetration steps of infection (5). Relatively little is known about the surface proteins of RS virus and there has been disagreement about the number and molecular weights of the glycoproteins present in this virus (6-10). However, the majority of reports in the literature have identified three glycopolypeptides in RS virus. No functional roles have yet been conclusively demonstrated for RS virus surface proteins although it is presumed that they are involved in adsorption and cell fusion activities. Thus it is very important to characterize these proteins in order to understand their role(s) in the infectious process and to develop means for control of this clinically important virus. In the experiments described here we have identified and partially characterized three viral-specific glycopolypeptides in purified virions and infected cells having molecular weights of approximately 90,000, 50,000 and 20,000. The effects of the drug tunicamycin as well as the effects of endoglycosidases on the synthesis and electrophoretic mobilities of these proteins were studied.

RS Virion Polypeptides. When ^{3}H-amino acid (^{3}H-AA) labeled purified virions grown in either CV-1 or HEp2 cells
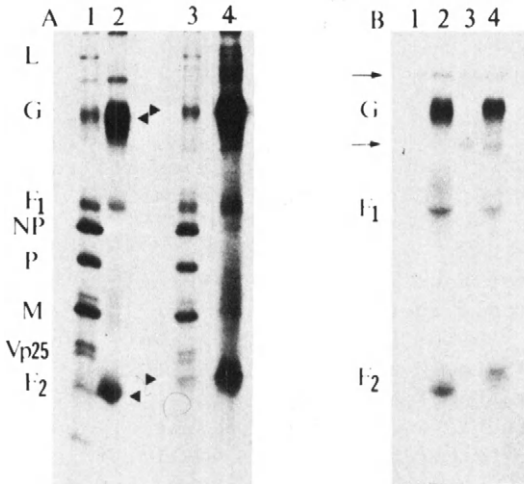
FIGURE 1 (A).   Comparison of [³H] amino acids ([³H]AA)
and [³H] glucosamine-labeled proteins of purified RS virus
(Long) grown in CV-1 and HEp2 cells.   Virus labeled with
[³H]AA or [³H] glucosamine (20 μCi/ml) was harvested at 48 hr
p.i. and purified from the media of infected cell cultures
(11).   Virus pellets were resuspended in Laemmli PAGE sample
buffer (12).   Samples were electrophoresed in 10% polyacryla-
mide slab gels at 40 mA.   Lane 1, [³H]AA-labeled RS virions
from CV-1 cells; lane 2, [³H]-glucosamine-labeled virions
from CV-1 cells; lane 3, [³H]AA-labeled RS virions from HEp2
cells; lane 4, [³H] glucosamine-labeled virions from HEp2
cells.   (B) Immunoprecipitation of [³H] glucosamine-labeled
polypeptides from uninfected (lanes 1 and 3) and infected
(lanes 2 and 4) CV-1 and HEp2 cell lysates.   The procedure of
Wechsler et al. (13) was used except that immune complexes
were precipitated using IgGsorb (The Enzyme Center, Boston,
MA).

were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-
PAGE), eight viral proteins were consistently observed (Fig.
1A, lanes 1 and 3).   Three glycoproteins labeled with [³H]
glucosamine (G, F1 and F2) were identified in purified virions
(Fig. 1A, lanes 2 and 4) and could be purified on lentil lec-
tin sepharose columns (14).   The three glycoproteins could be
labeled equally well in HEp2 cells with [³H] mannose (data
not shown).   Additional viral proteins labeled with [³H] glu-

cosamine did not bind to lentil lectin sepharose columns sug-
gesting that they were not glycosylated (14). The eight poly-
peptides identified were assigned letter designations and had
approximate molecular weights of 180,000 (L), 89,000 (G),
48,000 (Fl), 42,000 (NP), 34,000 (P), 28,000 (M), 25,000 (Vp-
25), and 21,000 (F2). The F2 protein was not always seen in
virions labeled with ^{3}H-AA but was always heavily labeled
with [^{3}H] glucosamine. The two smaller glycoproteins (Fl and
F2) have been shown to be disulfide bonded subunits of an ap-
proximately 70,000 dalton protein that is the most likely
candidate for the fusion protein of RS virus (10, 14).

Because of observed differences in the apparent quanti-
ties and labeling characteristics of the large glycoprotein
(G) in virions grown in HEp2 cells as compared to virus grown
in BSC-1 (monkey kidney) cells, Pringle et al. (9) proposed
that this protein might be a host cell glycoprotein induced
by RS virus replication. In order to investigate this possi-
bility we examined immunoprecipitates of ^{3}H-glucosamine la-
beled uninfected and RS-infected HEp2 and CV-1 (monkey kidney)
cell extracts by SDS-PAGE (Fig. 1B). All three glycoprotein
species were immunoprecipitated from infected cell lysates of
both cell types but not from uninfected cell lysates, suggest-
ing that all three glycoproteins are viral encoded proteins.
An interesting observation was that the G and F2 proteins of
HEp2 and CV-1 grown virions and cell lysates migrated differ-
ently (Fig. 1A and B). This phenomenon was seen consistently
and is most likely the result of differences in glycosylation
and processing of these polypeptides in the two cell lines.

Effects of Tunicamycin on Viral Infectivity and Viral
Proteins. The drug tunicamycin (TM) is a specific inhibitor
of N-acetyl-glucosamine-lipid intermediates and inhibits gly-
cosylation of nascent proteins (16, 17). To determine the
effects of this drug on the infectivity of released RS virus
we infected HEp2 and CV-1 cells at a multiplicity of 1 and
treated with either 0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, or
4.0 μg/ml TM. At 48 hr p.i. no CPE was seen in TM-treated
cultures whereas the untreated monolayers exhibited extensive
syncytial formation. Culture fluids were harvested and virus
was titrated as previously described (11). The results (Ta-
ble 1) demonstrated that release of infectious RS virus from
CV-1 cells was more sensitive to TM treatment than was the re-
lease of infectious RS virus from HEp2 cells.

Similar results were obtained when purified [^{3}H]AA- or
[^{35}S] methionine-labeled virus from untreated HEp2 and CV-1
cells was compared to that from TM-treated cells (0.5 - 2.0
μg/ml). The recovery of labeled virus particles from HEp2
cells was reduced by 40-60% whereas recovery of labeled virus
particles from the media of treated CV-1 cultures was almost

completely inhibited (approximately 5% recovered). The poly-
peptides of [^{35}S] methionine-labeled virions grown in untreat-
ed HEp2 or CV-1 cells and cells treated with 1.0 μg/ml TM were
examined by non-reducing SDS-PAGE (Fig. 2A). No polypeptides
in virions from TM-treated HEp2 cells co-migrated with either
G or Fl, 2 (Fig. 2A, lane 2). Two faint bands migrated more
slowly than the G protein at approximately 100,000 and 94,000
daltons and might represent aggregates. However, two addi-
tional, more heavily labeled, protein bands which probably
represent unglycosylated forms of the G and F proteins were
observed (Fig. 2A, arrows lane 2). One band migrated at ap-
proximately 80,000 daltons and could conceivably be the un-
glycosylated form of the G protein. The other band migrated
at approximately 55,000 daltons and could represent the un-
glycosylated fusion protein. Poor recovery of RS virus from
TM-treated CV-1 cells did not allow observation of similar
proteins (Fig. 2A, lane 4).

Incorporation of [^{3}H] glucosamine into virion glycopro-
teins was almost completely inhibited by 1 μg/ml tunicamycin.
In order to observe the effects of TM on incorporation of [^{3}H]
glucosamine in infected cells we labeled uninfected and infec-
ted CV-1 cells treated with TM at 0, 0.5, 1.0, and 2.0 μg/ml.
Labeled polypeptides were immunoprecipitated from cell ex-
tracts and analyzed by SDS-PAGE under reducing conditions
(Fig. 2B). Incorporation of [^{3}H] glucosamine into host cell
and virion polypeptides was inhibited with increasing concen-
tration of TM. Although TM significantly reduced the incor-
poration of [^{3}H] glucosamine into viral glycoproteins it was
not completely inhibited. Incorporation of label into host

TABLE 1

EFFECTS OF TUNICAMYCIN TREATMENT ON RELEASE
OF INFECTIOUS RS VIRUS FROM INFECTED CELLS

| TM (μg/ml) | CV-1 | | HEp2 | |
	PFU/ml[a]	PFU/cell[b]	PFU/ml[a]	PFU/cell[b]
0	5.75×10^5	1.15	1.5×10^8	150.0
0.1	6.5×10^2	0.0013	9.1×10^7	94.0
0.25	1.0×10^3	0.002	6.0×10^4	0.06
0.5	7.0×10^2	0.0014	2.2×10^4	0.022
0.75	8.0×10^2	0.0016	0.8×10^3	0.0018
1.0	8.0×10^2	0.0016	2.0×10^4	0.020
2.0	5.5×10^2	0.0011	1.6×10^3	0.0016
4.0	2.5×10^2	0.0005	3.6×10^4	0.036

[a]PFU/ml values are averages of two assays per sample on
HEp2 cells.

[b]PFU/cell determination made on basis of 5×10^5 CV-1
and 1×10^6 HEp2 cells/60 mm dish.
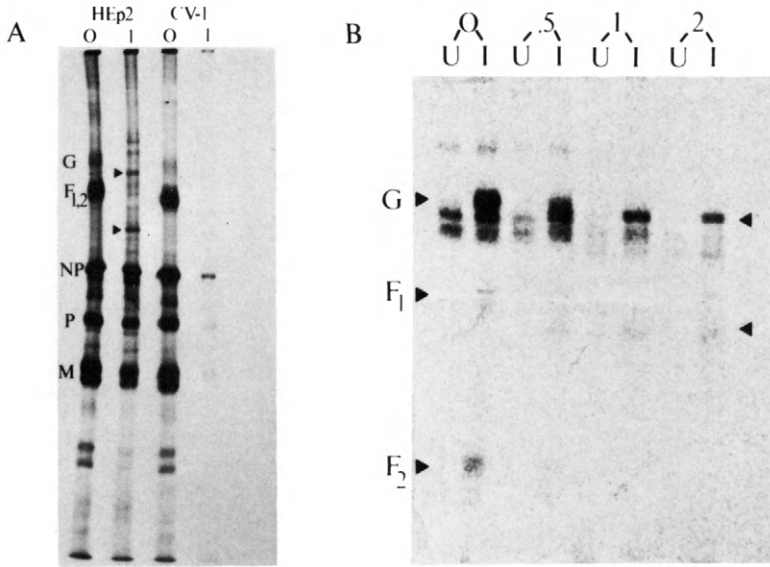
FIGURE 2A. Non-reducing SDS-PAGE analysis of ^{35}S-methionine labeled polypeptides of virions from untreated and TM-treated HEp2 and CV-1 cells. Lane 0, RS virus polypeptides from virions grown in untreated cells; lane 1, viral polypeptides from cells treated with 1 μg/ml TM.

FIGURE 2B. Effects of TM treatment on incorporation of [3H] glucosamine into proteins of uninfected and infected CV-1 cells. Cells were labeled for 24 hr and harvested. U, uninfected; I, infected. Numbers (0, .5, 1, and 2) refer to the concentration of TM in μg/ml.

cell proteins was almost completely inhibited. Labeling of the G protein was less sensitive to the effects of TM than labeling of F1 and F2. In TM treated cells the G protein migrated at about 80,000 daltons and the F1 at about 41,000 daltons. The F2 protein was not detected in TM treated cells. The G and F1 proteins migrated faster in cells treated with TM (arrows), suggesting that although these proteins contained reduced amounts of carbohydrate, they were still labeled. Thus, it is possible that some TM-resistant glycosylation of RS glycoproteins occured even at relatively high concentrations of the drug. However, two observable biological functions of the virus (infectivity and cell fusion) were inhibited by relatively low doses of TM (Table 1).

Endoglycosidase Treatment of [3H]AA-Labeled Virion Poly-
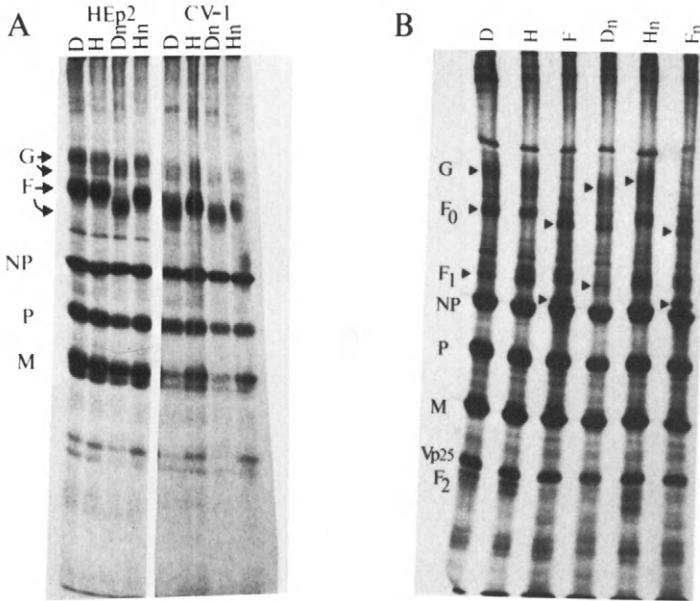
FIGURE 3. Endoglycosidase treatment of [^{3}H]AA-labeled virions. Virions were incubated with enzyme at 37°C for 40 hr. An equal volume of 2X PAGE sample buffer was added and samples were electrophoresed in 10% polyacrylamide gels under either non-reducing (A) or reducing conditions (B). Fig. 3A: HEp2, virions grown in HEp2 cells; CV-1, virions grown in CV-1 cells. Lanes D, virions treated with endo-D; lanes H, virions treated with endo-H; lanes 3 and Dn, virions treated with both endo-D and neuraminidase; lanes Hn, virions treated with endo-H and neuraminidase. Fig. 3B: [^{3}H]AA virions grown in HEp2 cells were analyzed. D, endo-D treated; H, endo-H treated; F, endo-F treated; Dn, Hn, Fn, samples were treated with endoglycosidase and neuraminidase.

peptides. Additional information concerning the nature of RS glycoproteins was obtained by treating [^{3}H]AA labeled puri-fied virions with either endoglycosidase D (endo-D), an enzyme which cleaves complex carbohydrates (18), endoglycosidase H (endo-H), an enzyme which cleaves high mannose carbohydrates (19), and endoglycosidase F (endo-F), an enzyme which cleaves both complex and high-mannose carbohydrates(20). Treatment of labeled virions with either endo-D or endo-H had no effect on the migration of either the G or Fl, 2 proteins of virions grown in HEp2 cells when electrophoresis was carried out un-der non-reducing conditions (Fig. 3A, lanes D and H) but there appeared to be a change in the migration of G and Fl, 2 pro-

teins of virions grown in CV-1 cells treated with endo-D
(lane D). Glycoproteins of virions grown in HEp2 cells were
made susceptible to digestion by endo-D by co-treatment with
neuraminidase (lane Dn) suggesting that the glycoproteins of
virions grown in HEp2 cells contain sialic acid residues
which blocked the action of endo-D. Similarly, neuraminidase
treatment along with endo-D treatment of the glycoproteins of
virions grown in CV-1 cells seemed to enhance the action of
the enzyme (lane Dn). Endo-H treatment alone or with neura-
minidase did not alter the migration of the G or Fl, 2 pro-
teins of virions grown in either cell line (Fig. 3A, lanes H,
Hn). Therefore, the carbohydrate moiety of both the G and F
proteins of virus grown in either HEp2 or CV-1 cells appears
to be of the complex type. Analysis of endo-F treated viri-
ons, grown in HEp2 cells, by SDS-PAGE under reducing condi-
tions revealed altered migration of the apparent F0 and Fl
proteins (the F0 migrated faster and the Fl migrated slightly
slower) even without neuraminidase treatment (Fig. 3B, lane
F, arrows). The G protein essentially disappeared and a new
band of approximately 43,000 daltons (which migrated just
above the NP protein but below the Fl protein) was observed
(lanes F and Fn, arrows). Whether the 43,000 dalton protein
is a subunit or a cleavage product of G (or even Fl) is at
present unknown. Results of endo-D and endo-H digested viral
glycoproteins analyzed in reducing gels (Fig. 3B) were con-
sistent with the results obtained in non-reducing gels (Fig.
3A). We are attempting to further clarify the relationships
of these polypeptides to each other by pulse chase analysis
of viral proteins in infected cells and by comparison of pep-
tide maps of virion glycoproteins to viral proteins in infec-
ted cells. The data presented here suggests that the carbo-
hydrate moieties of the G and F proteins are of the complex
type and their presence on viral glycoproteins is necessary
for the presumed biological activities of these proteins (i.e.
infectivity and cell fusion)

ACKNOWLEDGEMENTS

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CHARACTERIZATION OF RABIES VIRUS RECEPTOR-RICH REGIONS AT PERIPHERAL AND CENTRAL SYNAPSES[1]

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MORPHOLOGIC STUDIES OF THE ACCUMULATION AND TRANSFER OF RABIES VIRUS AT PERIPHERAL AND CENTRAL SYNAPSES.

Rabies virus antigen and intact particles can be found in the region of neuromuscular junctions between one and six hours after infection. Co-localization of virus antigen with the motor end plate can be shown morphologically by immunoelectron microscopy and histochemically by co-localization with regions possessing high densities of the acetylcholine receptor (AChR) (1).

Peripheral synapses. Injection of rabies virus into either hindlimb muscles or facial muscles of the mouse is followed by accumulation of virus at synaptic junctions. Within one-two hours, the virus antigen is near motor end plates as revealed by dual fluorescent staining with anti-viral and anti-AChR reagents. The co-localization becomes more precise at six hours after injection at which time viral antigen is closely associated with the nerve-muscle junction. Thin-section electron microscopy has shown that, at this time, isolated intact particles are present on both the muscle and nerve sides of the neuromuscular junction. On the muscle side of the junction, particles were sometimes enclosed in vacuoles and in other cases were free. Within the nerve terminal itself, few intact particles were found. However, by immunoelectron microscopy, accumulations of viral antigen were found to be more widespread at the junctions as revealed by labeling with a protein A-gold conjugate. Gold label was found near the junctional folds and in the nerve terminal clustered near particle-like configurations (1) and Figure 1.
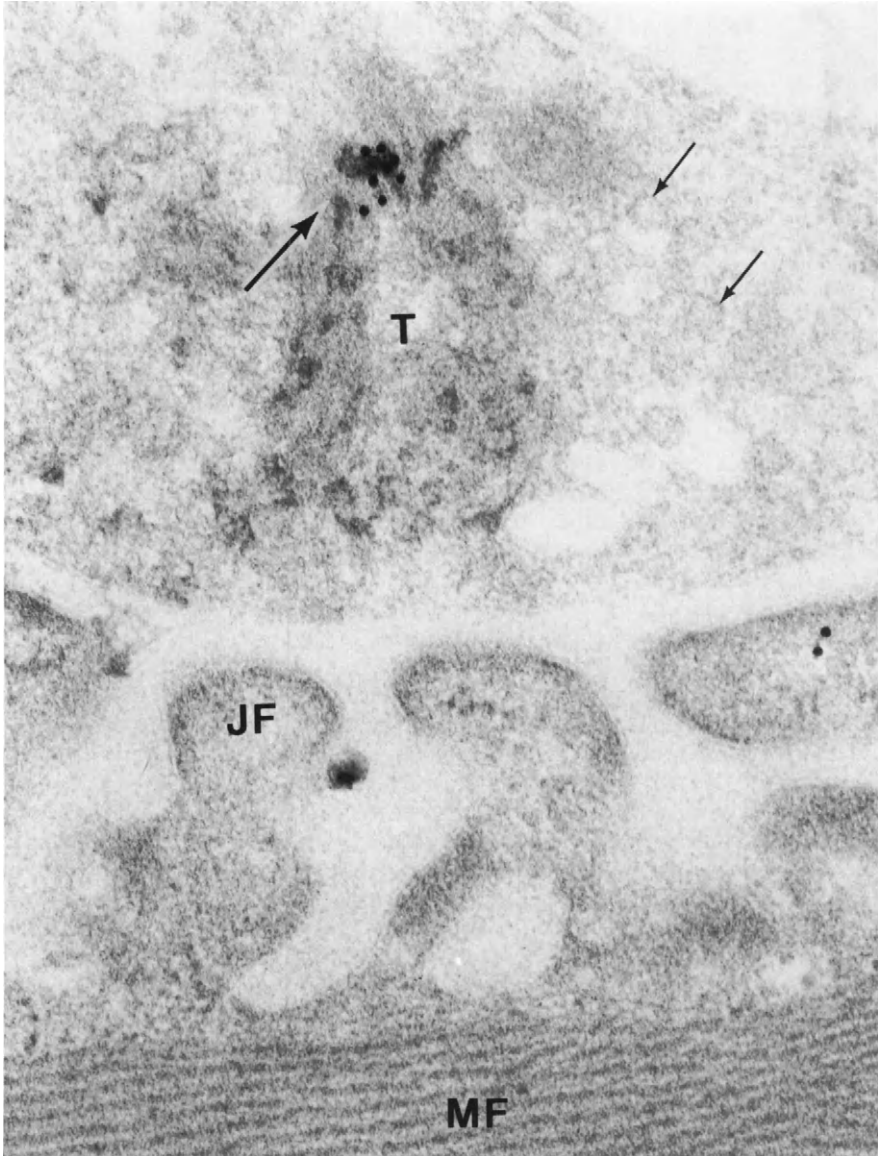
FIGURE 1. Protein A–gold labeled antigen at a neuromuscular junction. A rod–shaped profile (large arrow) with protein A–gold is in the nerve terminal (T) which has synaptic vesicles (small arrows). The muscle cell has junctional folds (JF) and myofilaments (MF). A few gold particles are associated with a junctional fold.   x94,000.

Gold labeled antigen was seen being actively engulfed by
neutrophils and lying within large vacuoles in macrophages
(2). Gold label was also found closely associated with
microtubules in large nerves. Virus particles apparently
reached the neuromuscular junction by diffusion through the
extracellular space near this site (1).

   Central synapses. Rabies virions were frequently found
at synaptic regions within the central nervous system either
free in the extracellular space or enclosed within vacuoles
at nerve terminals [Figure 2A]. Viral uptake was
morphologically similar to that at peripheral synaptic
junctions where particles were also enclosed in vacuoles
[Figure 2B].
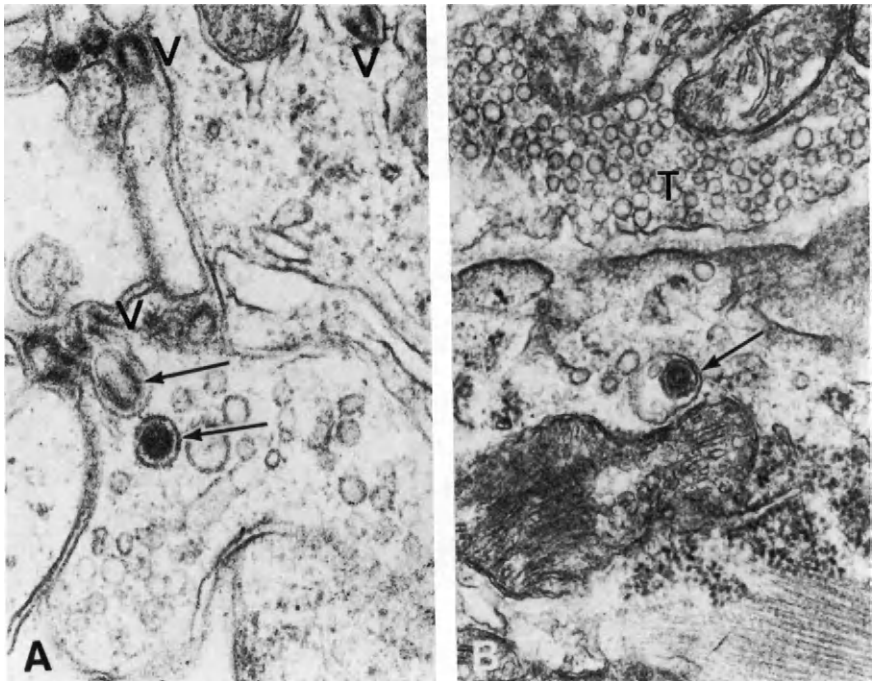
   FIGURE 2. Rabies virus particles at central and
peripheral synapses. A. Synaptic neuropil from the
cerebellum. Rabies virus particles within vacuoles (arrows)
in a terminal. Virions (V) also in the extracellular space
and budding from internal membranes. x68,900. B. A
particle (arrow) in the sarcoplasm of muscle. The junctional
area is defined by a synaptic vesicle-laden terminal (T).
x55,500.

Transfer of particles at brain synapses was frequently
observed.   Particles budded into coated pits consistent with
receptor-mediated endocytosis [Figure 3A].   Intact particles
and protein A-gold label were seen near postsynaptic
densities indicating accumulation of viral proteins. [Figure
3B].   To characterize the viral receptor at these sites, we
initially isolated synaptosomal fractions from infected brain
tissue (3).   Virions were attached to synaptosomal membranes,
but particles were not attached to contaminating myelin
figures or to mitochondria [Figure 3C].
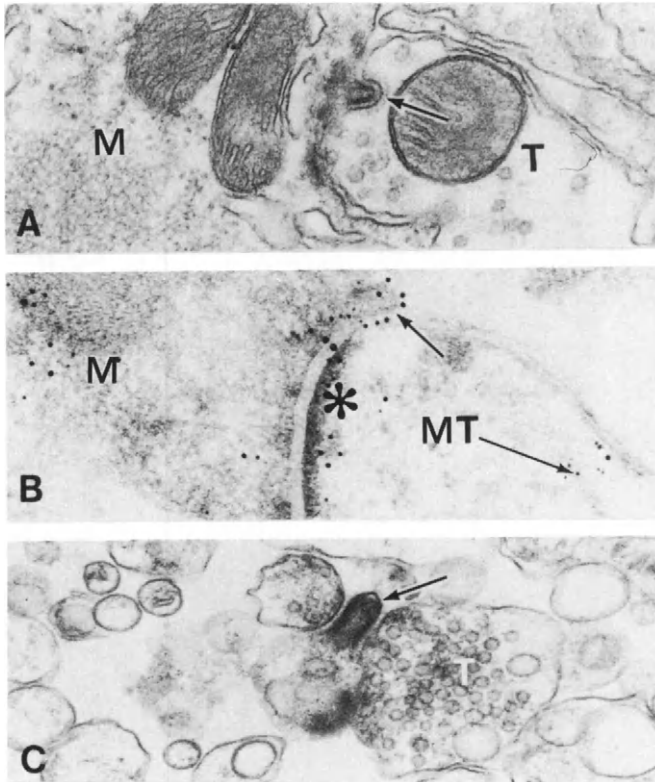
FIGURE 3. A.   Particle in a coated pit of a terminal
(T). Dense material is the postsynaptic surface.   Viral
matrix (M). x52,000.   B.   A particle (arrow) with gold
label in the junctional space. Labeled matrix (M) and labeled
postsynaptic density (*). x60,000 C.   Virion-synaptosome
association (arrow). Terminal (T). x74,500.

TRANSPORT AND PROCESSING OF VIRIONS AND VIRION-ASSOCIATED
PROTEINS.

      Rabies virions and virion-specific proteins have been
localized in both the peripheral and central nervous systems
using thin section electron microscopy and immunoelectron
microscopy. A protein A-gold conjugate has been used to
detect rabies virus antibody on thin sections of rabies
virus-infected tissue embedded in Lowicryl at low temperature
(2). Fixation was by paraformaldehyde and glutaraldehyde;
post-fixation with osmium tetroxide was omitted. Sections
were stained with uranyl acetate and lead citrate.

      Transport. Within hours after infection of muscle
tissue, gold labeled antigen was found in association with
microtubules in large peripheral axons in the inoculated hind
limb (1). Since a polyclonal anti-viral antibody was used in
these studies, protein A-gold label was found over the
glycoproteins, membrane and nucleocapsid proteins of intact
particles. Since intact particles were not found in
peripheral nerves at this early time, it was not possible to
determine which viral proteins were associated with the
microtubules. It is possible, however, that the gold label
was associated with intact virions which were not in the
plane of section and thus could not be morphologically
identified (2). Inhibitors of microtubular function have
been used in the past early after infection to show that
rabies virus or subunits thereof are transported by
axoplasmic flow (4,5). Viral matrices were not found within
axons but were found within terminals even at 6 hours.
      Within the central nervous system, gold labeled antigen
was found in association with microtubules and rough
endoplasmic reticulum, but not with neurofilaments or the
Golgi complex (2). The latter are not known to be directly
involved in axoplasmic transport. As mentioned earlier,
budding into coated pits at terminals was a prominent feature
of the transport process. This could result from assembly of
viral proteins at the post-synaptic surface or from receptor-
mediated endocytosis.
      In addition to membrane-associated transport, intact
virions were found either free or in vacuoles within axonal
and dendritic processes. Viral matrices were not found in
axons but were frequently seen in dendritic processes. Thus,
transport in axons was limited to axoplasmic flow whereas
transport in dendrites could occur either by axoplasmic flow
or by specific assembly at synaptic sites.
      Processing. After transfer of virions at synaptic

terminals, particles were often found with multivesicular bodies in the dendritic and cell body cytoplasm [Figure 4A]. Multivesicular bodies are thought to be an immature form of a lysosome (6). The presence of virions within these bodies is consistent with current hypotheses regarding the uptake and uncoating of enveloped viruses from this and other genera (7). Significant accumulations of gold particles were found associated with the cisternae of the endoplasmic reticulum near viral matrices. This observation was consistent with the current models of protein assembly.

IMMUNOCYTOCHEMICAL STUDIES ON RABIES VIRUS RECEPTOR-RICH REGIONS.

In earlier reports, we have suggested that sites containing high levels of the acetylcholine receptor may also contain a molecule which serves as a specific host cell receptor for rabies virus. This hypothesis is based on findings that ligands for the acetylcholine receptor and monoclonal antibody to a subunit of the receptor reduce viral attachment to muscle cells (1). As we have shown here, virus attachment also occurs at terminals in the central nervous system. Therefore, we have looked for acetylcholine receptor in the brain. Acetylcholine receptor has been previously shown to be present on the post-synaptic density of isolated synaptosomes (3). The purpose here was to determine whether the same molecule to which virus binds at peripheral synaptic regions is present at terminals in the central nervous system. The terminals of dendritic processes in the cerebellum of infected mice are sites where there are large concentrations of both intact virions and virion proteins as shown by protein A-gold label [Figure 4B]. Using an acetylcholine receptor monoclonal antibody which co-localized with virus on muscle cells (1), we found binding to proteins distributed both on the synaptic plasma membrane and within the terminal itself [Figure 4C]. Finding receptor in the terminal was an unexpected result which could suggest that the molecular address of the receptor may govern viral replicative events beyond attachment. Alternatively, the presence of receptor in the terminal may result from metabolic changes induced by the virus. Morphologic evidence of selective metabolic changes in infected neurons were found several days after infection at peripheral synapses (1). At degenerating neuromuscular junctions, nerve terminals had retracted from muscle tissue and only dense core vesicles remained intact. Co-localization studies using immunoelectron microscopy may resolve questions raised here.
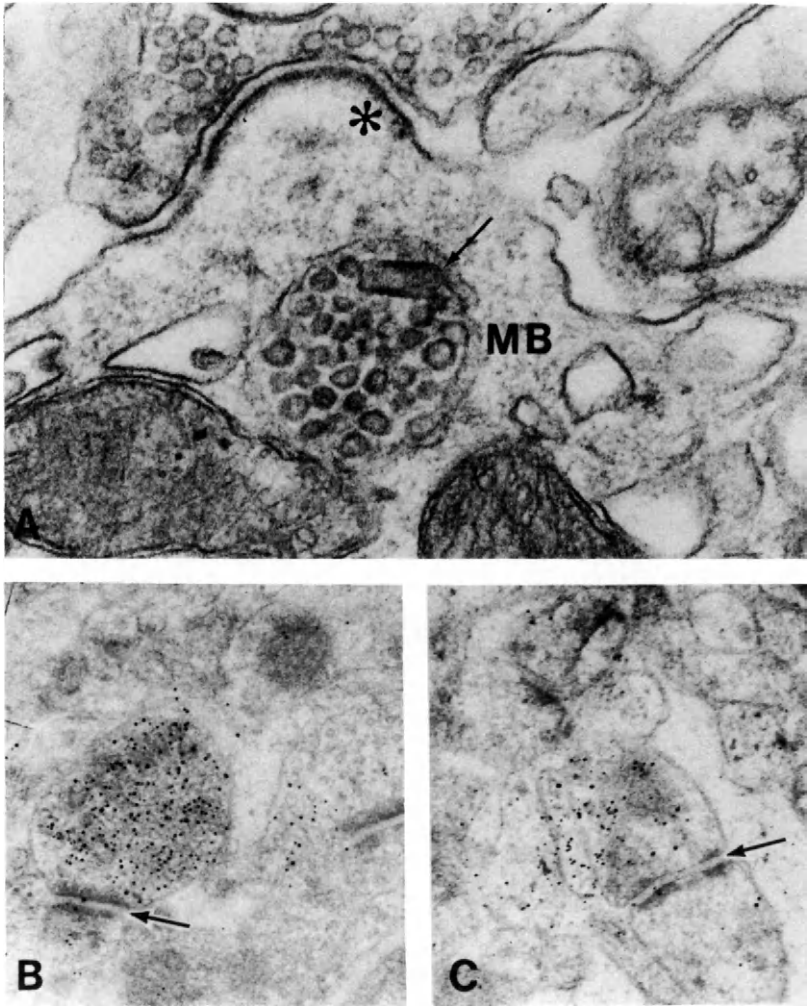
FIGURE 4. A. Rabies virion (arrow) in a multivesicular body (MB) in a dendritic process adjacent to a synaptic junction. Note the postsynaptic density (*) and synaptic vesicles in the terminal. x66,700. B. Protein A-gold label on viral particles and matrix in a terminal. Label is also present on synaptic membranes. Label also occurs on microtubules in the adjacent dendrite. x35,600. C. Terminal from infected cerebellum incubated with monoclonal antibody to the acetylcholine receptor and stained with protein A-gold. Label is on the synaptic membrane, but is largely in the terminal. Junctional space (arrow). x32,000.

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EARLY INTERACTIONS OF RABIES VIRUS WITH CELL SURFACE
RECEPTORS[1]

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The rabies virus provides an excellent model of neuro-
tropism whereby virus infects the central nervous system
(CNS) exclusively via peripheral nerves. Infection of
tissues outside the CNS, such as salivary glands, occurs by a
centrifugal spread of rabies virus from the CNS, again
through the peripheral nerves (1). This neurotropism imposes
a severe host range limitation in vivo although in vitro the
virus infects nearly all mammalian and avian cell types (2).
    Since it is known that the presence of cell surface
receptor sites is often a major controlling factor of tissue
tropism, we have investigated early interactions of rabies
virus with cells of neural and non-neural origin. We report
that rabies virus utilizes a rhabdovirus-common receptor in
its cellular interactions and pH-dependent fusion in vitro.

VIRUSES AND CELLS

    Cultures of BHK-21 clone 13 cells and mouse
neuroblastoma C1300 (NA) cells were used throughout these
studies (3). The ERA strain of fixed rabies virus, its
growth in BHK-21 cells, radiolabeling with [^{3}H]leucine or
[^{35}S]methionine, and purification has also been described
(5). The origin and purification of rabies nonpathogenic
variant RV194-2, vesicular stomatitis virus (VSV), West Nile
virus and reovirus type 3 are described elsewhere (4).

STANDARDIZATION OF BINDING ASSAY

BHK-21 or NA cells at 5 x 10^6 cells/ml were suspended in
minimal essential medium (MEM) containing 0.2% bovine serum
albumin (BSA), 0.05 M HEPES (pH 7.4), and DEAE-DEXTRAN
(DEAE-D; 50 μg/ml). Radiolabeled virus was added directly to
cells and incubated at 4°C until an apparent equilibrium was
reached (3 hours). Unattached virus was separated from cell-
associated virus by centrifugation and the distribution of
radiolabel between cell pellet and supernatant fractions
determined by liquid scintillation spectrometry. DEAE-D was
found to enhance attachment rate and was equally effective as
a cell pretreatment, suggesting an action of DEAE-D on the
plasma membrane rather than on the virus. These observations
confirm earlier studies by Kaplan et al. (5).
The virus-cell interaction did not require divalent
calcium or magnesium ions, nor was the intact cellular recep-
tor unit (CRU) sensitive to digestion by trypsin, chymotryp-
sin, pronase or sialic acid-specific enzymes. Attachment was
pH- and temperature-dependent. A greater level of attachment
was observed at low pH but did not correlate well with an
increase in infectivity.
Attachment of fixed rabies virus to BHK-21 cells obeyed
the laws of mass action under the standard binding conditions
used. The number of virus particles that attached per cell
increased linearly as the ratio of the concentration of virus
particles and cells was increased with respect to each other.

SATURATION AND COMPETITION FOR CELL SURFACE RECEPTORS

Saturation was demonstrated by binding ^{3}H-labeled
virus probe to the cell surface in the presence of increasing
concentrations of unlabeled homologous virus particles
(Figure 1). The point at which increasing input multiplici-
ties failed to cause a continued linear increase in specifi-
cally attached virus defines saturation of cell surface
receptors. From five individual experiments it was estimated
that saturation was complete at approximately 3-15 x 10^3
attached virions per cell.
Competition was demonstrated using excess unlabeled ERA
strain rabies virus, ERA strain nonpathogenic variant RV194-2
and VSV, all of which competed with ^{3}H-labeled ERA virus for
receptor sites on NA and BHK-21 cells, causing 70-82% inhibi-
tion of probe with 100 μg of competing virus. Non-competi-
tive ligands included purified rabies virus glycoprotein (G),
rabies soluble antigen (G_s) and reovirus type 3 at 100 μg of
virus. West Nile Virus caused only a 27% inhibition, further
suggesting the existence of separate receptor sites for the
rhabdoviruses.
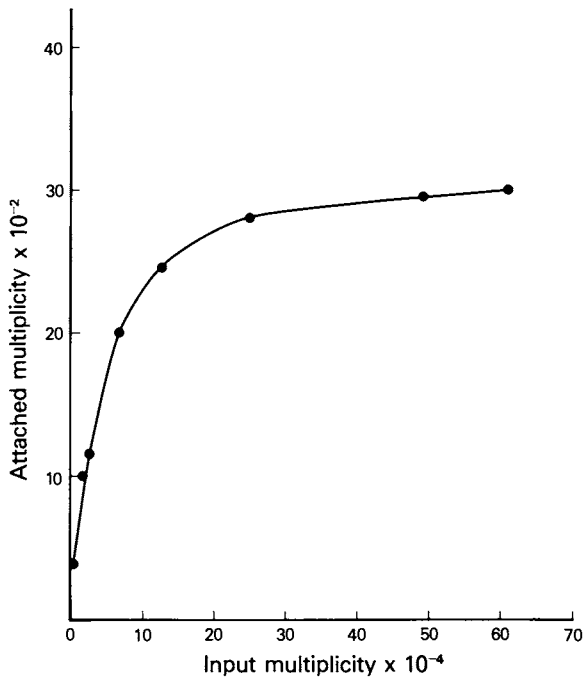
FIGURE 1.   Saturation of BHK-21 cells with ERA virus.
Bound virus was determined after a 3-hour incubation at 4°C.

SOLUBILIZED RECEPTOR ACTIVITY

    A soluble extract was prepared by treating BHK-21 cells
in suspension with 50 mM octyl-β-D-glycopyranoside (OG
extract) (6) which blocked the attachment of rabiolabeled
virus to BHK-21 cells in the presence or absence of DEAE-D
(Figure 2). The binding-inhibitory fraction of the OG
extract was resistant to proteases, boiling, and freeze-thaw
cycles but sensitive to phospholipases. Unlike the result
with whole cells, we found the OG extract inhibition to be
destroyed by neuraminidase.

MEMBRANE FUSION BY RABIES VIRUS

    To study adsorptive endocytosis, we have examined the
fusion capabilities of rabies virus by exposing infected
BHK-21 cells to mildly acidic pH (7). A suspension of 2.5 x
10^5 BHK-21 cells/ml in MEM containing 10% fetal calf serum
and approximately 5 x 10^5 PFU/ml ERA virus (which caused the
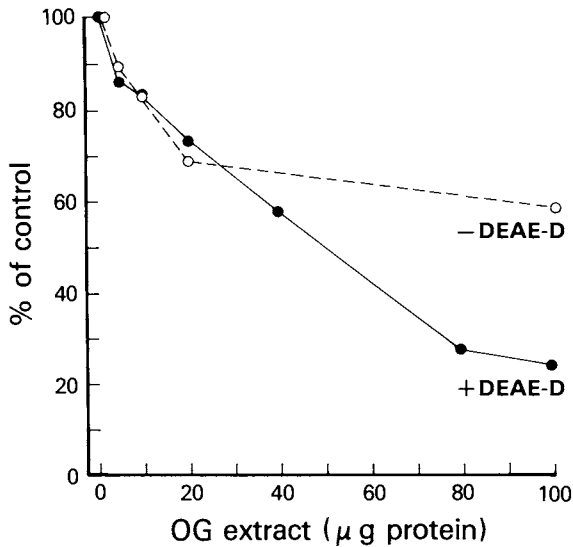
FIGURE 2.   Inhibition of ERA virus binding by an OG
extract from BHK-21 cells.   BHK-21 cells were suspended in
attachment medium with or without 50 µg/ml DEAE-D.   Increas-
ing amounts of dialyzed OG extract were added to the cell
suspension followed by [^{35}S]methionine-labeled ERA virus.
Virus bound after 3 hours at 4°C was determined and is
expressed as a percentage of a control culture which received
no OG extract.

development of rabies virus-specific antigen in 100% of the
cells within 24 hours) was cultured on plates containing
coverslips and incubated for 24 hours at 37°C.   Cover-slips
were removed, washed in PBS, and exposed for 60 seconds to
warmed MEM buffered to pH 5.8 or 7.0 with 10 mM MES and 10 mM
HEPES.   Cells were replaced in warmed MEM containing 0.2%
BSA, 50 mM HEPES, pH 7.4, and incubated for 30 minutes at
37°C, then fixed and stained.   As shown in Figure 3, infected
cells exposed to pH 5.8, but not pH 7.0, were extensively
fused.   This polykarion development which occurred only in
infected cells could be inhibited by monoclonal antibodies
directed to the rabies virus glycoprotein.   The pH threshold
for triggering fusion was 5.9.

