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The Chemical and Immunochemical Identity of Amyloid

ABRAHAM RIMON[1]

With 5 Figures

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I. Introduction

Amyloidosis, first described by ROKITANSKI (1842), is characterized by the deposition in the heart, tongue, liver, spleen, kidneys, adrenals and, to a smaller extent, in other tissue, of a whitish interstitial substance that resembles starch both by its physical appearance and by its response to staining with iodine solution. It was these properties of the substance which prompted VIRCHOW (1853 and 1854) some 120 years ago, to suggest the name "amylid" for it, although soon after amyloid was found to be essentially a protein (FRIEDREICH and KEKULÉ, 1859; KUHNE and RUDNEFF, 1865).

As a disease, amyloidosis finds its expression in a variety of clinical forms. It sometimes appears as a primary illness, but mostly it is associated with other disorders that have nothing in common. Accordingly, several clinical types of amyloidosis have been described (HELLER et al., 1964; MISSMAHL, 1969; COHEN, 1967) which need not concern us here. We should note, though, that amyloid has been induced in experimental animals by the repeated injection of a variety of unrelated substances like casein, Freund's adjuvant, $CdCl_2$ solution, bacterial endotoxin, and others. However, the relation of experimental amyloidosis to any known type of human amyloidosis has so far not been conclusively established (SORENSON et al., 1966; COHEN and CATHCART, 1972; COHEN and SHIRAHAMA, 1972). Many histochemical reactions have been found

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to be characteristic for amyloid since the time of VIRCHOW. Some of these reactions are listed below:

1. Metachromatic effect with crystal violet (BRAUNSTEIN and BUERGER, 1959) or toluidine blue (WAGNER, 1955; WOLMAN, 1971; COOPER, 1974)

2. A green birefringence in polarizing microscope after staining with Congo red or Sirus red (BENNHOLD, 1922; MISSMAHL and HARTWIG, 1952; COOPER, 1974)

3. Positive staining with p-dimethyl-aminobenzaldehyde (p-DMAB), which is characteristic for materials rich in tryptophan (COOPER, 1969)

4. Fluorescent effect following staining with thioflavin T or thioflavin S (SAEED and FINE, 1967; WALDROP et al., 1973)

Apart from these histochemical criteria, amyloid is also characterized by a typical fibrillar ultrastructure first described by COHEN and CALKINS (1959) and by SPIRO (1959). This structure is a common feature for amyloid from many different sources and although fibrils have been observed in various sizes, the most commonly reported dimensions are 40–140 Å in width and up to 10,000 Å in length (SOHAR et al., 1967; SHIRIHAMA and COHEN, 1965 and 1967; GLENNER et al., 1968; PRAS et al., 1969; HEEFNER and SORENSEN, 1962).

A different ultrastructure of amyloid was reported by BLADEN et al. (1966), who observed relatively small (about 90 Å in a diameter) pentagonal structures, many of which were organized in rods of various lengths up to 2300 Å. Such rods, however, constitute only a minute percentage of the amyloid material that can be extracted from amyloidotic tissue, so that at present there is no doubt that a fibrillar ultrastructure is characteristic for the bulk of amyloid substance.

The listed parameters have been of great diagnostic value in the identification of amyloid. From a chemical standpoint, however, they have been rather perplexing inasmuch as some of the purportedly "characteristic" histochemical properties of amyloid tend to vanish during its purification from amyloidotic tissue. Thus, one is never sure whether some purified preparations of amyloid actually represent what was originally identified as amyloid in the tissue. As one author puts it: "The definition of amyloid is man-made and no absolute yardstick for comparison is available" (WOLMAN, 1971). It would seem that a differentiation has to be made between *amyloid complex* as it appears in the tissue and *amyloid substance* as a pure chemical entity. Amyloid complex is comprised of numerous components and consequently exhibits all the aforementioned parameters, whereas the purified substance displays only a part of these parameters.

It is the identity of this purified entity that concerns us here. We shall, therefore, disregard the clinical and many other interesting aspects of amyloid or amyloidosis, which are outside the scope of this review and for which the interested reader is referred to the several excellent reviews published in recent years (COHEN, 1967; FRANKLIN and ZUCKER-FRANKLIN, 1972a; GLENNER et al., 1973).

II. Purification of Amyloid

Attempts to purifiy amyloid substance from amyloidotic tissue are hampered by the fact that amyloid cannot be dissolved by conventional solvents of proteins (NEWCOMBE and COHEN, 1965). Formerly, therefore, investigators have employed rather drastic solvents such as alkaline solutions (HASS and SCHULTZ, 1940; WAGNER, 1955; CALKINS and COHEN, 1958), or 8M urea (BENDITT et al., 1962). These procedures yielded a nonhomogeneous product with different sedimentation coefficients ranging from 1–23 S, and various electrophoretic mobilities. Such inconstancy of the product made it practically impossible to gain insight on the properties of the native protein.

Current procedures for the elution and purification of amyloid all employ as starting material a saline homogenate of amyloidotic tissue. The method of COHEN and CALKINS (1962, 1964) relies on differential centrifugation whereby the homogenate, spun at 10000 rpm, produces at the bottom of the tube a three-layered sediment, with the topmost layer especially rich in amyloid fibrils. This top layer can then be separated and purified by repeated resuspensions and sedimentations.

KIM et al. (1967) further separate the "top layer" product by sucrose gradient centrifugation and then treat it with collagenase, 10% NaCl, and 3% ammonium hydroxide, which dissolves or destroys any contaminants but leaves the amyloid fibrils intact. A similar approach is employed by SRI-RAM et al. (1968) who suspends the top layer sediment in 2M urea and treats it first with pepsin at pH 1.8, and then with trypsin and chymotrypsin at pH 8.0.