DISCUSSION

     These data suggest a specific, rhabdovirus-common CRU
exists on the plasma membrane of susceptible cells in
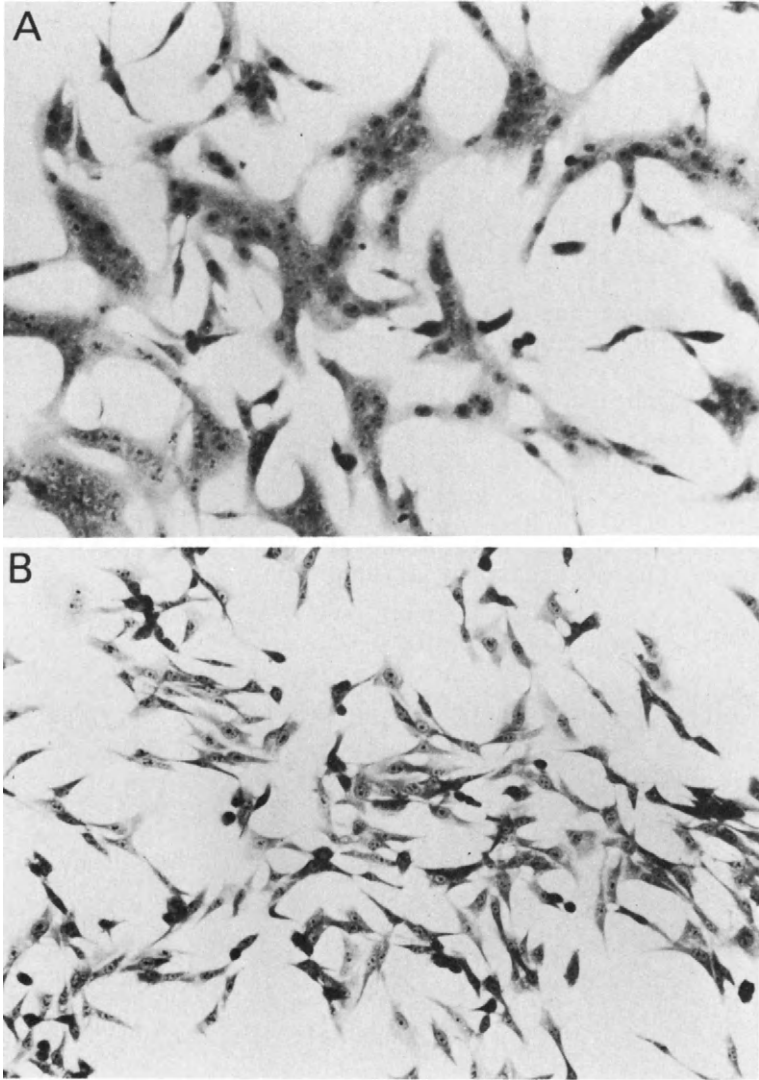
FIGURE 3.   Fusion of ERA virus-infected BHK-21 cells by brief exposure to MEM, pH 5.8 (A).   Replicate cultures exposed to MEM, pH 7.0 (B), failed to fuse.

culture. The areas of similarity between VSV and rabies
virus are summarized below. VSV attaches to a limited number
(approximately 4×10^3) of high affinity sites on Vero cells
(8), a number which correlates well with the $3\text{-}15 \times 10^3$ sites
for rabies virus on BHK cells. VSV and rabies virus attach-
ment to cells was independent of pretreatment of cells with
proteolytic enzymes or the ommission of Mg^{+2} or Ca^{+2} from the
binding medium (4, 6, 9). In both systems, isolated viral G
failed to block whole virus attachment (4, 9, 10), yet VSV
was capable of competing with rabies for its receptor on
BHK-21 and NA cells. An OG extract of susceptible cells,
which was sensitive to phospholipases, could block specific
attachment of either virus (6; Figure 2), suggesting that a
lipid component had binding activity. We are currently
investigating whether this component represents a functional
receptor. Finally, both VSV and rabies virus promote
cellular membrane fusion with a similar pH threshold (7;
Figure 3).

The nonpathogenic variant of ERA strain rabies virus,
RV194-2, recognized and competed for the same CRU as its
virulent parent virus. This suggests that in cultured cells,
an alteration of cellular receptor specificity does not
represent the mechanism of attenuation.

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MICROINJECTION OF MONOCLONAL ANTIBODIES TO VESICULAR
STOMATITIS VIRUS NUCLEOCAPSID PROTEIN INTO HOST CELLS:
EFFECT ON VIRUS REPLICATION[1]

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MICROINJECTION OF ANTIBODIES INTO CELLS

Microinjection of antibodies into cells is a powerful
technique to interfere with distinct viral (1) or cellular
(2) functions. It is also attractive to use this technique to
dissect viral replication and maturation at the molecular
level: the living cell can be used as an experimental
system, it is possible to inject large quantities of anti-
bodies, the half life of the injected antibodies is usually
longer than 24 hours (3), and injection of antibodies as such
does not alter cellular ultrastructure (4) nor biofunction
(5). In the present communication, we analyze aspects of the
role of the vesicular stomatitis virus (VSV) nucleocapsid
protein N in viral transcription and replication. We compare
effects of monoclonal antibodies either introduced into cells
by manual needle microinjection (6, 7) or mixed into cell-
free transcription/replication systems.

MONOCLONAL ANTIBODIES THAT DISTINGUISH BETWEEN TWO POOLS OF
CYTOPLASMIC N PROTEIN

Mouse monoclonal antibodies were obtained by standard
procedures after immunization with a preparation of nucleo-
capsid free N protein obtained after high salt treatment of

purified VSV nucleocapsids (serotype Indiana). Antibodies
were purified to homogeneity by affinity chromatography on
N protein attached to CNBr-activated Sepharose 4B, and
binding specificity was tested on VSV proteins separated by
poly-acrylamide electrophoresis and transferred to
nitrocellulose papers.

Two anti-N antibodies were selected for these studies
based on their pattern of immunofluorescent staining of N
protein in VSV infected Madin Darby bovine kidney cells.
Antibody 1 shows cytoplasmic clusters of N protein and a weak
diffuse fluorescence (Figure 1a). Antibody 2 shows only the
diffuse pattern of the N fluorescence (Figure 1b). These two
antibodies recognize different epitopes (data not shown), and
it is, therefore, conceivable that the epitope recognized by
antibody 2 is not accessible on clustered N protein. Since
electron microscopic studies show that clustered N protein
may represent nucleocapsids (Shinishi Ohno, unpublished), we
can postulate that antibody 1 recognizes nucleocapsids and
free N protein and antibody 2 only free N protein.

EFFECTS OF MICROINJECTION OF ANTI-N ANTIBODIES ON VIRUS
YIELDS

If the above hypothesis of a differential recognition of
nucleocapsids and free N protein is correct, then microinjec-
tion of antibody 1 should protect cells against subsequent
infection because antibody 1 would bind to infecting nucleo-
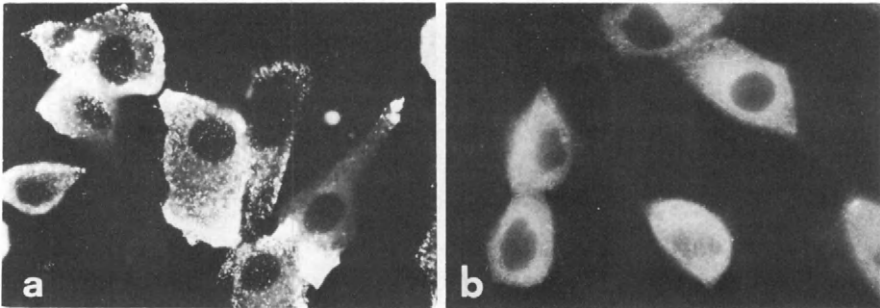
FIGURE 1. Immunofluorescent staining of N protein. Con-
fluent monolayers of Madin Darby bovine kidney cells were
fixed with 2% formaldehyde and permeabilized with 0.05%
Triton X 100 5 hours after infection with VSV at a multi-
plicity of ≃ 0.5 pfu/cell. They were then incubated with
mono-clonal antibodies (50 µg/ml), washed, and incubated with
a goat anti-mouse IgG-fluorescein conjugate. a) monoclonal
antibody 1, b) monoclonal antibody 2.

capsids and inhibit primary transcription. Microinjection of
antibody 2 should not interfere with primary transcription,
but the antibody should bind to newly synthesized N protein,
thus making it unavailable for binding to viral RNA which may
inhibit viral replication. We first injected antibodies at a
concentration of 5 mg/ml (equivalent to $\simeq$ 2×10^{6} molecules/
cell) into bovine kidney cells which were subsequently
infected with 10 pfu/cell of VSV.   Immunofluorescent double
staining for injected antibody and viral G protein 4 hours
after infection showed that of 56 cells injected with
antibody 1, none expressed G; of 55 cells injected with
antibody 2, 42 expressed G at low levels and 12 showed no G;
and of 42 cells injected with antibody 3, a control antibody
made to an unrelated synthetic peptide (8), all except one
expressed G at high levels. To test the effect of antibodies
on virus yields, antibodies were injected at the same
concentration into all of approximately 100 cells grown on
coverslips. Cells were subsequently infected as above and
supernatants were titrated for infectivity at several time
points after infection. Results are shown in Figure 2. Cells
injected with antibody 1 made negligible amounts of virus,
cells injected with antibody 2 produced peak infectivities at
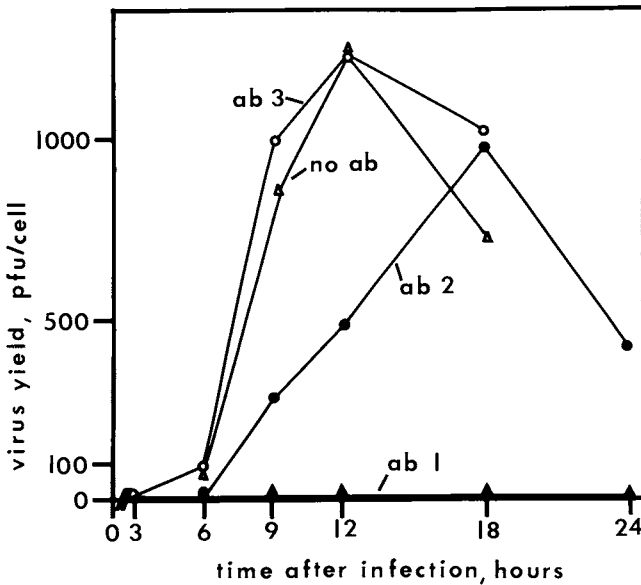
FIGURE 2. Cumulative virus yields in microinjected
cells.   Plaque titration of supernatant fluids was done in
baby hamster kidney cells.

18 hours, and cells injected with control antibody 3 had a
peak infectivity at 12 hours, similar to cells not injected
with antibody. Thus, antibody 1 inhibited and antibody 2
delayed viral replication.

EFFECT OF ANTIBODIES ON IN VITRO TRANSCRIPTION OF VSV

    The small number of cells that could be injected in
reasonable time made biochemical studies in the living cell
unfeasible. We, therefore, tested the effect of the two
antibodies in standard in vitro transcription assays (9).
Detergent disrupted VSV was mixed with various concentrations
of antibodies and incubated with the four nucleotide triphos-
phates, one of which was radiolabeled (^{3}H-UTP, 2 Ci/mmol).
Trichloroacetic acid precipitable transcripts were counted.
Results are shown in Figure 3. Antibody 1 (15 μg/ml)
inhibited transcription by more than 80%, whereas neither
antibody 2 (17.5 μg/ml) nor antibody 3 (67.5 μg/ml) had any
effect. Partially purified antibody 2 tested at concentra-
tions of antibodies of up to 330 μg/ml had, likewise, no
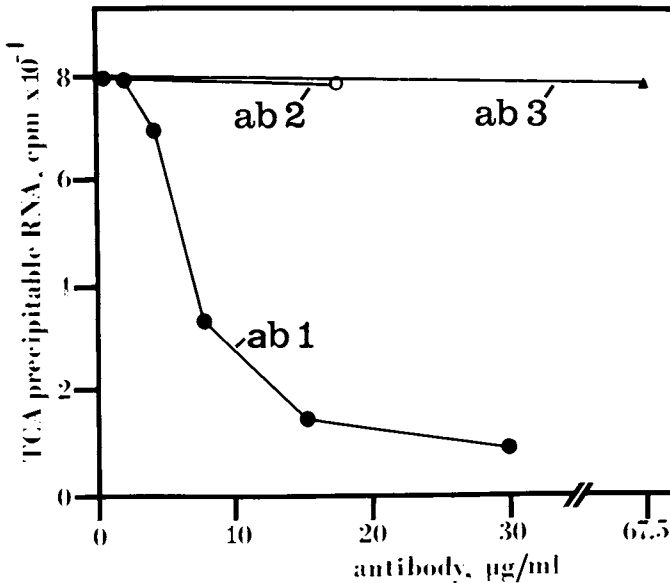
    FIGURE 3. Effect of antibodies on in vitro transcription
of VSV. Detergent disrupted virus (4 μg protein/100 μl) was
incubated with antibodies and 10 U/100 μl of RNasin, 1 mM
ATP, 200 μM CTP, 200 μM GTP and 50 μM ^{3}H-UTP for 90 minutes
at 30^{0}C. TCA precipitable RNA was counted in a Beckman
scintillation counter.

effect (data not shown). Thus, the delay in viral growth in
cells injected with antibody 2 cannot be explained by an
effect on primary transcription.

EFFECT OF ANTIBODIES ON IN VITRO REPLICATION OF VSV

    An in vitro system, including micrococcal nuclease
treated rabbit reticulocyte lysate and intracellular VSV
defective interfering (DI) nucleocapsid templates, supports
the synthesis of DI genome length RNA when viral protein
synthesis is programmed by added viral mRNAs (10). In the
absence of viral mRNA, the intracellular DI nucleocapsids
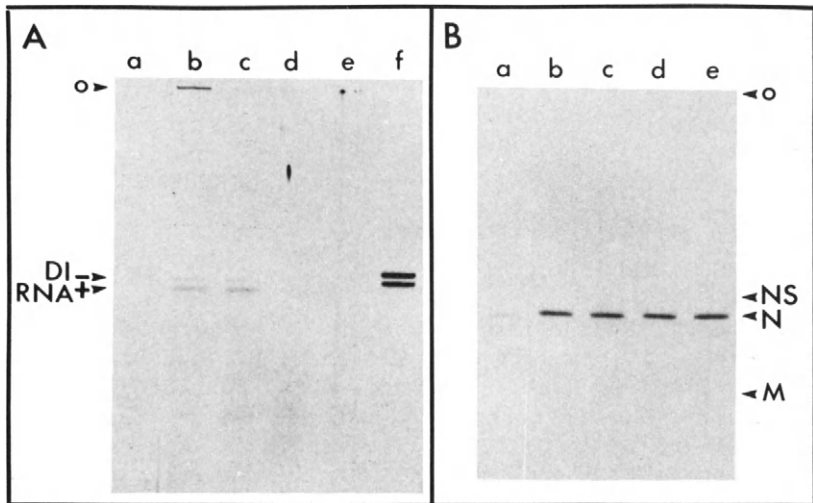
    FIGURE 4. In vitro RNA replication and protein synthesis
in the presence of monoclonal antibodies to N protein.
Reaction mixtures (13) containing a constant amount of
hybrid-selected N mRNA, ^{3}H-UTP and ^{35}S-methionine were
incubated at 30^{0}C for 45 min.   Anisomycin (25 µM), the
respective   antibody,   and   purified   intracellular   DI
nucleocapsids were added and the incubation was continued for
2¼ hours. Deproteinized RNA products (A) were electrophoresed
in a 6 M urea agarose gel (10), and protein products (B) were
analyzed by PAGE (14). (a) No added mRNA, no antibody, (b) N
mRNA, no antibody, (c) N mRNA, antibody 3, (d) N mRNA,
antibody 2, (e) N mRNA, antibody 1, (f) in vivo labeled DI
intracellular nucleocapsid template RNA.

produce only a 46 base leader RNA. Hybrid-selected N mRNA
was used to program protein synthesis in this <u>in</u> <u>vitro</u>
system. The results showed that a source of N protein alone
was sufficient to promote genome length RNA synthesis by the
enzymatically active templates (11) (Figure 4, reactions a
and b). These data and the finding of others (12) lead to the
prediction that antibodies to the N protein would inhibit
replication <u>in</u> <u>vitro</u>. In fact, both antibody 1 and 2
inhibited genome length RNA synthesis when added to reactions
programmed with hybrid-selected mRNAs. Control antibody 3 had
no effect (Figure 4, reactions c, d and e). A source of free
N protein is required for RNA replication, but not for tran-
scription; the N genome complex is the template for both
reactions. Therefore, the finding that antibody 2 inhibits
replication, but not transcription, is consistent with the
hypothesis that this antibody recognizes only free N protein.
The mechanism by which antibody 1 inhibits replication may
involve interaction with both free and nucleocapsid-bound N
protein.

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THE COILING OF VESICULAR STOMATITIS VIRUS NUCLEOCAPSIDS
AT THE INNER SURFACE OF PLASMA MEMBRANES:
IMMUNOLOCALIZATION OF THE MATRIX PROTEIN

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Nucleocapsids of vesicular stomatitis virus (VSV) are loosely coiled or extended in the cytoplasm of infected cells and tightly coiled in the virion. Thin-section electron microscopy has shown that tight coiling occurs during viral budding from cellular membranes (1). However, the molecular mechanisms of this coiling are not yet understood, and little ultrastructural information on this process is available. Here, we present high resolution views of nucleocapsid coiling at the cytoplasmic surface of plasma membranes as it would be seen by an observer localized inside the cell.

PLATINUM-CARBON REPLICAS OF THE CYTOPLASMIC SURFACE OF PLASMA
MEMBRANES

Cytoplasmic surfaces of plasma membranes of VSV infected baby hamster kidney cells were prepared by a modification of the technique originally described by Büechi and Büchi (2). Cells were grown on glass coverslips and incubated for six hours after infection at 37^0C. They were then vigorously rinsed in ice cold physiological buffer (100 mM KCl, 5mM $MgCl_2$, 2 mM EGTA and 30 mM Hepes, pH 7.1). This resulted in the removal of cell bodies from the coverslip, leaving behind basal plasma membranes with their cytoplasmic surfaces exposed. Fixation with 1% glutaraldehyde in the above buffer was followed by a brief rinse in 15% methanol in distilled water. The coverslips were then quickly hand-dipped into liquid propane/2-methyl butane (3:1) cooled by a liquid nitrogen bath. Etching and platinum-carbon rotary shadowing were performed in a Balzers 301 freeze-fracture device according to Heuser and Kirschner (3).

MORPHOLOGY OF VSV NUCLEOCAPSIDS ON THE INSIDE OF PLASMA
MEMBRANES

     Embedded between numerous clathrin sheets, clathrin
baskets and cytoskeletal elements, we observed nucleocapsids
in three different conformations: tightly coiled, tortuous
and linear. These different conformations were either
separate or attached to each other (Figures 1-2). The
bullet-shaped, tightly coiled structures consisted of a nose
cone of 5 to 6 turns of increasing diameter followed by a
variable number of turns of 42.5 + 2.5 nm diameter. These
structures resembled nucleocapsid "skeletons" as derived from
octylglucoside treated virus particles (4) and were never
found in non-infected cells. The apex of the nose cone was
observed free of uncoiled material and attached to the plasma
membrane. In addition, the coils forming the nose cone were
often separated from adjacent strands in the coil (Fig. 1 and
2), probably the result of physical stress induced during the
coverslip rinsing. Stereo views demonstrated that many tight
coils were embedded in the membranes. Surface subunits (33 +
3 A in diameter) formed a lattice along the helical coils
which had a periodicity of approximately 5 nm. The distance
between subunits along the cylindrical portion of the tight
coils was approximately 17 A. The nose cones appeared to
have a tighter subunit packing. Uncoiled tortuous material
was consistently found attached to the base of the tight
coils and most likely represents nucleocapsid that was not
yet tightly packed. Several tight coils were often found
associated with one tortuous complex. Stereo views show that
the tortuous complexes often projected away from the plane of
the membrane. The surface underlining tortuous material was
more granular than the surrounding cytoplasmic surfaces. In
addition, linear nucleocapsids were observed traversing into
or out of tortuous complexes. These linear structures were
often in close association with the plasma membrane. The
linear portions of the nucleocapsids had a morphology similar
to that of negatively stained nucleocapsids prepared from
cells or virions (5) and could easily be distinguished from
the thicker microtubules and intermediate filaments. The
distinction between microfilaments and linear nucleocapsids
could be made on the basis of differences between subunit
morphology and periodicity. Nucleocapsid subunit periodicity,
approximately 37 A, was less than the microfilament subunit
spacing, approximately 55 A. Depending on the angle of
viewing, linear nucleocapsids had a width of 37 to 87 A while
the more symmetrical microfilaments were between 85 and 100
A.
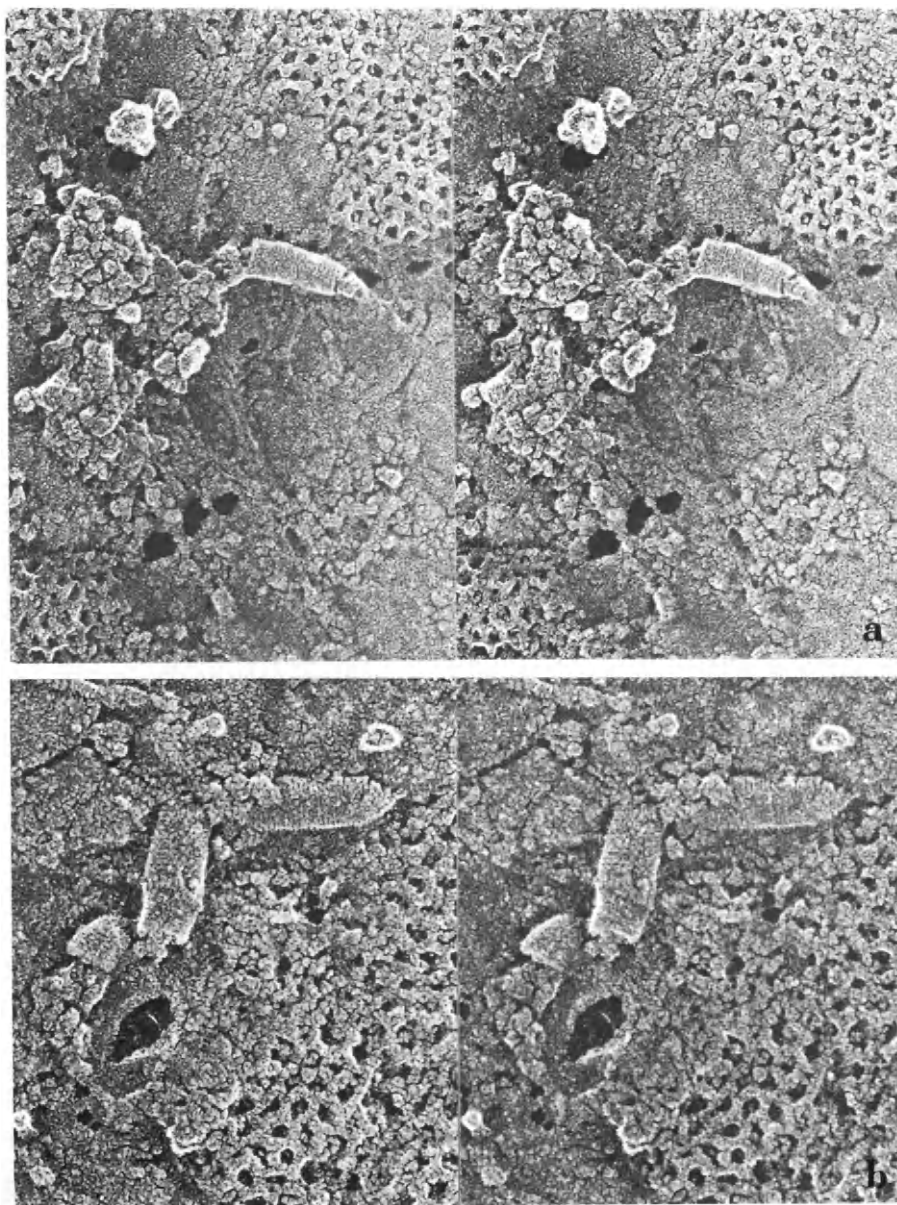
FIGURE   1.   Stereo   views   of   nucleocapsids   on   the
cytoplasmic side of basal plasma membranes. (a) note the
tortuous complex attached to the base of the tight coil whose
apex is attached to the membrane (127,500x).   (b) varying
degrees of nucleocapsid coiling (172,500x).

IMMUNOLOCALIZATION OF VSV MATRIX PROTEIN

     The exact localization of the viral matrix (M) protein
in the virus and in the cell is still controversial. When
viral membranes are solubilized by low ionic strength deter-
gents, the M protein remains tightly associated with the
nucleocapsid "skeletons" and is involved in maintaining the
tightly coiled "skeleton" conformation (6). Other studies
suggest that the M protein is located on the inner surface of
the viral membrane (7, 8, 9) or that it even traverses the
lipid bilayer (10). However, the amino acid sequence of the M
protein does not indicate any long hydrophobic or nonpolar
domains that would suggest membrane association (11).
Therefore, we wanted to immunolocalize the M protein on the
cytoplasmic side of the plasma membrane and the different
nucleocapsid structures described above.
     As a specific immunoreagent, we used polyclonal rabbit
antibodies which were raised against M protein purified by
SDS polyacrylamide gel electrophoresis and were affinity
purified on M protein coupled to CNBr-activated Sepharose 4B.
Purified antibodies were specific for M protein as tested by
immunoblotting procedures (data not shown). They were coupled
to 5 nm colloidal gold particles according to standard
methods (12). This conjugate was used on plasma membrane
fragments either before or after fixation with 1% glutaralde-
hyde. To enhance the observed frequency of nucleocapsid tight
coils on the plasma membrane, infected cells were incubated
with 15 μg/ml of Concanavalin A in the above buffer for 2
hours at 4°C and then rinsed as above. Figure 2 represents
views from the immunolocalization. The colloidal gold parti-
cles which appear as white dots on these reversed image
micrographs were only found on tortuous nucleocapsids and not
on the surface of tight coils, linear nucleocapsids, cyto-
skeletal elements, or the plasma membrane.

CONCLUSIONS

     Tortuous nucleocapsid was found at the bases of bullet-
shaped tight coils of varying lengths while the apex of their
nose cones, free of uncoiled nucleocapsid, were closely asso-
ciated with the plasma membrane. This consistant observation
suggests that the nucleocapsid coiling initiates at a mem-
brane attachment site and then proceeds unidirectionally with
nose cone formation followed by a helical cylinder. It
further suggests that the tortuous conformation is necessary
for the development of the tight coils or, conversely, is the
result of tortional stress induced by the tight coiling.
     The M protein is immunologically labeled only on the
tortuous nucleocapsids. Two different scenarios of nucleocap-
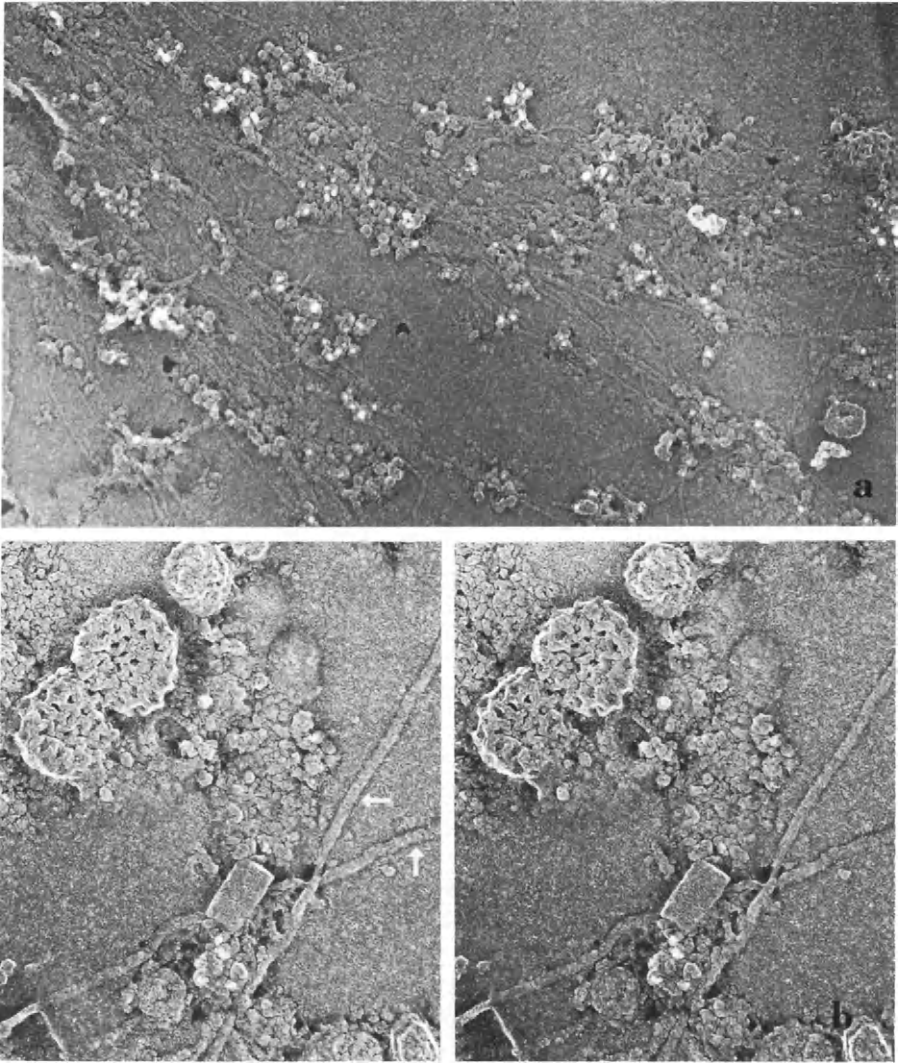
FIGURE 2. Immunolocalization of M protein on the cytoplasmic side of the plasma membrane. (a) low magnification view of linear and labeled tortuous nucleocapsids. Note the close association between the linear nucleocapsids and the membrane (72,000x). (b) stereo view of a tight coil and labeled tortuous nucleocapsid, arrows indicate fibers with the dimensions of intermediate filaments (120,000x).

sid coiling may have led to this M protein localization: twisting of nucleocapsids would expose sites on the nucleocapsid that enable the M protein to bind; alternatively, M protein could bind to membrane bound nucleocapsid and induce a twisted nucleocapsid conformation which would then favor tight coiling. Matrix protein could then cross-link adjacent turns in the helix. What molecular interaction would allow initial binding of M protein to nucleocapsid is not clear. We are currently investigating whether the cytoplasmic portion of the viral G protein has any role in binding of nucleocapsid to the plasma membrane. It is evident that our present results do not distinguish between these two scenarios. However, it is clear that under our experimental conditions the M protein cannot be localized on the inner side of the plasma membrane or on the surface of the tight coils and may therefore, not play a role in the association of nucleocapsids with membranes.

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INTERACTIONS OF VIRAL PROTEINS WITH MURINE LYMPHOCYTES[1]

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SYNTHETIC SUBUNIT VIRAL VACCINES

Progress in the development of synthetic subunit viral vaccines has been reported (1). Small peptides have been isolated from immunogenic proteins by enzymatic or chemical cleavage, tested for their ability to elicit antibodies that react with the isolated protein and the virion, their amino acid sequence determined, and the synthetic peptide made by the Merrifield technique (2). A number of investigators have used these procedures to synthesize biologically and chemically active peptides. For example, a synthetic peptide consisting of 20 amino acids corresponding to a portion of the MS-2 coat protein elicits antibodies which efficiently neutralize MS-2 infectivity (3); a number of synthetic peptides containing greater than 10 amino acids and corresponding to peptides of the surface protein of hepatitis B virus elicit antibodies in rabbits which precipitate the 23,000 and 28,000 dalton envelope proteins of Dane particles (4); synthetic peptides of the influenza virus hemagglutinin protein elicit antibodies that react with the complete hemagglutinin protein in an ELIZA test (5); and a 20 amino acid peptide of VP1 of foot and mouth disease virus will protect mice and guinea pigs against challenge with live foot and mouth disease virus (6). Thus, small synthetic peptides can be made which elicit antibodies that react with the complete antigen, and in some cases neutralize infectivity and protect animals against challenge with live virus. These synthetic immunogenic peptides should be useful as effective, inexpensive subunit vaccines.

For the development of these synthetic subunit vaccines a rapid, inexpensive in vitro assay system for determining the ability of isolated viral proteins and their peptides to stimulate the cells of the immune system would be useful. Recently it has been demonstrated that viruses and their isolated antigens can stimulate lymphocytes in vitro. This has been shown for herpes simplex virus types 1 and 2 (7,8,9), influenza virus (10) and its hemagglutinin (11), vesicular stomatitis virus and its glycoprotein (12,13,14), Sindbis virus and its glycoproteins (15), and Sendai virus and its glycoproteins (16). In vitro mitogenesis is a rapid, inexpensive assay system for measuring the ability of viruses, their isolated proteins and peptides to activate lymphocytes. The requirements for various cell populations and the cooperation of B and T cells in the mitogenic response are easily measured in this in vitro assay. Thus, it would be useful for studying the interaction of viral proteins and peptides alone, chemical modifications of these antigens and combinations of antigens to determine their ability to elicit a mitogenic response in vitro. Proteins and smaller peptides that are active in this in vitro mitogenesis assay can be tested either alone or attached to carrier molecules for immunogenicity in vivo. Those mitogenic peptides that neutralize infectivity will be candidates for synthetic subunit viral vaccines.

In this communication, we present data that show that the immunogenic glycoprotein (G) of VSV, cyanogen bromide peptides and tryptic peptides of the G protein activate murine spleen cells in vitro. In addition, we demonstrate that the purified F glyco-protein of Sendai virus is the major mitogen associated with the virion. The use of an in vitro assay system and the mitogenic activity of these proteins and their peptides as model systems for the development of synthetic subunit vaccines are discussed.

PEPTIDES OF THE VSV GLYCOPROTEIN

Mitogenic Activity of Cyanogen Bromide Peptides of the G Protein. Since we have established that the G protein is the major mitogen associated with VSV (13), we attempted to determine if peptides of the G protein are also mitogens. The G protein was isolated from purified virus by Triton X-100 extraction as previously described (13) and cleaved with cyanogen bromide according to the procedure of Stanworth and Turner (17). Cleavage of the G protein was monitored by PAGE on 14% polyacrylamide gels in the presence of 1% SDS and 1% 2-mercaptoethanol (18). Treatment with cyanogen bromide removed essentially 80-90% of the band migrating as G protein and yielded 9 to 10 peptides smaller than native G protein. This result is consistant with the potential number of cyanogen bromide peptides present in the G protein as determined by its amino acid sequence (19).

Various concentrations of the G protein or cyanogen bromide-treated G protein, along with known mitogens LPS, Con A, and VSV, were incubated with murine spleen cells and then assayed for

TABLE 1
LYMPHOCYTE ACTIVATION BY
CYANOGEN BROMIDE (CB)-TREATED G PROTEIN[a]

Mitogen	µg/Well	^{3}H-Thymidine Incorporation	
		Average CPM	S.I.[b]
None	– –	5,122	– –
LPS	10	69,285	12.2
Con A	0.12	109,265	21.3
HKCC-VSV	5	24,468	4.8
G Protein	1	17,418	3.4
	5	31,609	6.2
	10	55,972	10.9
CB-treated	1	21,020	4.1
G Protein	5	53,878	10.5
	10	37,875	7.4

[a]   Spleen cells from BALB/c female mice were used; standard error of the mean was less than 10%.
[b]   S.I.= stimulation index: cpm experimental/cpm control.

mitogenesis 48 hr after culture initiation. The data presented in Table 1 show that the mixture of cyanogen bromide peptides of the G protein is at least as active as the untreated G protein in the mitogen assay. At 1 and 5 µg/well, the mixture of cyanogen bromide peptides of G are more mitogenic than the same concentrations of untreated G protein, indicating that cleavage of the G protein changed the optimal concentration of G protein for mitogenesis from 10 to 5 µg/well. Both the G protein and the mixture of cyanogen bromide peptides of the G protein are more mitogenic than VSV, a result similar to our previous finding (13,14).

Mitogenic Activity of the Tryptic Peptides of the G Protein. The VSV glycoprotein, isolated as described above, was treated with an equal amount of trypsin. This treatment reduced all of the band migrating as the uncleaved G protein to peptides which were smaller than trypsin.   This result is consistent with the size of tryptic peptides present in the G protein (19).
To determine if tryptic peptides of the G protein are mitogens, various concentrations of untreated or trypsin-treated G protein were incubated with murine spleen cells and then assayed for mitogenesis. The data in Table 2 show that at 0.5 and 1.0 µg/well, a

mixture of tryptic peptides of the G protein are approximately 4 times as stimulatory as the same concentrations of untreated G protein, suggesting that cleavage has shifted the optimal concentration for mitogenesis from 10 to 1 µg/well. Alternatively, the lower mitogenic activity of trypsin-treated G protein at 5 and 10 µg/well may be due to the effect of trypsin on the lymphocytes. The data in the lower portion of Table 2 show that increasing concentrations of trypsin decrease mitogenesis, possibly by removing cell surface receptors for the mitogens.

The data from these experiments demonstrate that one or more of the cyanogen bromide and tryptic peptides of the G protein are mitogenic for murine spleen cells. We are presently attempting to isolate the cyanogen bromide and tryptic peptides in order to determine the mitogenic activity of individual peptides. On the basis of size and partial amino acid sequence data we should be able to determine which portions of the G protein have mitogenic activity. Mitogenic peptides will be injected into mice to determine their immunogenicity and their ability to elicit neutralizing antibodies for infectivity and mitogenicity of the virion.

SENDAI VIRUS GLYCOPROTEINS

We have demonstrated that a mixture of the Sendai virus glycoproteins HN and F are mitogenic for murine spleen cells (16). The mitogenic response of the glycoproteins has two components, a T cell-independent B cell response and a T cell dependent B cell response. To determine if these different mitogenic responses reside in one or both of these Sendai virus glycoproteins we have separated the two glycoproteins and begun to characterize their mitogenic activities.

Mitogenic Activity of the Sendai Virus HN Glycoprotein. The glycoproteins of Sendai viruses were isolated by solubilization in Triton X-100 and the HN glycoproteins separated from the F glycoprotein by adsorption to and elution from gluteraldehyde treated RBC (20). The adsorbed and eluted material contained only HN as judged by PAGE. Various concentrations of the purified HN glycoprotein, along with known mitogens, were incubated with lymphocytes from CBA/J mouse spleens and mitogenic activity was determined at various times after culture initiation. The data in Table 3 show that the isolated HN glycoprotein is mitogenic at 48 and 72 hr after culture initiation with 0.5 and 1.0 µg/well. However, the stimulation index was only slightly above 3.0, which is the lowest value considered significant.

Mitogenic Activity of the Sendai Virus F Glycoprotein. The F glycoprotein was isolated from Sendai virus as described above. PAGE of the unadsorbed material indicated that the F glycoprotein was the only Sendai virus protein present. Contaminants derived

TABLE 2
LYMPHOCYTE ACTIVATION BY
THE TRYPSIN-TREATED G PROTEIN[a]

| Mitogen | µg/Well | ^{3}H-Thymidine Incorporation | |
		Average CPM	S.I.[b]
None	- -	1,079	- -
LPS	10	33,691	31.2
Con A	0.12	133,580	123.8
G Protein	0.5	7,908	7.3
	1	10,982	10.2
	5	26,053	24.1
	10	41,156	38.1
Trypsinized	0.5	37,193	34.5
G Protein	1	41,521	38.5
	5	35,688	33.1
	10	25,785	24.0
Trypsin	10	3,136	2.9
	25	1,603	1.5
	50	814	0.8
	100	519	0.5

[a,b]Same as in Table 1.

from the gluteraldehyde-treated RBC were present, but were not mitogenic in our assay. Various concentrations of the F glycoprotein, along with standard mitogens, were tested for their mitogenic activity in mouse splenocytes. The data in Table 4 show that the purified F glycoprotein is mitogenic for murine splenocytes. Significant mitogenicity occurs at all concentrations and times tested. Optimal activity occurs at 72 hr after culture initiation with 0.5 and 1.0 µg/well. The stimulation index of approximately 14 is about 3 times greater than that for the HN glycoprotein and is comparable to that of the isolated VSV glycoprotein (14).