PRAS et al. (1968) found that the solubility of amyloid in aqueous solutions is extremely dependent on the salt concentration. Accordingly they start with a saline homogenate of amyloidotic tissue which is then repeatedly extracted with saline to remove soluble proteins and extraneous matter. The insoluble residue is next extracted several times with distilled water, yielding a colloidal solution of reasonably pure amyloid fibrils. Figure 1 demonstrates the result of salt and water extractions of amyloidotic tissue. A further purification is attained by precipitating the amyloid with 10% NaCl and treating it with 90% acetone (ZUCKERBERG et al., 1972). Similar methods have also been developed by other investigators (PIRANI et al., 1964; SCHLEYER, 1967; ASHKENAZI et al., 1969), and although none of these methods produces an absolutely pure protein, many investigators are satisfied that the product is pure enough for analytical work.

III. Chemical Composition of Amylid

FRIEDREICH and KEKULE (1859) were first to suggest that amyloid is a protein or predominantly a protein. This suggestion was subsequently confirmed by numerous investigators (KUHNE and RUDNEFF, 1865; MAYEDA, 1909; EPPINGER, 1922). A considerable amount of work was invested in the identification of the protein and other constituents of amyloid. Histochemical and immunochemical procedures were used to reveal the presence in the amyloid

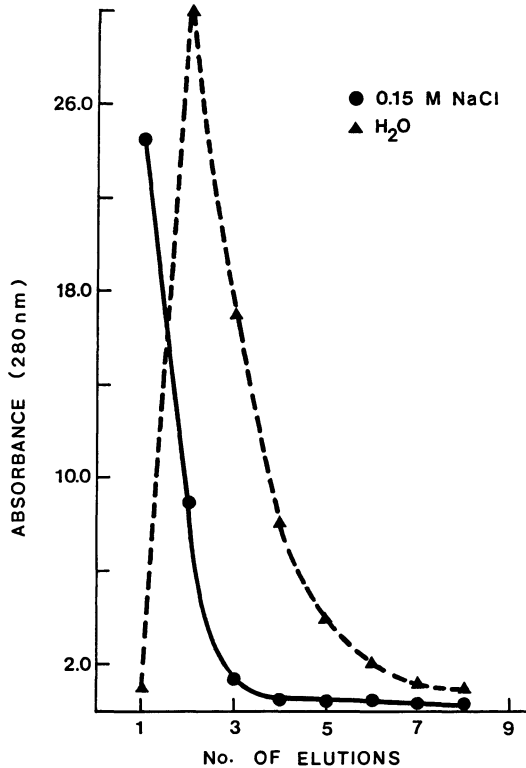

Fig. 1. The effect of salt and water extractions on amyloidotic tissue. A sample of tissue homogenate was repeatedly extracted with 0.15 M NaCl, removing most salt-soluble proteins. Amyloid was then eluted by repeated extractions with distilled water. The elution pattern was followed by measuring absorbence of supernatant at 280 nm (from FLEMINGER, 1974)

complex of mucopolysaccharides, lipids, and several plasmatic components. The best results obtained with some purified preparations showed amyloid to have a nitrogen content of 14–15 %, a sugar content of 1–2 % and a lipid content of 2–3 % (COHEN, 1966; KIM et al., 1967; ZUCKERBERG et al., 1972).

Although the presence of lipids in amyloid complex was reported by several authors (STARK and McDONALD, 1948; LETTERER et al., 1955; BATTAGLIA and PERNIS, 1957; KIM et al., 1967). they do not seem to form an integral part of the amyloid fibril since the fibril is morphologically unimpaired after thorough extraction with organic solvents. Moreover, the lipids in an amyloid preparation were found to drop from 10–19 % to 2,4 % upon purification and disappeared entirely after extraction with an organic solvent and proteolysis (KIM et al., 1967). Nevertheless, some of the characteristic properties attributed to amyloid are still prevailing under these conditions.

The presence of sugars in amyloid complex has been repeatedly demonstrated by histochemical procedures and led many investigators to believe that mucopolysaccharide is an integral part of amyloid structure. This belief re-

Table 1. Carbohydrate content of purified amyloid (% of dry weight)

Hexose	Hexosamine	Uronic acid	Sialic acid	Reference
3.0–4.0	1.2–1.7		0.6–2.0	PIRANI et al., 1964
1.96 ± 0.52	1.77 ± 1.39	0.67 ± 0.29	0.84 ± 0.80	COHEN, 1966
1.17—1.34	0.18—0.26	0.23—0.30	0.10—0.13	PRAS et al., 1968

Table 2. Amino acid composition of partially purified amyloid preparations as reported by several laboratories. Original data were converted into g amino acid per 100 g protein for the sake of comparison

Amino acid	SCHMITZ-MOORMANN, 1964b	COHEN, 1966	PRAS et al., 1968	BENDITT et al., 1972
Asp	7.8	9.3	8.4	11.3
Thr	3.5	3.8	6.2	3.0
Ser	7.0	4.9	8.2	5.1
Glu	12.9	11.8	12.4	12.3
Pro	6.6	4.6	6.3	4.5
Gly	3.5	5.6	5.9	5.5
Ala	5.1	5.8	6.3	6.6
Cys	0.4	1.6	1.1	1.4
Val	3.5	4.9	7.2	4.1
Met	0.8	2.5	0.9	2.6
Ile	5.6	4.0	3.5	4.2
Leu	10.5	7.8	7.7	6.5
Tyr	8.8	4.7	5.1	5.6
Phe	8.8	5.4	4.3	7.2
Hyp	—	0.0	0.0	0.5
Lys	12.5	6.4	6.3	6.6
His	5.2	2.2	2.3	2.5
Arg	3.3	7.7	4.9	10.3
Trp	2.9	1.7	—	—

ceived support from the fact that the typical staining properties of amyloid are indeed characteristic for glycoproteins or mucopolysaccharides and also from the consistent chemical identification of various sugars in the amyloid complex (ODDI, 1894; KRAWKOW, 1898; HASS, 1942; KLENK and FAILLARD, 1955; MEYER et al., 1956; BERTELSEN and CHRISTENSEN, 1959; OKUYAMA and TURUMI, 1963; SCHMITZ-MOORMANN, 1964a). However, PRAS et al. (1968) and ZUCKERBERG et al. (1972) found less than 2% sugars in purified amyloid preparations. ROTTMANN (1969) studied the composition of amyloid extracted from duck's liver and identified in it galacturonic acid, glucuronic acid, glucose, galactose, galactosamine glucosmaine, mannose, and xylose.

Some quantitative data are summarized in Table 1. It seems that sugars, like lipids, are constituents of the amyloid complex and are present in small quantities also in purified amyloid fibrils. It is doubtful, whether they are essential to the fibril structure.

Table 3. Plasma proteins found in amyloid complex

Protein	Reference
Immunoglobulin	VAZQUEZ and DIXON, 1956; MELLORS and ORTEGA, 1956; CALKINS et al., 1958; HOROWITZ et al., 1965; SCHULTZ et al., 1966; HUSBY and NATVIG, 1972b
Complement	LETTERER et al., 1960; VOGT and KOCHEM, 1960; LACHMAN et al., 1962; SCHULTZ et al., 1967; HUSBY and NATVIG, 1972b
Fibrinogen	LACHMAN et al., 1962; HOROWITZ et al., 1965
Rheumatoid factor	LACHMAN et al., 1962
β_1-globulin	KOCHEM, 1966
Lipoprotein	KASUKAWA et al., 1966; SCHULTZ et al., 1967
α_1-globulin (P-component)	CATHCART and COHEN, 1966

Amino acid analysis of amyloid protein has in many cases been carried out on preparations which were too contaminated to enable meaningful interpretation. Nevertheless, it has been possible to deduce that amyloid is completely different from other extracellular fibrillar proteins like collagen and elastin, insofar as it lacks hydroxyproline, hydroxylysine, and desmosine (LETTERER et al., 1955; SCHMITZ-MOORMANN, 1964b; PIRANI et al., 1964; COHEN, 1966). This conclusion gains support from an analysis of more purified preparations which, on the whole, fails to show any similarity between amyloid and other plasmatic or tissue proteins (TRISTRAM and SMITH, 1963; PRAS et al., 1968) (Table 2).

Many amino acid analyses have been performed on low molecular weight fragments of amyloid. These analyses have revealed the following amino acids to be the primary ones: glutamic acid, aspartic acid, glycine, alanine, serine, and occasionally arginin and leucine. On the other hand, there is very little or no cystein and methionine (GLENNER et al., 1970; BENDITT et al., 1971; FRANKLIN et al., 1972; BENDITT et al., 1972; ZUCKERBERG et al., 1972).

The N-terminal amino acid of amyloid was investigated by SKINNER and COHEN (1971) on amyloid obtained from 14 different patients falling into three categories of the disease: primary amyloidosis, secondary amyloidosis, and amyloidosis associated with myeloma. These investigators detected aspartic or glutamic acid in some of the amyloids, but with no correlation to the clinical category. In five cases the terminal amino acid was unidentified and might have been pyrolydone carboxylic acid. A trace amount of serine was also found at the terminus.

The physical properties of purified amyloid have been little investigated which is not surprising in view of the difficulties involved with a colossal molecule like that of amyloid, insoluble as it is in conventional solvents. One of the few studies on its sedimentation behavior has revealed a marked difference between fresh and aged amyloid as well as extreme variations between different

amyloid preparations. Of 8 preparations studied, 4 had an $S_{w, 20}$ value of 40–45 for fresh and 70–95 for aged amyloid, 3 had a sedimentation constant of about 8 S when fresh and 9.5–13.5 S when aged, and the remaining preparation had a sedimentation constant of 74 S even when fresh (PRAS et al., 1968; PRAS et al., 1969). The isoelectric point of amyloid was deduced from the binding of dyes to it, and was reported to be 4.5–5.0 (CARNES and FORKER, 1956; GOLDBERG and DEANE, 1960).

IV. Components of Amyloid Complex

That collagen or elastin are not components of the amyloid complex has already been established from its amino acid analysis (COHEN, 1966). Furthermore, it has been shown that amyloid fibrils are not destroyed by collagenase even after 48 hrs incubation (COHEN and CALKINS, 1964). On the other hand, several normal plasma proteins were identified in the amyloid complex either by reacting amyloidotic preparations with antisera specific to various plama proteins or by cross-reacting plasma proteins with antiserum against amyloid. The components thus identified in amyloid complex are listed in Table 3. While most of these components were previously recognized as plasma proteins, the last on the list, a specific α_1-globulin named "P" component, was strictly related to amyloid substance. It could be demonstrated in amyloidotic but not in healthy tissue (CATHCART and COHEN, 1966; CATHCART et al., 1967), and was found to be identical to the pentagonal ultrastructure demonstrated by BLADEN et al. (1966) in amyloid preparations. P-component was recently purified and characterized by SKINNER et al. (1974) who found it to be a protein of 180000 molecular weight with a unique N-terminal amino acid sequence that was not similar to any of the sequences previously reported for amyloid fragments or amyloidrelated proteins (see Section E). Therefore, the relation of P-component to amyloid or to the low molecular fragments derived from it is still obscure. Clearly, the participation of a plasma protein in the structure of amyloid, if conclusively established, should prove of primary importance to an understanding of the pathogenesis of amyloidosis. Of special interest in this concern are the experiments reported by HOROWITZ et al. (1965) who used immunofluorescent methods in an attempt to detect the presence of fibrinogen and of IgG in amyloidotic tissue from a myeloma patient with hypergammaglobulinemia and from a familial Mediterranean fever patient with hyperfibrinogenemia. Although they succeeded in demonstrating the presence of fibrinogen as well as IgG in the sick tissue from both patients, the amount of fibrinogen was higher in the hyperfibrinogenemia case whereas the amount of IgG was higher in the hypergammaglobulinemia case. One must, therefore, concur with their conclusion that "amyloid represents merely a deposition of any plasma protein which is present in high concentration."