The data presented here suggest that the isolated F glyco-protein contains the majority of the mitogenic activity associated with a mixture of the HN and F glycoproteins reported earlier (16). We are currently attempting to determine if the isolated F glyco-protein stimulates B cells or T cells or both, and if T cell help is required as was demonstrated for the mixture of HN and F glyco-proteins (16). In addition, we will prepare peptides of the F glycoprotein and determine its mitogenic potential. Any mitogenic

TABLE 3
LYMPHOCYTE ACTIVATION BY HN GLYCOPROTEIN

Mitogen	μg/well	CPM (^{3}H)THYMIDINE INCORPORATION[a]			
		48 hr	S.I.[b]	72 hr	S.I.
None	– –	6,737	(–)	713	(–)
LPS	20	35,721	(5.3)	6,695	(9.4)
Con A	0.125	157,466	(23.4)	25,795	(36.2)
HN	1.0	24,780	(3.7)	3,417	(4.8
	0.5	20,003	(3.0)	2,533	(3.6)
	0.25	17,410	(2.6)	2,045	(2.9)
	0.125	18,826	(2.8)	2,995	(4.2)

[a]Spleen cells from CBA/J female mice were used; standard error of three replicate samples was less than 10%. [b]Same as in Table 1.

TABLE 4
LYMPHOCYTE ACTIVATION BY F GLYCOPROTEIN

Mitogen	μg/well	CPM (^{3}H)THYMIDINE INCORPORATION[a]			
		48 hr	S.I.[b]	72 hr	S.I.
None	– –	1,094	(–)	581	(–)
LPS	20	15,106	(13.8)	18,618	(32.0)
Con A	0.125	125,466	(114.7)	150,795	(259.5)
F	1.0	7,535	(6.9)	8,319	(14.3)
	0.5	6,645	(6.1)	7,827	(13.5)
	0.25	6,216	(5.7)	6,569	(11.3)
	0.125	5,869	(5.4)	4,264	(7.3)

[a,b]Same as in Table 3.

peptides will be tested for immunogenicity in vivo and their ability to induce antibodies that neutralize mitogenicity of the F glycoprotein and infectivity of Sendai viruses.

Isolation of mitogenic peptides and correlation with in vivo immunogenicity will be the first step in a rational approach to the development of synthetic viral subunit vaccines. The in vitro mitogen assay is rapid and inexpensive, and we have demonstrated that viral proteins and their peptides are active in the system. Those that are active in the in vitro system should be tested in vivo for immunogenicity. Those that are immunogenic, either alone or attached to a carrier, and are small enough to be synthesized, should be sequenced and then synthesized. With the rapid advances in peptide synthesis, the era of inexpensive, synthetic subunit vaccines is rapidly approaching.

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HOST RANGE MUTANTS OF PIRY VIRUS: A NEW TYPE OF MUTANT IN DROSOPHILA

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I will recall the principles of selection of host range mutants in Drosophila. Flies were inoculated with one effecti- ve viral unit (cloning conditions). The observed frequency distribution of individual incubation times began as a Gaus- sian one and was extended by a tail due principally to physio- logical heterogeneity of flies but also to genetic heterogene- ity of viruses. An attempt at selecting mutants in the head of incubation distribution has been unsuccessful. On the contrary selection in the tail gave a great variety of mutants(1).

As selection was based on incubation time, it seemed pos- sible to select mutants specific of symptom production; in fact all mutants I have studied were mutants restricted in their capacity to invade drosophila fly. These mutants were called agD (affected growth in Drosophila). The majority seems to have abnormal cellular cycle in all drosophila cells, but tissular specificity is possible.

There are mutants restricted specifically in some droso- phila strains. tsl (F1 clone) is one of them. tsl is submitted to a general restriction in all flies but is specifically hig- hly restricted by an allele of ref(3)A gene present in the Paris drosophila strain. A secondary mutant, obtained after one passage of F1 in a standard fly (hybrid Oregon ♀ x ebony ♂), F1SS4, appeared to have lost general restriction but re- tained specific one. Study of F1SS4 showed that in permissive drosophila strains incubation times are shorter than those of wild type (wt) original Piry virus. The same event has been observed a second time after a passage in an ebony fly of an unspecifically restricted agD mutant. Lastly, when I have searched directly for mutants restricted in some drosophila strains other than Paris strain, by selecting the tail of in- cubation distribution, an important fraction of mutants I found were more rapid than wt in appearance of symptom. I ha- ve interpreted these mutants as double mutants: a second mu-

tation which appeared and was selected during multiplication
of a restricted mutant might have an enhancing effect which
would not only rub out the effect of the first mutation but
also go beyond.
      We shall neglect the problem that these double mutants
had been obtained in all drosophila strains but Paris one, and
keep our attention on the fact that a new type of mutation
does exist which enhances the invading fly capacity of virus.
I shall call these mutants rgD (rapid growth in Drosophila).

      Direct Search of Single rgD Mutants. In the $\mathcal{S}$6 population,
multiplied in chick embryo fibroblasts (CEF), by selecting the
head of Paris flies' incubation-time distribution, I found
such a mutant out of 500 infective units (IU) approximately.
      The figure shows the relation of Mean Incubation Time
(MIT) in Paris flies to inoculum for the first rgD mutant PSP2
and the wt. Disposition of curves may be interpreted as resul-
ting of more rapid or more productive cellular cycle in all
cells (2).
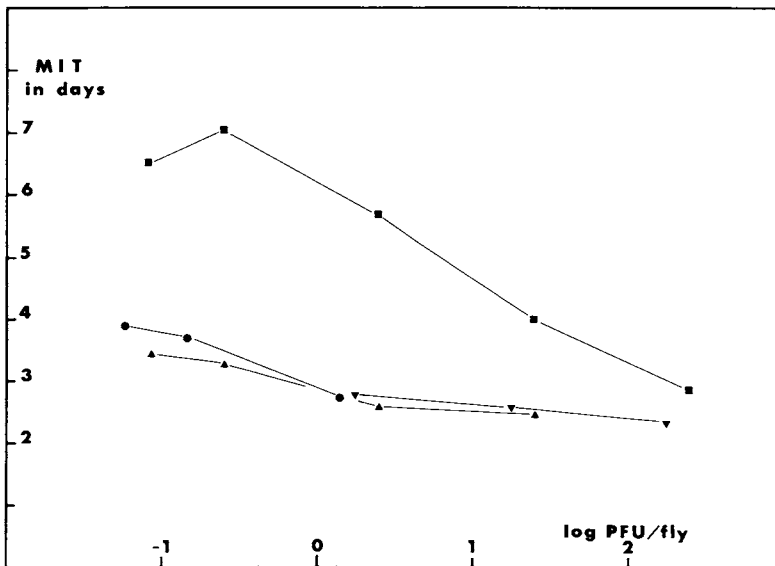
      FIGURE. Comparison of MIT, at 20° in Paris flies from the
same stock, for wt ■ , PSP2 ● and 20S3 ▲ mutants and for a
ts^{+} revertant of the last, 20S3r ▼ .

I noticed also a little increase (approximately 0.3 log unit) of the Relative Probability of Infection (RPI: ratio of the probability of infecting a fly to the probability of forming plaque in CEF) in Paris flies, but a great increase (1 log unit) in standard flies, so that RPI is the same for this mutant in Paris and standard flies (and in all strains used). I recall that Paris flies have been chosen as the most permissive for wt Piry virus, standard flies, hybrid Oregon x ebony, being the least. So, effects of diverse restrictive alleles (not still localized) on RPI are wholly rubbed out, but are still visible on incubation time.

The study of rgD mutants can be approached by two ways: genetic and physiologic; I have begun by genetical study.

GENETICAL STUDY OF rgD MUTANTS

Making up a Collection of Independent rgD Mutants. This collection obtained from 7 independent viral populations by selection in Paris and standard flies, is presented in table 1.

All nine rgD mutants have the same characteristics (MIT as a function of inoculum -see figure- and RPI) in Paris and standard flies, except F7S11 mutant. F7S11 shows peculiarities in Paris flies which may be interpreted as tissular specificity. Underlined mutants in table 1 are temperature sensitive (ts) in CEF (restrictive temperature 39°7); mutant 20S3 is leaky. Frequency of ts, 5/9, is high and exceeds random possibility (mean frequency of ts mutants in populations 5%), so that in some cases the two phenotypes rgD and ts should result from the same mutation. Reverting mutants for ts phenotype have been selected; for all ts,rgD mutants except 20S3 (see figure) this second mutation produces a reversion of the rgD phenotype.

TABLE 1

rgD MUTANTS SELECTED IN DIVERSE POPULATIONS MULTIPLIED IN CEF

Viral population	Selection in Paris	Selection in standard flies
S6	PSP2	S16
F7	-	F7S11 , F7S23
clone 12	-	-
clone 13	13P1	13S3
clone 14	-	-
clone 20	20P3	20S3
clone 21	-	21S3

TABLE 3
INTRACISTRONIC COMPLEMENTATION IN GROUP I

Mutant	Complementation by F4ts9	Complementation by 13P1	Complementation by S16
13P1	35	N.S.[a]	N.S.
S16	15.5	1.6	N.S.
VXER6	5.4	16.5	3.7
21PR7	4.0	17.7	1.6
F7S11	3.0	11.0 ; 8.4	N.S.
F11ts5	1.9	5.9	2.0
ts1(F1A)	1.9	63	52
F11ts8	1.2	104	39
14PR1	0.2	69	28
F4ts9	no compl.	144 ; 180	86
wt	"	69	45
ts20	"	81	39
F2ts6	"	100	49
20S3	"	49	-
13S3	"	50 ; 127	47

[a]N.S.: non significative
    Complementation by mutants is expressed as percent of
complementation by wt in the same experiment.

(for F4ts9 for example) and direct confirmation of homology
of established groups with VSV groups are in project.
    Group frequencies among spontaneous ts mutants, selected
as ts or agD, are similar to those observed for VSV Indiana
(recapitulated data in (7)). Concerning ts mutants selected as
rgD, difference in group frequencies has no signification be-
cause of the fewness of them; but the difference in frequency
of group I mutants complemented by F4ts9 (3/4 if one excludes
20S3, probably double mutant) is highly significative. Moreo-
ver, complementation between F4ts9 and 13P1 or S16 is very
effective in the two reciprocal ways (Table 3). Intracistronic
complementation in group I of rhabdoviruses is classical (8,7)
and high efficiencies have been observed with Chandipura (9).
    Table 3 gives results of group I intracistronic comple-
mentation experiments. All the mutants complemented by F4ts9
are presented and ordered in decreasing efficiency of comple-
mentation by F4ts9. Complementation by F4ts9, 13P1 and S16 is
expressed as percent of complementation by wt at comparable

multiplicity in the same experiment (irradiation 4000erg/mm^2).
We shall note that wt has a group I defect at 39°7 which is
efficiently complemented by irradiated wt virions (autocomple-
mentation). Four classes can be distinguished; 13Pl comple-
ments the majority of group I mutants as wt does (S16 in the
same class, appeared less effective), but a middle class
exists only partly complemented by both F4ts9 and 13Plmutants.
     Existence of this middle class is a second argument (the
first is the relation of efficiency of complementation by ir-
radiated wt to UV dose) to classify 13Pl and S16 in group I.
But 13Pl and S16 define clearly an under-group which corres-
ponds probably to a particular site of the L protein.

PHYSIOLOGICAL STUDY, PROJECTS.

     For a well host-adapted virus as wt Piry, especially in
Paris flies, there are many ways of losing its multiplying ca-
pacities, but certainly a few ones of becoming more efficient.
In fact rgD mutants actually appeared phenotypically all alike
(F7Sll seems to have a similar rapid multiplication but to be
restricted in a specific tissue commanding access to symptom
site).
     The efficiency of rgD mutations to correct (in fact over-
correct) diverse agD mutations and to correct effects of di-
verse restrictive alleles (especially for RPI) suggests that
mutations of this type enhance primary transcription. So, my
project is to study the cellular cycle of rgD mutants in cul-
tured drosophila cells and especially the primary transcrip-
tion.
     My provisional hypothesis (suggested by existence of
F7Sll and of a double mutant which seems rgD cryosensitive) is
that this enhancement is due to an association of L with an
host protein which renders transcription more effective. This
is only remaining to be proved.

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EARLY APPEARANCE AND CO-LOCALIZATION OF
INDIVIDUAL MEASLES VIRUS PROTEINS USING
DOUBLE-LABEL FLUORESCENT ANTIBODY TECHNIQUES

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In order to understand the sequence of events which
lead to the assembly of measles virus, it is necessary to
establish the precise time and location at which viral pro-
teins first appear and how they interact with one another
at the cell membrane during viral maturation. Norrby et al.
(1) used monoclonal antibodies directed against the hemag-
glutinin protein (HA), phosphoprotein (P), nucleocapsid
protein (NP), fusion protein (F) and matrix protein (M) of
measles virus and found differential localizations of some
of these proteins within infected cells. We have extended
these studies using mouse monoclonal and rabbit polyclonal
antibodies directed against individual viral proteins. Here
we present a sequential immunofluorescence analysis of the
emergence of viral proteins and, in some cases, their co-
localization at sites of viral assembly.
    Vero cells were infected with the Edmonston strain of
measles virus at an MOI of 10 which resulted in infection
of 90% of the cells and initiation of viral induced fusion
at 18 hrs after infection. Cells were fixed at 2 hr in-
tervals from 2 to 24 hrs after infection with periodate-
lysine-paraformaldehyde fixative (2), a fixative which pre-
serves both cell morphology and antigenic sites on pro-
teins.   To detect intracellular viral proteins, the cell
membrane was permeabilized by treatment with 0.05% triton-x
for 10 minutes.   The mouse antibodies used in indirect im-
munofluorescent experiments were monoclonals to the HA, NP,
P and M proteins (3, 4).   Two polyclonal antibodies were
raised in rabbits against: (a) purified HA protein and (b)
a synthetic 20 amino acid peptide (p20) constructed from
the nucleotide sequence of a region of the gene coding for

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the P protein (Bellini et al., this volume).   The F protein
was   not   investigated.   Conjugated   antibodies   used   for
double-labeling   experiments   were   goat   anti-rabbit   fluore-
scein   and   goat   anti-mouse   rhodamine.   None   of   the   anti-
bodies or conjugates stained uninfected cells.

Staining of the HA protein was first detected at 4 hrs
after infection (Fig. 1) in the form of a few bright gran-
ules and small dots scattered in the cytoplasm.   Prelimi-
nary EM analysis suggest that the small dots correspond to
HA   associated   with   the   membrane   of   smooth   vesicles
(Rickaert, unpublished observations).   By 6 hrs, HA protein
staining was predominantly found in the perinuclear area.
At 8 hrs, HA was localized throughout the cytoplasm and at
the cell surface where occasional punctate accumulation was
seen.   The   HA   glycoprotein   was   the   only   viral   protein
tested that could be stained in non-permeabilized cells or
in living cells.   NP was also first detected at 4 hrs in
bright dots scattered over the cytoplasm (Fig. 1).   With
time,   NP accumulated into larger and more abundant cyto-
plasmic dots.

Using   monoclonal   antibodies,   P   and   M   proteins   were
first detected at 8 hrs.   P protein formed bright cytoplas-
mic dots in a pattern similar to NP, while M protein stain-
ing was weak and diffuse.   After 8 hrs of infection, stain-
ing intensity of all proteins increased rapidly.

By   14   hrs   the   HA,   NP,   M   and   P   proteins   were   co-
localized into patches at the membrane, leaving surrounding
membrane areas free of stain.   Patches increased in number
and extent until cell fusion began at 18 hrs, and gradually
decreased thereafter.

Cytoplasmic   inclusions   known   to   contain   nucleocapsids
(5)   were   first   detected   at   12   hrs   after   infection.   Inclu-
sions could be stained with antibodies to NP and P proteins
but not with four different monoclonal antibodies to the M
protein (6-3 from W. Bohn; 16BB2, 16BB6 and 19CG6 from E.
Norrby).

Double-labelling of viral proteins at 18 hrs is shown
in Figs. 2 and 3 and the results are summarized in Table I.

Our results suggest that HA, a transmembrane glycopro-
tein, and NP protein, the major structural component of the
nucleocapsid, emerge simultaneously in the cytoplasm at 4
hrs postinfection and that HA is transported from ER and
Golgi regions to the surface within the next 4 hrs.   P,
another nucleocapsid associated protein, and M, the matrix
protein, were found later.   While it cannot be excluded
that P and M proteins may be synthesized at only low levels
early in infection, it seems more likely that their delayed
appearance may be more apparent than real.   Monoclonal
antibodies   bind   to   only   one   antigenic   epitope   and   with
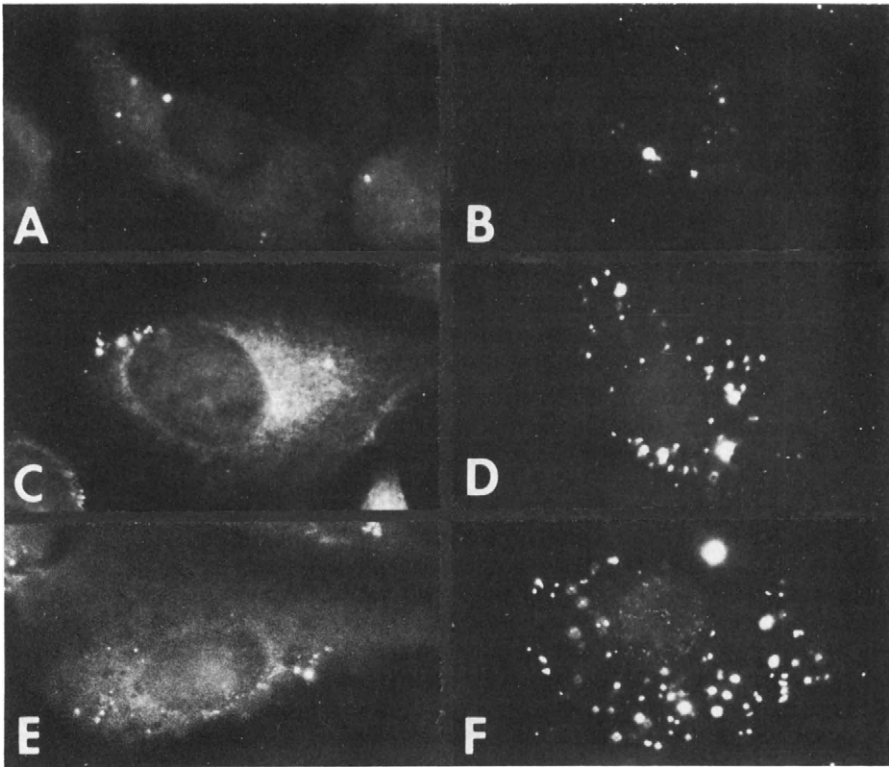
FIGURE 1. Immunofluorescence staining of HA and NP
proteins of measles virus in Vero cells at 4 hrs (A,B), 6
hrs (C,D) and 8 hrs (E,F) after infection. (A,C,E): mono-
clonal anti-HA; (B,D,F): monoclonal anti-NP.

TABLE 1
VIRAL PROTEINS IN CELLS 18 HOURS AFTER INFECTION

Immunofluorescent localization	Genome associated proteins		Envelope associated proteins	
	N	P	M	HA
Cytoplasm				
Inclusions	+	+	−	−
Diffuse small granules	−	−	+	−
Membrane				
Patches	+	+	+	+
Surface stain	−	−	−	+
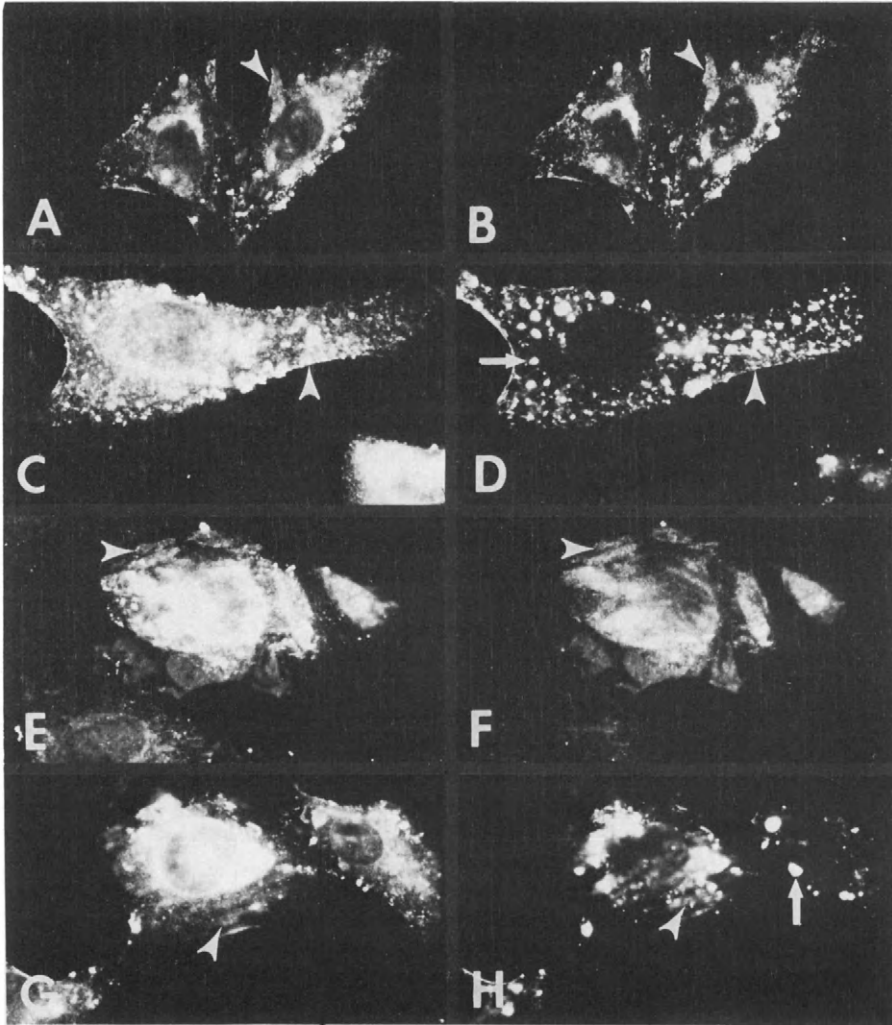
FIGURE 2. Double-label fluorescence in Vero cells 18 hrs after measles virus infection. Cells on left were stained with rabbit anti-HA (A, C, E, G) and identical cells on the right were stained with mouse monoclonal antibodies: (B) anti-HA, (D) anti-NP, (F) anti-M, (H) anti-P. Arrowheads indicate "patches" at membrane. Arrows indicate cytoplasmic inclusions. Note co-localization of all four proteins at membrane patches and lack of M staining of inclusions.
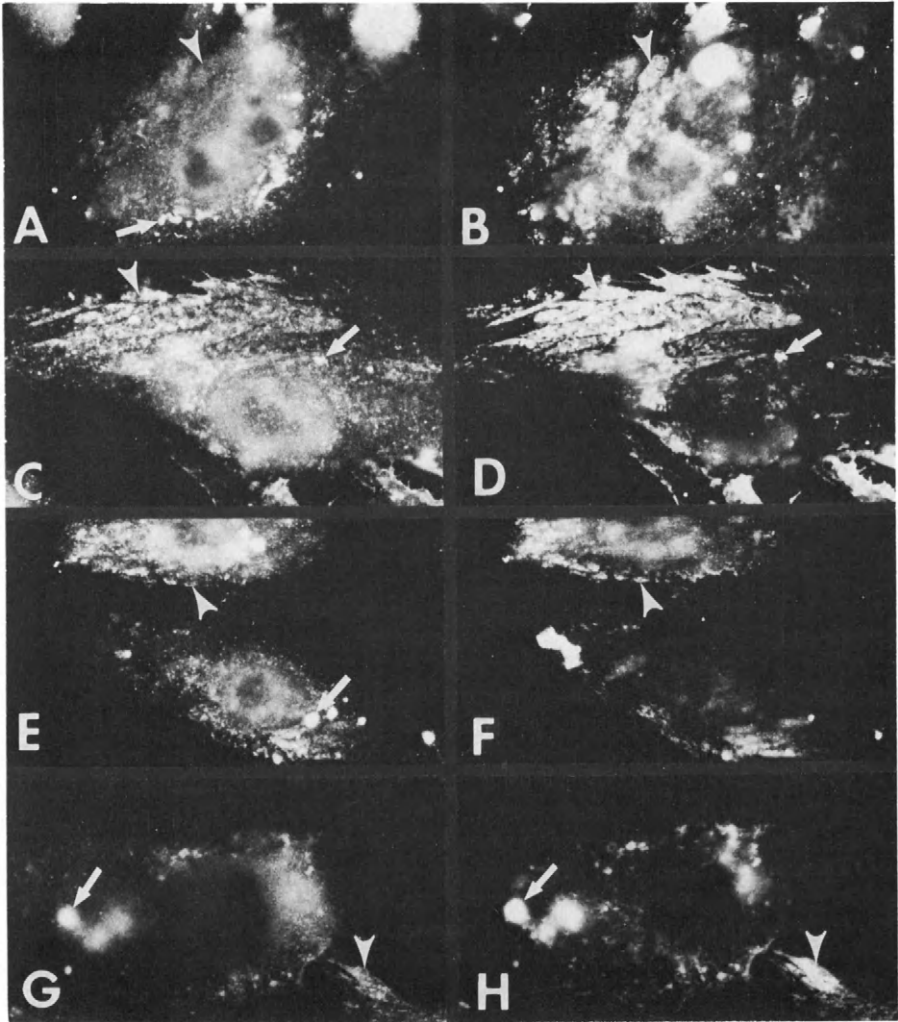
FIGURE 3. Double-label fluorescence in Vero cells 18 hrs after measles virus infection. Cells on left were stained with rabbit anti-p20 (A, C, E, G) and identical cells on right were stained with mouse monoclonal antibodies: (B) anti-HA, (D) anti-NP, (F) anti-M, (H) anti-P. Arrowheads indicate "patches" at membrane. Arrows indicate cytoplasmic inclusions. Note co-localization of all four proteins at membrane patches and lack of M staining of inclusions.

differing avidites. Monoclonal or polyclonal antibodies
with high affinity may be more sensitive probes for detec-
tion of early forms of P and M proteins. The most striking
observation at later stages of infection is the co-
localization of viral proteins in two main cellular sites:
(a) membrane patches containing NP, P, N and HA which prob-
ably represent active sites of transmembrane interactions
between these components in preparation for viral budding
(6); (b) cytoplasmic inclusions consisting of nucleocapsids
not incorporated into virion and containing NP and P pro-
teins but apparently not M. The interaction of M protein
with nucleocapsids in inclusions in paramyxovirus infec-
tions is controversial (7,8,9). Since M protein is known to
play a critical role in viral assembly (10), it is possible
that binding of M protein to nucleocapsid occurs only at
sites of viral budding at the plasma membrane.   EM
localization of M protein might elucidate this question.

ACKNOWLEDGMENTS

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CROSS-REACTION OF MEASLES VIRUS PHOSPHOPROTEIN
WITH A HUMAN INTERMEDIATE FILAMENT:
MOLECULAR MIMICRY DURING VIRUS INFECTION[1]

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Several mechanisms have been proposed to explain the
induction of autoimmunity in humans. One explanation is
infection by viruses. Viruses could induce autoimmunity
through shared determinants between virus structures and
molecules normally present on host cells, by deregulating
the host's immune system, or by causing the expression or
liberation of "normally sequestered" self antigens. Auto-
antibodies are commonly found in the circulation of virus-
infected individuals, both during and post infection. For
example, after infection with Epstein-Barr virus (1,2) anti-
bodies reacting with intermediate filament proteins, immuno-
globulin, or thyroglobulin could be detected. Similarly,
humans infected with hepatitis, herpes, mumps, or measles
viruses can produce antibodies to their own cytoskeleton
components (3-5), although the antibodies that reacted with
cytoskeletal proteins were not shown to bind to viruses (5).
Monoclonal antibodies (MAbs) provide useful reagents to
analyze unique determinants on viruses and on self constitu-
ents. During the derivation of such reagents we noted that a
large proportion of MAbs originally derived by immunization
of mice with purified measles virus react with self compo-
nents of human cells. We describe here one of these MAbs
with dual specificity; one that recognizes the phosphoprotein
of measles virus and an intermediate filament protein, M_r
52,000 found in uninfected cells.

Procedures. HeLa, Vero, L929, BHK_{21}, and human astrocyte cells were maintained in Eagles minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 1% glutamine and antibotics. Astrocytes derived from a human brain biopsy sample were positive for glial fibrillary acidic protein by immunofluorescent staining.

P3X63Ag8 mouse plasmacytoma cells (P3) were grown in Eagle's medium containing 10% FCS and 0.1 mM 8-azaguanine. Cloned hybridomas producing antibody to the measles virus nucleocapsid were from E. Norrby (Karolinska Institute, Stockholm) and those producing antibodies to the hemagglutinin were from D. McFarlin, (National Institutes of Health, Bethesda, MD). All other measles MAbs were generated after immunization of BALB/c mice with measles virus (6,7). The preparation, purification and use of measles virus (Edmonston strain) and the subsequent immunofluorescence, have been reported (8,9).

A preparation of Triton-X100-insoluble proteins (intermediate filament enriched) of HeLa cells was obtained as described by Pruss et al. (10).

SDS-gels were analyzed as described (8,9). Proteins from gels were transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) as reported by Towbin et al. (11). Strips were incubated with 5 ml of supernatant fluid from hybridoma or P3 cell culture (7) in 5 ml of 3% bovine serum albumin (BSA), 0.1% deoxycholic acid, 0.5% Nonidet P-40, 1% normal goat serum in PBS (binding buffer), or 20 μl of ascitic fluid derived from BALB/c mice injected with the hybridoma cells in 10 ml binding buffer. The strips were then incubated for 1 hr at 37°C, washed 3x with 3% BSA in PBS and incubated with 10 ml of binding buffer containing ^{125}I-labeled goat anti-mouse Ig. After 3 hr at 37°C, the strips were washed 3x with 10 ml of 1.5% BSA in PBS and dried overnight. Strips were mounted and exposed to Kodak X-RPI x-ray film the next day.

Detection of Dual-Reactivity by Immunofluorescence. The anti-measles virus MAb 2A-54-5, which reacted with measles virus infected and uninfected HeLa cells (Fig. 1), stained uninfected cells in a pattern similar to that reported by Franke et al. (12) for vimentin and cytokeratins, i.e., a network-like pattern in cells with a large cytoplasm-to-nucleus ratio (Fig. 1A, B). In contrast, a speckled pattern of labeling was observed with 2A-54-5 in large, rounded cells and staining was concentrated between dividing cells as shown in Fig. 2C and D. Similar patterns of reactivity with the MAb occurred in HeLa, Vero and the primary human astrocytes and was cell cycle dependent. No reactivity

was observed with BHK$_{21}$ or mouse L929 cells. Unlike the uninfected cells, cells infected with measles virus (Fig. 2A, B) showed a bright globular pattern that was more intense than the staining observed in uninfected cells. Many of the staining bodies could be seen by phase-contrast illumination as intracytoplasmic bodies (Fig. 2B). Immunofluorescent staining of uninfected cells with MAb 2A-54-5 was seen only when cells were fixed with acetone. This suggests that MAb 2A-54-5 reacts with a host specific determinant located inside the cell. The reactivity of MAb 2A-54-5 against infected cells could be removed by absorption with an intermediate filament preparation.
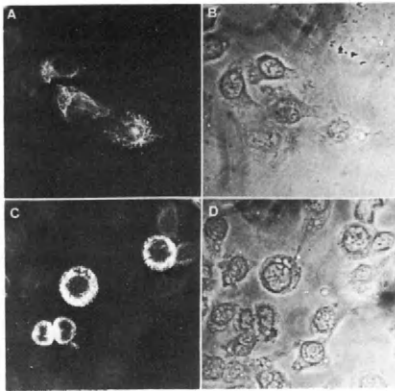
Figure 1. Network and speckled pattern of staining of uninfected HeLa cells (A-D) with antimeasles virus MAb 2A-54-5 (all x300). (A) Uninfected cells spread and stained in a network pattern. (B) Phase-contrast of A. (C) Uninfected cells in mitosis showing characteristic speckling. (D) Phase-contrast micrograph of cells in C.
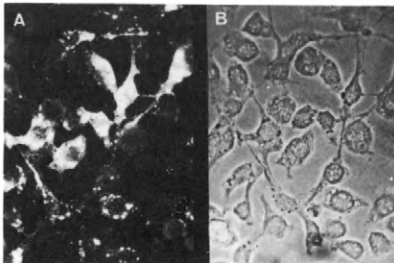
Figure 2. Globular staining of measles virus infected HeLa cells with MAb 2A-54-5. (A) Globular staining of the cytoplasm of measles virus infected cells. (B) Phase-contrast of the same field as in A. Note the cytoplasmic inclusion bodies.

Biochemical Characterization. We first identified the
cytosol component of measles virus infected cells that MAb
2A-54-5 detected. The results are shown in Figure 3. A
band at M_r 70,000 is visible in infected cell cytosol (lane
3) but not in uninfected preparations (lane 4). This band
migrated with the phosphoprotein of measles virus (lane
5). Cytosols from uninfected (not shown) or infected
cells failed to react with the second antibody alone or P3
supernatant fluid (lanes 1 and 2).
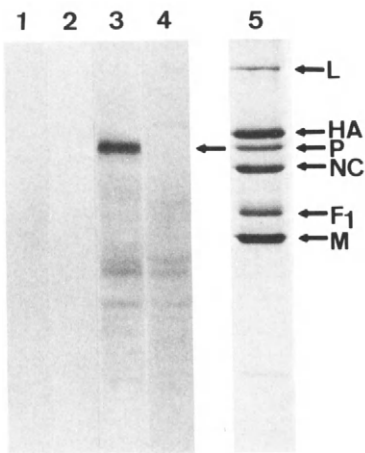
Figure 3. Immunoblots of re-
action of measles virus P pro-
tein with MAb 2A-54-5. Lanes:
1, infected cell cytosol trans-
ferred to nitrocellulose paper
incubated with labeled 2nd
antibody only; 2, the same in-
fected cell cytosol prepara-
tion incubated with P3 fluid
and then with labeled 2nd anti-
body; 3 and 4, infected cell cy-
tosol and uninfected cell cy-
tosol, respectively, incubated
with MAb 2A-54-5 and with lab-
eled 2nd antibody; 5, marker
purified measles virus.

Because cytosol preparations contain small amounts of
intermediate filaments, Triton-insoluble material (interme-
diate filament-enriched) from HeLa cells was prepared and
electrophoresed on SDS_4-gels. The proteins were then trans-
ferred to nitrocellulose paper and incubated with MAb spe-
cific for either measles virus phosphoprotein (3-60-5) or
HeLa cytoskeleton (3-3-2), and MAb 2A-54-5. As detected by
immunofluorescence, MAb 3-60-5 reacted with infected cells
in a globular pattern but not with uninfected cells, whereas
MAb against HeLa cytoskeleton (3-3-2) reacted with uninfected
and infected cells in the network-like staining pattern
indicative of intermediate filaments. MAb 3-60-5 did not
react with the Triton-insoluble preparation (Fig. 4, lane
2). MAbs 3-3-2 and 2A-54-5 both reacted with a M_r 52,000
protein (Fig. 4, lanes 3 and 4). Thus, MAb 2A-54-5 (Fig. 4,
lane 4) reacts with the same or similarly migrating interme-
diate filament protein as the 3-3-2 MAb (Fig. 4, lane 3).
The MAb against measles virus phosphoprotein (Fig. 4, lane
2) does not react with the M_r 52,000 protein and thus does
not recognize the same determinant as 2A-54-5.
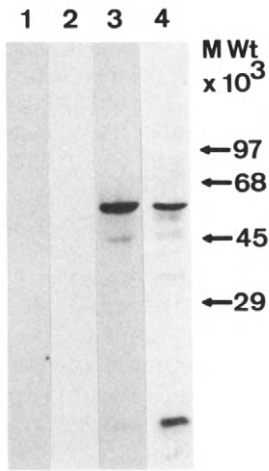
Figure 4. Immunoblots showing reaction of MAbs to intermediate filaments of HeLa cells. Lanes 1-4 represent strips to which intermediate filaments proteins were bound incubated with various MAbs followed by 2nd antibody. Lanes: 1, P3 fluid was incubated with the strip followed by labeled 2nd antibody; 2, strip incubated with MAb specific for measles virus P protein (3-60-5); 3, strip incubated with MAb to intermediate filaments (3-3-2); 4, strip incubated with MAb 2A-54-5. The prominant band is M_r 52,000.

To determine whether the phosphoprotein of measles virus detected by MAb 2A-54-5 was present both in the cytoplasm of infected cells and in virions, purified measles virus was disrupted, electrophophoresed on SDS gels, and the proteins were transferred to nitrocellulose paper and blotted (Fig. 5). No reaction was seen when the strip was incubated with labeled second antibody alone (lane 1), with P3 supernatant fluid (lane 2), or with MAb to intermediate filament (lane 3). MAb to measles virus nucleocapsid reacted with the nucleocapsid protein (lane 4). Reaction with measles virus phosphoprotein was seen when 2A-54-5 was incubated with the strip (lane 5). MAb to measles virus hemagglutinin reacted specifically with the viral hemagglutinin protein (lane 6). Lanes 7 and 8 are MAb 2A-54-5 and 3-60-5, indicating recognition of the same polypeptide.

In attempting to produce MAbs to measles virus, we obtained stable clones that secreted antibody reactive both with intermediate filaments of normal cells and with the phosphoprotein of measles virus. The M_r 52,000 intermediate filament protein was obtained as insoluble material from cells treated with high salt/Triton X-100 buffers and is probably vimentin or a cytokeratin (12). Our staining patterns with 2A-54-5 are similar to those presented by Franke et al. (12) who described the rearrangement of cytokeratins and vimentin during the cell cycle. The cross-reaction of the intermediate filament protein with the phospho-
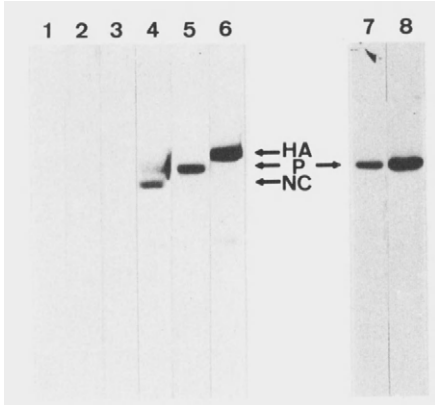
Figure 5. Immunoblots showing reaction of MAbs with purified measles virus. Lanes 1-8 are strips to which purified measles virus proteins are bound. Lane 1, reaction with the second antibody alone (affinity-purified ^{125}I-labeled goat anti-mouse Ig); the remaining strips were incubated with various MAbs and then the second antibody. Lanes 2, reaction with P3 supernatants; 3 MAb to intermediate filaments (3-3-2); 4, measles virus nucleocapsid MAb; 5, MAb 2A-54-5; 6, MAb to measles virus hemagglutinin; 7 and 8, MAb 2A-54-5 and MAb to measles virus P protein (3-60-5), respectively.

protein of measles virus was detected using immunofluorescence and immunoblotting. The 2A-54-5 MAb reacted with the phosphoprotein of measles virus both from infected cells and from purified virus. It is interesting that many of the intermediate filament proteins are phosphorylated as well as the phosphoprotein of measles virus.

There are two alternative explanations for the observed antigenic relationship between vimentin or cytokeratin and viral proteins. First, these cellular proteins may have certain sequences (or surface configurations) of amino acids in common with measles virus phosphoprotein. This relationship may be entirely fortuitous. Alternatively, parts of intermediate filament proteins may become incorporated into viral proteins in the course of virus replication. The observations of Blase et al. (13) and of Dulbecco et al. (14) on cross-reactive determinants between vimentin and tropomyosin (13) as well as vimentin and Thy-1 (14) and of Pruss et al. (10) of common antigenic determinant shared by all classes of intermediate filaments support the concept of molecular mimicry. This notion of molecular mimicry discussed by Lane and Koprowski (15) has several important biological implications. Recently Wood et al. (16) described an antibody that reacts with neurons, cardiac muscle cells, and Trypanosoma cruzi. Those investigators suggested that a common antigen seen by this antibody may be important in the pathogenesis of trypanosomiasis. A similar scenario may occur with viruses that share epitopes with cellular proteins. In this instance,

an immune response against virus may result in formation of
cross-reacting antibodies that bind to "normal" cell surface
determinants and result in the modulation of cellular function
(17,18) or lysis of the cell with complement or lymphocytes
bearing an Fc receptor. Cells lysed by virus infection or
immune attack expose intracellular determinants. The reaction
of autoantibody with intracellular components might yield
secondary pathogenic sequalae during viral infection. Hence
the reaction of autoantibody with self molecules may increase
injury at the site of viral infection or at distant sites due
to trapped immune complexes (19).
     Autoimmunity associated with virus infection could occur
by several mechanisms in addition to "molecular mimicry". Vi-
ruses may act as polyclonal B cell activators (20-26), there-
by expanding a preexisting clone that reacts with "self". Vi-
ruses may also cause the shedding of antigens not ordinarily
seen by the host immune system (27). Similarly, viruses can
potentiate or augment the response to many antigens (28,29).
Any of these mechanisms may explain the appearance of auto-
antibodies during and after virus infections (1-5, 30) and
perhaps the origin of many so-called "natural" antibodies
(31-33).

ACKNOWLEDGEMENTS

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MEASLES VIRUS INFECTION OF HUMAN PERIPHERAL BLOOD
LYMPHOCYTES: IMPORTANCE OF THE OKT4[1] T-CELL SUBSET[1]

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INTRODUCTION

    Measles virus has been know to infect human lymphocytes
and studies employing mainly lymphoblastoid cell lines have
indicated that both T and B cells can be infected
(1,2,3,4,5). Lucas (6) has shown that measles virus can
produce a silent infection in unstimulated peripheral blood
lymphocytes (PBLs). We have reported previously, that
infection of unstimulated PBLs results in the production of
high levels of virus-induced alpha interferon associated with
low levels of detectable virus (5). Expression of viral
antigens on cell surface membranes was induced by either
incubation of these infected cells with a mitogen or with a
non-mitogenic anti-leukocyte interferon serum that
neutralized the virus-induced interferon. These treatments
produced a concommitant increase in the production of
infectious virus. To extend our previous observations we
have investigated the replication of measles virus in T-cell
subsets. We will show that measles virus preferentially
infects the T-cell subset with an OKT4 (helper/inducer)
phenotype. Infection of purified T-cell subsets results in
higher viral yields form OKT4^{+} cells compared to simarlarly
infected OKT8^{+} lymphocytes (suppressor/cytotoxic
subpopulation). The decreased measles virus titers from
infected OKT8^{+} cells is associated with an increase in the
production of virus-induced alpha interferon. Finally, the
observation that measles virus preferentially replicates in
the OKT4^{+} T-cell subset is discussed in terms of the
importance of this T cell population in measles
virus-lymphoctye regulation with particular emphasis to
Multiple Sclerosis.