Another controversial point is the specificity of the antibodies produced against amyloid. There is good reason to believe that amyloid fibril per se is not immunogenic. This has been clearly shown in more than one study (SRI-

RAM et al., 1968; FRANKLIN and PRAS, 1969), and since most of the immunochemical identifications of protein components in amyloid complex have employed antisera against the intact fibrils it is quite likely that the antibodies in such antisera detect the components of the *amyloid complex* rather than the *amyloid protein* itself. Only in recent years have studies been carried out which utilize antibodies elicited by immunizing rabbits with low-molecular weight fragments of amyloid. The antibodies thus produced were specific to some of the antigenic determinants of the amyloid protein (FRANKLIN and PRAS, 1969; GLENNER et al., 1969; CATHCART et al., 1970; CATHCART et al., 1971; FRANKLIN and ZUCKER-FRANKLIN, 1972b; HUSBY and NATVIG, 1972a; RUKOSUEV, 1973; LEVIN et al., 1973; SHAPIRA et al., 1973). This approach has already produced very valuable information, as two groups of investigators identified a serum component that was antigenically related to the amyloid protein. This component was found in 50–88% of various pathologic sera as well as in 7% of normal sera tested by LEVIN et al. (1973). HUSBY et al. (1973; 1974a; HUSBY and NUTVIG, 1972a) confirmed these observations and were able, in some cases, to detect the serum component 2–3 years prior to the clinical diagnosis of amyloidosis. Partial characterization of this component showed it to be an α-globulin with a molecular weight of 80000–100000. In a more recent study, ROSENTHAL and FRANKLIN (1975) used a highly sensitive radioimmunoassay technique and found the serum component to be present in various concentrations in all human sera. A marked increase in concentration was noted in aging individuals as well as in patients with myeloma, macroglobulinemia, lymphoma, carcinoma, rheumatoid arthritis, and tuberculosis. It is suggested that the serum component thus discovered might be a normal serum protein that increases in quantity under pathologic conditions and in such cases serves as a precursor for the amyloid fibrils, or at least that class of amyloid that is characterized by "protein A" subunit (see next section for further discussion of protein A and other amyloid subunits).

Antibodies to low-molecular weight fragments of amyloid were also used to investigate the relation of immunoglobulins to the amyloid protein and helped to establish that some amyloids consist of immunoglobulin light chains (ISERSKY et al., 1972; HUSBY and NATVIG, 1972b). These findings have also been substantiated by direct biochemical analysis.

V. Low-Molecular Weight Fragments of Amyloid

A considerable advance in our understanding of the composition of amyloid was made possible in recent years by the study of low-molecular fragments of amyloid. PRAS et al. (1969, 1972) reported on two different fragments. One was obtained by degradation of amyloid in dilute alkaline solution, using 0.1 N NaOH at room temperature. It was termed DAM (degraded amyloid). The other was a product of acid degradation, using 0.02 N HCl at 60° C, and was called ASF (acid soluble fraction). Their molecular weights were estimated at about 35000 and 7000 respectively. DAM and ASF fragments were originally prepared from the tissue of patients with familial Mediterranean fever but

Table 4. Some of the typical low-molecular weight fragments derived from amyloid of various sources

Fragment	Disease	Molecular weight	Sequence prototype	Reference
Protein A[a]	Tuberculosis	8000	AA[c]	BENDITT et al., 1962; 1971
DAM	Various	35000		PRAS et al., 1969
Fibril protein X	Primary amyloidosis	7500	B–J, ϰI	GLENNER et al., 1969; HARADA et al., 1971
Fibril protein VIIIb	Epidermolysis bullosa	18300	B–J, ϰI	GLENNER et al., 1969; HARADA et al., 1971
ASF	Familial Mediterranean fever	8000	AA	PRAS et al., 1972
MALAF	Familial Mediterranean fever	16500	AA[b]	PICK et al., 1972
Protein F	Familial Mediterranean fever	8000		ZUCKERBERG et al., 1972
Protein AS	Rheumatoid arthritis	5000	AA	HUSBY et al., 1972b
Fibril protein IV	Rheumatoid arthritis	5000	AA	EIN et al., 1972
Fibril protein Tew	Multiple myeloma G		B–J, ϰII	TERRY et al., 1973
Protein AR	Primary amyloidosis	16000	AR	HUSBY et al., 1974b

[a] Of human or monkey origin.
[b] A. PICK, personal communication.
[c] Protein A, as well as ASF and AS, are now termed AA according to the nomenclature accepted in the Intern. Symp. on Amyloidosis held in Helsinki in August 1974. The serum component related to AA is termed SAA.

other classes of amyloidosis were equally good sources (FRANKLIN et al., 1972; HUSBY et al., 1972b; HUSBY et al., 1973). Another group of investigators studied amyloid from a monkey and several human patients, all classified as secondary amyloidosis associated with a severe inflammation (BENDITT et al., 1962; BENDITT and ERIKSEN, 1966; BENDITT et al., 1971; BENDITT and ERIKSEN, 1972). They treated amyloid with 6 M urea and 0.01 N acetic acid and separated the mixture by gel filtration chromatography, isolating a fragment of about 8000 molecular weight, that was termed "protein A." GLENNER et al. (1969, 1972 and HARADA et al., 1971) degraded amyloid by 6 M guanidine-HCl and separated the mixture by sequential gel filtrations into several fragments ranging from 5000–31000 molecular weight. These fragments, that were referred to as "amyloid fibril proteins," were obtained from several patients of both primary amyloidosis as well as the secondary type, including amyloidosis associated with multiple myeloma.

Several other fragments have been studied by other investigators using various chemical methods for the degradation of the amyloid molecule (PICK et al., 1972; ZUCKERBERG et al., 1972; EIN et al., 1972; HUSBY et al., 1972b; SHAPIRA et al., 1973; HUSBY et al., 1974b). A list of some of the fragments

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Bence Jones Ker (κI)[a]	Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-Ala-Ser-Val
Amyloid X[b]	Asp-Ile-Gln-Met-Thr-Gln-Ser-*Ala*-Ser-Ser-Leu-Ser-Ala-Ser-Val
Amyloid VIII-b[b]	Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-Ala-Ser-Val
Bence Jones Tew (κII)[c]	Asp-Ile-Val-Met-Thr-Gln-Ser-Pro-Leu-Ser-Leu-Pro-Val-Thr-Pro
Amyloid Tew[aa]	Asp-Ile-Val-Met-Thr-Gln-Ser-Pro-Leu-Ser-Leu-Pro-Val-Thr-Pro

[a] MILSTEIN, 1966. [b] GLENNER et al., 1973. [c] PUTNUM et al., 1973. [aa] TERRY et al., 1973.

Fig. 2. N-terminal amino acid sequence of low-molecular weight fragments ("amyloid fibril protein") derived from *immunoglobulin-related amyloids*, and Bence Jones proteins of two prototypes. The first 15 positions are displayed for comparison

reported in the literature and their main properties is presented in Table 4. They all have close physicochemical characteristics but divide sharply into two groups when their antigenicity and amino acid sequences are examined. Two of the "amyloid fibrin proteins" studied by GLENNER'S group were reported to have an amino acid sequence almost identical to the amino-terminal portion of a Bence-Jones protein of the κI type (GLENNER et al., 1971b). A similar homology was found by PUTNAM et al. (1973) and by TERRY et al. (1973) who studied an amyloid protein and a Bence Jones protein of the κII type derived from the same patient (Fig. 2).

On the other hand, protein A studied by BENDITT et al. and protein ASF studied by PRAS and FRANKLIN revealed a unique sequence unsimilar to any known Bence Jones protein (BENDITT et al., 1971; HERMODSON et al., 1972; FRANKLIN et al., 1972; LEVIN et al., 1972). In fact, no equivalent sequence could be found among the human proteins hitherto analyzed as displayed in the *Atlas of Protein Sequence and Structure* (DAYHOFF, 1972). Several other fragments studied by other investigators proved to have an amino acid sequence identical to protein A (Figs. 3 and 4) (EIN et al., 1972; PICK, 1973; HUSBY et al., 1972b; SLETTEN and HUSBY, 1974).

On the basis of these data, one can classify amyloids into two classes: the *immunoglobulin-related* type, which is characterized by fragments of a Bence Jones-like sequence, and the *immunoglobulin-nonrelated* type, which is characterized by fragments revealing the unique sequence of protein A, or ASF. The immunoglobulin-related fragments were purified in a variety of molecular sizes ranging from 7500–18000. The second type of fragments seem to be more uniform in size; their molecular weight is about 8000. They consist of 76 amino acids, and lack cystein.

A third type of amyloid was discovered by HUSBY et al. (1974b) in a patient with primary amyloidosis. A low-molecular weight fragment (termed "protein AR") isolated from this amyloid was of the nonimmunoglobulin type but differed from protein A in that its amino acid sequence was neither similar to Bence Jones protein nor to protein A (Fig. 3). Antibodies raised against protein

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Human protein A[a]	Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Gln-Ala-Phe-Asp-Gly-Ala-Arg
Monkey protein A[a]	Arg-Ser-*Trp*-Phe-Ser-Phe-Leu-Gly-Gln-Ala-*Tyr*-Asp-Gly-Ala-Arg
Protein ASF[b]	Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Gln-Ala-Phe-Asp-Gly-Ala-Arg
Protein AS[c]	Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Gln-Ala-Phe-Asp-Gly-Ala-Arg
Fibril protein[aa]	Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Gln-Ala-Phe-Asp-Gly-Ala-Arg
Protein AR[bb]	Asp-Phe-Met-Leu-Thr-Gln-Pro-His-Val-Ser-Gln-

[a] BENDITT et al., 1971; HERMODSON et al., 1972. [b] FRANKLIN et al., 1972; LEVIN et al., 1972. [c] HUSBY et al., 1972b; SLETTEN and HUSBY, 1974. [aa] EIN et al., 1972. [bb] HUSBY et al., 1974b.

Fig. 3 N-terminal amino acid sequence of low-molecular weight fragments derived from various *immunoglobulin-nonrelated amyloids*. The first 15 positions are displayed for comparison

```
  1             5               10                15
H2N-Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Gln-Ala-Phe-Asp-Gly-Ala-Arg-
                20              25                30
    Asp-Met-Trp-Arg-Ala-Tyr-Ser-Asp-Met-Arg-Gln-Ala-Asn-Tyr-Ile-
                35              40                45
    Gly-Ser-Asp-Lys-Tyr-Phe-His-Ala-Arg-Gly-Asn-Tyr-Asp-Ala-Ala-
                50              55                60
    Lys-Arg-Gly-Pro-Gly-Gly-Ala-Arg-Ala-Ala-Gln-Val-Ile-Ser-Asn
                65              70                75
    Ala-Arg-Gln-Asn-Ile-Gln-Arg-Leu-Thr-Gly-Arg-Gly-Ala-Gln-Asp-
    76
    Ser-COOH
```