RESULTS

    Human PBLs isolated on Ficoll-Hypaque gradients and
infected with the Edmonston strain of measles virus at an MOI
of 1.0 were incubated with a 1:500 dilution of a potent
anti-leukocyte interferon serum for 6 days. At that time
cells were stained for the presence of cell associated
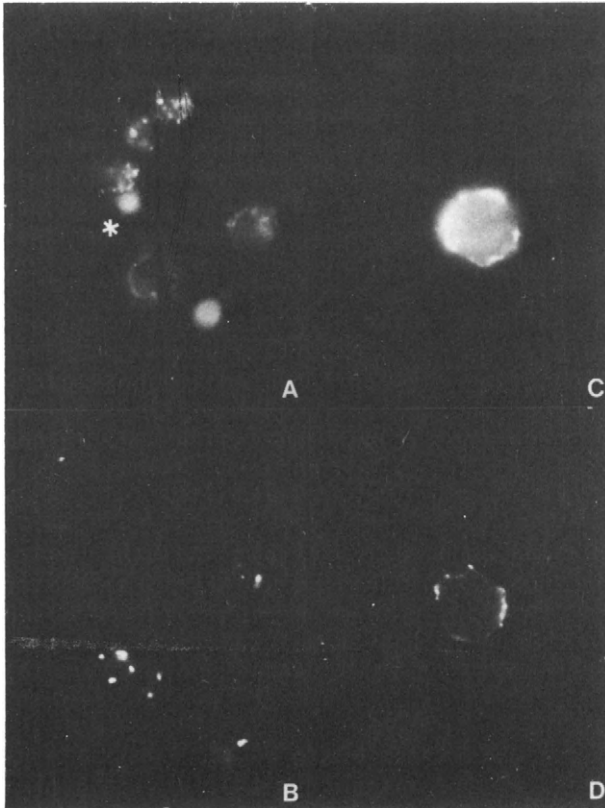
    Figure 1. a) RITC-human anti-measles virus, b) same
cells, FITC-OKT8, c) RITC-human anti-measles virus, d) same
cell, FITC-OKT4

measles virus antigens (human SSPE serum coupled to rhodamine (RITC) conjugated goat anti-human IgG) and phenotypic expression of either OKT4 or OKT8 determinants (mouse OKT4 or OKT8 antisera coupled to fluorescein (FITC) conjugated to sheep anti-mouse IgG). Using immunofluorescence procedures any cell expressing measles virus antigens stained red and could be classified as an OKT4 or OKT8 cell (staining green). These results are shown in Figure 1.

The use of the anti-interferon serum enabled us to detect measles virus antigens on infected PBL cell membranes (5). In Figure 1, the cell infected with measles virus (Panel C) was the same cell reacting with the OKT4 antibody (Panel D). Infected PBLs were rarely of the OKT8 phenotype. The asterisk in Figure 1, Panel A denotes the presence of the OKT8$^+$ cell in Panel B. None of the infected cells in Panel A were stained by the OKT8 antibody (Panel B). These data would suggest that measles virus infection of human PBLs was preferential for the OKT4$^+$ T-cell subset relative to the OKT8 population.

To confirm this T cell preference for measles virus replication we isolated purified populations of OKT4 and OKT8 cells. We employed a lymphocyte panning procedure whereby sheep resette forming cells (E$^+$) were positively selected for either OKT4 or OKT8 by incubation with the appropriate monoclonal antibody and subsequently plated on plastic plates coated with an F(ab')$_2$ rabbit anti-mouse IgG. Adherent cells were removed and the surface phenotype of these lymphocytes were determined on the FACS-IV Fluorescence-Activated Cell Sorter. The purity of these T-cell populations are shown in Table 1.

TABLE 1
PURITY OF HUMAN T-CELL SUBSETS: ANALYSIS BY FLUORESCENCE-
ACTIVATED CELL SORTER

	OKT8+	OKT4+
OKT3[a]	96%	95%
OKT4	5%	94%
OKT8	93%	5%

[a] All determinations are relative to a negative control of mouse ascites fluid produced by the P3X63 myeloma cell line.

TABLE 2
MEASLES VIRUS PRODUCTION IN HUMAN T-CELL SUBSETS (PFU/ml)

	OKT8+	OKT4+
Unstimulated [a]	8.0×10^3	2.7×10^4
Pokeweed mitogen	3.5×10^4	4.0×10^5
Anti-leukocyte interferon	8.0×10^3	2.3×10^5
Mock anti interferon	8.3×10^3	2.1×10^4

[a] Lymphocyte subsets cultured at a concentration of 2 x 10^6 cells/ml. Virus harvested at 4 days post infection. PWM - final concentration 1:50; Anti-interferon - final concentration 1:500; mock anti-interferon - final concentration 1:100.

   To determine if measles virus can preferentially replicate in one T-cell subset, purified T cell populations were infected at an MOI of 1.0 and incubated with either PWM, an anti-interferon serum, a mock anti-interferon preparation or left unstimulated. Four days later supernatants were harvested and assayed for infectious virus. Unstimulated, "silently" infected T-cell subsets produced low levels of virus although even in this group there was an indication that more virus was produced in the OKT4^{+} population (Table 2). Stimulation of infected PBLs with PWM had been shown to augment the production of measles virus (5,6). The stimulatory effect of PWM on these infected T-cell subsets was greatest for the OKT4^{+} cells compared to the OKT8^{+} population. The addition of a non-mitogenic anti-leukocyte interferon serum to "silently" infected PBLs also activated these lymphocytes to produce large amounts of virus (5). Incubation of infected T cell subsets with this anti-interferon serum resulted in no increase in virus production from infected OKT8^{+} cells and an approximately one log increase in infectious measles virus from the infected OKT4^{+} population (Table 2). A control serum with no antibody to interferon had no effect on virus production and virus titers from this group were similar to unstimulated, infected T-cell subsets. Again, even in this latter group, more virus was produced from infection of OKT4^{+} cells. These data coupled with the immunofluorescent pattern seen with infected PBLs (Figure 1) strongly suggest that measles virus appears to replicate preferentially in the OKT4^{+} T-cell subset relative to the OKT8^{+} population.

TABLE 3
ALPHA INTERFERON PRODUCTION IN MEASLES VIRUS INFECTED
HUMAN T CELL SUBSETS (INTERFERON UNITS/ml)a

	OKT8+	OKT4+
Unstimulated b	320	160
Anti-leukocyte interferon	0	0
Mock anti- interferon	320	160
Uninfected	0	0

a One laboratory unit of interferon is equal to .05
international units.

b Lymphocytes cultured at a concentration of 2 x 10^6
cells/ml; supernatants assayed for interferon at 4 days post
infection.

We had shown that low levels of infectious measles virus
produced by unstimulated, infected PBLs was associated with
the production of alpha interferon (5). We wished to
determine if increased levels of interferon of infected
OKT8+ cells could also be associated with low yields of
measles virus from this T-cell subset. Purified T-cell
subpopulations were infected with measles virus at an MOI of
1.0 and supernatants were harvested 4 days later to be
assayed for interferon. Interferon was determined by CPE
reduction of an EMC virus challenge on GM2504 cells. The
results are shown in Table 3. Unstimulated, measles virus
infected OKT8+ cells consistently produced twice as much
alpha interferon as comparably treated OKT4+ cells.
Incubation of these lymphocytes with an anti-leukocyte
interferon preparation (which augmented the production of
infectious measles virus from infected OKT4+ cells, Table
2), completely neutralized this virus-induced interferon. A
control antisera had no inhibitory effect. Unstimulated
cells produced no interferon (Table 3).

DISCUSSION

The purpose of this study was to examine measles virus
replication in lymphocyte subpopulations. Numerous reports
have indicated that measles virus can persist in
lymphoblastoid cell lines with either B or T cell
characteristics (1,2,3,4). Infection of peripheral blood
lymphocytes (PBLs) may represent a more relevent model for
the study of measles virus replication in vivo. Studies have

shown that measles virus can replicate in human PBLs
(1,2,5,6,7). Unstimulated, infected PBLs produced low levels
of infectious virus and failed to express viral antigens on
cell surface membranes. These cells produced high levels of
virus-induced alpha interferon (5). Treatment of these
silently infected PBLs with mitogen or an anti-leukocyte
interferon serum produced a productive infection.

    To extend these findings the present studies have
examined measles virus replication in T-cell subsets. Our
results would suggest that measles virus preferentially
replicates in T lymphocytes with an OKT4$^+$ phenotype. This
is based on two observations. First, by employing a
dual-labelling immunofluorescent procedure we were able to
detect measles virus antigens associated predominantly in
those infected PBLs co-staining with the OKT4 monoclonal
antibody and not OKT8 (Figure 1). Secondly, infection of
purified T cell subsets (Table 1) resulted in higher virus
yields from OKT4$^+$ cells compared to infected OKT8$^+$ cells
(Table 2). Taken together, these data strongly suggest that
the preferred lymphocyte population for measles virus
replication is the OKT4$^+$ T cell subset.

    We had demonstrated previously that virus-induced alpha
interferon was associated with measles virus persistence in
PBLs (5). Infection of purified T-cell subsets also induced
alpha interferon (Table 3), although considerably less than
infected PBLs. In the percent study, separated T cell
populations were greater than 90% pure when stained by the
monoclonal antibody to which it had been positively selected
(Table 1). Less than 1% of the cells in each purified
population reacted with monoclonal antibodies that detect
macrophages (data not shown). It is known that macrophage
and NK cells are important co-factors in the production of
interferon in PBLs (8). This may account for the higher
yields of alpha interferon from infected PBLs (5) compared to
similarly infected T cell subsets (Table 3). Our data also
indicated that OKT8$^+$ cells produced twice as much
virus-induced alpha interferon as infected OKT4$^+$ cells
(Table 3). This may be associated with lower virus yields
from OKT8$^+$ cells (Table 2), however it could not fully
account for this phenomena since an anti-leukocyte interferon
serum which augmented the production of measles virus from
infected-PBLs (5) and purified OKT4$^+$ cells had no effect on
infected OKT8$^+$ cells (Table 2).

    The ability of measles virus to replicate in OKT4$^+$ T
cells can permit some interesting speculation. Firstly, this

may suggest that measles virus is capable of persistence in T
cells with an OKT4 phenotype. Immunological activation of
these cells might then specifically augment the immune
response to this virus. Secondly, antigen presentation may
occur on the surface of OKT4 cells allowing recognition of
viral antigen in association with histocompatability antigens
that occur on this subset. Lastly, it has long been
suggested that measles virus may play a role in the
pathogenesis of Multiple Sclerosis (9,10) a disease in which
abnormalities of T-cell subsets have been reported
(11,12,13,14,15,16,17). With regards to this present study,
OKT4$^+$ cells have been implicated in MS both with augmented
amplifier activity for specific antibody production (9,18)
and possibly with lesion progression (19,20). The
significance of these observations to our findings is
uncertain at this point.

ACKNOWLEDGMENTS:

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TREATMENT OF EXPERIMENTAL MUMPS VIRUS MENINGOENCEPHALITIS USING MONOCLONAL ANTIBODIES

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INTRODUCTION

Viral invasion of the central nervous system (CNS) is com-
mon in the course of mumps. While this is usually subclinic-
al, mild signs and symptoms of meningeal involvement are not
infrequent and encephalitis with severe CNS damage does occur
(1). There is some evidence that the administration of hyper-
immune immunoglobulin (Ig) beneficially modifies the subse-
quent course of human mumps (2), and in a newborn hamster mod-
el of mumps menigoencephalitis, the development of specific
humoral immune responses to mumps virus correlates well with
the fall of free virus content in brain (3). Given the avail-
ability of monoclonal antibodies to mumps virus (4), we sought
to determine whether or not the passive administration of such
defined Ig could exert a protective effect on suckling ham-
sters previously infected with an otherwise lethal dose of a
neurovirulent mumps virus strain, and to determine the mecha-
nism of any observed protection. Our data show that some mon-
oclonal antibodies have therapeutic potential against other-
wise fatal viral infections of the CNS.

MATERIALS AND METHODS

Virus. The Kilham strain of mumps virus was plaque puri-
fied three times, expanded in CV-1 cells and aliquots stored
at -70°C prior to use. Stock virus contained $10^{7.1}$ plaque
forming units (PFU)/ml when assayed on CV-1 cell monolayers as
previously detailed (5). The virus inoculum used contained
$128LD_{50}$ as determined from survival data at day 14 after inoc-
ulation of newborn hamsters with serial decimal dilutions of

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this virus stock.

Animals. Pregnant multiparous Syrian hamsters were obtained from Charles River (Wilmington, MA) during the second week of gestation and housed separately. All pups were under 24 hrs. of age when inoculated with 25ul virus into the parietal region of the right hemisphere. Treatments were administered in 0.1ml volumes by intraperitoneal (IP) injection at the various times indicated. The treatment protocol was not varied within a given litter, only between litters which were randomly selected for a given treatment. Census was taken daily. Deaths occuring within the first 72 hrs. of life were attributed to non-specific causes and excluded from both experimental and control groups from further analysis. Animals removed from the litters for histologic or virologic analyses were handled as censored data at the time of harvest. All censored animals were randomly selected appropriate with the experimental protocol. For most experiments, observations were terminated between 40 and 50 days after infection, at which time all surviving animals were censored.

Monoclonal Antibodies. Mouse monoclonal antibodies were generated and characterized as previously detailed (4), and are designated by the specific virus polypeptide which they precipitate, their order of isolation, and IgG isotype. The antibodies were either purified from tissue culture supernatants with protein A sepharose or ascitic fluids from Balb/C mice were used directly.

Virus Titers. Clarified 10% homogenates (wt/wt) of individual hamster brains were prepared in Hank's basic salt solution (HBSS) from four animals at each of the indicated time intervals as previously detailed (3) and stored at $-70^{\circ}C$ until titered. All plaque assays were performed in triplicate on CV-1 monolayers (5).

Morphology. Eight experimental and two control animals were killed by perfusion-fixation with 4% paraformaldehyde solution on days 4, 6 and 10 after infection. The brains were sectioned coronally and embedded in paraffin for standard histology and immunohistochemical localization of virus antigen. Mumps virus nucleocapsid protein was localized on deparaffinized sections using NP4-1k ascitic fluid as primary antibody at a 10^{-5} dilution. Goat anti-mouse IgG conjugated to horseradish peroxidase was used at a 10^{-2} dilution as seeking antibody in an indirect technique. The reaction product was generated with diaminobenzidine as previously detailed (6).

     Statistics. Survival curves were constructed according to the method of Kaplan and Meier and treatment dependant differences between the survival curves sought using a nonparametric comparison test for multiple samples with censored observations (7). Group contrasts were performed using an overall level of significance ≤ 0.01 in all analyses.

     Distribution of Labeled Monoclonal Antibody. HN2-2ak purified on protein A sepharose was radiolabeled with ^{125}I using iodogen, and free iodine removed by a second purification on protein A sepharose with extensive washing of the column prior to elution of the iodinated IgG. Two infected pups each were injected with equal amounts of iodinated antibody, either 24 or 80 hrs. after infection and killed 24 hrs. later by perfusion-fixation with 4% paraformaldehyde solution. Brains and other organs were then removed and autoradiographs prepared (8).

RESULTS

     Newborn hamsters inoculated with an intracerebral dose of $10^{5.5}$ PFU of plaque purified Kilham strain mumps virus and treated with a single 0.1ml IP injection of HBSS alone developed uniformly fatal disease (Fig. 1).

The slope of the survival curve for control animals was both reproducible and quite steep. Fifty percent of the control animals were dead by day 11 of the infection and there were no survivors beyond day 12 in the cumulative control group of 127 animals, 80 of which were uncensored. However, the single IP inoculation of relatively small amounts of selected monoclonal antibodies was shown to shift the survival curves markedly to the right, while other monoclonal antibodies showed little or no beneficial effect. The monoclonal antibody HN-2ak has hemagglutination inhibition (HAI) and neutralization (Nt) activities when measured in vitro and immunoprecipitates the 78K mol.

FIGURE 1
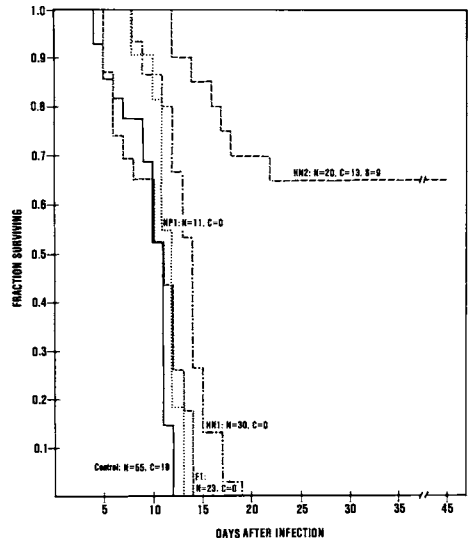
wt. glycoprotein of mumps virus. In vivo, a single IP inoc-
ulation of 100ug of HN2-2ak monoclonal antibody 12 hrs.
after infection showed considerable protective effect (p $\leq$
0.001) with 65% of treated animals surviving to 45 days af-
ter infection (Fig. 1). Some animals surviving the acute in-
fection appeared to die from obstructive hydrocephalus. This
was especially common at or about the time of weaning (day 22-
24). However, most animals which survived to day 40 showed
neither altered appearance or behavior. In other experiments,
selected animals were followed for more than three months.
Some of these proved endrocrinologically intact as evidenced
by pregnancy and subsequent healthy litters.

In contrast, a single treatment with 100ug of the mono-
clonal antibody HN1-3k which also precipitates the 78k mol.
wt. glycoprotein and has measurable HAI and Nt activity in
vitro exerted a statistically significant protective effect
(p $<$0.001) but did not produce long term survivors. Finally,
single 100ug injections of the monoclones F1-2ak, directed to
the 60K mol. wt. fusion glycoprotein, and NP1-2ak, directed
against the 68k mol. wt. nucleocapsid protein, both of which
lack measurable biologic activity in vitro, failed to benefi-
cially effect the survival curve (Fig. 1).

The effect of a single IP treatment with 100ug of
HN2-2ak, $10^{3.5}$ units of antibody as determined by end
dilutional ELISA, given at different times after the
initial infection was then
explored. The most drama-   FIGURE 2
tic effects were seen with
treatment 12 hrs. after
infection with 65% of the
animals surviving through-
out the period of obser-
vation. However, treat-
ment at 24, 36 and 48
hrs. after infection also
prolonged survival over the
HBSS treated control group
(p $\leq$ 0.001). It therefore
appeared that the course of
an established virus
encephalitis could be
substantially modified
with selected monoclonal
antibodies.

The above studies were
all undertaken with mouse
monoclonal antibody purified
from tissue culture super-

natants. This limited the quantity of antibody available for
study. Neither HN1-3k nor HN2-2ak grow well in the peritoneal
cavity of Balb/C mice. Therefore, a new monoclone was select-
ed for the remaining studies. This monoclone, HN8-1k also has
HAI and Nt activity in vitro. The effect of varying treatment
dosage was explored with a single IP injection given 24 hrs.
after the standard infection.
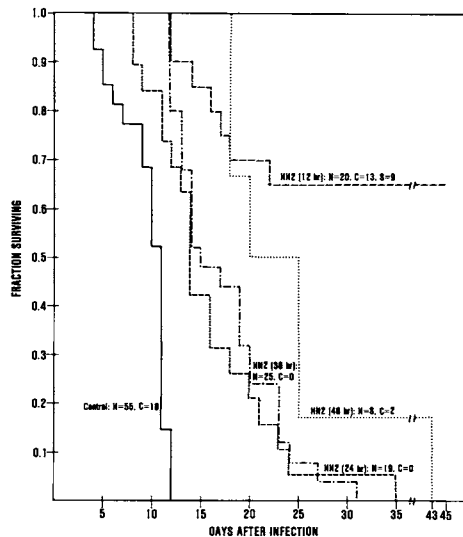
Animals received either (A) $10^{3.8}$, (B) $10^{2.8}$ or (C) $10^{1.8}$ units of antibody (Figure 3). A dose response relationship was apparent with substantially improved survival at all doses ($p \leq 0.05$) and an increased fraction of long term survivors with the more concentrated doses.

In order to further explore the mechanism of protection by HN8-1k, infected animals were treated with a single inoculation of HN8-1k ($10^{3.6}$ units of antibody) or HBSS, 24 hrs. after infection and followed for survival, virus growth curves, histology and the distribution of viral antigen in brain. As anticipated, HN8-1k again afforded significant protection ($p \leq 0.001$) with only 30% overall mortality at 1.5 months (Fig. 4).

Free virus titers were substantial in these animals with $10^{4.37}$ PFU/gm recoverable from brain tissue at 24 hrs. after infection. (Fig. 5). Viral titers rapidly peaked in HBSS treated control animals (filled squares and fitted curve) and remained high until death. In contrast, few animals treated with HN8-1k (filled circles)

FIGURE 3
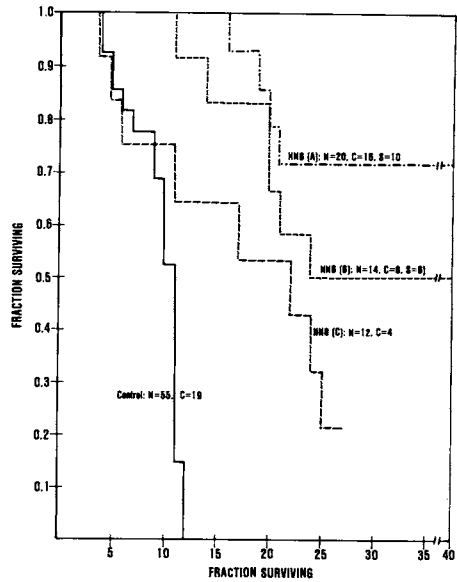
FIGURE 4
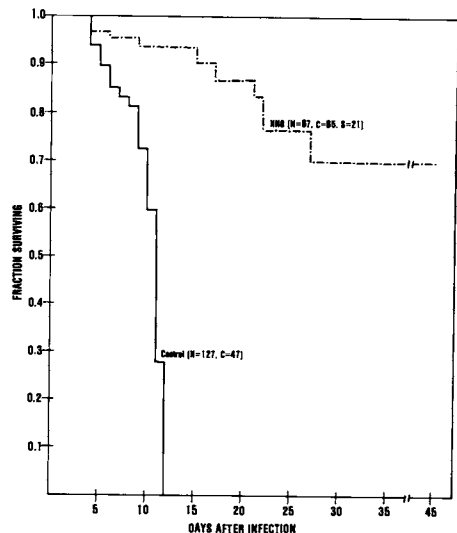
had virus recoverable at
the dilutions assayed
(11/28) and when virus
was found it was generally
at lower titer than from
that recovered from con-
trols. Similarly, all of
the control animals (6/6)
showed viral nucleocapsid
antigen widely disseminated
throughout brain by day 4
which subsequently further
increased in distribution.
Most HN8-1k treated animals
(13/18) had only limited or
no detectable mumps virus
nucleocapsid antigen in
brain at any time, and when
present it was restricted
to scattered ependymal
cells. Of those HN8-1k
treated animals with viral
antigen in brain parechyma
(5/18) only two had morpho-
logic evidence of infection
comparable to matched con-
trols. Finally, the dis-
tribution of injected mono-

FIGURE 5

clonal antibody was sought in a pilot study. Two infected
animals were inocculated on each of days 1 and 5 post-in-
fection, with 125I HN2-2ak and killed by perfusion-fixation
24 hrs. later. Autoradiography localized activity in well
perfused brains primarily to meningeal and parenchymal vas-
culature of the choroid plexus.

DISCUSSION

Monoclonal antibodies have recently been used to protect
animals from the lethal effects of a variety of experimental
viral infections of the CNS including herpes simplex virus
(9,10), Sindbis virus (11), Venezuelan equine encephalitis
virus (12), and Semliki Forest virus (13) infections. In
these model infections, monoclonal antibody has been adminis-
tered prior to establishing the infection. While extremely
useful studies in delineating protective effects of antibodies
which exist in vivo that were unexpected from the known func-
tional activities of the antibodies as measured in vitro, they
provide only limited insight into how such molecules might mo-
dify the course of natural infections. The present studies,
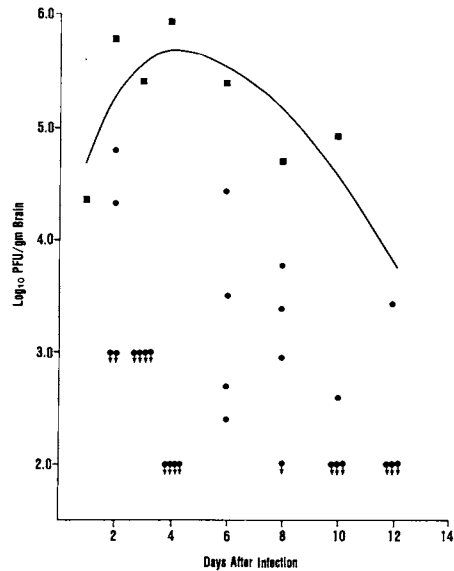
like these using monoclonals to influenza virus (14) and mea-
sles virus (15) provide preliminary evidence that selected
monoclonal antibodies may beneficially modify the course of
established CNS infections.

In the mumps virus-hamster meningoencephalitis model,
heterologous mouse monoclonal Ig directed against one or more
epitopes on the HN glycoprotein of mumps virus would appear to
diffuse into the CNS across vascular endothelium of the cho-
roidal, meningeal, and parenchymal vasculature to reach cells
within the CNS. At a minimum, these defined Ig prevent the
spread of infection by neutralizing progeny virus shed from
ependymal and choroidal epithelium infected up to 48 hrs. pre-
viously. These conclusions are based on the preliminary auto-
radiographic studies which localize labelled Ig to the above
anatomic sites within the CNS, the effects on survival of ap-
propriate monoclonal Ig administered as late as 48 hrs. after
infection, the restricted sites of viral antigen in the brains
of treated animals, and the markedly diminished viral titers
in the brains of most treated hamsters. Which domains of the
polypeptides of mumps virus are most important for inducing
humoral responses that are protective from infection and re-
infection, what restrictions for Ig entry and spread within
the CNS may exist during the course of encephalitis, and the
role of complement and antibody dependent cell mediated cyto-
toxicity mechanisms in the therapeutic effects seen with these
monoclonal Ig are now approachable problems with this model
system.

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BIOCHEMICAL ASPECTS OF CHEMILUMINESCENCE INDUCED BY SENDAI VIRUS IN MOUSE SPLEEN CELLS[1]

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Sendai and influenza viruses induce an immediate, short-lived burst of luminol-dependent chemiluminescence (CL) in mouse spleen cells that is triggered by the interaction of the envelope glycoproteins with the membranes of the responsive subpopulation of cells (1,2,3). In the case of Sendai virus, the chemiluminescent response depends upon both the hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins, the bulk of the light emission being associated with the action of the latter (2,3). Chemiluminescence reflects the generation by the cells of unstable oxygen species (e.g. O_2^-, H_2O_2, OH·) that react with the easily oxidized chemiluminogenic probe luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (reviewed in 4). The emission of photons from this reaction can be monitored in a conventional liquid scintillation counter (LSC) operated in the "out-of-coincidence" mode. An understanding of virus-induced CL is significant for several reasons. Firstly, being an event associated with the action of HN and F, an understanding of its origin and mechanism would help to elucidate biochemical events associated with adsorption and penetration of the virus into its host cell. Secondly, if it reflects the interaction of the virus with phagocytic cells, the biochemistry of CL might help to elucidate some aspects of the pathogenesis of virus infection such as the impairment of immune responsiveness and the appearance of systemic symptoms associated with locally restricted virus multiplication (5).

In this paper, we report the bivalent cation dependence of virus-induced CL and present evidence that indicates a prominent role for arachidonic acid metabolism in this process.

CL Induced by Sendai Virus. Fig. 1 shows a typical CL response of mouse spleen cells to Sendai virus. CL increased rapidly on addition of virus to the cells, reaching a peak at approx. 2-3 min. post-infection and declining thereafter to prestimulation levels at 20-40 min. post-infection.
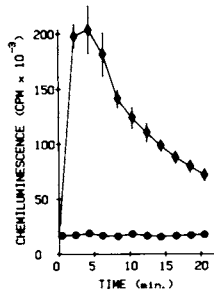
FIGURE 1. The CL response of mouse spleen cells to Sendai virus. Spleen cells and virus were prepared as described previously (3). CL was measured in a modified Kontron MR 300 DPM liquid scintillation counter using the settings for tritium with the dead time correction and coincidence circuits switched off. Data were recorded and analyzed as described (3). To 3×10^6 cells suspended in 750 μl Hanks' balanced salt solution (HBSS) were added 390 hemagglutinating units of virus. Zero time represents the addition of virus to the spleen cells. Three samples of virus-stimulated cells and one sample mock-stimulated with PBS were studied. The points represent the means and the bars standard deviations. ◆——◆ Virus-stimulated cells; ●——● mock-stimulated cells.

The Role of Bivalent Cations in the Generation of CL. To investigate the role of bivalent cations, cells were suspended in HBSS lacking either Ca^{2+} or Mg^{2+}, or both. Fig. 2 shows that omission of Mg^{2+} from the medium did not decrease CL stimulated by Sendai virus; it was inhibited more than 75%, however, in medium lacking Ca^{2+} but supplemented with Mg^{2+}. When EGTA (0.1-4 mM) was included in the Ca^{2+}-free assay medium, CL was not decreased further, suggesting that the residual CL was generated independently of trace amounts of Ca^{2+} present in the assay medium. In medium lacking both Ca^{2+} and Mg^{2+} and containing EGTA and EDTA (1.0 mM) to bind trace amounts of bivalent cations, CL was similar to that observed in the medium lacking Ca^{2+} alone. To compare virus-induced CL with that evoked by a stimulus known to be Ca^{2+}-dependent, similar experiments were carried out using the Ca^{2+} ionophore A23187 at a

final concentration of 1 μM. The ionophore stimulated CL with
a time course similar to that of the virus-induced response
(results not shown). However, in contrast to virus-induced CL,
the response evoked by the ionophore was completely abrogated
in the absence of extracellular Ca^{2+}. Fig. 2 also shows that
Mg^{2+} exerted an inhibitory effect on CL induced by the iono-
phore; the light emission in the presence of Ca^{2+} alone ex-
ceeded that generated when both Ca^{2+} and Mg^{2+} were present.
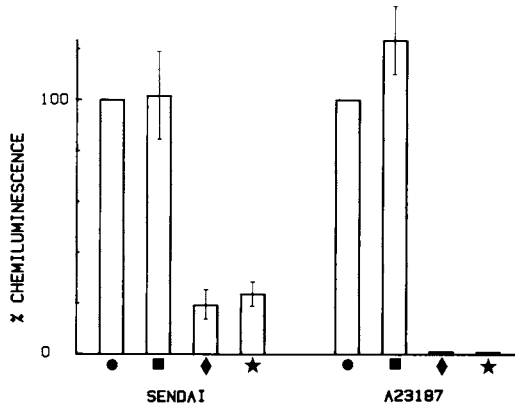
FIGURE 2. The effects of Ca^{2+} and Mg^{2+} on Sendai virus-
induced CL. Spleen cells were washed twice in Ca^{2+}/Mg^{2+}-free
HBSS. Ca^{2+} or Mg^{2+} (final concentration 1 mM) were added to the
cell suspension at 30 min. and EGTA or EDTA at 10 min. before
stimulation with the virus. The different ionic conditions did
not alter the kinetics of the CL response and integrated light
emission over 20 min. was compared in each case. CL in the pre-
sence of Ca^{2+} and Mg^{2+} is taken as 100%. Columns represent
integrated light emission and bars represent the standard
errors of the respective means. Values from 6 and 5 separate
experiments are compared for virus- and ionophore-stimulated
CL, respectively. The response observed in the Ca^{2+}-depleted
buffers was significantly different at the $p < 0.05$ level. ● con-
trol, Ca^{2+} and Mg^{2+} at 1 mM; ■ Ca^{2+} alone; ◆ Mg^{2+} alone plus
EGTA; ★ no Ca^{2+} and Mg^{2+}, but EGTA and EDTA present.

Glucose Requirement of the CL Response. CL associated
with the respiratory burst in phagocytic cells is thought to
originate from a membrane-spanning NAD(P)H oxidase located
within the plasma membrane (6,7). The electrons required for
the reduction of oxygen originate mainly from the oxidation of
glucose via the oxidative segment of the pentose phosphate
pathway. To investigate the glucose dependence of virus-induced
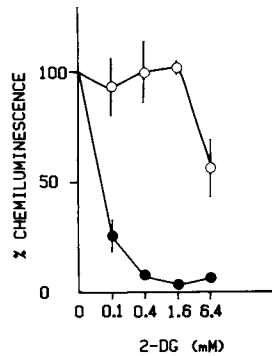
FIGURE 3. The effect of 2 deoxy-D-glucose on virus-induced CL. Points represent the means of integrated light emission curves (20 min) of 6 separate experiments and bars represent the standard errors of the means. ●——● No D-glucose; ○——○ D-glucose present (5 mM).

CL, we compared the responses of cells incubated in the presence and absence of glucose. Preliminary experiments had shown that the extent of CL is dependent upon the time of preincubation of cells in glucose-free medium; it begins to decrease when the period of preincubation exceeds 1 h. The decrease in CL is not accompanied by a decrease in cell viability as assessed by the Trypan Blue exclusion test (results not shown).

These observations suggested that cellular light emission has a requirement for glucose that can be met partly by mobilization of glycogenolytic intermediates from endogenous stores. To investigate this possibility, cells were prepared in medium containing glucose, further incubated in this medium for 30 min at 20°C, washed twice and incubated thereafter for 60 min at 37°C in glucose-free medium. Under these conditions, the CL produced was similar to that observed in control cells incubated in a glucose-supplemented medium. However, when the glucose analogue 2-deoxy-D-glucose (2-DG) was added to the cells 10 min before virus (i.e. after 50 min incubation in the glucose-free medium), CL was decreased in a concentration-dependent manner (Fig. 3). The inhibitory effect of 2-DG was practically eliminated when D-glucose was present in the assay medium (Fig. 3), suggesting that the inhibition by 2-DG was due to interference with D-glucose metabolism. In contrast to 2-DG, the transportable but non-metabolizable glucose analogue 3-O-methyl-D-glucose did not affect CL when tested over the

same concentration range (result not shown). Since this ana-
logue, unlike 2-DG, is not phosphorylated by hexokinase, it
would be unlikely to compete with the metabolism of glyco-
genolytically-derived intermediates.

The Role of Phospholipase Metabolism. The release of
arachidonic acid from membrane phospholipids and its subse-
quent metabolism via the cyclooxygenase and lipoxygenase path-
ways represents a major regulatory network in the inflammatory
response to a wide variety of pathogens. The former pathway
leads to the production of prostaglandins and thromboxanes and
the latter to leukotrienes and HETE's. In both pathways, high-
ly unstable epoxy- and hydroperoxy-intermediates are formed,
respectively, and it has been suggested that these interme-
diates may play a role in the generation of CL in various sys-
tems (8,9). To investigate the role of arachidonic acid meta-
bolism in virus-induced CL, we used a variety of metabolic
inhibitors (see Fig. 4).

MEMBRANE PHOSPHOLIPIDS

PHOSPHOLIPASE A_2 $\vdash \left[\begin{array}{l} \text{U3585} \\ \text{PG } E_1 \end{array} \right.$

ARACHIDONIC ACID

$\left. \begin{array}{l} \text{ASPIRIN} \\ \text{INDOMET} \\ \text{ETYA} \end{array} \right\}$ — CYCLOOXYGENASE   LIPOXYGENASE $\vdash \left[\begin{array}{l} \text{ETYA} \\ \text{NDGA} \end{array} \right.$

PROSTAGLANDINS   LEUKOTRIENES
THROMBOXANES   HETE'S
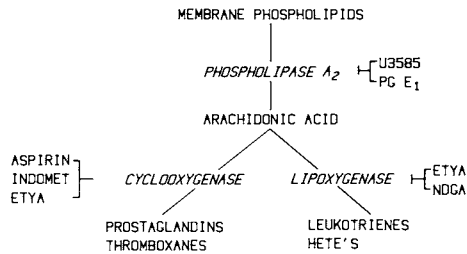
FIGURE 4. The metabolism of arachidonic acid and the
sites of action of the inhibitors used.

Of these inhibitors, U3585, prostaglandin E_1, ETYA and
NDGA inhibited CL in a concentration-dependent manner, whilst
aspirin had no significant effect and indomethacin at high
concentration only affected CL (Fig. 5). Control experiments
suggested that none of the inhibitors used interfered with the
binding of ^{3}H -uridine-labelled Sendai virus to mouse spleen
cells, nor did they quench the chemical reaction between H_2O_2
and luminol (except for NDGA, which quenched H_2O_2 stimulated
luminol CL by 50% whilst decreasing cellular CL by 97% when
used at 10 µM).
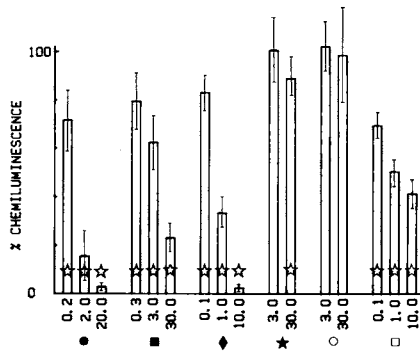
FIGURE 5. Effect of inhibitors of arachidonic acid meta-
bolism on Sendai virus-induced CL. The columns show light
emission integrated over 20 min in the presence of inhibitors
at the concentrations indicated in μM. Values represent the
means $\pm$ SEM of 5 separate experiments with U3585 and 6 sepa-
rate experiments with the other inhibitors.   ☆ Indicates
significant difference at the $p \leq 0.05$ level (t-test).   ●
U3585, ■ ETYA, ◆ NDGA, ★ Indomethacin, ○ Aspirin, □
Prostaglandin E_1.

DISCUSSION

     In this paper we have characterized the metabolic requi-
rements of Sendai virus-induced CL in mouse spleen cells. The
results show that CL depends on: (a) glucose metabolism and
(b) the presence of external Ca^{2+} but not Mg^{2+}; and (c) that
there is a correlation between the magnitude of the CL res-
ponse and phospholipase A_2 and lipoxygenase activities.
     Within this conceptual framework, two reactions have been
shown to lead to CL. An NAD(P)H oxidase located at the surface
of phagocytic cells is known to be associated with the pro-
duction of reactive oxygen species that generate CL in the
presence of luminol (4,6,7). More recent evidence indicates
that lipoxygenase activity may also lead - either directly or
indirectly - to CL, most likely through the decomposition of
highly unstable hydro-peroxy-intermediates generated during
the formation of HETE's and leukotrienes (8,9) or through the
activation of NAD(P)H oxidase by one of these metabolic inter-
mediates and/or products. To definitely attribute the source
of reactive oxygen species to one or other or both of these
pathways, it will be necessary to carry out additional experi-
ments. The evidence for the involvement of the lipoxygenase
pathway and glucose catabolism in Sendai virus-induced CL is

indirect and the specificities of the inhibitors used in the
present study must be checked by assaying for product forma-
tion using independent methods such as ion-pair HPLC (10).
Moreover, although we have checked the effects of all the in-
hibitors used in the present work on a luminol-H_2O_2 reaction
in a cell-free system, the possibility that they might cause
interference with the reaction between luminol and the (un-
known) reactive oxygen species responsible for virus-induced
CL cannot be excluded with certainty. In spite of these limi-
tations, the results of the present study are of interest in
several respects. Firstly, two recent papers have shown that a
correlation exists between glucose metabolism and lipoxygenase
activity (as measured by product formation) similar to that
observed in the present experiments (10,11), and it was sug-
gested that lipoxygenase activity and the respiratory burst
are interrelated phenomena with mutual regulatory activity
(10). Secondly, the dependence of CL on exogenous Ca^{2+} can be
explained in terms of the requirement for this cation of
phospholipase A_2 (12). Several observations suggest that the
reactions underlying CL favour membrane-membrane fusion. Thus,
lyso-phospholipid products that result from the activation of
phospholipase A_2 are known to be fusogenic and the reincorpo-
ration into the membrane of lipoxygenase products (e.g. HETE's)
(13) is likely to lead to increased membrane fluidity. How-
ever, Sendai virus has been reported to fuse with artificial,
enzyme-free membranes (14), suggesting that factors in addi-
tion to those described in this paper may contribute to enve-
lope-cell membrane fusion.

　　　Finally, it seems likely that the biochemical reactions
responsible for generating chemiluminescence may play a role
in the pathogenesis of virus infection. The metabolism of
arachidonic acid leads to the production of several highly
potent mediators of inflammation (e.g. leukotrienes, HETE's),
and reactive oxygen metabolites released from phagocytic cells
on activation by the virus might contribute significantly to
tissue damage.