Fig. 4. The complete amino acid sequence of ASF from a patient with familial Mediterranean fever (from LEVIN et al., 1972). Variations in positions 52, 53, 57, 68, 69, and 71 were found in other low-molecular fragments of same amyloid class (EIN et al., 1972; SLETTEN and HUSBY, 1974). Protein A prepared from monkey varied from ASF in positions 3, 11, 25, 30, 31, 46, 52, 67, 69, 71 and 76 (HERMODSON et al., 1972)

AR revealed specific antigenic determinants and did not cross-react with other amyloid preparations or with immunoglobulins or Bence Jones proteins. These antibodies, however, detected in the patient's serum a component antigenically related to AR. It is speculated that this component might be the precursor of this type of amyloid in the same way that a Bence Jones protein or a serum component related to protein A might be the precursors for these two types of amyloid (LEVIN et al., 1973; HUSBY and NATVIG, 1974; HUSBY et al., 1974a; HUSBY et al., 1974b). In a survey of 352 pathological sera tested by HUSBY et al. (1974a), 16 sera were found to contain a protein AR–related component whereas 149 contained a protein A–related component.

Perhaps the most striking property of the low-molecular weight fragments of amyloid is the ability of some of them to polymerize spontaneously, under

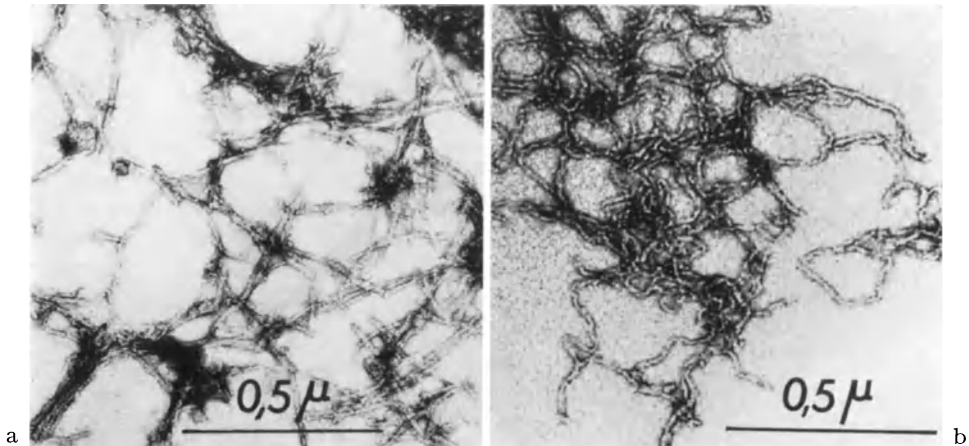

Fig. 5. Electron microscopic comparison of (a) native amyloid fibrils (prepared from a familial Mediterranean fever patient) and (b) fibrils reconstructed from F-protein. Negative staining with uranyl acetate. Magnification ×66000 (from ZUCKERBERG et al., 1972)

certain conditions, into fibrils that resemble the typical morphology of amyloid fibrils (Fig. 5) (ZUCKERBERG et al., 1972; PRAS and RESHEF, 1972). This property points to the primary role of these fragments in the fibrilar structure of amyloid and suggests that, at least, protein A type fragments are true subunits of amyloid in the sense that they polymerize to form the parent molecule.

VI. Relation of Amyloid to Immunoglobulin

Of all the plasma components that are possibly involved in the structure of amyloid, most attention has been paid to the immunoglobulins. If they, or part of them, could be shown to constitute the amyloid protein, then amyloidosis could be mediated through immunological mechanisms and there would be support for the view that amyloid deposits result from an interaction of an antibody with an unknown, possibly pathologic, antigen (LETTERER, 1926; LOESCHCKE, 1927). Indeed, many investigators reported on the presence of immunoglobulins as well as complement components in the amyloid complex (Table 3), although the interpretation of these findings has been questioned (PAUL and COHEN, 1963; HOROWITZ et al., 1965).

Another set of observations that related amyloid to immunoglobulins is the close association of amyloidosis with multiple myeloma. MAGNUS-LEVY'S (1931, 1934, 1952) original report on this matter was repeatedly verified by other investigators on all types of myelomas, including the rare myeloma D (NASHEL et al., 1973). OSSERMAN et al. (1964; PICK and OSSERMAN, 1968; ISOBE and OSSERMAN, 1974) compared clinical and laboratory data of 27 amyloidotic patients and found plasma cell dyscrasias in most of them. CATHCART et al. (1972)

came to a similar conclusion after a study of 62 patients with amyloidosis, in most of whom they found one kind or another of immunoglobulin abnormality. Although, in a recent study, LIMAS et al. (1973) maintain that the association of amyloidosis with multiple myeloma is not as close as previously believed, the data on this subject are still very suggestive that abnormal immunoglobulin products are involved in the formation of amyloid deposits.

A more direct demonstration of the relation between immunoglobulins and amyloid came primarily from the work of GLENNER and his colleagues on the low-molecular weight fragments of amyloid that was reviewed in the previous section. The identity of the amino acid sequence of some of these fragments with a known Bence Jones protein leaves no doubt as to the immunoglobulin origin of the amyloid protein in these cases.

Moreover, immunizing rabbits with low-molecular weight fragments of amyloid elicited antibodies specific to antigenic determinants of amyloid proteins (FRANKLIN and PRAS, 1969; CATHCART et al., 1970; CATHCART et al., 1971; FRANKLIN and ZUCKER-FRANKLIN, 1972b; HUSBY and NATVIG, 1972a; LEVIN et al., 1973). The use of these antibodies showed an unequivocal antigenic relation between amyloid protein and immunoglobulins in many cases where the direct sequence analysis was not practical (HUSBY and NATVIG, 1972a, 1972b; ISERSKY et al., 1972).