ACKNOWLEDGEMENTS

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TRANSCRIBING VSV$_{NJ}$ DI PARTICLE AND ITS BIOLOGICAL ACTIVITIES

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Defective interfering (DI) particles contain only a portion of the genetic information of the parental standard virus [1,2]. DI particles are not capable of self replication in infected host cells, however, they can be amplified when they are coinfected with the parental standard virus. Coinfection of cells with the standard virus and DI particles results in suppression of standard virus production. This phenomenon is known as viral interference [1,2]. The molecular mechanisms of DI particle mediated viral interference is not yet known.

The genetic contents of a substantial number of VSV and Sendai virus DI particles have been characterized [2,3,4]. It is clear that the biological activities of DI particles are related to the sequences present in the DI genome [2,5].

DI particle genomes represent true deletions of the standard virus genome at either the 3' or 5' terminus of the genome. The most abundant DI particles of RNA viruses represent the 5' terminus of the standard virus. A recent report has described the isolation of 3' DI particles of Sendai virus [4] and we have isolated several 3' DI particles of VSV$_{IND}$ [6]. One well defined DI particle of VSV$_{IND}$, designated as DI-LT, represents the 3' half of the genome [7,8,9]. The genome of this DI particle contains genes for N, NS, M and G proteins including the leader RNA sequences. It can be transcribed by the endogenous RNA polymerase activity yielding 4 functional messenger RNAs and leader RNA [10,11]. The DI-LT interferes with replication of standard virus from both Indiana serotype (homotypic interference) and New Jersey serotype (heterotypic interference) of VSV [5]. In contrast, all 5' DI particles are capable of interfering only with the replication of the standard virus of their own serotype [1,2,5]. This paper deals with the isolation and partial characterization of a New Jersey serotype DI particle which interferes with the replication of the standard virus of both New Jersey and Indiana serotypes of VSV.

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Generation of DI 121 from VSV$_{NJ}$.   The prototype of 3' DI particles, DI-LT, has been isolated from a heat resistant strain of VSV$_{IND}$[12]. We used the same strategy to isolate 3' DI particles of New Jersey serotype. We chose VSV$_{NJ}$ (Ogden) because the DI-LT which originated from VSV$_{IND}$ interferes with the replication of the standard virus of heterotypic VSV$_{NJ}$ (Ogden) [5].   The virus was heated at 45°C for 30 mins, appropriate dilutions were made and used to infect rat cells transformed with B77 strain of avian sarcoma virus (R(B77)). The cells were overlayed with 0.9% agar and incubated at 40.5°C overnight.   Plaques arising from the heat treatment were selected and made subsequent plaque isolations were made by repeating the same procedure. After three consecutive plaque isolations, the virus was propagated in R(B77) cells. We made subsequent high multiplicity of infection passages of the virus until we detected an appropriate concentration of DI particles. It took approximately five 15-hour passages to obtain adequate quantities of DI particles. Fig. 1 illustrates the sucrose gradient purification of DI particles from both VSV$_{IND}$ and VSV$_{NJ}$. The DI particle from New Jersey serotype, DI-121, sedimented close to the standard virion and relatively free of other DI particles. In contrast, DI-011 and DI-LT preparations had some minor contamination of other DI particles. The DI-121 of VSV$_{NJ}$ sedimented at the same position as DI-LT, thus, DI-121 may have genomic RNA of approximately 50% that of the standard virus genome since DI-LT represents 50% of the standard viral genome.
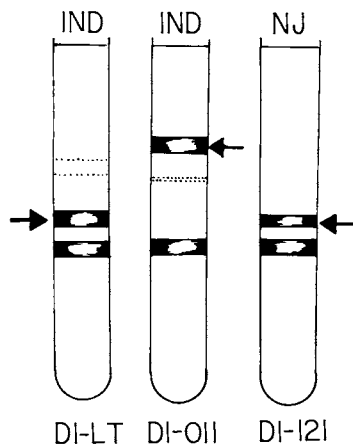
FIGURE 1. Sucrose gradient purification of DI particles and the standard virions. Samples of lysates were centrifuged at 81,000 x g for 75 minutes, resuspended in 0.2-0.3 ml of PBS containing 1M NaCl and layered on a linear 5-30% sucrose gradient made in PBS. After centrifugation at 110,000 x g for 35 minutes at 5°C in a Spinco SW41 rotor, the visible DI particles (with arrows) and the standard virions (the bottom bands) were collected.

Endogenous RNA transcriptase activity of DI-121. DI particles representing the 5' terminus of the standard VSV$_{IND}$ synthesizes RNA products of 46 nucleotides in length when the endogenous RNA polymerase reaction is carried out in vitro. There is a strong termination signal which prevents polymerase read-through (2). In contrast, the 3' DI particle, DI-LT, synthesizes the leader RNA and four functional messenger RNAs (10,11).
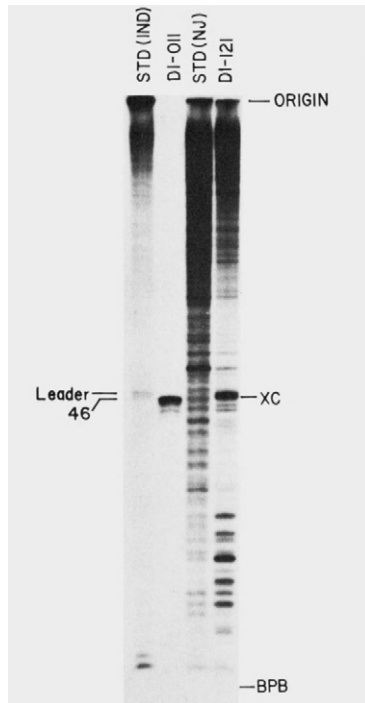
FIGURE 2. Polyacrylamide gel electrophoresis of RNA transcripts. RNAs were synthesized in vitro in the presence of -32p GTP by DI-011, DI-121, and the standard virions of both VSV$_{IND}$ and VSV$_{NJ}$. The resulting RNA products were purified by Sephadex G-50 chromatogrpahy followed by extraction with phenol and chloroform. The 32p GTP-labeled RNA products were analyzed by electrophoresis in 12% polycacrylamide gel containing 8M urea.

Figure 2 shows the analyses of RNA transcripts from in vitro polymerase reaction. VSV$_{IND}$ standard virus synthesizes 47 nucleotide leader RNA and large transcripts whereas, DI-011 synthesizes predominantly the 46 nucleotide products without larger RNAs. In contrast, DI-121 synthesizes large transcripts similar to that of VSV$_{NJ}$ standard virus (Fig. 2). There are numerous RNA products which are much smaller than messenger RNAs. It is unlikely tht these smaller products are the result of post-transcritional degrada-

tion since VSV$_{IND}$ standard virus shows predomintly large RNA transcripts under the same conditions of preparation. It is possible that VSV$_{NJ}$ polymerase terminates RNA transcription much more frequently. The 46 nucleotide DI products yields 21 and 13 oligonucleotides after ribonuclease T$_1$ digestion. In contrast, the leader RNA of VSV$_{IND}$ has 28 nucleotides which can be identified easily in 20% polyacrylamide-urea gels after ribonuclease T$_1$ digestion (Fig.3). We have digested RNA transcripts of both VSV$_{NJ}$ (Ogden) and DI-121 with ribonuclease T$_1$ and analyzed in the 20% polyacrylamide gel. A comparison of the digests of the RNA transcripts of DI-121 and the standard virus shows no significant differences betwen the standard virus of VSV$_{NJ}$ and DI-121 particles (Fig. 3).
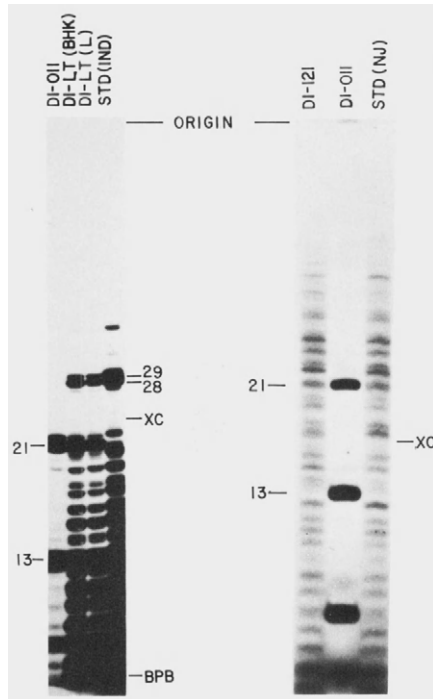
FIGURE 3. Polyacrylamide gel electrophoresis of RNA transcripts after ribonuclease T$_1$ digestion. The ^{32}P GTP-labeled RNA products from Fig. 2 were digested with ribonuclease T$_1$ and analyzed in 20% polyacrylamide gel containing 8M urea.

Homotypic and heterotypic interference by DI-121. The DI-LT, the 3' DI particle isolated from the heat resistant strain of VSV$_{IND}$, interferes the replication of the standard viruses of both Indiana and New Jersey serotypes [5]. The biological activities of DI-121 have been investigated to determine whether this DI particle also interferes with the replication of standard virus of both

TABLE 1.
Interference by DI-011 and DI-121 on production
of the standard virus of VSV$_{IND}$ and VSV$_{NJ}$

	VSV$_{IND}$ PFU/ml (% control)	VSV$_{NJ}$ PFU/ml (% control)
Standard virus alone	3.2×10^9 (100%)	1.3×10^9 (100%)
Standard virus + DI 011	1.5×10^7 (0.5%)	1.0×10^9 (76.9%)
Standard virus + DI 011 (Anti-VSV$_{IND}$-Ab)	1.3×10^9 (40.6%)	9.0×10^8 (69.2%)
Standard virus + DI 011 (Anti-VSV$_{NJ}$-Ab)	1.2×10^7 (0.4%)	8.5×10^8 (65.4%)
Standard virus + DI 121	5.6×10^7 (1.8%)	2.7×10^7 (2.1%)
Standard virus + DI 121 (Anti-VSV$_{IND}$-Ab)	1.5×10^8 (4.7%)	4.2×10^7 (3.2%)
Standard virus + DI 121 (Anti-VSV$_{NJ}$-Ab)	1.8×10^9 (56.3%)	8.0×10^8 (61.5%)

The gradient purified DI particles were treated with either anti-VSV$_{IND}$-antibody or anti-VSV$_{NJ}$-antibody. The antibody treated DI particles were layered on a 10% sucrose made in PBS and 0.5 ml of 65% sucrose cushion in Spinco SW50.1 rotor and centrifuged at 189,000 x g for 90 minutes at 5°C. The pelleted antibody treated DI particles were mixed with standard virus and infected with multiplicity of infection of 10 PFU/cell. The progeny virus was harvested after 8 hours of infection and tested for infectivity by plaque assay.

serotypes since DI-121 resembles the DI-LT both in terms of its size and biochemical properties. It is clear from the data presented in Table I that over 95% of infectious virus production of standard viruses of both Indiana and New Jersey serotypes was inhibited by DI-121. Furthermore, DI-121 interferes with replication of the standard virus of VSV_{IND} and VSV_{NJ} at about the same level (Table I). Thus, the DI-121 is capable of both homotypic and heterotypic interference.
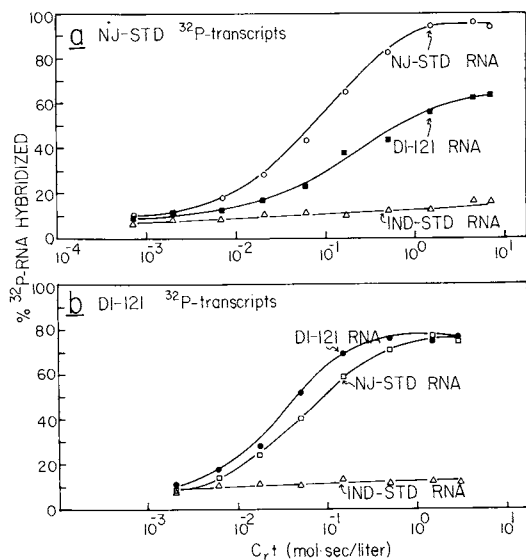
FIGURE 4. Kinetics of hybridization of in vitro transcripts of VSV_{NJ} standard virion and DI-121 to different VSV RNAs. $32P$ GTP-labeled transcripts from in vitro transcription of VSV_{NJ} standard virion (4000 cpm/sample) and DI-121 (3500 cpm/sample) were incubated at 63°C in 25 u liters of annealing buffer containing 0.3M salt sealed in 25 u liter micropipettes with 300ng of RNAs of VSV_{IND} standard virion, VSV_{NJ} standard virion, DI-011, or DI-121. At different times, samples were withdrawn and were frozen. At the end of all incubations, the extent of hybridization was determined by ribonucleases A and T_1 digestion in 0.3M salt. The results are expressed as the proportion of total $32P$ RNA hybridized at a given $C_r t$ value in 0.3M salt (RNA concentration in moles/liters X time of incubation in seconds).

The origin of DI-121. Since the size and biological activities of DI-121 resembles the DI-LT, it was necessary to prove that this DI particle originated from VSV_{NJ} rather than VSV_{IND}. The gradient purified DI particles were treated with either anti-VSV_{IND}-antibody

or anti-VSV$_{NJ}$-antibody, centrifuged through 10% sucrose and pelleted on 65% sucrose cushion to remove unbound antibodies. The pelleted antibody treated DI particles on the 65% sucrose cushion were mixed with known amounts of the standard virus and infected. The progeny virus was harvested after 8 hours of infection and tested for infectivity by plaque assay. As can be seen in Table I, the DI-011 activity for interference can be neutralized by anti-VSV$_{IND}$-antibody but not by the anti-VSV$_{NJ}$-antibody. In contrast, DI-121 activity for interference was neutralized by anti-VSV$_{NJ}$-antibody only. Thus, DI-121 contains surface antigens of VSV$_{NJ}$.

To further ensure that DI-121 is indeed originated from VSV$_{NJ}$ rather than being a pseudotype representing genomic RNA from Indiana serotype and surface antigens derived from VSV$_{NJ}$, hybridization experiments have been carried out. A significant amount of ^{32}P-labeled RNA transcripts of VSV$_{NJ}$ hybridized with RNAs from the standard virus of VSV$_{NJ}$ and from DI-121 (Fig. 4a). A reciprocal experiment was also carried out (Fig. 4b). The transcripts of DI-121 hybridized with RNAs from the standard virions of VSV$_{NJ}$ and from DI-121. However, the transcripts failed to hybridize to RNAs of VSV$_{IND}$. These results demonstrate that DI-121 genome is derived from VSV$_{NJ}$.

Summary. The DI-121 isolated from heat resistant strain of VSV$_{NJ}$ inteferes with the replication of standard viruses of both Indiana and New Jersey serotypes of VSV. It synthesizes RNA transcripts similar to that of the standard virus of VSV$_{NJ}$. We are currently analyzing the nucleotide sequences of DI-121 genome in order to determine the relationship between the biological activities and nucleotide sequences.

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RECOMBINATION EVENTS DURING THE GENERATION
OF DI RNAS OF VSV

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The model for the generation of defective interfering
(DI) particles of Vesicular stomatitis virus (VSV) proposes
aberrant replication events rather than breakage and reunion
of RNA molecules (1, 2). The central feature of this copy
choice model is that the polymerase interrupts RNA synthesis,
detaches from the template and moves with the nascent
daughter strand attached to it to another position on the
same or a different template where RNA synthesis resumes,
thereby extending the nascent chain. With respect to genomic
sequences retained and the degree of self-complementarity,
four classes of DI particles can be distinguished: deletion,
panhandle, snapback and compound DIs.   The nucleotide
sequence analysis of the L gene (Harmison et al., this
volume) enabled us to identify the sites of rearrangement
involved in the generation of DI genomes. In this communi-
cation, we describe their structural organization and discuss
whether termination and/or resumption of RNA synthesis are
specified by the primary sequence of the RNA template.

STRUCTURAL ORGANIZATION OF DI GENOMES

Figure 1 shows the structure of DI-LT, O11, T, T(L) and
611 as compared to VSV and the positions where the DI and L
gene sequences diverge.

Deletion DI RNA. Hybridization studies revealed that
DI-LT is a true deletion mutant in which most of the L gene
is deleted, while the N, NS, M and G genes are retained (3).
The site of the deletion was identified by sequencing cDNA
clones of the relevant regions of the DI and the parental

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genome (4). Comparison with the complete sequence of the L gene demonstrates that the deletion spans from positions 341 to 6190 of the genome involving 5848 nucleotides. It is important to note, that DI-LT represents a mixture of at least 2 DI particles of similar size, DI-LT$_1$ and DI-LT$_2$ (3).

Snapback DI RNA. Earlier studies established that the genome of the snapback DI 011 consists of a region which is homologous to the genomic 5' end for about 1 kb and which is covalently linked to its exact complement (5). Comparison of the published sequence of about 70 bases before and behind the turnaround point (5) with the sequence of the cDNA clones of the L gene reveals, that the last base of the (-) sense strand of DI 011 corresponds to position 1167 of the VSV genome.

Panhandle DI RNA. The panhandle DI particles T, T(L) and 611 have retained different amounts of genetic informa-tion from the 5' half of the genome and share at their 3' end a 45 (DI-T) or 48 (DI-T(L) and 611) nucleotides long sequence which is complementary to the 5' end (6) -- the panhandle region (Figure 1, a'). The sequences beyond the panhandle regions were determined for about 80-100 bases from the 3' end of the DI RNAs using the chemical sequencing method described by Peattie (7). Homology to the L gene sequence was found to extend to positions 2163 with DI-T, 3641 with DI-T(L) and 4417 with DI 611 RNA.
The locations of the recombination sites within the L gene of all the DI particle RNAs described above are consis-tent with the overall size of the DI particle genomes, based on hybridization data and oligonucleotide fingerprint mapping. We, therefore, assume that no additional severe
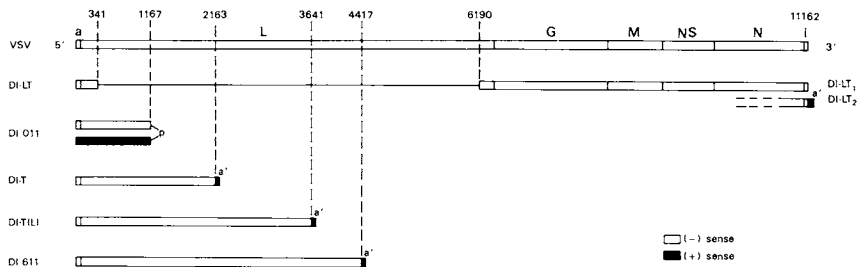
FIGURE 1. Maps of the genomes of VSV, DI-LT, DI 011, DI-T, DI-T(L) and DI 611. Black bars represent sequences of DI particle genomes not present in the VSV (-) sense sequence.

rearrangements have occurred in the RNA of these DI particles. The complete sequences of the genomes of DI 011, T, T(L), and 611 can, therefore, be deduced from the primary structure of the L gene.

SEQUENCE ANALYSIS OF THE RECOMBINATION SITES

The sequences of the genomic recombination sites involved in the generation of DI-LT, 011, T, T(L) and 611 are shown in Figure 2a-c. By comparing these data, we did not detect the existence of a general termination or resumption signal. This result, however, does not mean that the construction of DI particle genomes occurs in all cases independently of the primary structure of the VSV genome. In fact, in the following part, we present evidence that a particular sequence may be involved in the origin of the deletion DI-LT and another sequence may specify the events leading to the panhandle DIs T, T(L) and 611. We propose that different sequences play a role in the generation of some DI particle genomes by exerting their influence through different mechanisms.

Origin of Deletion DI RNA. The sequence immediately before the start of the deletion in DI-LT contains two hexa-nucleotides ATCTGA and GATTGG, marked by boxes in Figure 2a. Interestingly, these groups are also found about 6000 bases downstream at the end of the deletion. In addition, the spacing of the hexanucleotides in both regions is very similar: the number of bases between the hexanucleotides is in the same range (10 and 13 bases) and also the distance between the sequence GATTGG and the base flanking the deletion at each site differs only little (5 and 7 bases).
This arrangement suggests a model for the generation of DI-LT which may involve annealing of the nascent RNA strand to the template before synthesis resumes (Figure 2a). Unique for this scheme is that a sequence at the end of the nascent RNA chain determines the resumption site of RNA synthesis, while the termination cannot be addressed to a specific sequence at this time.

Origin of Snapback DI RNA. The recombination site involved in the generation of DI 011 does not reveal that a particular sequence may play a role in the origin of this DI particle (Figure 2b).
In order to explain the exact complementarity of the DI 011 genome, two models have been discussed (5). One of these models depends on the presence of a small region of dyad symmetry in the RNA template. It was suggested that an unencapsidated transcript of this region could potentially

self-anneal and that the polymerase then copies back the
nascent chain. Our sequencing data clearly rule out this
model, because the sequence in the template before and behind
the position corresponding to the turnaround point are not
self-complementary. Our data are, however, consistent with
the second model, which suggests replication across a
replication fork (5).

Origin of Panhandle DI RNA. The similarity in the
length of the stem regions of DI-T, T(L) and 611 was
explained by involvement of a specific polymerase recognition
site with the sequence GGUCUU which is located 43-48 bases
from the 5' end of the VSV genome (6). Based on this copy
back model (8, 9) the recombination sites within the L gene
are presumptive termination sites. There is no evidence that
termination may be specified by sequence information (Figure
2c).
   Because of a repeat of two bases (AA) at the termination
and resumption sites involved in the generation of DI-T(L)
and 611, the 48th and 47th base from the 3' terminus of these
DI particle genomes could also be encoded in the case of DI-
T(L) by positions 3640 and 3641 of the L gene and by
positions 4416 and 4417 in the case of DI 611. Thus, the
added 3' terminal regions of these DI particle RNAs are
identical in size as the predominant 46 nucleotides long (-)
strand leader RNA (10). The same region of DI-T consists of
45 nucleotides.
   A model for the origin of these DI particles can be pro-
posed which involves as a first step the synthesis of (-)
leader RNA. Instead of releasing the transcript the poly-
merase detaches with it from the (+) strand template and
extends it by several thousand bases after resuming synthesis
at a (-) strand nucleocapsid. In this model, the termination
of RNA synthesis presumably can be addressed to the sequence
3'-GAA(A)-5' which is considered to function as part of a
stop signal in VSV (11), while the resumption seems to occur
randomly. This scheme is similar to the proposed model for
the generation of the compound DI-LT$_2$ (12). Although the
copy back model (6, 8, 9) and the model suggested here are
contrary, they have in common that a sequence signal plays a
role, specifying either the resumption or the termination of
RNA synthesis.
   In a modified form both models may also be applicable
for DIs with larger panhandle regions (13). Based on the
first model, the hexanucleotide GGUCUU which can be found at
positions 43-48 and also at positions 156-161 from the 5' end
of the genome is a necessary prerequisite for resumption of
RNA synthesis, but the resumption site is less localized than
assumed. Based on the second model, occasional readthroughs
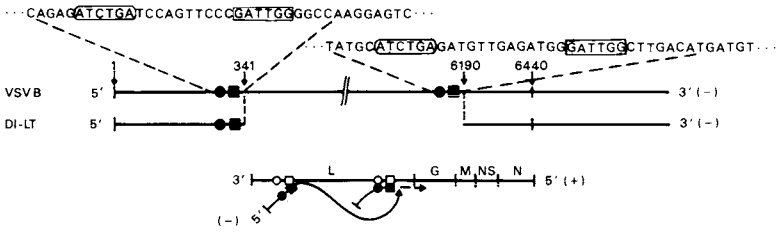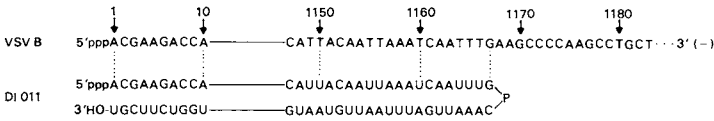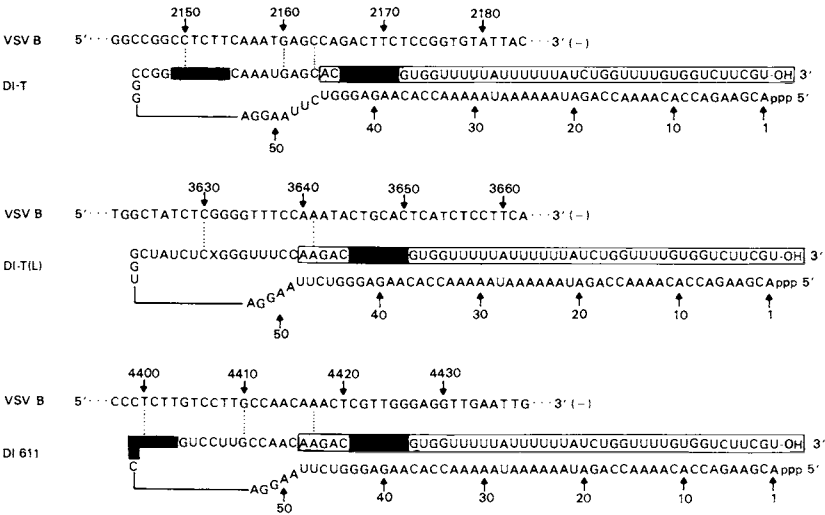
FIGURE 2. Recombination sites involved in the generation of DI particle genomes.

through the stop signal at the end of (−) leader RNA region could account for larger panhandles.

At the end of the 5' region, we found in the case of DI-T and DI 611, but not DI-T(L), the hexanucleotide CCUCUU which is also repeated at the beginning of the stem region in all three DI RNAs. If this sequence exerts an effect then the origin of DI-T(L) follows a different pattern than DI-T and DI 611.

The recombination sites reveal that in some, but not all cases, the termination and/or resumption of RNA synthesis which lead to DI particle generation may be due to the primary structure of the VSV genome. In addition, if sequences are involved they share no homology and operate by different mechanisms. Thus, it seems that the generation of DI particles of VSV has no strict requirement for sequence information. We propose that each site in the genome has the potential to give rise to DI particles through a copy choice mechanism, unless other yet unknown factors are competing. However, we also propose, that sequences play a role by modulating the process of DI particle generation in a quantitative manner in that they favor the construction of a particular type of DI particle. The large number of pan-handle particles with stem regions of 45-48 bases, for example, may be explained by this model.

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STRUCTURE AND GENERATION OF DELETION
MUTANTS OF VESICULAR STOMATITIS VIRUS[1]

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One of the defective interfering (DI) particles generated
by the heat-resistant strain of vesicular stomatitis virus
(VSV-HR) contains genetic information from the 3' half of the
genome (1-3). This DI particle (DI-LT; DI 0.46) is the only
true internal deletion mutant of VSV to be identified (4-7).
DI-LT retains both parental termini, whereas other VSV DI
particles contain a short nonparental sequence at the 3' end
which is the complement of the parental 5'-terminal sequence
(8). Our previous analyses indicate that DI-LT contains 320-
350 nucleotides of the 5'-terminal parental sequence (5).
    Because DI-LT contains genetic information from the 3'
half of the genome and retains the 3'-terminal sequence it can
synthesize the leader and mRNAs for the nucleocapsid (N),
phospho- (NS), matrix (M), and glyco- (G) proteins (9, 10).
In contrast, DI-LT2 (DI 0.50) is also derived from the 3' half
of the VSV genome but does not retain the 3'-terminal sequence
and does not synthesize mRNA (11).
    When the DI-LT mRNAs are resolved in a 1.5% agarose gel
containing 6 M urea at pH 3.0, the messages for the NS, M, and
N proteins comigrate with the corresponding mRNAs synthesized
by VSV-HR.   Surprisingly,   only very little of the
polyadenylated DI-LT mRNA comigrates with authentic G message.
Instead, a new larger and relatively abundant message is
observed (G*) (12).   Structural analyses of the G* RNA
indicate that it contains an estimated 250-nucleotide
transcript of the remnant polymerase (L) gene covalently
linked to the 3' end of a transcript of the G gene. This

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message is not interrupted by an intervening poly(A) (13),
but contains a 3' poly(A) tail most probably encoded by the
remnant L gene polyadenylation signal (12).

SEQUENCING OF THE G* TRANSCRIPT

     In order to understand the underlying reasons for the
synthesis of the G* message, I have cloned and sequenced a
complementary DNA (cDNA) copy of this abnormal polycistronic
RNA. These experiments have pinpointed the location of the
deletion within the genome of the transcriptionally-active DI-
LT particle. A comparison of this sequence with previously
published sequences for the transcriptionally-inactive DI-LT2
(14, 15), for the wild type virus (16-20), and for other
unrelated DI particles derived from the 5' half of the genome
(21) has revealed some structural features shared by all DI
particles.   These observations suggest a single unified model
for the generation of DI particles by VSV. This model may
have broader implications for the regulation of RNA synthesis
by the negative strand viruses.
     cDNA was prepared from total intracellular poly(A)$^+$ mRNA
that had been extracted from BHK cells at 4.5 h after
infection with DI-LT. Since most, if not all, DI-LT stocks are
mixtures of the transcriptionally-active DI-LT and the -
inactive DI-LT2 (4, 5), the cloning and sequencing of cDNA
prepared from poly(A)$^+$ mRNA ensured that only sequences from
the biologically expressed cistron were examined.
     After cloning the message cDNA by standard techniques into
the Pst 1 site of the E. coli plasmid pBR322, recombinant
colonies were identified by hybridization to radioactive
plasmid p011 DNA. Plasmid p011 contains cloned sequences
equivalent to the 368 nucleotides at the precise 5' end of the
(-) sense VSV genome (14). Of several clones obtained, the
largest, containing approximately 450 nucleotides of DI-LT-
specific sequences, was chosen for further analysis.
     The viral-specific insert in this plasmid was sequenced by
the chemical method of Maxam and Gilbert (22). Examination of
the sequence indicated that it could be aligned with the
sequence of the VSV-San Juan G message (17) beginning at
nucleotide 1429 (numbered from the 5' end of the normal
message). The sequence of the cloned G* cDNA was homologous
with that of G message, with the exception of four single base
changes, up to nucleotide 1618; the two sequences diverged
after that position (not shown).
     The divergent sequence aligned with the sequence beginning
at position 259 from the 3' end of the L mRNA (318 from the 5'
end of the genome). Since the nucleotides at position 1618

```
                                  a              b
                                         |
(1)              -3'..AGUUGAACACUAU|GGUACGA..5'-
                 +5'..UCAACUUGUGAUA|CCAUGCU..3'+

                                         |
                                         |

                                  a              d
                                         |
(2)              -3'..AGUUGAACACUAU|CUAGAGA..5'-
                 +5'..UCAACUUGUGAUA|GAUCUCU..3'+

                                         |
                                         |

                                  c              d
                                         |
(3)              -3'..CCCUUGACCUAGU|CUAGAGA..5'-
                 +5'..GGGAACUGGAUCA|GAUCUCU..3'+

                                         |


(4)              -3'..UUUUGAAACUAGG..5'
                 +5'..AAAACUUUGAUCC..3'+
```
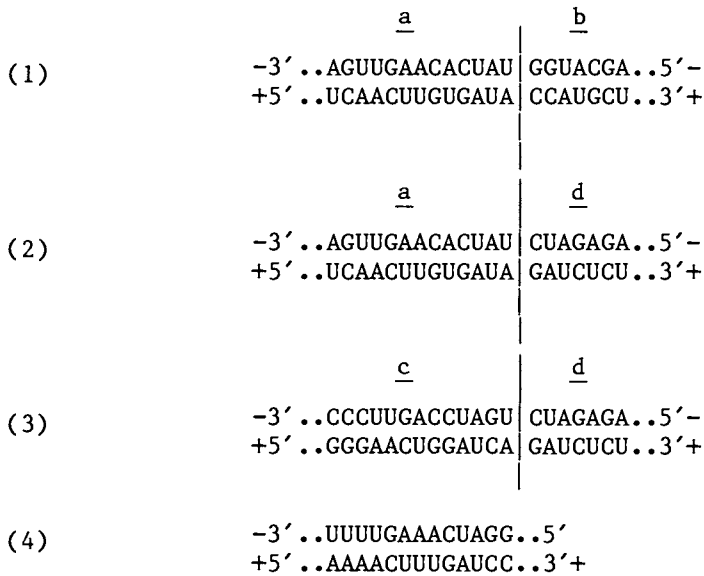
FIGURE 1.   Sequences surrounding the deletion in DI-LT.
The sequences in the (1) G,   (2) G*, and (3) L cistrons
surrounding the deletion point (|) have been aligned.   The
sequences a and d are conserved in DI-LT; the segment of the
genome bounded by sequences b and c is deleted. (4)   The
sequence at the 5' end of the L gene which terminates with a U
at position 60 from the 5' end of the genome.

from the 5' end of G message and at position 259 from the 3'
end of L message are both adenosine residues, the base at
position 1618 of G* could have been derived from either side
of the deletion.
    As a result of the large deletion in the DI-LT genome, the
last 54 nucleotides of the G cistron including the
polyadenylation signal, the intercistronic dinucleotide, and
all but the last 258 nucleotides of the polymerase gene have
been removed.   Because the normal regulatory signals are no
longer present at the junction between the G and L cistrons,
an open transcription unit has been created in DI-LT which
extends from the 3' end of the G cistron up to the remnant L
gene polyadenylation signal.   However, since the deletion is
downstream from the normal translation termination signal, the
protein encoded by G* is not affected (not shown).
    Comparison of the sequences present at the borders of the
deletion in the G and L cistrons reveals some elements of
similarity (Figure 1).   The sequence 3'..UUGA..5' is found in
both the conserved region of the G cistron (a) and in the

```
                        a              d

                        |
DI-LT          -3'..AGUUGAACACUAU|CUAGAGA..5'-
                        |

                        |
DI-LT2         -3'..CUACAAUUGUAGU|ACCGGGG..5'-
                        |

                        |
DI-0.50        -3'..ACAAUUGUAGUAC|CXXGGUU..5'-
                        |
```
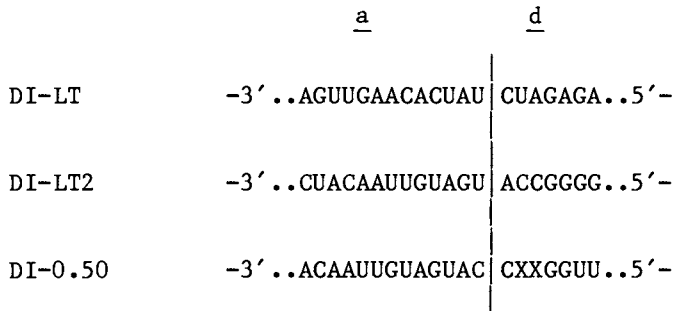
    FIGURE 2. Comparison of deletion mutants. The sequences
surrounding the deletion in the DI particles have been aligned
at that point (|). DI-LT2 sequence from ref. 14; DI-0.50
sequence from ref. 15. X, unidentified nucleotide.

deleted region c of the L cistron. A similar sequence has
also been shown to be present at the 3' end of both the leader
and L genes (16, 20) where it may play a role in transcription
termination. Several other elements of homology are also
observed at the borders of the deletion (Figure 1).
    Yang and Lazzarini (14) and De and Perrault (15) have
sequenced an internal deletion mutant(s) containing a deletion
whose boundaries are entirely within the polymerase gene. By
virtue of the techniques used to obtain those sequences (14,
15) it is likely that they are derived from the
transcriptionally-inactive DI-LT2 (DI 0.50).

MODEL FOR THE GENERATION OF DI PARTICLES

    A comparison of the three sequences at the boundaries of
the internal deletions reveals no obvious elements of homology
in the primary structure (Figure 2). Note, however, that De
and Perrault (15) have located their deletion two bases down-
stream from the position at which Yang and Lazzarini have
placed it. Although the primary sequences show no
similarities, homology is detected between the potential
secondary structures of these regions (Figure 3). Assuming
only standard A:U, G:C base pairing, the conserved a sequence
in both DI-LT and DI-LT2 can be drawn as a short base-paired
hairpin which is located just up stream from the deletion
point (Figure 3). (If G:U pairing is also permitted, then
slightly different hairpins could be drawn which encompass
the deletion points.)   The conserved d regions can also be
formed into small hairpins. The resulting DI genomes can,
thus, be folded into very similar secondary structures.

A. DI-LT

(1)   a   b   (2)   c   ↓   d

```
       G                                    U
      / \                                  / \
     U:A          |                       G:C
     U:A          ↓                       A:U
                                          U:A
-3'..UAG:CACUAUGGUACGA..5'-      -3'..CCUUGACC:GAGA..5'-
```

(3) a   ↓ d
```
          G     U-C
         / \ U-C
        U:A A:U
        U:A U:A
-3'..UAG:CAC:GAGA..5'-
```

B. DI-LT2

(1) a   b   (2) c   d
```
     A-U                                G-G
     A:U                                C:G
     C:G                                C:G
     A:U  |                             A:U
     U:A  ↓                      ↘ A:U
-3'..CUC:GUACAGUU..5'-           -3'..UCUGAGGA:UAG..5'-
```

(3) a   d
```
     A-U
     A:U
     C:G G-G
     A:U ⌐C:G
     U:A ⌊C:G
-3'..CUC:GUA:UUAG..5'-
```
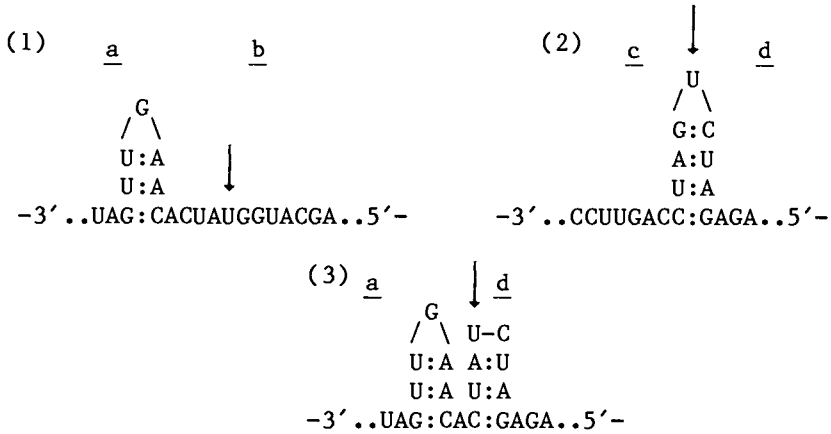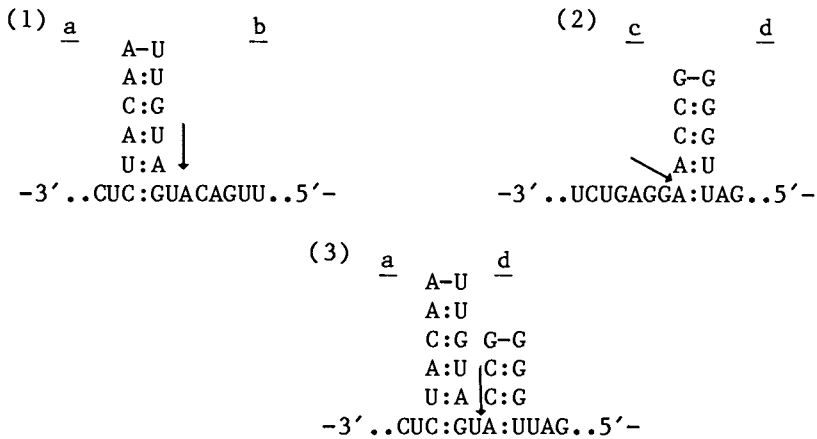
FIGURE 3. Secondary structure at deletion sites. The sequences of the (-) sense 42S genome surrounding the deletion points have been folded into hairpins using standard base pairing (:). Structures (1) and (2) are the upstream and downstream regions, respectively; structure (3) is the (-) sense sequence of the resulting DI particle. Sequences for DI-LT2 from ref. 14. Arrow, deletion point.

Thus, for the first time, two independently generated DI particles can be shown to be related, not by primary sequence, but by the potential to form very similar secondary structures. A model based on these observations suggests that

(1)   (2)

```
                    A                                          U
                   / \                                        / \
                  U:A                                        A:U
                  U:A /                                      A:U /
       +3'..AAUGUUAAUUUAG:C..5'+              -5'..UUACAAUUAAAUC:G
                                                             |
                                              +3'..AAUGUUAAUUUAG:C
                                                             U:A
                                                             U:A
                                                              \ /
                                                               A
```
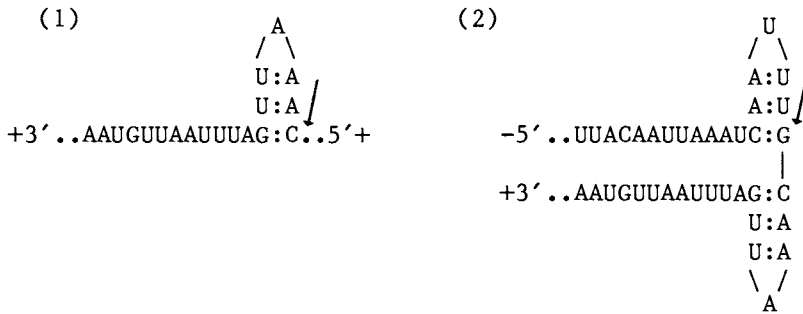
FIGURE 4. Structure of DI-011. (1) The sequence of the (+) sense 42S template at the site of premature termination (19) with potential hairpin. (2) The structure of the resulting DI-011. <u>Arrow</u>, site of premature termination.

secondary structure elements may play an important role in the generation of DI particles. The model predicts that the polymerase with attached nascent chain may be prematurely ejected from the template at or very close to sequences having the potential to form a small base-paired hairpin. The polymerase may subsequently reattach to a template at a another site possessing a hairpin sequence and then resume RNA chain elongation.

This model is also applicable to snapback type DI particles such as DI-011 which contains approximately 1700 nucleotides of covalently linked and complementary sequences from the 5' end of the VSV genome (19). The sequence of the (+) sense strand at the site where the polymerase prematurely detached from the template can be written as a small base-paired hairpin which is similar in structure to that found at the boundaries of the deletion in DI-LT (Figure 4). However, instead of reattaching to a template RNA, the polymerase appears to have recognized a hairpin structure at the 3' end of the nascent chain to resume RNA synthesis. The resulting DI-011 genome contains two small hairpin sequences which are tandomly arranged in virtually same way as they are in the internal deletion mutants. Thus, a single model accurately predicts the generation of DI particles with structures as diverse as those found in DI-LT and DI-011.

Since panhandle type DI particles such as DI-T differ from snapback DIs only in the length of the sequence that was copied from the nascent chain, it is likely that the generation of these DI particles is also predicted by the model. Most panhandle DI particles are generated when the polymerase resumes synthesis at a position 45-48 nucleotides

```
                                                          G
   (1)          59            /              (2)        / \
                 G           ↙                          U:A
                / \    A-U                              U:A
               U:A    A:U                               U:A
               U:A    G:C /                             G:U
        -3'..UUUUU:ACUAG:UGGGAGAACACC..5'-        -3'..AG:UCA..5'-
                      45        40                     83    70
```
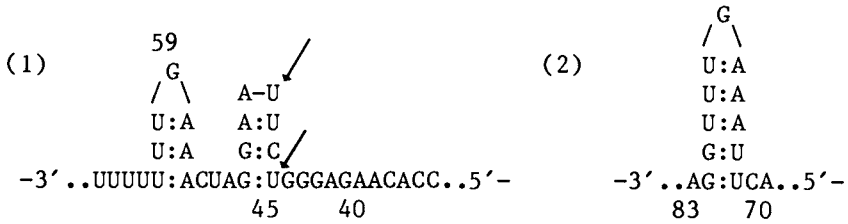
FIGURE 5.   Structures at resumption points.   (1) The most common "fall-back" site (21) results in 45-48-nucleotide panhandles.   (2) Possible site from which 70-nucleotide panhandles (23) would be generated.   Numbering is from the 5' end of the 42S RNA.   Arrows, sites of resumed synthesis.

from the 5' end of the nascent genome (21).   Schubert et al. (21) suggest that the sequence in this region resembles a normal polymerase binding site and this implies that the generation of DI particles is sequence-specific.   The model presented here suggests that this sequence may be more relevant to DI formation because it forms one arm of a small hairpin (Figure 5). As shown by Kolakofsky (23), DI particles containing panhandle sequences ranging from perhaps 70 to 200 base pairs can be isolated.   While the primary genomic sequences at these positions show no direct relationship to the sequence at position 45-48, they are related by the ability to form similar secondary structures (Figure 5). Since only limited sequencing data is available for panhandle type DIs at the sites of premature termination (21), it is not presently possible to analyze these sequences directly. Nevertheless, the model predicts that these sequences also contain small potential hairpins.

The model presented here may have broader implications for the regulation of viral RNA synthesis.   Hairpin sequences are located at both the end of the leader and L genes, positions at which the polymerase is known to terminate synthesis, and at each of the intergenic junctions in the VSV genome (not shown). While it is has not been demonstrated that such hairpins can form in RNA in the environment of a nucleocapsid, the presence of these structures, even if only transient, may be sufficient to regulate RNA synthesis.   Consequently, elements of secondary structure may punctuate the viral genome and serve as attenuators and enhancers of RNA synthesis by the negative strand viruses.

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SENDAI VIRUS DI RNA SPECIES CONTAINING 3'-TERMINAL GENOME FRAGMENTS[1]

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VARIETIES OF DI GENOMES

Defective interfering (DI) virions are deletion mutants. To survive, they require the replicative machinery provided by a co-infecting standard virus whose replication they, in turn, depress. Determining the mechanisms by which DI virions are generated, survive and interfere with replication of the standard virus are matters of special interest.

A variety of DI RNA genomes have been derived from non-segmented negative strand RNA viruses. The most common species found in vesicular stomatitis virus (VSV) and also found in Sendai virus is the copy-back type (1,2), in which only a 5'-terminal portion of the genome is conserved, the 3' end being replaced by a complementary copy of a portion of the 5' terminus. However, both viruses have also yielded DI genomes that retain both parental termini; examples are the LT RNA of vesicular stomatitis virus (VSV) (3) and the fusion DI RNAs of Sendai virus (4). LT RNA is deleted exclusively in a major 3' portion of the L gene, whereas Sendai virus fusion DI RNAs have lost all of their internal genes and intergenic sequences. These fusion DI RNAs retain a 3' fragment of the NP gene attached to the 3'-terminal leader RNA template on one side of the deletion and a 5'-terminal portion of the L gene on the other side.

An interesting fusion DI strain of Sendai virus is our
strain 7, which contains three subgenomic RNA species, 7a,
7b, and 7c, whose electrophoretic mobilities indicate
molecular weights of 1.24, 0.70, and 0.55 x 10^6 (about 26%,
15% and 11% of the standard virus genome), respectively (5).
Oligonucleotide mapping had indicated that RNA species 7b
retained at least part of the leader RNA template, but
revealed no sequences characteristic of the adjacent NP gene
(4). Direct RNA sequencing later showed that all three DI
RNAs in strain 7 had genomic 3' termini (5). Here, we
report that the minimum amount of 3' terminus retained by any
member of this DI RNA family is 100 nucleotides.

SEQUENCES OF SEPARATED FUSION DI RNA SPECIES

By direct RNA sequencing (6) and by dideoxynucleotide
sequencing with a synthetic primer (7), we determined the
3'-terminal 70 bases of RNA 7a and 100 bases of RNAs 7b and
7c. These were identical to the genomic 3'-terminal sequence
(Fig. 1; Gupta, Re and Kingsbury; Morgan, Gupta, Re and
Kingsbury, this volume). Guanine residues at positions 19
and 46 define a sequence whose first 26 bases are identical
to the sequence of T1-oligonucleotide 7 (4) extracted from a
2-dimensional mapping gel (our unpublished data). Indirect
evidence had indicated that oligonucleotide 7 was part of the
positive-strand leader template. Our sequencing data have
now confirmed this deduction.

At bases 56 to 65, there is 3'-UCCCAGUUUC (Fig. 1),
homologous (where underlined) to the consensus sequence that
starts each of the next 4 genes (Gupta, Re and Kingsbury,
this volume). This is the start of the NP gene, according to
sequence data on a complete NP gene clone (Morgan, Gupta, Re
and Kingsbury, this volume) and data of D. Kolakofsky
(personal communication).

Because the Sendai virus positive strand leader RNA has
not yet been sequenced, the 5' terminus of the leader RNA
template is not known. However, considering the many homolo-
gies in the organization of the Sendai virus and VSV genomes
(5,8,10; Gupta, Re and Kingsbury, this volume), it may be
base 51, since bases 52 through 55 are $(A)_4$ and a four- to
five-base A-rich sequence intervenes between the leader RNA
template and the start of the N gene in two VSV serotypes
(9).

```
          10        20        30        40        50
           I         I         I         I         I
 3'-UGGUUUGUUCUCUUCUUUGCACAUACCUUAUAUAUUACUUCAAUCUGUCCU


          60        70        80        90        100
           I         I         I         I         I
   AAAA UCCCAGUUUCAUAGGUGGGACUCCUCGUCCAAGGUCUGGGAAACG


          110       120
           I         I
   AAACGACGGUUUCAAGUGCUAC
```
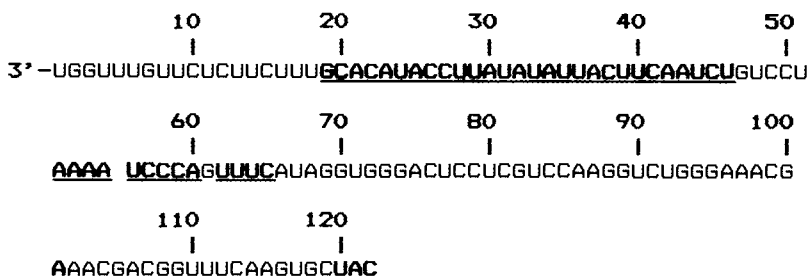
FIGURE 1. Sendai virus 3'-terminus. The positive strand
leader template is thought to end at base 51. The high-
lighted sequence at bases 19 to 46 is Tl-oligonucleotide 7
(4). The presumed intervening sequence (A)$_4$ is at bases
52-55 and the NP gene starts at bases 56-65. The A residue
at base 101 marks the first deletion site in a DI RNA
species of strain 7. The UAC at position 120 is the first
translational anticodon.

FURTHER SEQUENCING OF MIXED FUSION DI RNAs

     To expedite the task of learning the extent of 3'
terminus conservation in the family of strain 7 fusion DI
RNAs, we used the mixture as templates for dideoxynucleotide
sequencing. Our primer was a ^{32}P-labeled dodecamer comple-
mentary to bases 13 to 24. We expected the sequence of the
mixture to be homologous to the virus genome up to the point
where one of the DI RNAs contained a mutation, either by
base substitution or at the site of deletion. As shown in
Fig. 2, the mixed DI RNA sequence matched the virus genome
to position 100. This is evidently a deletion site, since
subsequent positions in the sequencing ladders contain extra
bands, missing bands, and anomalous band spacing, marked in
Fig. 2 starting with the asterisk at position 101 and con-
tinuing with arrowheads.
     Thus, at least one strain 7 DI RNA appears to retain
only 100 nucleotides of the genomic 3' terminus. This may
be RNA 7b, since 7b did not contain any NP-specific oligo-
nucleotides (4). However, we have not examined 7a or 7c by
oligonucleotide mapping. Taking the start of the NP gene as
the U in position 56, this DI RNA species contains only the
first 44 bases of the NP gene, falling 20 bases short of the
first UAC initiation anticodon at position 120 (Fig. 1).
The other two DI RNAs in the mixture may possess con-
siderably more of the NP gene, since the sequencing gels

contained traces of the genomic sequence up to about 300
bases from its 3' end (Fig. 2; other data, not shown).

FUSION DI GENOMES, TRANSCRIPTS, PROTEINS

      Our results reveal that all of the DI RNAs of strain 7
include the signal for initiation of NP mRNA transcription.
Tl-oligonucleotide mapping studies had shown that RNA 7b also
contains 5'-terminal L gene sequences and it seems likely
that all three of these RNAs possess the gemonic 5' terminus,
since no DI RNA lacking the 5' terminus has been isolated
from a nonsegmented negative strand RNA virus (1).   We have
shown that the L gene termination signal lies in positions 58
to 66 from the 5' end of the virus genome (10).   Since the
molecular weights of all three strain 7 DI RNAs are great
enough to include much more of the L gene, we expect that
they all contain L gene termination signals.
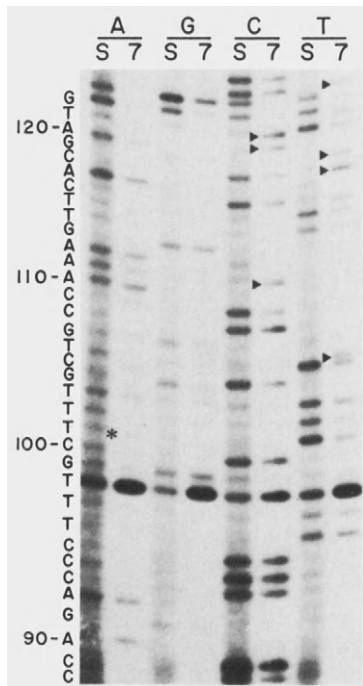
FIGURE 2.   3'terminal sequences of the Sendia virus genome
and mixed strain 7 DI RNAs.   S: genome; 7: DI RNAs.   The
asterisk and arrowheads denote loci that differ from the
genomic sequence.

Possessing both an NP gene start signal and an L gene stop signal, a fusion DI RNA species would have all of the sequence information necessary to direct synthesis of a fusion transcript. If such transcripts appear in cells infected with strain 7, their impact on the replication of the standard virus genome can be determined. Furthermore, depending on the availability of an NP initiation codon and the reading frame of the L gene fragment, fusion DI transcripts might specify fusion proteins containing N-terminal fragments of the NP protein and C-terminal fragments of the L protein. (Other reading frames of the L gene fragment might also be translatable, yielding fusion proteins with missense C-terminal regions.)   It seems unlikely that any such chimeric proteins could influence the outcome of infection, but the possibility is worth examining. We note in this context that members of the major class of DI RNAs of influenza virus, representing internally deleted RNA segments 1 to 3 (specifying the P proteins), possess transcription start and stop signals and have been transcribed in vitro (11), so a similar chain of events is conceivable in that case, as well.

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LONG-TERM PERSISTENCE BY VESICULAR STOMATITIS VIRUS
IN HAMSTERS[1]

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RNA and DNA viruses are being implicated with increasing
frequency in pathological changes associated with chronic
degenerative diseases. Since in many instances disease be-
comes apparent only many years after the initial virus infec-
tion, studies of viral persistence in animals are needed. As
a model system of long-term viral persistence, we have been
studying persistent infection of Syrian hamsters by vesicular
stomatitis virus (VSV).
     Acute infection of inbred LSH hamsters, following intra-
peritoneal (i.p.) injection of 100 plaque-forming units (pfu)
of VSV, Indiana serotype, results in death of 90% of the ani-
mals (1); however, lethality can be attenuated by coinjection
of defective interfering (DI) particles or polyI:polyC (2).
We previously have shown (3) that adult hamsters that survive
an acute infection of VSV become persistently infected and
that VSV could be recovered from brain, spleen, and liver tis-
sue homogenates as long as 8 months post-infection. All of
the hamsters from which virus was recovered had high titers of
anti-VSV neutralizing antibodies at the time of sacrifice and
some hamsters became transiently or permanently paralyzed in
one limb within two weeks of VSV injection. The present re-
port summarizes additional observations concerning long-term
persistence of VSV in hamsters.

_____

LONGEVITY AND CLINICAL STATUS

In our original study (3) only 5 hamsters were kept for an extended period following VSV infection and, aside from those that were partially paralyzed, no changes in the health of the infected animals were noted. It was of interest, therefore, to follow a larger number of animals to determine if VSV infection would alter the life expectancy of hamsters and/or result in any clinical manifestations of disease. At $2\frac{1}{2}$ months of age, 42 LSH hamsters received an i.p. injection of 10^4 pfu of VSV plus either 10^{10} DI particles or 100 μg of polyI:polyC. Figure 1 shows that at 14 months post-infection (age $16\frac{1}{2}$ months) only 48% of the hamsters were living. Although it was impractical to follow a large number of uninfected hamsters during this same time period, we know that hamsters can live to be 2 to 3 years old in captivity. It appeared, therefore, that VSV infection during early adulthood shortened the life expectancy of LSH hamsters and that an increase in the rate of death occurred at approximately 9 months post-infection.

In this same group of 42 hamsters we observed: (i) hemorrhage from the nose and anus prior to death--a feature of death due to acute systemic VSV infection in hamsters; (ii) kidney disease, as evidenced by dramatic fluctuations in urine output; and (iii) neurological disease, as evidenced by peri-
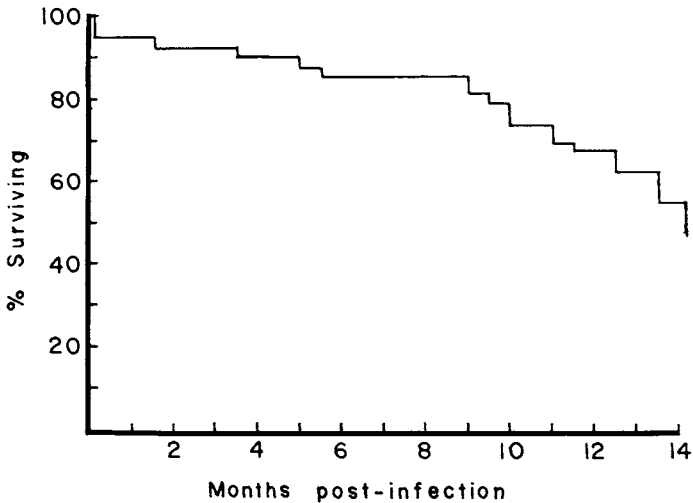
Figure 1. Mortality of LSH hamsters persistently infected with VSV.

odic seizures and tremors with accompanying changes in social
behavior.

HISTOPATHOLOGY

    Histologic examination of tissues taken from animals 4 to
16 months post VSV infection have revealed several pathologic
changes. Changes seen in livers and kidneys included inflam-
matory cell infiltrates (Figure 2A), foci of hepatocellular
necrosis, and dilated or congested tubules in kidneys. In
liver tissue from an animal sacrificed 13½ months after VSV
infection, foci of necrotic cells that resembled active sites
of infection were positive for VSV antigens by indirect immu-
nofluorescence assays with a hyperimmune rabbit anti-VSV anti-
serum. The pathology of the liver and kidney sections that
were examined was consistent with immune complex disease, but
this has not been verified.
    Lesions in the central nervous system (CNS) were seen
only in the gray matter where the most prominent evidence of
disease was a marked cellular drop-out in both the brain and
spinal cord. Spongiform lesions were seen most often in the
hippocampus (Figure 2B), but in some animals other areas of
the brain also were affected. There was not only neuronal
cell drop-out but also neuronophagia with round cells adjacent
to neurons in the pons of the brain of an LSH hamster sacri-
ficed one year post-infection. The subsequent demonstration
by indirect immunofluorescence of VSV antigens in the pons of
this same animal indicated that these lesions probably were a
result of VSV infection. These studies showed that pathologic
lesions that are associated with VSV antigens are prominent in
liver, kidneys, and the CNS of hamsters persistently infected
with VSV.

IMMUNOCOMPETENCE

    We showed above that VSV establishes persistent infec-
tions in hamsters and that many months after infection vari-
ous organs contain cells that express VSV antigens. It is
important to identify ways by which VSV can escape host immu-
nosurveillance mechanisms. Escape from immune elimination
could be accomplished either by specific failure or inactiva-
tion of components of the immune system or by viral mutation
to avoid immune surveillance. As stated above, all hamsters
from which VSV was isolated after long-term infections had
titers of VSV neutralizing antibodies comparable to those
found in animals 2 to 4 weeks post-infection. To determine if
anti-VSV antibody titers decreased or fluctuated following
initial VSV infection, we periodically obtained serum from
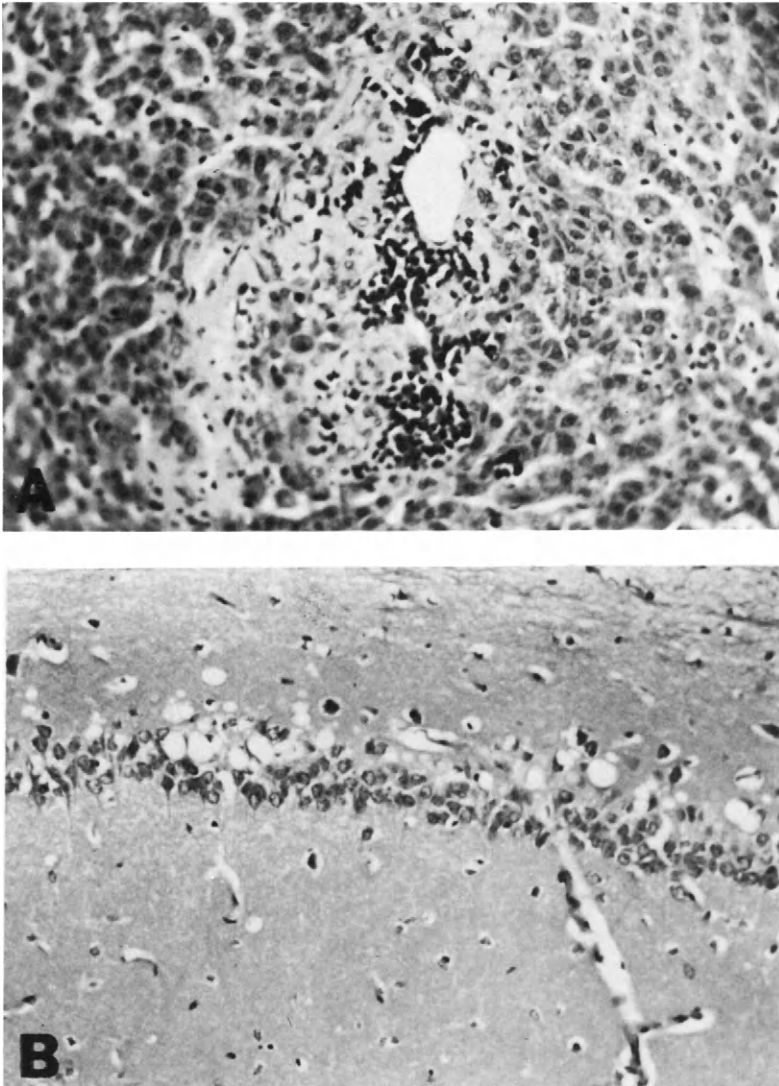
Figure 2.   (A) Inflammatory cell infiltrate in liver
parenchyma near a portal vein, found in an LSH hamster sacri-
ficed 6 months post-infection.   (B) Neuronal cell drop-out in
the hippocampus of the same LSH hamster as in A.   This animal
had experienced at least 3 seizures in the month preceding
sacrifice.   Hemotoxylin and eosin, x400.

animals via the retro-orbital plexus and assayed the sera for
neutralizing antibodies in plaque-reduction assays (3). Fig-
ure 3 shows antibody titers in 4 animals from one month to 16
months post-infection. It is obvious that following VSV in-
fection neutralizing antibodies to VSV remain at high levels
for the remainder of the animals' lives. These data indicate
that not only are VSV-specific B cells synthesizing antibodies
but also that helper T cells are functional during VSV per-
sistence.

   To determine if hamsters persistently infected with VSV
can eliminate VSV-infected cells, we injected 10^7 BHK cells or
VSV-persistently infected BHK cells (BHK-VSV) subcutaneously
into inbred LSH and random-bred LVG hamsters that had received
an i.p. injection of VSV 6 to 11 months earlier. Table 1
shows that irrespective of the status of the animal (normal or
VSV-infected), hamsters cannot reject large numbers of BHK
tumor cells, whether normal or VSV-infected. The only excep-
tion was an uninfected LVG control that did not allow tumor
formation, presumably due to killing of the BHK-VSV cells by
cytotoxic cells. It is assumed that inbred LSH hamsters are
unable to reject BHK tumor cells (derived from random-bred
hamsters) because LSH hamsters and all random-bred hamsters
tested (4) have the same major histocompatibility haplotype.
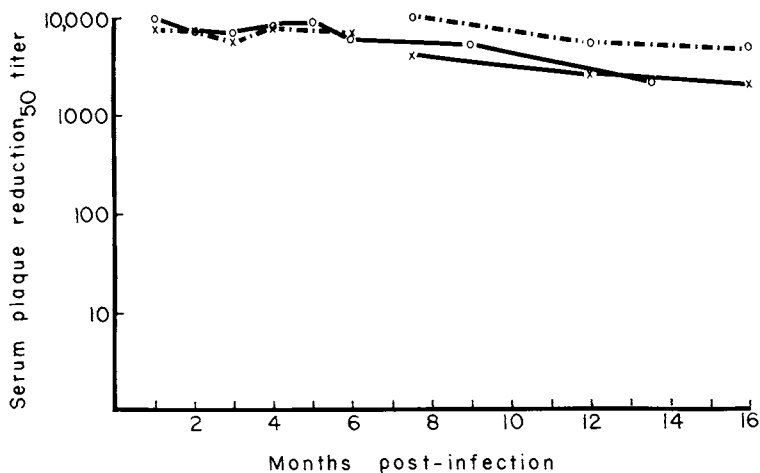
   Figure 3. VSV neutralizing antibody titers in sera from
hamsters infected with 100 pfu of VSV. The different combina-
tions of symbols and lines represent serum titers in 4 animals
at different times post-infection. Titers are the dilution of
serum that reduced VSV plaques by 50%.

TABLE 1
GROWTH OF TUMOR CELL LINES IN NORMAL AND
VSV-INFECTED HAMSTERS[a]

Tumor cells	Hamster strain	Hamsters infected	Total no.	No. with tumors
BHK	LSH	–	3	3
		VSV	6	6[b]
	LVG	–	1	1
		VSV	4	4
Total			14	14
BHK-VSV	LSH	–	4	4[b]
		VSV	6	6[b]
	LVG	–	5	4
		VSV	5	5
Total			20	19

[a]See text.
[b]One tumor in this group grew to a diameter of approx-
imately 2 cm and then regressed.

To determine whether the tumors that grew from the inocula of
BHK-VSV cells contained VSV, cell lines were established from
19 excised tumors; 7 of the tumors were from animals that were
normal at the time of tumor cell inoculation and 12 were from
VSV-persistently infected animals. Indirect immunofluores-
cence assays and superinfection experiments with the 19 cell
lines revealed that essentially all of the VSV infected cells
in the inoculum had been eliminated. Of the 19 cell lines
tested by immunofluorescence for VSV antigens, 16 were nega-
tive and 3 had less than 0.1% positive cells. In superinfec-
tion experiments all of the cell lines gave yields of VSV com-
parable to the yield of VSV on uninfected BHK cells (data not
shown). Thus, host defense systems of VSV-persistently infec-
ted hamsters apparently can recognize and lyse VSV-infected
cells, suggesting that the persistent state is not maintained
via VSV-specific immunosuppressive mechanisms. We conclude
that VSV-persistently infected hamsters are immunocompetent
with respect to VSV.

CHARACTERIZATION OF RECOVERED VIRUSES

    Since there appeared to be no obvious defect in immune
reactivity to VSV in VSV-persistently infected hamsters, we
looked for changes in the recovered viruses. VSV, serologic-

ally identical to the inoculated virus (VSV_O), was cloned by
serial plaque isolations. The clones were analyzed by Tl
oligonucleotide mapping, for temperature sensitivity (ts), and
for alterations in plaque morphology. Of 18 clones tested for
genomic mutations by Tl mapping, 11 clones had maps identical
to VSV_O and 7 had one or two spot differences. Phenotypic
analyses of the clones showed that greater than 90% were ts at
39.5^OC and 4 clones with VSV_O Tl maps were small-plaque mu-
tants (data not shown). The fact that Tl mapping did not re-
veal mutations in virus clones that were phenotypically diffe-
rent from VSV_O is not surprising since Tl mapping identifies
only 10% of VSV nucleotide changes. We conclude that VSV does
accumulate genomic mutations during in vivo persistence, anal-
ogous to earlier findings (5,6).

DISCUSSION

    We have shown that VSV persists in various organs of in-
bred and random-bred hamsters following i.p. injection of the
virus. Whether one or all of the resulting disease states are
caused directly by VSV cytopathic effects or are immunopatho-
logical is not yet known. We do have evidence that autoanti-
bodies are present in sera from some persistently infected
hamsters. It will be interesting to see if autoimmunity is
elicited in hamsters due to the polyclonal B-cell activation
property of VSV (7) or to crossreaction of antibodies to VSV
with cellular components (8). The VSV persistent state exists
in hamsters despite continual production of VSV neutralizing
antibodies. VSV apparently does not undergo major antigenic
changes to escape immunosurveillance since antiserum to VSV_O
neutralizes recovered viruses (3) and gives positive immuno-
fluorescence in VSV-persistently infected tissues. The con-
tinued high production of VSV neutralizing antibodies and the
apparent lack of VSV-specific immunosuppression in these ani-
mals could be explained if VSV replicates in and inactivates
a subset of hamster suppressor T cells as has been demonstra-
ted for VSV in the murine system (9). The persistence of VSV
despite a functional immune system and the failure to accumu-
late antigenic mutations suggests that VSV may escape immune
attack through viral mutations that alter replication and/or
maturation. Perhaps VSV does accumulate debilitating muta-
tions during long-term persistence in hamsters since we have
failed to isolate virus from tissues taken 13 months post-
infection that were positive for VSV antigens by immunofluor-
escence. The VSV-hamster system provides a unique model for
studying chronic degenerative disease and the effects of per-
sistent infection on the immune system in adult immunocompe-
tent animals with a cloned, biochemically well-characterized
virus.

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PERSISTENT INFECTIONS OF BHK-21 CELLS WITH RABIES VIRUS[1]

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Three BHK-21 cell lines persistently infected with the CVS strain of rabies virus have been established from independent cell clones. They have been maintained at 37°C for 2 years. Production of plaque forming units (PFU) was low, irregular and heterogeneous (small plaque variants, temperature sensitive and semi lethal mutants). It ceased definitively between the thirthieth and the sixthieth passage depending on the cell line.

After that period, cells continued to release variable amounts of slowly replicating non-plaque forming units and non-infectious particles. The presence of defective interfering (DI) particles and interferon in the supernatant of the cell cultures was not evidenced using a technique which allowed to detect at least 10^4 to 10^5 DI particles or 1 interferon unit/ml. Immunofluorescence staining with fluorescein conjugated anti-nucleocapsid antibodies revealed that nearly every cell was positive. The fluoresceing pattern was identical to that of CVS acutely infected (AI) cells suggesting that the N protein was still preferentially associated into nucleocapsids.

Intracellular viral proteins and RNA syntheses have been studied and results are presented hereunder.

INTRACELLULAR VIRAL PROTEINS

Immunoprecipitation of intracellular viral proteins were performed with serum from CVS immune mice (Fig. 1). From AI cell lysates, the two forms of the glycoprotein, G_1 and G_2, were precipitated as well as the nucleocapsid protein N and the matrix protein M_1. From PI cell lysates only N and M_1 proteins were precipitated. The ratio between the N and M_1 proteins was the same in AI cells and in two PI cell lines (D, F). In PI
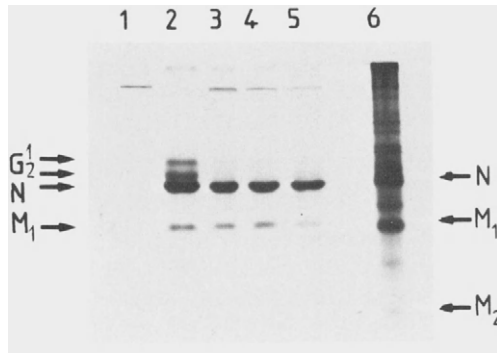
FIGURE 1.   Immunoprecipitation with mouse antiserum of
lysates from uninfected cells (lane 1), 44 hours-CVS-infected
cells (lane 2) and PI cells D, F, J (lanes 3, 4, 5).
    10^5 cells were labelled for 20 hours with 40 µCi of (^{3}H)-
lysine. They were the disrupted in lysis buffer (0.5 % NP_{40},
0.15 M NaCl, 50 mM Tris HCl pH 7.4) and sonicated for 15 sec.
Cell debris were removed by centrifugation at 10,000 rpm in an
Eppendorf centrifuge. Cell lysates were incubated overnight at
4°C with serum from adult mice inoculated with inactivated CVS.
Then anti-mouse globulins were added for 4 hours. The complex
was spun down, washed twice with buffer (0.15 M NaCl, 15 mM
Tris HCl pH 7.4, 5 mM EDTA, 5 % sucrose, 1 % NP_{40}), disrupted
in Laemmli buffer, and analyzed in a 10 % polyacrylamide SDS
gel. Lane 6 shows viral proteins in AI cells .

cell line J it was two times lower (Fig. 1, lane 5). In the
three PI cell lines, the G protein was undetectable and so re-
presented less than 10 % of the amount present in AI cells.
    Immunoenzymatic tests with monoclonal antibodies n° 101-1
and 509-6 (1) recognizing sites I and II of the glycoprotein
(2) also showed a restriction of this protein. In order to
point out a low amount of G protein in PI cells, (^{3}H) glucosa-
mine labelling was performed for 20 hours. Cell extracts were
analyzed on a 8-12 % polyacrylamide SDS-gel (Fig. 2). In unin-
fected cells no glycosylated protein migrated at the same pla-
ce as the viral glycoprotein. Therefore minor amounts of G
protein should have been detectable in PI cells. However no si-
gnificant amount of the glycoprotein could be detected.
    The L and M_2 proteins were visible in AI cells on longer
exposed autoradiograms. In PI cells only the L protein was seen.
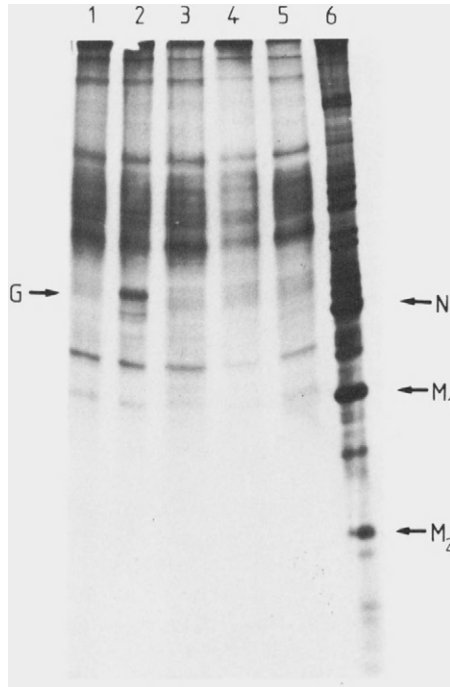
FIGURE 2.   (^{3}H)-glucosamine labelled proteins
     Uninfected cells (lane 1), 6 hours CVS-infected cells (la-
ne 2) and PI cells D, F, J (lanes 3, 4, 5) were incubated for
20 hours in the presence of 50 μCi of (^{3}H) glucosamine. Cell
were disrupted in Laemmli buffer and analyzed in an 8-12 %
polyacrylamide SDS-gel.Lane 6 : marker proteins.

TRYPTIC MAP OF N AND M_1 PROTEINS

     Whether or not N and M_1 underwent genetical evolution in
PI cells was studied by establishing the tryptic peptide map
of the proteins at the eightieth passage. Since it is possible
that viral material evolves independently in each cell, a glo-
bal extract may contain an heterogeneous viral protein popula-
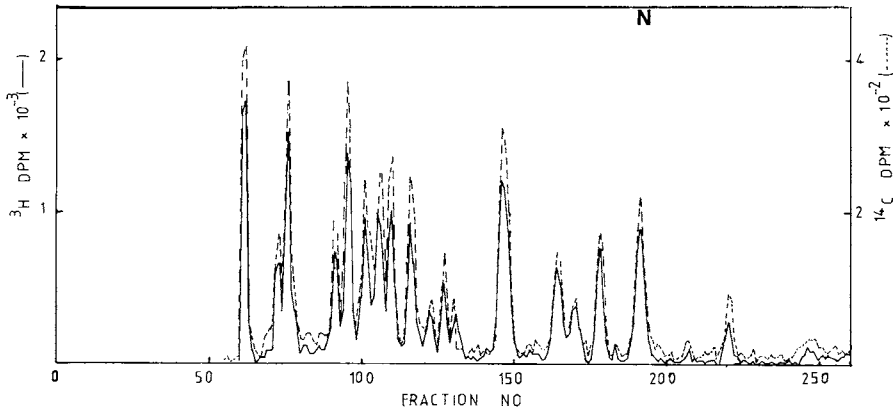
FIGURE 3.   Tryptic peptide map of N protein extracted
from PI cell line J.
    (^{3}H) lysine labelled N protein was prepared from PI cells
by immunoprecipitation with mouse antiserum. (^{14}C) lysine la-
belled N protein from purified virions was eluted from a 10 %
polyacrylamide SDS gel. (^{3}H) and (^{14}C) proteins were mixed,
reduced with dithiothreitol (O.1 M), alkylated with iodoaceta-
mide (O.25 M) and incubated with trypsin (TPCK, Worthington).
Tryptic peptides were separated by a pH gradient from 2.4
(O.1 M pyridine acetate) to 5.2 (2 M pyridine acetate) on a
chromobead P (Technicon) column (3,4).
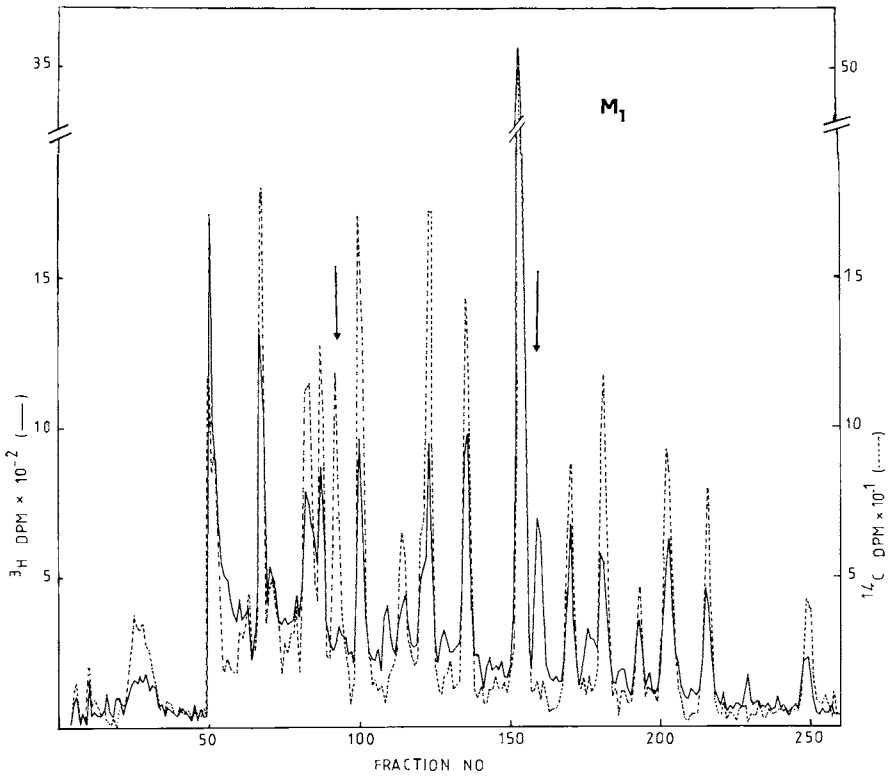
FIGURE 4.   Tryptic peptide map of M_1 protein extracted
from PI cell line J.
    (^{3}H) lysine labelled protein from PI cell and (^{14}C) lysi-
ne labelled protein from purified virions were prepared and
digested as explained in Fig. 3.

tion. To avoid this problem, cell extracts were performed from recently cloned PI cells. (^{3}H) labelled proteins were obtained from PI cell lysates by immunoprecipitation with mouse antiserum. (^{14}C) labelled proteins were prepared from purified CVS virions.

Fig. 3 presents the peptide map of N protein extracted from PI cell line **J** or from virions. No difference appeared between the two patterns. The same results were obtained with N proteins extracted from the other two PI cell lines.

On the contrary peptide pattern of M_1 protein from PI cell line J exhibited several modifications indicated by arrows on fig. 4. Tryptic maps performed with M_1 purified from other PI cell lines also revealed several modifications, although in other peaks (data not shown).

RNA CONTENT OF INTRACELLULAR VIRAL NUCLEOCAPSIDS

We have analyzed RNA from intracellular viral nucleocapsids in order to see if it was full size (42S) or defective. (^{3}H) uridine or (^{32}P) Na_3PO_4 labelled intracellular nucleocapsids from PI or AI cells were purified on a CsCl gradient and their RNA were analyzed on a sucrose gradient (fig. 5). PI nucleocapsids from AI cells contained an RNA which sedimented as a single peak. In the three PI cell lines, a major peak of (^{3}H) RNA sedimented as the single peak of (^{32}P). In two cases (PI cell lines D and J) additional RNA of smaller and heterogeneous size was also detected (fig. 5 (2) and (3)). (^{32}P) radioactivity at the top of the gradient probably corresponds to the N protein which is phosphorylated in vivo (5).

DISCUSSION

Long-term persistent infection with rabies virus was characterized by a relatively stable amount of N protein (detected with fluorescein-conjugated antinucleocapsid antibodies) in PI cells in the course of several passages. Analysis by tryptic peptide maps revealed that this protein did not undergo a great evolution. On the contrary M_1 protein presented several modifications in its peptide pattern indicating that some evolution of the protein was compatible with the maintenance of the PI state. L protein was also detectable in PI cells by radiolabelling but in too low an amount to be further analyzed. Persistence of L, N, and M_1 proteins is not surprising since those proteins are thought to be necessary for viral transcription and replication. G protein could never be detected either by immunoprecipitation, by immunoenzymatic test or by specific labelling with (^{3}H) glucosamine, and thus represented less than
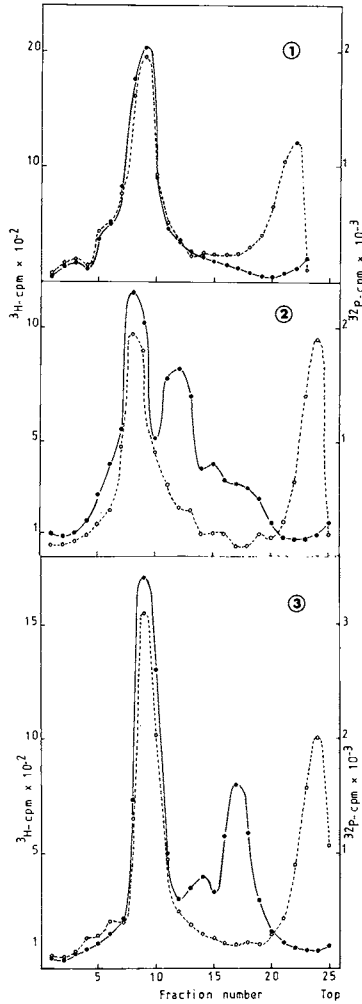
FIGURE 5.   Nucleocapsid RNA from PI cells.
      PI cells were labelled in presence of 2 µg/ml of Actino-
mycin D with 100 µCi of (^{3}H) uridine for 20 hours.
      Eight hours-CVS infected cells were labelled in presence
of 2 µg/ml of Actinomycin D with 700 µCi of (^{32}P) Na$_3$PO$_4$ for
20 hours in a medium without phosphate.
      Intracellular nucleocapsids were purified on a CsCl gra-
dient (20-40 %) (13) and pelleted. Nucleocapsids from PI cells
(●——●) and from AI cells (●·····●) were mixed, treated with 0.5 %
SDS· and analyzed on a 5-23 % sucrose gradient.
      (1),(2), (3) : viral RNA from PI cells F, D, J.