Further evidence that amyloid fibrils might be formed from immunoglobulin fractions was supplied by experiments in which "synthetic" fibrils were made from Bence Jones proteins. Several laboratoris reported on the production of amyloid-like fibrils from Bence Jones proteins after limited proteolysis with pepsin or with renal lysosomal proteases (GLENNER et al., 1971a, 1974; SHIRAHAMA et al., 1973; LINKE et al., 1973a, 1973b; TAN and EPSTEIN, 1972; EPSTEIN et al., 1974). In one study (GLENNER et al., 1971a) three Bence Jones proteins of the $\varkappa$-type and 2 of the λ-type were tested but only the two λ-proteins formed amyloid-like fibrils. In another study (LINKE et al., 1973a) 61 λ-proteins and 14 $\varkappa$-proteins were tested. Nine of the λ-proteins and one of the $\varkappa$-proteins formed fibrils. The fact that only some and not all Bence Jones proteins produce fibrils under these conditions suggests that certain structural prerequisites are necessary for a Bence Jones protein to convert into a fibril. A distinction between "amyloidogenic" and "non-amyloidogenic" protein has, indeed, been suggested (GLENNER, 1973). These assumed structural prerequisites for an amyloidogenic protein are not restricted to immunoglobulin light chains, since the in vitro formation of amyloid-like fibrils was also demonstrated with fractions of IgG heavy chains (PRUZANSKI et al., 1974).

It should be added that, although the involvement of immunoglobulins in the structure of some amyloids is well documented now, not all of the amyloids are related to immunoglobulins, as pointed out in the previous section. It is unlikely that immunoglobulins are involved in the structure of the other classes of amyloids. These are formed from precursors of yet unknown nature (EIN et al., 1972; LEVIN et al., 1973; HUSBY et al., 1974a; HUSBY and NATVIG, 1974).

VII. Conclusions

On reviewing the data on the chemical nature of amyloid, a clear distinction should be made between the *amyloid complex* as it appears in the tissue and the pure *amyloid protein* that can be extracted from it. Such a distinction is very helpful in evaluating some of the conflicting data that were published through the years. In the search for the identity of amyloid, many plasma proteins were detected in that complex but none of them, apparently, is a constituent of the pure fibrilar protein that represents the amyloid substance. The amyloid complex certainly has its own intrinsic significance as the natural pathologic product that deposits in the sick tissue. However, to gain an insight into the chemical nature of the pure amyloid protein it has been necessary to study low-molecular weight fragments of the purified amyloid protein.

Studies along this line have shown that the fibrilar protein can be easily degraded by a variety of chemical means into relatively small fragments, some of which can polymerize back into a fibril. Therefore, one is inclined to regard them as actual subunits of amyloid, i.e., monomeric forms of the fibrilar polymer. Several classes of low-molecular weight fragments were recognized. One class is identical in amino acid sequence to the N-terminal portion of certain prototypes of Bence Jones proteins. The amyloid derived from this class of fragments is, therefore, an "immunoglobulin-related" amyloid. Two other classes, protein A and protein AR, differ from each other but are both "nonrelated" to immunoglobulins. Thus, a substantial chemical heterogeneity among amyloids has been demonstrated. In fact, one can expect the immunoglobulin-related amyloids to exhibit the same degree of heterogeneity as do myeloma proteins or Bence Jones proteins and a similar heterogeneity probably prevails among the immunoglobulin-nonrelated amyloids. In addition to the two types that have already been reported, several more can be expected to be discovered in the near future.

Investigations carried out with low-molecular weight fragments led, through a series of immunochemical experiments, to the detection of circulatory serum components which are antigenically related to either A-type or AR-type amyloid. It has been suggested that these serum components might act as precursors for the respective amyloid fibrils, in analogy to Bence Jones proteins which are probably the precursors of the immunoglobulin-related amyloids.

The demonstration in vitro that amyloid-like fibrils were formed in a test tube after proteolysis of some Bence Jones proteins makes it conceivable that similar in vivo processes cause the formation of amyloid in the tissue. Suggestions to this effect have been made (Glenner, 1972). The exact mechanism of amyloid formation, however, will require a great deal of further research.

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The λ Repressor and its Action

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With 9 Figures

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I. Introduction

Repressors are a class of proteins which regulate the expression of specific sets of genes by inhibiting their transcription. This concept of genetic regulation was proposed by JACOB and MONOD (1961) and was based on genetic experiments on the expression of the lactose genes of *Escherichia coli* and the regulation of the lytic functions of the coliphage λ. The general principle of negative regulation in its present form is as follows: an operon consists of a set of adjacent genes which are coordinately transcribed or repressed. The operon is transcribed by RNA polymerase which recognizes a transcription initiation site called the promoter. A repressor protein turns off the transcription of the operon by binding specifically to a DNA site called the operator which precedes

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the genes of the operon, and interfering with either the binding of RNA polymerase to the promoter or the initiation of transcription. In response to a specific chemical or physical stimulus, the cell produces a specific inducer substance which inactivates the repressor and allows induction or derepression of the operon.

The prototype system is that of the lactose operon which consists of three genes whose products are required for the utilization of lactose. In the absence of lactose these gene products are superfluous. The transcription of the *lac* operon is prevented by the *lac* repressor binding to the *lac* operator (DE CROMBRUGGHE et al., 1971). Entrance of lactose into the cell results in the accumulation of allolactose, an isomer of lactose (JOBE and BOURGEOIS, 1972). Allolactose can bind both to free or to operator-bound repressor, inducing an allosteric transition in the repressor protein to a form with much lower affinity for the operator. The result is that allolactose acts an an effector signalling the presence of lactose in the cell, inactivating the repressor and inducing the expression of the *lac* operon.