10 % of what is found in AI cells. The amount of M_2 protein
seemed also to be restricted in PI cells (as shown by labelling
and immunoprecipitation).

The requirement of functional G and M_2 does not seem to be
necessary for the maintenance of PI state. Several authors also
reported modification (or restriction) of maturation proteins
in PI cells (6, 7, 8, 9). In SSPE disease the disappearance of a
membrane protein have also been reported (10, 11, 12).

ACKNOWLEDGMENTS

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MEASLES VIRUS PERSISTENT INFECTIONS :
MODIFICATION OF FATTY ACID METABOLISM

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INTRODUCTION

Measles virus belongs to the paramyxovirus group and in common with several of the other members of this group can give rise to persistent infections both in vitro and in vivo. Several years ago, we established a measles virus (Hallé strain) persistent infection in BGM (African green monkey kidney) cells (1). Initially the cultures produced high yields of infectious virus, but after 2 years of culture (150 passages), no infectious virus could be detected. Small quantities of virus particles continued to be released and these contained the normal complement of virus structural proteins (2).

Examination of the persistently infected cells (BGM-P) by immunofluorescence with polyclonal serum revealed that $\geqslant$ 95 % of the cells contained virus antigens, although the intensity of the staining varied with either the cell passage or time after trypsinisation. ^{125}I-iodination of the cell surface proteins and subsequent analysis by immunoprecipitation and polyacrylamide gel electrophoresis showed that both of the virus envelope antigens, hemagglutinin (HA) and the hemolysin (F) were expressed at the cell surface. When BGM-P cells were radiolabelled with ^{35}S-methionine and the virus-induced proteins analysed by the same techniques, the same spectrum of virus-induced proteins was found as in lytically infected cells (2). No modification in their electrophoretic mobility was noted.

MONOCLONAL ANTIBODY

The availability of measles virus monoclonal antibody to 5 of the 6 virus structural proteins (3) enabled us to probe further the comparison of the distribution of virus antigens in lytically (BGM-L) and BGM-P cells. The main

differences observed in immunofluorescence studies were seen
with the antinuclear protein (NP) and L-monoclonal anti-
bodies, FIGURE 1. As many as 50 % of the BGM-P cells contai-
ned intranuclear virus inclusion bodies and these could only
be stained with anti NP-monoclonal antibody. After cell
passage (trypsinisation), the intranuclear inclusions were
not found for a further 24 h, after which they accumulated,
reaching a maximum by the third day. It is presumed that
cells containing intranuclear virus antigens are in a termi-
nal state and are lost during cell passage. In BGM-L cells,
intranuclear antigens were observed only during late infec-
tion (28 h) and even then were limited to not more than 5 %
of the cells.

Immunofluorescence studies on BGM-P cells with a
single anti-L monoclonal antibody indicated that during the
evolution of the persistent infection there was a gradual
reduction of the protein detected. It was not established if
there was less L-protein synthesised or if there was a loss
of the epitope.

LIPID COMPOSITION

We were perplexed by our observations that similar
quantities of virus envelope antigens were synthesised and
expressed at the cell surface of BGM-P cells as in BGM-L
cells without leading to cell lysis. As lysis is also
intimately associated with the lipid composition, we exa-
mined the plasmic membranes for lipid changes.

The total lipids were extracted from monolayer cul-
tures of uninfected (BGM), BGM-L and BGM-P cells and the
individual phospholipids separated by thin layer chromato-
graphy (4). There were no major differences in the phospho-
lipid composition of the 3 systems examined.

The fatty acid composition of the total phospholipid
was examined by gas-liquid chromatography. There was a 25 %
increase in the palmitic acid ($C_{16:0}$) content and a 30 %
decrease in oleic acid ($C_{18:1}$) in BGM-P compared to BGM and
BGM-L cells. When the individual phospholipids were sepa-
rated, the increase of $C_{16:0}$ was found to be associated with
phosphatidylcholine (PC). The decrease in $C_{18:1}$ was asso-
ciated with both major phospholipids, PC and phosphatidyl-
ethanolamine (PE). There was also an accumulation of ara-
chidonic acid ($C_{20:4}$) in the PE fraction (50 % more than
BGM) and a decrease in BGM-L cells.

Typical distribution of virus proteins
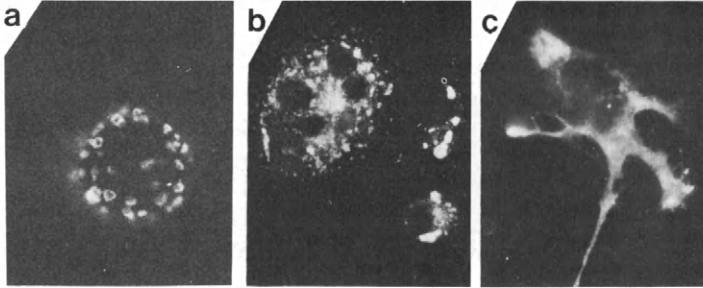
Modification of antigen distribution
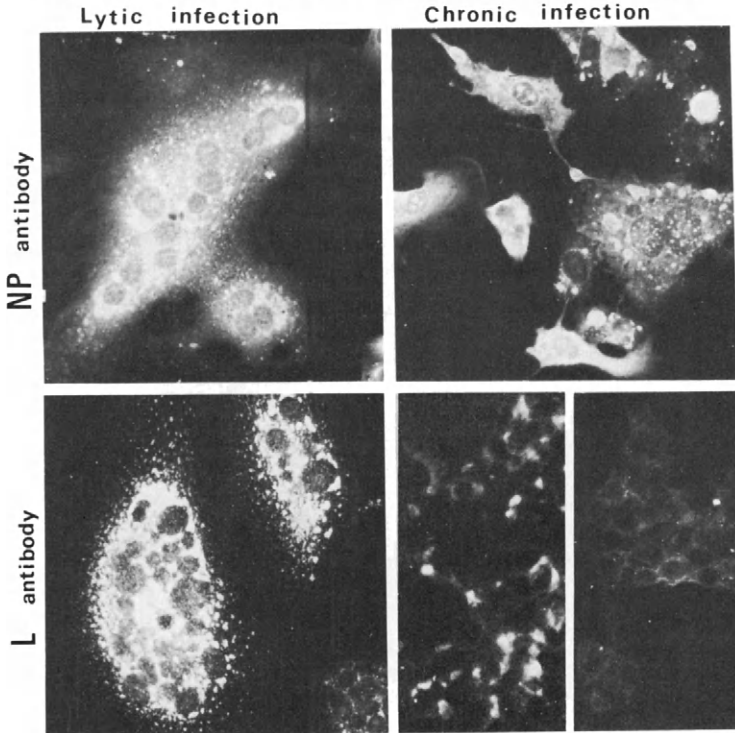
FIGURE 1. Immunofluorescence studies with measles virus monoclonal antibody. a. Membrane fluorescence with anti-HA (magnification X2000). b. Anti-NP on fixed cells. c. Anti-L fixed cells.

LIPID METABOLISM

     To study if the modification of the fatty acid compo-
sition of the cell membrane of BGM-P cells was due to
changes in the fatty acid metabolism we radiolabelled the
cells with two fatty acids. (a) ^{3}H-C$_{20:4}$ was chosen because
its composition is altered in BGM-P cells and is incorpo-
rated into the C-2 position of the glycerol moiety. (b)
^{14}C-stearic (C$_{18:0}$) whose composition is unchanged in BGM-P
and is incorporated in the C-1 position of glycerol.
     Monolayer cultures of BGM, BGM-L and BGM-P cells were
labelled with ^{3}H-C$_{20:4}$ and ^{14}C-C$_{18:0}$ for 2, 4 and 6 h. The
lipid was extracted and the incorporation of radioactivity
determined. There was no difference in the uptake of the two
isotopes into the different systems examined. The lipid
extracts were resolved into their neutral and polar species
by one dimensional thin layer chromatography and the parti-
tion of the ^{3}H and ^{14}C isotopes determined, FIGURE 2.
^{3}H-C$_{20:4}$ and ^{14}C-C$_{18:0}$ were incorporated into the neutral
lipids of BGM-P, 10 times and 2 times respectively, greater
than into BGM or BGM-L cells. To locate the increase in
incorporation of the isotopes, the neutral lipids were frac-
tionated and the distribution of radioacitivity determined,
TABLE 1.

TABLE 1
DISTRIBUTION OF ^{3}H-C$_{20:4}$ AND ^{14}C-C$_{18:0}$
IN NEUTRAL LIPIDS

^{3}H -arachidonic acid

	BGM	BGM-L	BGM-P
Cholesterol esters	0.38+0.09	0.41+0.10	3.05+0.40(***)
Triglycerides	0.33+0.08	0.28+0.04	21.49+1.56(***)
Free fatty acids	0.07+0.01	0.15+0.04	3.20+0.49(***)
Diglycerides	0.73+0.14	0.54+0.08	3.81+0.36(***)
Cholesterol	1.09+0.24	1.34+0.23	2.33+0.35(*)
Monoglycerides	0.17+0.02	0.19+0.03	0.60+0.36

^{14}C -stearic acid

	BGM	BGM-L	BGM-P
Cholesterol esters	2.55+0.82	1.87+0.38	2.97+0.84
Triglycerides	5.07+1.05	5.75+0.92	28.31+4.19(***)
Free fatty acids	0.05+0.02	0.06+0.03	0.37+0.15
Diglycerides	3.97+0.43	3.65+0.57	5.36+0.98
Cholesterol	2.91+0.47	3.04+0.47	2.36+0.51
Monoglycerides	0.50+0.21	0.25+0.08	1.42+0.53

     Results are expressed as the percentage dpm in phos-
pholipid + S.D., in the total lipid. Table reproduced with
permission from Biochem. J. (1983) 214, 665-670.
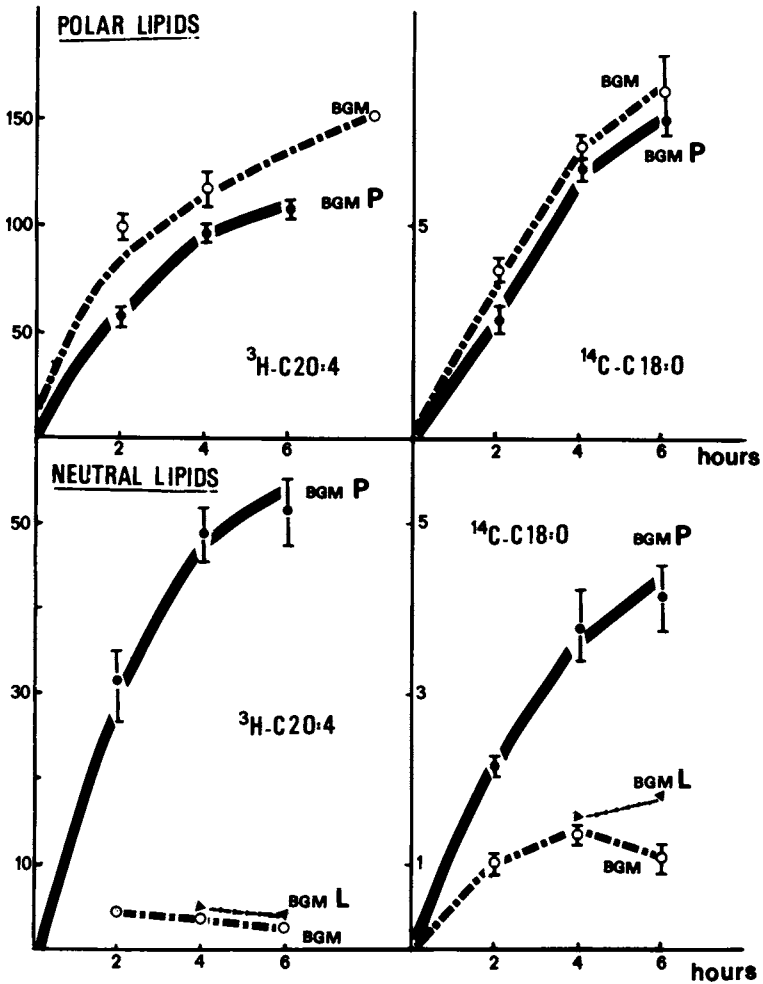
FIGURE 2. Incorporation of ^{3}H-arachidonic acid and ^{14}C-stearic acid into uninfected, lytically and persistently infected BGM cells. Reproduced with permission from Biochem. J. (1983) 214, 665-670.

In BGM-P, there was a 63-67 fold increase in the incorporation of $C_{20:4}$ into the triglycerides and a 37-61 fold increase into the free fatty acids. $C_{18:0}$ was also increased, but not by the same order of magnitude.

Analysis of the distribution of the isotopes into the polar lipid fraction, TABLE 2, showed that compared to BGM, the BGM-P cells had a reduced incorporation of $^3H-C_{20:4}$ into PC, PE and phosphatidylinosine and $^{14}C-C_{18:0}$ was also reduced by up to 50 % in PC. To verify that the radioactive precursors were incorporated into the correct position in the diacyl molecule, the radiolabelled phospholipids were hydrolysed with phospholipase A2 and the released fatty acid analysed. 94 % of the ^3H was found at the C-2 position and 87 % of the ^{14}C at the position C-1 position.

<div align="center">

TABLE 2

DISTRIBUTION OF $^3H-C_{20:4}$ AND $^{14}C-C_{18:0}$
IN PHOSPHOLIPIDS

</div>

	BGM	BGM-L	BGM-P
20:4			
Lysophosphocholine	0.1+0.1	0.2+0.1	0.1+0.1
Sphingomyelin	0.1+0.1	0.2+0.1	0.1+0.1
Phosphatidylcholine	31.2+0.9	27.9+0.7	24.6+0.6(**)
Phosphatidylinosine	33.4+0.4	30.5+0.7(*)	21.8+0.4(***)
Phosphatidylserine	3.2+0.2	4.8+0.4	3.8+0.1
Phosphatidylethanolamine	28.9+0.8	33.2+1.6	14.2+1.0(***)
Diphosphoglyceride	0.4+0.1	0.3+0.1	1.1+0.2
18:0			
Lysophosphocholine	0.5+0.1	0.4+0.1	0.4+0.1
Sphingomyelin	1.4+0.1	2.6+0.4	0.4+0.1
Phosphatidylcholine	45.2+1.1	45.6+1.4	19.7+1.1(***)
Phosphatidylinosine	19.4+0.7	19.5+0.7	15.0+1.2(*)
Phosphatidylserine	9.6+0.6	10.0+0.8	6.5+0.8(*)
Phosphatidylethanolamine	18.3+0.4	16.8+0.8	14.6+0.9(**)
Diphosphoglyceride	3.0+1.5	2.1+0.5	7.0+2.1

Results are expressed as the percentage dpm in phospholipid + standard deviation, in the total lipid.

(***) Signifies that there is a significant difference from the corresponding value for uninfected BGM cells.

DISCUSSION

Calculation of the relative specific activity of the two fatty acids in the phospholipids, which is a measure of the relative turnover, showed there was a parallel decrease in incorporation into PC of BGM-P for both fatty acids. This presumably results from a slower turnover of the entire diacylglycerol molecule. In contrast in PE of BGM-P, there is a large reduction in the turnover of $C_{20:4}$ compared to $C_{18:0}$. This would explain why we found an accumulation of $C_{20:4}$ in PE of BGM-P cells by chemical analysis. In the BGM-L cells, there was an increase in $C_{20:4}$ turnover.

It has been reported that the transmethylation of PE to PC is rendered insensitive during measles virus persistent infections (5). Although the N-methylation pathway of PE is relatively limited in this type of cell (6) and its inhibition could not account entirely for the accumulation of $C_{20:4}$ in neutral lipids, its biological implications are extremely important. The decrease in secretable $C_{20:4}$ could at the level of the animal lead to a metabolic disease. It may therefore be important to re-investigate the ethiology of certain of the metabolic disorders.

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ASSEMBLY OF MEASLES VIRUS NUCLEOCAPSIDS
DURING LYTIC AND PERSISTENT INFECTIONS[1]

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Persistence in cell culture may arise from subacute in-
fections involving either temperature sensitive viruses or
defective interfering particles. There is evidence, though,
that the maintenance of persistence may result from a
disruption in normal morphogenesis of virus particles in the
cells (1). In this study we describe a model system for
studying the morphogenesis of measles virus in both lytic and
persistent infections in culture.

The general inhalation anesthetic, halothane (2-bromo-2-
chloro-1,1,1-trifluoroethane), reversibly inhibits measles
virus replication (2). Halothane vapors do not inactivate
measles virions nor do they prevent entry of the virus into
cells. During lytic infection in the presence of halothane,
however, neither infectious virus nor measles virus nucleo-
capsids are produced and virus-induced cell fusion is
inhibited. It appears that the assembly of measles virus
particles is adversely affected by halothane exposure (2).
By characterizing virus products synthesized in infected
cells in the presence and absence of halothane, we are gain-
ing insight into the mechanism by which measles virus
particles are assembled.

LYTIC INFECTION

Vero cells lytically infected with measles virus at low
multiplicity of infection were treated with actinomycin D,

labeled with [³H] uridine, and treated with 1.8-2.5% halo-
thane in an atmosphere of 95% oxygen and 5% carbon dioxide.
Halothane concentrations were assessed by gas chromatography.
Concentrations of halothane of 1.8% or above are sufficient
to inhibit completely the synthesis of infectious measles
virus in Vero cells.
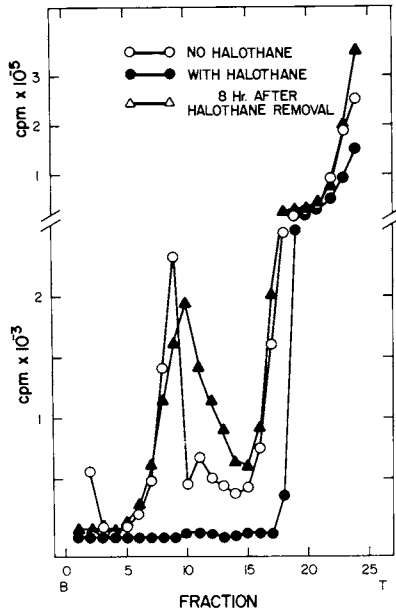
FIGURE 1. Nucleocapsid synthesis in the presence and
absence of halothane.

Nucleocapsid Assembly. Nucleocapsids were isolated by
the procedure of Kiley et al. (3) by rate zonal centrifuga-
tion in 15-40% sucrose gradients. Cells exposed to halothane
for 24 hours were harvested without removing the halothane.
These were compared with cells treated for 24 hours but
harvested 8 hours after removal of halothane, as well as with
untreated infected cells (Figure 1). Halothane treatment
completely inhibited the incorporation of ³H labeled RNA into
nucleocapsids, but nucleocapsids were synthesized soon after
halothane removal. There was a significant shift in the size
distribution of nucleocapsids to slower sedimenting particles
following halothane removal from the acutely infected cells.
RNA isolated from the peak fractions of the samples by
treatment of the nucleocapsids with 1% SDS at 65C for 30 sec
was subjected to centrifugation in 15-30% sucrose gradients

(Figure 2). RNA isolated from the halothane treated infected cells was predominantly of a smaller size (18-28S as compared with 50S) than that of the untreated infected cells.
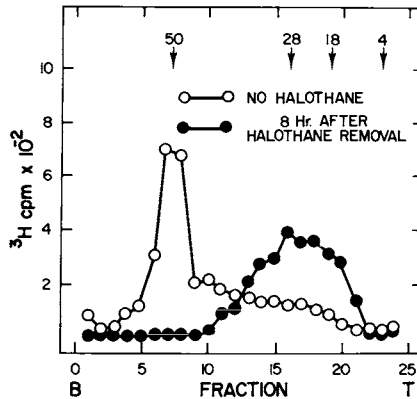
FIGURE 2. Size distribution of measles virus RNA synthesized after halothane treatment.

Preliminary experiments have indicated that measles virus RNA may be synthesized in the presence of halothane but not packaged into nucleocapsids (Figure 3). When [^{3}H] uridine was added to infected cells only after halothane removal, no labeled RNA was incorporated into nucleocapsids. However, if the labeled uridine was present during anesthetic treatment but not after anesthetic removal, the level of labeled nucleocapsid material recovered was comparable to the levels found when [^{3}H] uridine was present throughout the experiment.

Virus Protein Synthesis. In order to get a clearer picture of the effect that halothane has on virus replication and morphogenesis, infected cells were labeled with [^{35}S] methionine. Cell lysates were immunoprecipitated and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on 9% gels (Figure 4). Such experiments indicated that the amount of the measles virus P protein was greatly reduced in the presence of halothane. In addition, there was a slight decrease in the amount of NP, the nucleocapsid protein, in the presence of halothane. All other virus polypeptides were present in nearly normal amounts. Even though no syncytia formation occurs in the presence of halothane, a polypeptide of the molecular weight of F was detected in normal amounts.
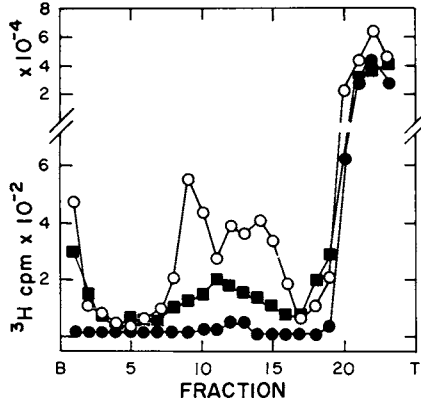
FIGURE 3.   RNA synthesis in the presence of halothane.
(o) No halothane.   (■) Label during halothane treatment.
(●) Label following halothane removal.
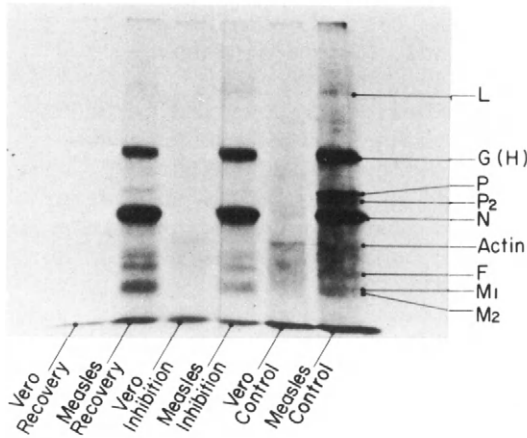
FIGURE 4.   Protein synthesis in the presence and absence
of halothane.

PERSISTENT INFECTION

Vero cells persistently infected with the Schwarz strain
of measles virus (4) were compared with the lytically
infected cells to determine what differences might exist in
the mechanism of virus morphogenesis and maturation in
chronically infected cells.   The persistently infected cells
were labeled with [^{3}H] uridine in the presence of actinomycin
D either with or without exposure to halothane.   Nucleocap-

sids and RNA were isolated as previously described for
lytically infected cells. Though the size distribution of
nucleocapsids in the persistently infected cells is similar
to that found in a lytic infection, there is a shift toward
more slowly sedimenting nucleocapsids (Figure 5).
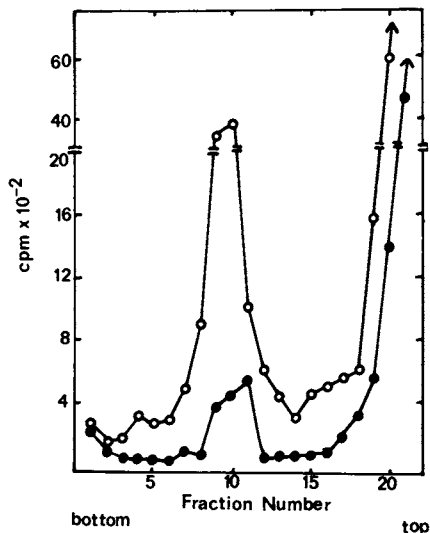
    FIGURE 5. Nucleocapsids isolated from cells undergoing
persistent (●) and lytic (o) infections with measles virus.

    Nucleocapsid assembly. Analysis of [^{3}H] uridine labeled
nucleocapsids produced in the persistently infected cells in
the presence of actinomycin D and halothane indicated that
nucleocapsids were assembled in the presence of the
anesthetic for at least 24 hours. If halothane treatment con-
tinued for approximately 45 hours, however, there was a
significant inhibition in nucleocapsid assembly. (Compare
Figure 6A and Figure 7.) Those nucleocapsids assembled in
the presence of halothane contain a significant population of
slow sedimenting particles (Figure 6A). When halothane was
removed after 24 hours, there was an increase in the
proportion of slow sedimenting nucleocapsids evident as early
as 4.5 hours after removal of the halothane and continuing
through 21 hours after anesthetic removal (Figure 6B, C).
The size of RNA isolated from the peak fractions of these
nucleocapsids appeared to be consistent with the size of the
nucleocapsid particles from which the RNA was isolated.
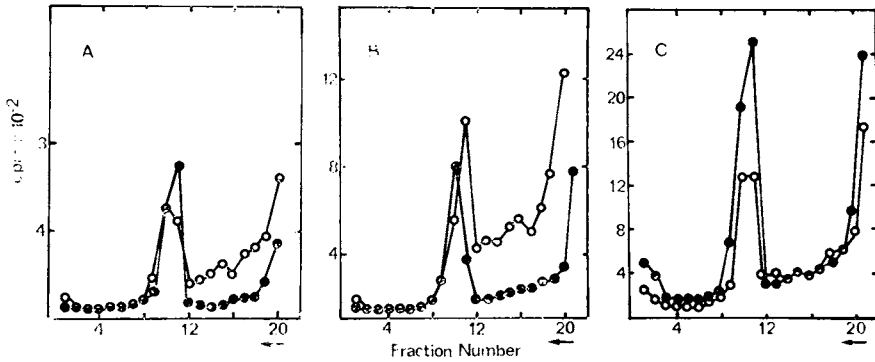
FIGURE 6. Nucleocapsid synthesis in persistently infected cells in the presence of halothane and after its removal. (●) No halothane, (o) with halothane. A. Cells were treated with 2.16% halothane for 24 hours and harvested for nucleocapsid isolation without removal of the anesthetic. B. Treated with halothane and harvested 4.5 hours after halothane removal. C. Treated with halothane and harvested 21 hours after halothane removal. The arrows indicate the direction of sedimentation in the gradients.

ASSEMBLY AND MATURATION OF VIRUS PARTICLES

In the presence of halothane, RNA synthesis continues while the synthesis of the viral protein NP is inhibited to a limited degree. Even though both major components of the ribonucleoprotein are available, they do not assemble into subviral cores. SDS PAGE analysis indicates that the synthesis of the viral P protein is substantially and reversibly inhibited in the presence of halothane. Another possible explanation is that, for some reason, the P protein is more labile than the other viral proteins in the presence of halothane; therefore, P may be preferentially degraded. It is the only viral protein affected to such a large degree. The P protein may be essential in regulating the assembly of viral RNA and NP into the ribonucleoprotein complex.

In addition to nucleocapsid assembly and production of infectious particles, measles virus syncytium formation is inhibited in halothane treated cells. The site of action of the anesthetic in inhibiting cell fusion does not appear to be at the level of polypeptide synthesis since the F protein is found in cell lysates from halothane treated cells. Halothane, though, is known to affect the membrane of cells (5,6). A reorganization of the cell plasma membrane
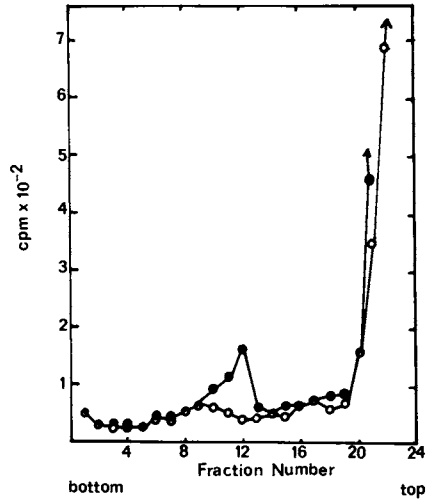
FIGURE 7. Nucleocapsid synthesis in persistently infected cells after prolonged halothane treatment. Cells were treated with halothane for 24 hours. The treatment was repeated at 24 hours and cells were finally harvested 21 hours after the second halothane exposure (45 hours total exposure) for nucleocapsid isolation. [^{3}H] uridine was present only during the last 21 hours of treatment. (●) No halothane, (o) with halothane.

structure in such a way that the F protein is inactive could occur. It is possible that these membrane alterations are influenced by chemical changes in the cellular cytoskeleton. Halothane has been shown to disrupt both the microtubules and microfilaments of certain cell lines (7,8).

In persistently infected cells a different situation occurs. Since preexisting viral proteins are present in the cells before halothane treatment, all proteins needed for the assembly of RNA and protein into nucleocapsids are available. Only after prolonged anesthetic treatment when the proteins that were synthesized before halothane treatment have been utilized in the assembly of ribonucleoprotein cores is further assembly inhibited.

In both lytically and persistently infected cells, when halothane is removed and nucleocapsid assembly occurs, there is an increase in slowly sedimenting RNAs. One possible explanation for the presence of these 18-28S RNAs is that they are encapsidated positive strand RNAs.

Studies aimed at determining the polarity of the slow sedimenting RNA along with studies to clarify the effect of halothane on the synthesis of viral proteins in the persis-

tently infected cells are currently underway. By exploiting
the ability of halothane to inhibit measles virus replication
reversibly, we have been able to examine various aspects of
virus assembly. We anticipate that subsequent studies will
provide valuable information on the relationship between
mechanisms for virus assembly and maintenance of persistent
infections.

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SYNTHESIS OF MATRIX PROTEIN IN A SUBACUTE [1]
SCLEROSING PANENCEPHALITIS CELL LINE

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INTRODUCTION

Subacute sclerosing panencephalitis (SSPE) is a slowly progressing, fatal disease of the human central nervous system (CNS), associated with measles virus persistence. The disease is characterised by an elevation of anti-measles antibody titres in both serum and CSF (1). Virus nucleocapsids are present as inclusion bodies in the cells of the CNS (2) but budding virus has not been observed and infectious particles have never been detected. The patient shows a low immune response toward matrix (M) protein, and direct examination of infected brain has failed to detect this molecule (3,4). All this evidence points to a defect in virus maturation which is associated with a lesion in the production of M protein. These features of SSPE have been reviewed (5,6). The defect in virus maturation may be to some extent host-controlled, since co-cultivation, or fusion of cells from infected tissue, with cell lines susceptible to measles infection, has sometimes resulted in the rescue of an infectious budding virus (7). More often virus rescue is only partially successful and a cell-associated cytopathic agent is obtained. These cell lines are known as SSPE cell lines, and many reports have confirmed that they also possess a lesion in the synthesis of M protein (8, 9). We have recently described two such SSPE cell lines established by co-cultivation of SSPE patient brain with Vero cells, in which matrix protein was not produced (10). In one (N-1), mRNA specific for M protein was present but unable to give rise to the normal protein by translation (11). In a second cell line (MF), we were unable to

detect mRNA sized molecules with matrix protein-related
sequences in the polyadenylated RNA fraction. We therefore
concluded that failure to produce M protein in the MF cells was
accomplished by a defect in mRNA formation or stability (10).

The MF cell line is unusual in that morphological features
normally associated with virus maturation have been reported
(12). Both the formation of an "inner leaflet" associated with
M protein combination with the plasma membrane (13), and
budding particles have been observed. These were found to be
non-infectious (12). Our previous study (10) utilized
high-passage MF cells (above 120) and we were therefore inter-
ested in examining this apparent maturational phenomenom in
relation to the expression of M protein in early passage cells.

RESULTS

MF cells were revived from liquid nitrogen storage at
various passage levels. We examined A(+) RNA, prepared from
these cells, by the technique of Southern blotting using
nick-translated plasmids containing M protein mRNA sequences
(14). A messenger RNA molecule carrying matrix protein
sequences was found to be present in one of these cultures
(passage 60: figure 1A, track 1) and it was of similar size to
that observed in a productive Edmonston virus infection (track
2). We have described another SSPE cell line (N-1) in which M
protein mRNA was readily detectable but not utilized correctly
in translation reactions, thus it was possible that the mRNA
detected in MF cells might not function in protein synthesis.
We therefore used the A(+) RNA fraction analysed in Figure 1A,
in an in vitro translation reaction. The virus-specific
products of this reaction were identified by immune
precipitation and separated on an SDS polyacrylamide gel
(figure 1B). Polyadenylated RNA extracted from a productive
measles virus infection was used as a positive control , and
from uninfected Vero cells as a negative control. M protein was
detected among the translation products of both MF and Edmonston
virus-infected cell mRNAs. Thus we conclude that this mRNA was
functional in vitro.

The lesion in M protein production within SSPE patient
brain is known to be to some extent host-controlled since
cell-fusion or co-cultivation experiments have resulted in the
expression of this protein (7). A host- controlled defect in
translation has been postulated in a laboratory-produced SSPE
virus persistent infection, since matrix protein produced by in
vitro translation could not be detected within the persistently
infected cells (5). We therefore prepared 35-S methionine
labelled extracts from these early passage cells and from
uninfected Vero cells and examined them for the presence of
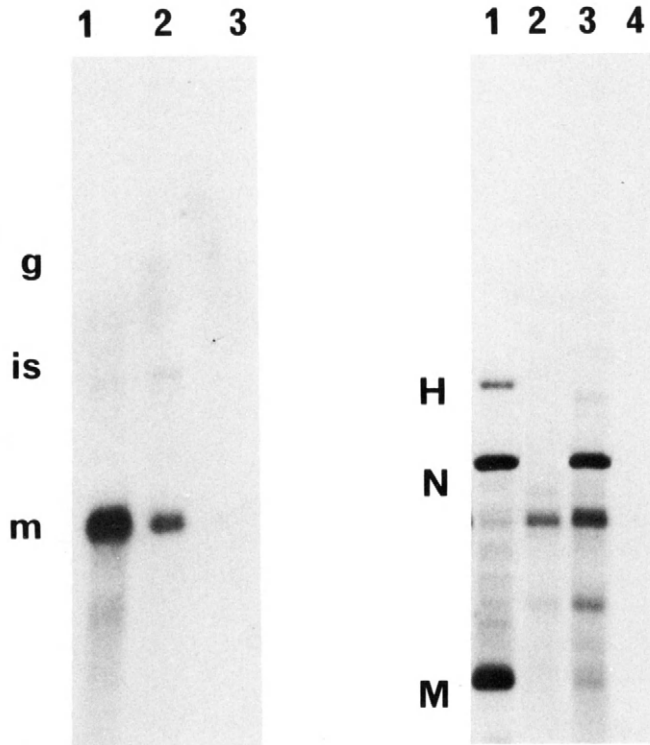
Figure 1.
Panel A.   Polyadenylated RNA was extracted from:track 1,
Edmonston virus-infected Vero cells; track 2, MF cells; track
3, uninfected Vero cells, and separated by electrophoresis on a
Mops buffered 1.5 % agarose gel. RNA was transferred to a nitro-
cellulose   filter   and   hybridised   to   32-P   labelled
nick-translated plasmid containing sequences derived from the
matrix protein mRNA. (g), genome; (is),   intermediate-sized
RNA; (m), mRNA.
Panel B.   Immunoprecipitated protein products formed by trans-
lation of the mRNAs analysed in panel A. track 1, Edmonston
virus-infected Vero cell mRNA; track 2, uninfected Vero cell
mRNA; track 3, MF cell mRNA; track 4, No mRNA addition.
Haemagglutinin (H), Nucleocapsid protein (N) and matrix protein
(M) were identified by reference to molecular weight markers.
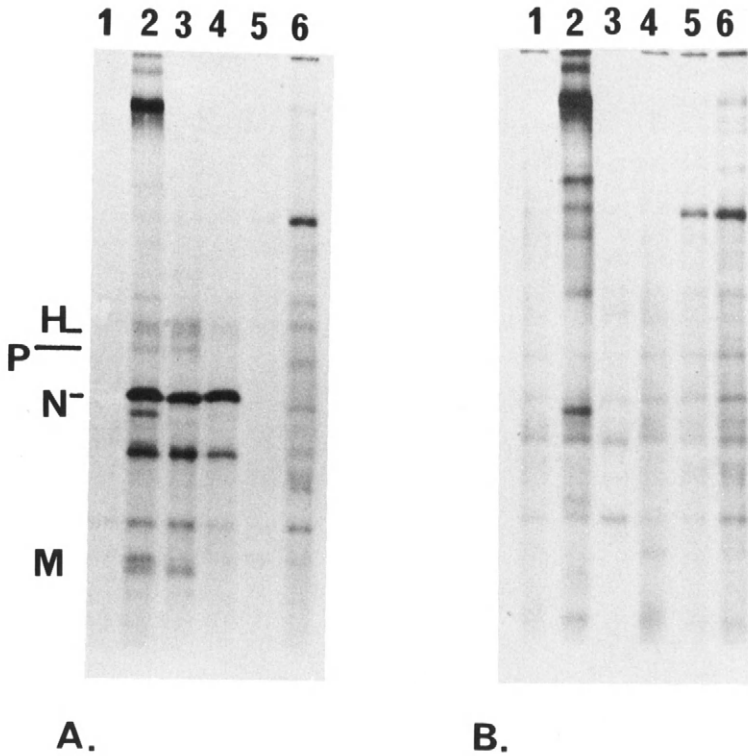
Figure 2.
Immunoprecipitation of proteins from cell lysates. MF cells
(panel A) and uninfected Vero cells (panel B) were labelled
with 35-S methionine and processed for immunoprecipitation. In
each panel proteins were immunoprecipitated by: 1, preimmune
rabbit serum; track 2, hyperimmune rabbit anti-measles virus
serum; track 3, serum from patient MF; track 4, CSF from patient
MF; track 5, control monoclonal antibody; track 6, monoclonal
antibody directed against measles virus M protein.

matrix protein by immune precipitation (figure 2). Serum and
CSF are still available from the patient from whom the MF cell
line was originally derived, and these were used in conjunction
with rabbit hyperimmune anti-measles virus serum, and a mono-
clonal antibody specific for measles virus matrix protein.
Rabbit preimmune serum and a monoclonal antibody directed
against corona virus antigen provided negative controls.

     Matrix protein was produced by the MF cell line and was
immunoprecipitated by both the hyperimmune rabbit and human
sera. It was not however detected using the monoclonal
antibody, or the human CSF. The failure of CSF to immunoprecip-
itate this peptide was expected since the MF patient suffered
from clinically normal SSPE, and antibodies to this protein
are lacking in the CSF. We therefore concluded that in this
culture of SSPE MF the production of mature functional mRNA for
matrix protein led to the successful expression of M protein.
In other cultures this protein was not detectable presumably
due to the lesion in the production of the corresponding mRNA
previously reported (10).

DISCUSSION

     The results presented here raise several interesting
questions. Firstly the production of M protein during the early
passage MF cells that can be inferred   from the previous
morphological description, is supported by the immunoprecipi-
tation of a matrix protein sized polypeptide formed in infected
cells and in in vitro translation reactions. However this
protein failed to react with monoclonal antibody directed
towards measles virus Edmonston matrix protein. The reasons for
this are unclear but this situation could arise from strain
differences between the virus which originally established SSPE
in the patient, and the Edmonston virus used for monoclonal
antibody production. Such differences have been detected
between measles virus haemagglutinin proteins by a variety of
biological techniques (16). It seems likely that more than one
strain of measles virus is capable of giving rise to SSPE, and
we have observed nucleoproteins synthesised in vitro, using RNA
extracted from two SSPE patients brains, to be of different
sizes, a finding we also interpret in terms of virus strain
differences .Alternatively this failure to react with
monoclonal antibody could have arisen from mutations
accumulating during persistence (17), and which have already
been demonstrated in the measles virus matrix protein (18).

     Budding particles have also been demonstrated in the early
passage MF cells (12) but these were found to be non-infec-
tious. It is generally assumed that restoration of matrix
protein synthesis is all that is required during attempts to

rescue virus from SSPE patient brain, but the observations
reported in the early characterization of the MF cell line
suggest this may not be the case. Another SSPE cell line is
known in which a lack of infectious virus was associated with a
defect in H protein expression on the cell surface (9).
Defective expression of H protein activity was also observed in
the early passage MF cells (12). We have also observed a marked
variation in the levels of nucleocapsid protein and its mRNA at
various passages. Presumably adequate synthesis of all virus
proteins is required for successful release of infectious
particles. The MF patient lacked antibodies in the CSF directed
against M protein, therefore this protein was probably not pro-
duced within the brain. Perhaps this block was overcome when
tissue was co-cultivated with Vero cells and the maturation
process previously described (12) could then occur. However,
cells are not available from the passages used in that report.
We observed only one MF culture in which matrix protein was pro-
duced. Furthermore other samples of the same passage cells
failed to produce M protein when revived. However the data
reported here indicate that MF cells possess the capacity to
produce a mRNA which is active in translation. The mechanism
which normally prevents the expression of this capacity is
unknown, but it is possible that physiological trauma involved
in the attempts to rescue virus from infected tissue, or cell
revival from liquid nitrogen storage, is important in the
successful circumvention of this block.

ACKNOWLEDGEMENT

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MATRIX (M) PROTEIN ALTERATIONS INDUCED BY TLCK IN CELLS ACUTELY AND PERSISTENTLY INFECTED WITH MEASLES VIRUS[1]

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TLCK (n-alpha-p-tosyl-L-lysine chloromethyl ketone-HCl) blocks proteolytic cleavages by trypsin and is often used to detect precursor-product relationships of proteins. When measles virus is grown in the presence of TLCK, the viral M (matrix) protein has an apparent molecular weight 1,000–2,000 daltons larger than normal as determined by SDS-PAGE (1). This suggests that M protein might normally be cleaved, by a trypsin like enzyme, from a slightly larger precursor polypeptide. This is particularly interesting, because in some cell lines persistently infected with measles virus, and in some viruses isolated from patients with the slowly progressive neurological disease SSPE (caused by a persistent infection with measles virus) the measles virus M protein has a similarly altered apparent molecular weight (2,3,4). If the normal M protein is cleaved from a larger M protein precursor molecule, then mutations interfering with this presumptive processing might account for the larger M protein often associated with measles virus persistence.

No other evidence for a possible M protein precursor has been reported. Therefore, we studied the effects of TLCK in more detail to see if a precursor polypeptide to the M protein could be conclusively identified. We confirm here that TLCK alters the electrophoretic mobility of the measles virus M protein; however, the mechanism is unrelated to protection of a precursor M protein from proteolytic cleavage. Thus, there is no evidence for a measles virus M protein precursor. More importantly, we found that TLCK affects the mobility of some viral and cellular proteins, even in the cold and in the presence of detergents. Thus, TLCK and related compounds should not be used as protease inhibitors in buffers.

Synthesis of measles virus proteins in the presence of TLCK resulted in changes in the electrophoretic mobilities of the viral M and P proteins and cellular actin (A) (Figure 1). M and actin synthesized in the presence of TLCK (M' and A') migrated more slowly than standard M and A, while the TLCK-induced P (P') migrated more rapidly than standard P. TLCK also caused a decrease in overall protein synthesis.
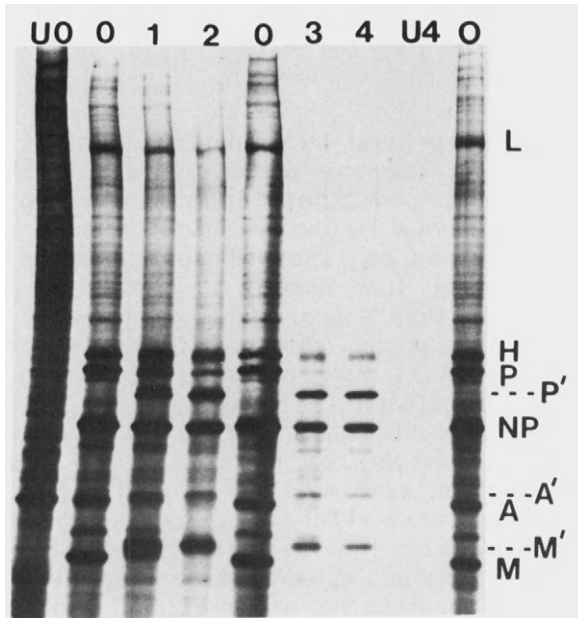
Figure 1. Measles virus proteins synthesized in the presence of TLCK. CV-1 cell monolayers were infected with wild type measles virus and labeled with $[^{35}S]$methionine (5,6). TLCK and $[^{35}S]$methionine were added simultaneously. After 15 min. the monolayers were suspended in gel sample buffer, run on SDS-PAGE (8), and processed for fluorography. Viral proteins: H, hemagglutination protein [80,000 daltons (80K)]; P, nucleocapsid associated phosphoprotein (70K); NP, nucleocapsid protein (60K); M, matrix protein (37K); A, cellular actin (43K). P', M' and A' are TLCK-induced forms of P, M, and A. L, involved in transcription (200K) and F_1 (41K) and F_2 (20K), the 2 components of the fusion protein are not consistently detected and were not analyzed. Gel lanes: 1,2,3,4, infected and labeled in the presence of 1,2,3 or 4mM TLCK respectively; 0, infected, no TLCK; U0, uninfected, no TLCK; U4, uninfected, 4mM TLCK.
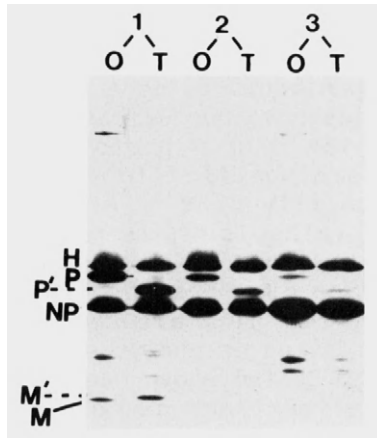
Figure 2. TLCK-induced M' and P' are not immune precipitated by sera with reduced affinity for M and P. Infected CV-1 cell monolayers were labeled, treated with TLCK, suspended in lysing buffer, divided into aliquots, immune precipitated with sera having different specificities for M and P proteins (9), run on SDS-PAGE, and processed for fluorography. Lanes: O, no TLCK; T, 4mM TLCK; 1, hyperimmune rabbit sera with affinity for all measles proteins; 2, a human SSPE sera with reduced affinity for M; 3, a human SSPE sera with reduced affinity for P and M.

Immune precipitations with human sera having selective specificities for different viral proteins demonstrated that the TLCK-induced M' and P' bands were related to M and P (Figure 2). Serum with low affinity to M did not precipitate either M or M' (lanes 2-O, 2-T). Likewise, serum with reduced affinity to P and M precipitated reduced amounts of P' and M' (lanes 3-O, 3-T). The relatedness of M', P' and A' to M, P, and A respectively, has also been demonstrated by peptide mapping with Staphlococus aureus protease V8 (5).

Figure 1, lane 1 shows that 1mM TLCK produces an M protein with a migration rate intermediate between standard M protein and the 4mM TLCK-induced M protein. This was unexpected. If protection from proteolytic cleavage was the sole mechanism of TLCK-induced changes in M, then lower concentrations of TLCK (1mM compared to 4mM) would be expected to produce smaller amounts of the larger (i.e., protected) form of M, rather than an M with an intermediate mobility. The finding of an intermediate sized M suggests a

multistep mechanism, thus arguing against protection from a
single cleavage event.   Further experiments described below
confirmed that the TLCK-induced changes were not due to the
ability of TLCK to inhibit proteolytic cleavages.

     Pulse-chase experiments using a pulse labeling period
as short as 20 minutes have not demonstrated any changes in
the mobilities of the M or P proteins of measles virus
(6,7).   Thus, any proteolytic cleavages that alter the
electrophoretic mobility of M or P must occur within 20
minutes following synthesis of the presumptive precursor
protein. The ability of TLCK to protect against this
hypothetical cleavage must also be limited to this time
span. Figure 3 shows that TLCK altered the mobilities of M
and P proteins synthesized as early as 4 hours prior to the
addition of the TLCK. By the above reasoning, the mechanism
involved in these alterations cannot be protection of a
precursor protein from proteolytic processing.
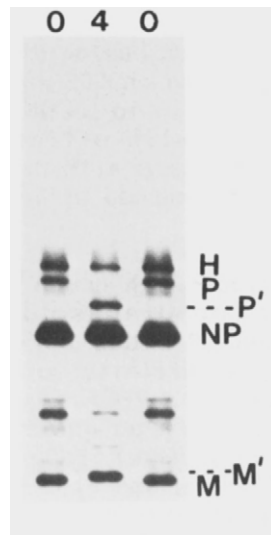
     Figure 3. Effect of TLCK on pre-existing measles virus
proteins.   Infected cells were labeled for 3 hr. and then
chased for 90 min. (5). TLCK was added during the final 30
min. of the chase period.   The monolayers were harvested,
immune precipitated and run on SDS-PAGE.   Lanes: 0, no TLCK;
4, 4mM TLCK.
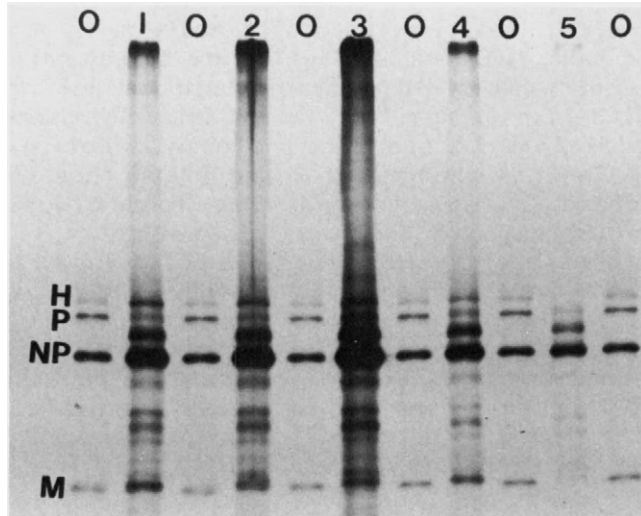
Figure 4. Alterations of viral proteins by TLCK at 0°C and in the presence of NP40, SDS, or azide. Measles infected CV-1 cells were labeled with [^{35}S]methionine for 3 hr. The cells were suspended in Tris buffer and aliquots placed in test tubes. The aliquots were all placed in an ice bath. Some aliquots received NP40 at a final concentration of 2%, sodium azide at a final concentration of 0.1M, or SDS at a final concentration of 0.2%. After 10 min. incubation with these additions, TLCK at a final concentration of 4mM was added to all the aliquots except the one shown in lanes 0. After 30 min. incubation at either 37°C or 0°C, 1 volume of 2X immune precipitation lysing buffer was added to each tube (9) and the samples were immune precipitated and subjected to SDS-PAGE and fluorography. Gel lanes: 0, no TLCK, 37°C; 1, TLCK, 37°C; 2, TLCK, 0°C; 3, NP40 and TLCK, 37°C; 4, sodium azide and TLCK, 37°C; 5, SDS and TLCK, 37°C.

To further investigate the conditions under which TLCK could induce changes in the mobility of M and P, we incubated previously labeled measles virus proteins with TLCK at 0°C, in the presence of the detergents NP40 and SDS and in the presence of sodium azide. TLCK-induced changes of P and M were detected in all cases (Figure 4, lanes 1-5).

We have previously reported further evidence that the TLCK-induced changes in M and P are due to factors other than inhibition of a trypsin-like cleavage (5). The general

protease inhibitor PMSF, which like TLCK inhibits trypsin, does not affect the electrophoretic mobility of M or P. On the other hand, TPCK and ZPCK which are structurally similar to TLCK, but inhibit alpha-chymotrypsin and not trypsin, do induce TLCK-like changes in P and M (5). This demonstrates that inhibition of a specific protease is not involved in the TLCK-induced changes of P and M and thus that these TLCK-induced changes are not due to protection of a precursor protein from proteolytic cleavage.

    The persistently infected HeLa cell line K11 has an M protein with an electrophoretic mobility similar to that of the TLCK-induced M' protein of wild type (wt) virus. We had originally wondered if the alteration of the M protein in these persistently infected cells might be analogous to the TLCK-induced alteration of the wt M protein. Figure 5 shows that TLCK altered the K11 M protein in much the same way as it had the wt M protein, that is, it increased the
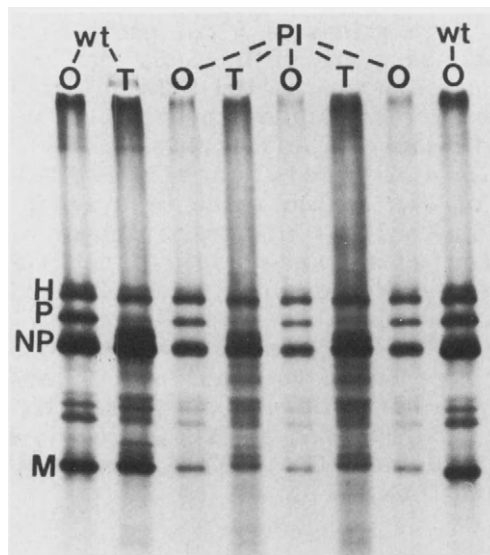
    Figure 5. Effect of TLCK on the M protein of persistently infected HeLa cells. Persistently infected HeLa cells (K11) (4) and HeLa cells acutely infected with wt virus were labeled for 3 hr. with [^{35}S]methionine and then incubated with 4mM TLCK for 30 min. The cells were harvested, immune precipitated and run on SDS-PAGE. Gel lanes: wt, wt infected cells; PI, persistently infected cells; O, no TLCK; T, 4mM TLCK.

apparent molecular weight by 1,000-2,000 daltons. Thus, the
TLCK-induced alteration of M in wt virus seems to be
unrelated to the alteration in mobility sometimes seen in
persistently infected cells.

Although TLCK affected the mobility of M protein by an
as yet undetermined mechanism, it occurred to us that the
TLCK-induced alteration of the M protein might be used as a
marker for gel bands related to M. ^{32}P labeling experiments
reported several years ago concluded that only the P and NP
measles virus proteins were phosphorylated (6). More recent
studies have demonstrated a very faint ^{32}P labeled band
migrating just above the main [^{35}S]methionine labeled M
protein band (7). By analogy with other paramyxoviruses,
but without any further evidence, this band was assumed to
be a phosphorylated form of M. Figure 6 shows that this
faintly phosphorylated band was affected by TLCK in the same
way as the main [^{35}S]methionine labeled M protein band,
i.e., in the presence of TLCK, the phosphorylated band
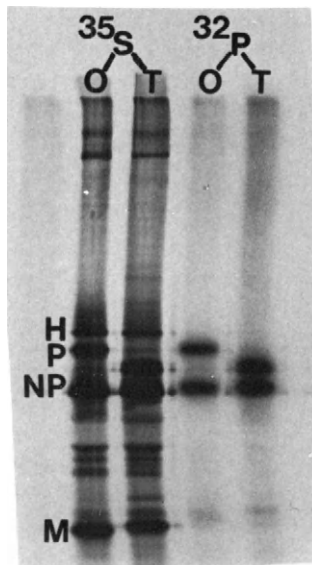
Figure 6. Effect of TLCK on ^{32}P labeled measles virus
proteins. Infected cells were labeled for 3 hr. with either
[^{35}S]methionine or ^{32}P. The monolayers were then chased for
30 min. either with or without 4mM TLCK added to the medium.
The monolayers were harvested, immune precipitated, and run
on SDS-PAGE. Lanes: ^{35}S, labeled with [^{35}S]methionine; ^{32}P,
labeled with ^{32}P; O, no TLCK; T, 4mM TLCK.

migrates slightly more slowly than the corresponding band
not treated with TLCK. To our knowledge, this is the first
suggestion, other than co-migration, that this faint ^{32}P
labeled band might be related to M. However, this is by no
means conclusive evidence, and should be interpreted
cautiously.

The results presented here show that TLCK can alter the
electrophoretic mobility of several proteins, including the
measles virus M protein, by a mechanism that is unrelated to
protection of protein precursors from processing by
proteolytic cleavage. It is possible that TLCK induces
conformational changes of M and P that are reflected in
changes in electrophoretic mobility. Binding of TLCK to M
and P might be involved. This possibility has not yet been
tested due to the unavailablility of isotopically labeled
TLCK. In any event, our results indicate that as yet there
is no evidence for an M protein precursor.

More importantly our findings should be kept in mind
when interpreting past and future reports of precursor-
product relationships in which TLCK or related compounds
were assumed to act solely by blocking proteolytic
cleavages. In addition, since TLCK altered the
electrophoretic mobilities of some proteins even in the cold
and in the presence of detergents, TLCK or related compunds
should not be used as protease inhibitors in buffers.

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MECHANISMS OF RSV DI PARTICLE INTERFERENCE[1]

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We have previously shown that defective interfering (DI)
particles of respiratory syncytial virus (RSV) not only
interfere with replication of standard virus in HEp-2 cells
but can also protect cells from virus induced cytopathology
(1). And we have used this cell protecting ability to
develop a quantitative biological assay for these DI
particles (2). During the studies described here defining
the role of DI particles in modulating virus infection on
undiluted passage, we observed a third biological activity
with the potential to attenuate or alter RSV infections –
interferon (IFN) induction.

UNDILUTED PASSAGE

Dynamics of Infectious Virus, DI Particle and IFN
Production. To determine the role of DI particles in
modulating an in vitro model of RSV infection, plaque
purified RSV was used to initiate four series of undiluted
passages in HEp-2 cells. Culture fluid was examined for
infectious virus, DI particles, temperature sensitive (ts)
virus and IFN.
      The results shown in Fig. 1 are representative of all
four series in that infectious virus titer dropped by
passage 5 and that this drop coincided with the appearance of
DI particles and a marked increase in IFN. A trough of
infectious and DI virus was seen at passage 6 followed by a
rebound at passage 7. For three of the four series a similar
trough in IFN production occurred at passage 6 followed by a
rebound at 7. Thereafter oscillating cycles of infectious
and DI virus and IFN were produced throughout the remainder
of the passages. Both alternate and cocycling of the three
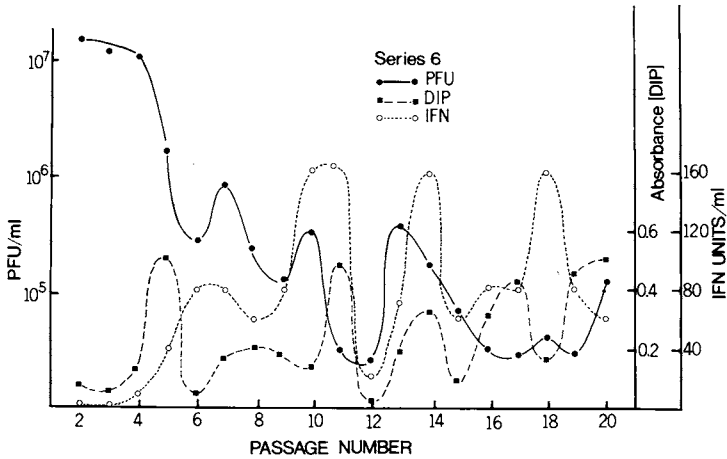
FIGURE 1. Undiluted passage of RSV in HEp-2 cells.
Four undiluted passage series were initiated with four
different clones of RSV which had each been plaque purified
3X. Flasks containing 2×10^6 HEp-2 cells were infected with
1 ml of culture fluid from the preceding passage for 2 hr at
32°C with rocking. Inoculum was removed. Flasks were fed
with 10 ml VGM (MEM + 5% fetal bovine serum). After 72 hr at
37°C, culture fluid was centrifuged to remove cells and the
supernatant frozen until it was analyzed for infectious virus
by plaque assay at 37°C, and for ts virus by plaque assay at
32, 37 and 39°C. DI particles were analyzed by colorimetric
assay (std. virus moi = 1) following 60 sec exposure to UV to
inactivate infectious virus which might otherwise mask DI
activity (2). Cell protecting activity in colorimetric assay
was due to DI particles and not to IFN because in each sample
it was inactivated with RSV antiserum but not pre-immune
serum. IFN was analyzed by filtering samples through a 0.2 µ
filter (Amicon Sterilet) which retains infectious and DI
virus but allows IFN to pass through. Two-fold dilutions of
the filtrate were evaluated for ability to interfere with VSV
cytopathology in human foreskin fibroblasts (3). A 50%
end-point determination and internal IFN standards were used
to estimate the quantity of IFN present. This filterable
interfering activity active against VSV was characterized as
βIFN by neutralization with anti-human βIFN serum but not by
anti-human αIFN serum.

activities occurred but no consistent pattern was seen beyond
passage 7. No ts virus able to grow at 32 °C but not at 37°C
or 39°C was detected.

When means of infectious and DI virus and IFN levels at
each passage were determined for all four series together, a
striking uniformity over the first seven passages is apparent
(Fig. 2). Because these series were initiated with four
different virus clones obtained by plaque purification (3X),
they represent four independent generations of DI particles.
Thus in HEp-2 cells, DI can be rapidly and reproducibly
generated for RSV and once they are generated their effects
are predictable.
     This presentation also emphasizes the pattern of
appearance of DI particles by passage 5 coinciding with an
increase in IFN and drop in infectious virus followed by a
further drop in all three activities and then a rebound.
Beyond passage 7, DI particles and IFN are produced in
relatively stable amounts which are significantly greater
than those in initial passages. Infectious virus is also
produced at stable levels but in significantly reduced
amounts than in initial passages. These results conform to
the model proposed by Huang and Baltimore (4,5) for
interactions of DI and infectious virus on continued passage
in that once DI are generated, further passage results in
continued balanced production of both DI and infectious
virions.
     Our inability to detect ts virus supports DI as the
major determinant of infectious virus modulation in these
undiluted passages. Further support comes from the
observation that both DI particle and IFN levels exhibited a
significant negative correlation with infectious virus
produced in the subsequent passage (Table 1).

     Establishment of Persistent Infections (PI). With
continued feeding, two of the four cultures at passage 5 and
all of the cell cultures (with two exceptions) beyond passage
5 survived and developed into PI stable in culture for a
minimum of eight weeks. These PI were characterized by
scattered foci of viral cytopathology, continued shedding of
infectious virus and periodic crises. Thus on continuous
passage, once DI appear, PI can be readily established.
Determination of the role of DI particles and of IFN in
establishing and maintaining these PI is in progress.

     Role of IFN. Both IFN in the virus inoculum and that
produced during the passages might be contributing to the low
infectious virus yield and low cytopathology of these
undiluted passages. However, it is unlikely that IFN in the
inoculum exerts an inhibitory effect because only a 2 hr
adsorption period was allowed. Greater than 2 hr exposure to
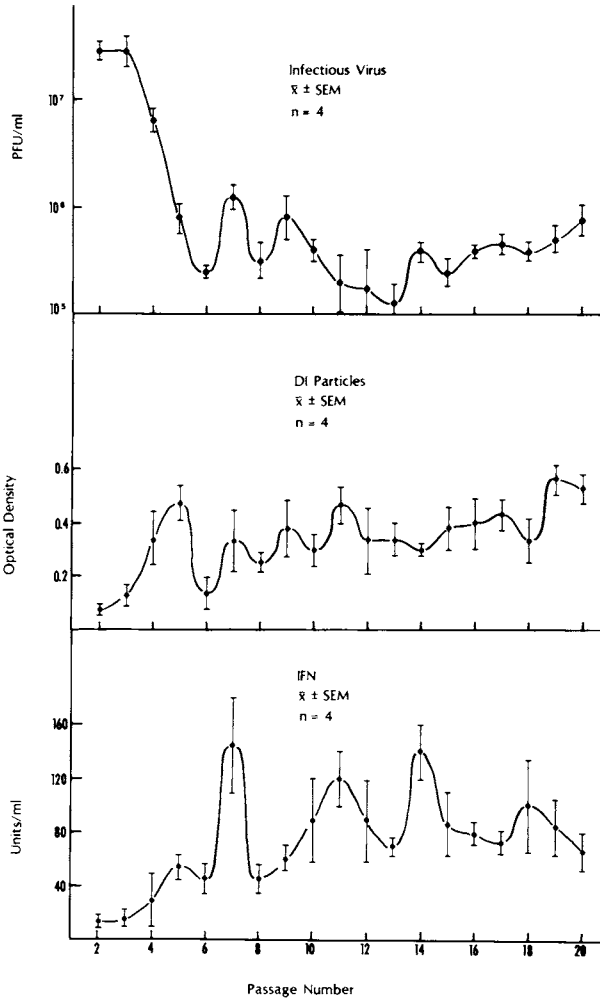
FIGURE 2. Summary of infectious virus, DI particles and
IFN production on undiluted passage of RSV in HEp-2 cells.
Means of infectious virus, DI particles and IFN at each
passage for the four passage series described in Fig. 1 were
determined and are shown bracketed by standard error of the
mean.

cells was found to be required for the IFN (0.2 μ filtrate)
in DI preparations to provide cell protection (Table 2).
     To determine whether the IFN produced during the
passages was exerting antiviral activity, antiserum to

TABLE 1

CORRELATIONS BETWEEN INFECTIOUS VIRUS, DI PARTICLES AND IFN
PRODUCED ON UNDILUTED PASSAGE[a]

	Series	r
Log DI particles vs	1	-.92[*]
log subsequent pfu	2	-.67[*]
(DF = 34)	4	-.50[*]
	6	-.10
Log IFN vs log	1	-.71[*]
subsequent pfu	2	-.74[*]
(DF = 34)	4	-.74[*]
	6	-.78
Log DIP vs log IFN	1	.65[*]
(DF = 36)	2	.45[*]
	4	.41[*]
	6	.42[*]

[a]Levels of DI and infectious virus and IFN in the same
sample and in the subsequent passage for all of the passages
of the series described in Fig. 1 were evaluated for
association by determining correlation coefficients (r).
    [*] $P < 0.01$

βIFN was added to four of the passages and infectious virus
yields and cytopathology evaluated relative to the same
passages without antiserum and to cultures infected with
similar mois of standard virus alone (Table 3).
Neutralization of extracellular IFN did result in an increase
in infectious virus yield but not to the levels produced by
similar mois of standard virus alone. Cell protection was
decreased only minimally. Therefore, we conclude that the
IFN induced during the passages does contribute to
interference with standard virus replication and
cytopathology, but it is not the only mechanism by which this
is accomplished.

ENHANCED IFN INDUCTION BY DI RSV

    The increase in IFN production coinciding with the
appearance and amplification of DI particles in these
undiluted passages suggests that the newly generated DI

TABLE 2
EFFECT OF FILTRATION ON RSV DI PREPARATION INTERFERENCE

Challenge virus	DI prep adsorption time	Filtration (0.2 µ)	Percent of maximum cell protection in colorimetric assay[a]
VSV	2 hr	−	3
		+	5
	24 hr	−	100
		+	91
RSV	2 hr	−	100
		+	11
	24 hr	−	100
		+	42

[a]Duplicate wells containing 1.5×10^5 HEp-2 cells were
exposed to 0.1 ml RSV DI preparation either prior to or
subsequent to filtration through a 0.2 µ (Amicon Sterilet)
filter for either 2 or 24 hr. Wells were then challenged
with RSV (moi = 1) or VSV (moi = 0.1). Virus inoculum was
removed and wells were fed with 1 ml VGM and incubated at
72 hr at 37°C until virus control wells containing standard
RSV or VSV alone exhibited maximum cytopathology. Cell
protection was assessed by incubating wells with neutral red
dye (33 mg/L) for 2 hr at 37°C. Dye was removed, plates were
washed and dye taken up by cells remaining on monolayer was
extracted and determined spectrophotometrically at 540 nm.
Percent of maximum cell protecting activity by the DI
preparation for each virus was determined.

particles are responsible for the enhanced IFN induction.
The ability of standard RSV alone to induce IFN is uncertain
as yet. Initial studies suggest that when standard virus
does induce IFN, it does so only at relatively high moi (5 vs
0.05-0.5 pfu/ml), relatively late in replication (>36 hr) and
that DI particles can be detected. Standard virus pools
contain relatively low amounts of IFN (<10 U/ml) compared to
DI preparations (100 U/ml). Finally, the positive
correlation observed for DI particles and IFN levels in the
same passage supports an interdependent relationship between
IFN and DI particle synthesis (Table 1). These observations
suggest that DI particles are better IFN inducers than
standard virus alone. Whether these DI particles alone can

TABLE 3
EFFECT OF ANTI-βIFN ANTISERUM ON VIRUS YIELD AND
CELL PROTECTION OF UNDILUTED PASSAGES[a]

Inoculum	Infectious virus input (moi)	Log pfu		Cell protection % of cell control	
		No Ab	+ Ab	No Ab	+ Ab
Std. virus	.5	6.73		1	
	.1	6.49		3	
	.05	6.59		5	
	.01	6.56		38	
Series 2-P14	.3[b]	5.34	5.72	46	40
Series 2-P15	.3	5.58	6.23	51	45
Series 1-P7	.05	5.59	6.08	56	51
Series 2-P12	.03	5.11	5.77	95	95

[a]Wells containing 1.5×10^5 cells were infected with
either standard virus or the undiluted passage indicated in
0.1 ml for 2 hr at 32°C with rocking. Inoculum was removed,
wells were fed with 1 ml VGM and anti-βIFN antiserum
(obtained from J. Vilček, New York University School of
Medicine, NY) sufficient to neutralize 500 U of βIFN was
added to wells indicated. Wells were incubated at 37°C for
72 hr and then culture fluid analyzed for infectious virus by
plaque assay. Cell protection was determined as described in
Table 2 and is expressed here as percent of dye taken up by
uninfected cell controls.
[b]Determined by plaque assay of inoculum.

induce IFN or whether replication is required remains to be
determined.

MULTIPLE INTERFERING ACTIVITIES

    In summary, DI particles of RSV exhibit multiple
biological activities: reduction of infectious virus yield,
reduction of cytopathology and IFN induction. Each of these
activities has the potential to alter infection in vivo
either by attenuating the infection or establishing a PI just
as they do in the in vitro cell culture system described
here. Whether these DI particle mediated activities share a
common mechanism or pathway is not yet known. All could be
due to competition of the DI genome with the standard virus

genome for replicase. This could result not only in reduced
infectious virus yield but also in reduced synthesis of
m-RNAs coding for the viral proteins responsible for
cytopathology and shutoff of host macromolecular synthesis.

ACKNOWLEDGMENTS

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THE EFFECT OF VIRUS PERSISTENCE
ON PLASMA MEMBRANE BOUND FUNCTIONS
IN CNS DERIVED CELL LINES.

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In many neurological diseases with a virus
aetiology it is uncertain whether the virus has a
direct cytopathogenic effect on neural cells or
whether virus induced cytotoxic immune responses are
responsible for the cellular dysfunction character-
istic of such diseases. It is known that many negat-
ive stranded RNA viruses such as the paramyxoviruses,
rhabdoviruses, arenaviruses integrate virus glyco-
proteins into the host cell membrane before release
through budding. It has been postulated that these
virus proteins could induce specific or nonspecific
disturbances of the normal microstructure of the cell
membrane thus creating the molecular basis for an
impairment of specialized membrane bound cellular
functions (1, 2). We have looked at suitable CNS
derived model cells which were persistently infected
with different viruses to investigate this possib-
ility. The C6 rat glioma cell line (ATCC CCL107) was
chosen because they possess large numbers of ß-
adrenergic receptors for catecholamines. They were
persistently infected with measles (SSPE), canine
distemper (CDV) and lymphocytic choriomeningitis
viruses (LCM). The neuron model cell mouse neuro-
blastoma x rat glioma hybrid (108CC15) created by
Amano and Hamprecht (3) were chosen because they
possess membrane receptors for prostaglandins, ade-
nosine, catecholamines, acetylcholine, opiates and
other signal transmitting substances. These cells
were persistently infected with rabies virus. The
stimulation of these different receptors in both cell
lines with their appropriate agonists is followed by

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typical shifts of the intracellular cyclic AMP con-
centrations which can be easily measured. We have
concentrated on the study of ß-receptor function in
C6 cells and the opiate receptor and prostaglandin
E 1 systems in 108CC15 cells. In C6 cells the ß-
adrenergic receptor is linked to the adenylate
cyclase enzyme by a stimulating regulatory N_s protein.
Various effects on the different components of the
receptor adenylate cyclase system have been observed
following establishment of persistence with differ-
ent viruses in these cells (4, 5). In 108CC15 cells
the cyclic AMP synthesizing enzyme is under two
controls: there are stimulating receptors for cAMP
synthesis (like PGE_1 receptors) which stimulate
after occupation by the specific hormone via a
stimulatory coupling protein N_s. Other types of
receptors (like opiate receptors) inhibits this
stimulation if they are occupied by their specific
agonists via an inhibitory coupling protein N_i also
connected to the adenylate cyclase. Effects have also
been observed on different components of these two
systems in persistently rabies virus infected cells
(6, 7). The differing effects of SSPE, CDV and LCM
virus persistence in C6 cells is of particular
interest. These persistently infected cultures had
virus antigen inserted in 100 % of cells but the
effect of this insertion of foreign antigen on
membrane function differed considerably. There was a
large reduction in the accumulation of intracellular
cyclic AMP following stimulation of ß-adrenergic
receptors in paramyxo virus infected cells but no
effect in LCM infected cells. The paramyxo viruses
also differ in their effects. CDV infection results
in a 50 % decrease in the number of ß-adrenergic
receptors without any alteration in the binding
constant for the ligand to the receptor (5). However,
persistence of SSPE virus did not result in a
decrease in the number of receptors in the membrane
or an alteration in their binding properties. Neither
insertion of LCM virus proteins in the membrane did
not appear to play any role in cellular dysfunction
in respect to the ß-receptor adenylate cyclase system
studied. To clarify the role of virus glycoproteins
in the C6/SSPE system virus antigen was removed from
the cell membrane by the use of antiserum directed
against virus proteins as previously described (8).

MODULATION OF VIRUS PROTEINS IN C6/SSPE CELLS

        C6 cells persistently infected with the measles
SSPE virus (Lec) were grown in medium containing heat
inactivated serum from SSPE patients. This serum
contains antibodies which recognize all the measles
virus proteins with a reduced reaction against M
protein. These cells were passaged every 3 to 4 days
and the amount of viral antigen present in the mem-
brane and the percentage of cells displaying membrane
and intracellular antigen was measured at each
passage. The amount of viral antigen present in the
membrane was measured by freezing a trypsinized cell
suspension with saturating amount of human SSPE anti-
serum and (125)I-labelled anti-human IgG. Thereafter
cell associated radioactivity was measured. The per-
centage of cells containing intracellular and mem-
brane viral antigen was measured by staining fixed
and unfixed cell preparations, respectively by the
indirect   immunofluorescent   method.   Our   studies
demonstrated a gradual loss of viral antigen from the
cell membrane over a period of 6 passages (Fig. 1A).
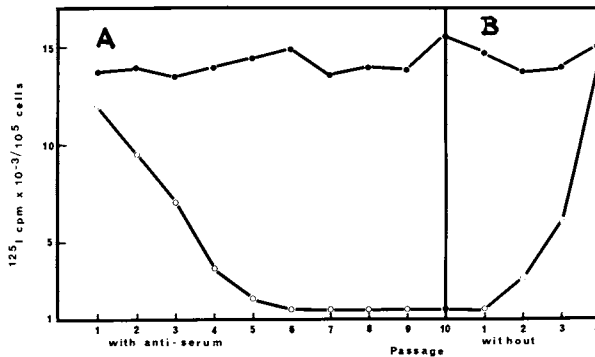
Figure 1
        Virus antigen inserted in the cell membrane was
measured by counts bound of (125)I-labeled anti-
human IgG to cells incubated with human anti SSPE
serum for the whole passage time. (A). Amount of
virus antigen present after antiserum treatment:
(●) C6/SSPE cells grown in antiserum for 1 hr only;
(o) C6/SSPE cells maintained in antiserum. (B)
Amount of virus antigen present after removal of
antiserum. (●) C6/SSPE cells grown in antiserum for
1 hr. only; (o) C6/SSPE cells released from anti-
serum treatment. Cells were passaged every 3-4 days.

At passage 6 the amount of radioactivity bound to C6
SSPE cells was equal to that binding to uninfected C6
cells grown in medium containing antiserum. At this
stage, however, approximately 30 % of cells still
contained intracellular viral antigen even though no
cells displayed positive staining of the membrane
antigens (Fig. 2).
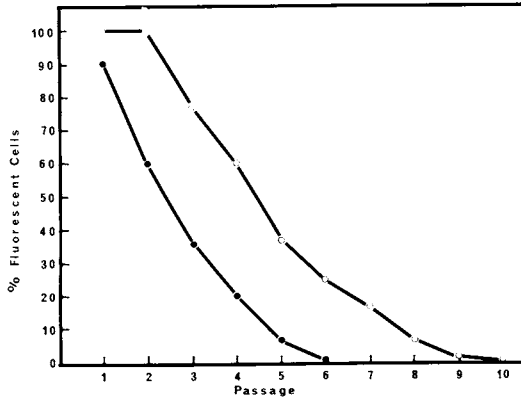
Figure 2
        Percentage of cells displaying virus specific
immuno fluorescence in (●) unfixed (membrane anti-
gens stained) and (o) acetone-fixed C6/SSPE cells
(intracellular viral antigens stained) maintained in
antiserum. Cells were passaged every 3-4 days.

When there was no detectable virus antigen of any
type present in the antiserum treated cells they were
washed in PBS and further incubated in medium without
antiserum. This was followed by a rapid recovery in
the expression of virus antigen on the cell membrane
and intracellularly (Fig. 1B).

EFFECT OF MODULATION ON HORMONE STIMULATED cAMP
RESPONSE

        To determine if the previously reported impair-
ment of the cyclic AMP response in C6/SSPE cells (5)
could be attributed to intracellular or membrane
virus antigens we examined cyclic AMP production at
various stages during virus antigen modulation.
Persistently infected cultures grown with or without

SSPE antiserum were stimulated via ß-adrenergic
receptors at every passage. with DL-isoproterenol
under the optimal conditions for cAMP synthesis.
Intracellular cAMP was expressed as a percentage of
the amount of cAMP produced in uninfected C6 cells
grown in medium containing an identical concentrat-
ion of measles antiserum (Fig. 3).
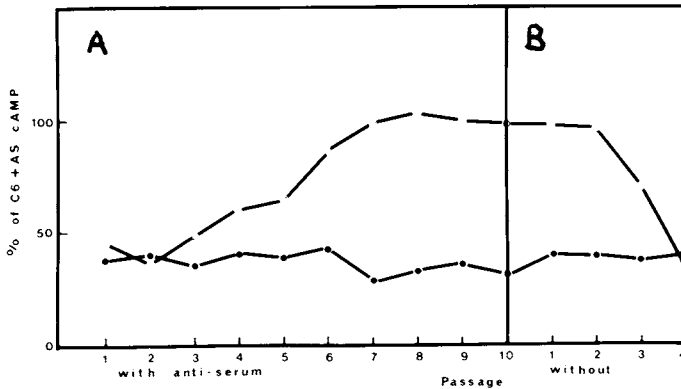
Figure 3

Measurement of isoproterenol stimulated cAMP syn-
thesis expressed as a percentage of control levels in
uninfected C6 cells grown in SSPE antiserum. (A)
After treatment with antiserum: (●) untreated
C6/SSPE cells; (o) C6/SSPE cells maintained in SSPE
antiserum. (B) After removal of antiserum: (●)
previously untreated; (O) previously treated with
antiserum. Cells were passaged every 3-4 days.

Over the initial 3 passages there was little differ-
ence between cAMP synthesis in antiserum treated and
untreated C6/SSPE cells. This was always less than
50 % of that of uninfected C6 cells. There was an
initial recovery in cAMP synthesis at passage 4,
where it was for the first time over 50 % of control
levels and it reached 100 % at passage 7. At this
stage there was no viral antigen present in the cell
membrane while approximately 25 % of cells still
showed distinct fluorescence in the cytoplasm
(Fig. 2). This clearly demonstrates that it is in-
sertion of viral antigen into the cell membrane and
not intracellular accumulation which is reponsible

for the impairment of the cAMP response in C6/SSPE
cells, and this is accomplished without alteration in
the total number of receptors or their affinity for
DL-isoproterenol. When antiserum treated cells were
grown in medium without antiserum as previously
described there was a rapid drop in cAMP levels com-
mensurate with the reappearance of virus antigen in
the cell membrane (Fig. 1B, 3B).

MEASUREMENT OF ADENYLATE CYCLASE ACTIVITY IN ANTI-
SERUM TREATED CELLS

     The mechanism by which virus antigen produced
this impairment in membrane function was unclear as
there was no alteration in receptor number or
affinity. We therefore measured the effect of virus
antigen on the cAMP synthesizing enzyme adenylate
cyclase. The fluoride ion stimulated activity of this
enzyme was determined in particular cell fractions
from uninfected C6 cells, C6/SSPE cells grown in
antiserum containing medium, and C6/SSPE cells grown
in antiserum containing medium until virus antigen
negative and then further passaged in normal medium so
that virus antigen was again produced. Figure 4 shows
that this enzyme activity was drastically reduced in
SSPE virus infected cells. However, adenylate
cyclase activity had recovered to control levels in
antiserum treated cells. Removal of antiserum then
led to a renewed depression in this enzyme activity.
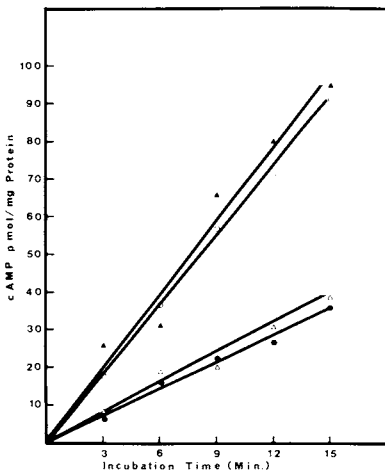
Figure 4
Measurement of the activity
of adenylate cyclase enzyme
by estimation of F^--ion
stimulated cAMP synthesis in
membranes isolated from (▲)
uninfected C6 cells, (△)
C6/SSPE cells, (o) SSPE
antiserum-treated C6/SSPE
cells, and (●) SSPE anti-
serum treated C6/SSPE cells
removed from antiserum.

CONCLUSIONS

These data indicate that measles virus glyco-
proteins impair cAMP synthesis through an effect on
the adenylate cyclase enzyme on its regulatory com-
ponent. It is interesting that in C6 cells persist-
ently infected with LCM virus there is no effect on
cAMP synthesis although virus glycoproteins are in-
serted in the cell membrane. This implies that simple
insertion of foreign proteins may not be sufficient
to cause membrane dysfunctions in virus infected
cells. It is likely that a specific interaction must
occur between viral membrane proteins and the
integral membrane protein components of the signal
transmitting receptor adenylate cyclase system. The
antibody directed loss of viral antigen from the cell
surface of a CNS derived cell described here may be a
mechanism for allowing establishment and maintenance
of viral persistence in the brain as postulated by
Fujinami and Oldstone (9). During such a persistence
there would be no insertion of glycoprotein in the
membrane causing an impairment of membrane function
or stimulating a cytotoxic immune response. However,
any drop in titers of modulating antiviral antibody
which would allow expression of viral antigen in the
membrane would lead to a rapid impairment of membrane
function and the possible development of clinical
symptoms. Studies on the mechanism of maintenance of
virus persistence at the RNA level are described in
another communication (Carter et al. this volume).

ACKNOWLEDGEMENTS

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