JACOB and MONOD (1961) argued that the life cycle of the temperate coliphage λ provides an entirely analogous example of negative control. Upon infection of a sensitive bacterium, phage λ can either proceed to multiply and lyse the cell, or it can inactivate itself by producing a repressor which binds to two operators and prevents the expression of the lytic genes of the phage. The phage DNA is then inserted into the bacterial chromosome and behaves as an integral part of it. The continued presence of repressor in the bacterium renders it immune to superinfection by additional λ phages since upon entering the cell their genes will also become repressed. Such a bacterium will not be immune to superinfection by other types of phage with different immunity characteristics, i.e., different operators and repressors. Bacteria carrying a repressed phage are called lysogenic because with a frequency of about one in 10^4 cell divisions, the phage becomes derepressed and proceeds to grow and lyse the cell. Derepression can be induced with high efficiency by a number of treatments which interfere with DNA replication, of which the most common is irradiation with ultraviolet light. These treatments lead to the accumulation of some substance which inactivates the repressor and allows gene expression.

This review attempts to summarize the salient aspects of the immunity system of λ and the present knowledge of the repressor and its action. The emphasis is on the developments which have occurred since the writing of earlier reviews (PTASHNE, 1971; EISEN and PTASHNE, 1971) and in many cases the approach taken is highly speculative.

II. Repressor and λ Regulation

A. Basic Control Features

The repressor is the product of the *c*I gene of λ. Phage carrying mutations in this gene are unable to lysogenize. Temperature-sensitive mutations in the *c*I gene result in a thermoinducible lysogen. Other mutations in this gene affect

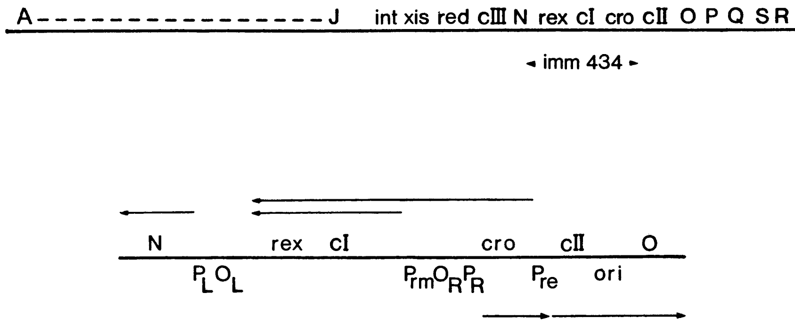


Fig. 1. Schematic diagram of λ genome. *N-O* region is shown enlarged below. Arrows indicate transcripts originating at P_L, P_R, P_{re} and P_{rm} promoters

the inducibility of a lysogen by ultraviolet radiation. KAISER and JACOB (1957) showed that the *c*I gene determines the immunity characteristics of the phage. By repeated crosses they isolated a hybrid phage in which a very small region of the λ DNA containing the *c*I gene was replaced by the corresponding region from the related phage 434. This region, within which λ and 434 fail to recombine, is called the immunity region and amounts to 5 % of the genome.The fact that the hybrid phage λ imm^{434} has the immunity properties of phage 434 implies that the immunity region contains both the repressor gene and its sites of action, the operators (Fig. 1).

The λ repressor exerts direct control over two operons located to the right and to the left of the *c*I gene. These operons are transcribed in opposite directions and therefore from opposite strands. They contain the early genes, active in the initial stages of phage growth in an induced lysogen or upon infection of a sensitive cell. To the right of *c*I are the *O* and *P* genes which are involved in DNA replication. To the left of *c*I are genes responsible for genetic recombination. The first gene of the left hand operon is the regulatory gene *N*. The product of this gene is required to activate transcription at three other sites: (1) to extend transcription of the left-hand operon beyond the *N* gene itself; (2) to extend transcription of the right-hand operon beyond the first gene (*cro*); and (3) to transcribe another regulatory gene, *Q*, whose function is to activate the late genes involved in the synthesis and assembly of the structural components of the phage particle. In the absence of *N* gene function, transcription initiates at P_R and P_L and stops after the *N* gene on the left or the *cro* gene on the right. The *N* gene product is therefore a positive regulator which is required for the activation of most of the remaining phage genes. It is through the *N* protein that the repressor exercises indirect control over the late genes.

The *cro* gene, also called *tof* or *fed*, is another regulatory gene which appears to act at three distinct sites to turn off the expression of the left-hand operon, the right-hand operon, and of the *c*I gene itself.

In addition to regulating the right- and left-hand operons, the repressor was thought at one time to exert a direct control on λ DNA replication. On

superinfection of a λ lysogen, phage λ fails to replicate even if all the necessary gene products are supplied by a coinfecting λimm^{434} phage (THOMAS and BERTANI, 1964). This replication inhibition is now believed to be an indirect effect due to repression of transcription of the region around the O and P genes. DOVE et al. (1971) have identified a site called *ori* located between genes *c*II and O, at which λ replication initiates. Mutants which are able to transcribe this region constitutively also fail to show replication inhibition. Conversely, mutations selected because they relieve replication inhibition also cause constitutive expression of the O and P genes. HAYES and SZYBALSKY (1973) have presented evidence indicating that transcription of the O-P region produces a species of RNA believed to act as a primer in *cis* for the initiation of λ DNA replication.

B. Operator Mutation: Virulence

λ mutants which are able to grow on a λ lysogen are called virulent. Such mutants, first described by JACOB and WOLLMAN (1954), were interpreted by JACOB and MONOD (1961) as lacking effective operators and therefore uncontrollable by the repressor. The classical virulent phage contains three mutations: v_2 in the left-hand operator (O_L) and v_1 and v_3 in the right-hand operator (O_R) (PTASHNE and HOPKINS, 1968). Upon infection of a λ lysogen, $\lambda\, v_2$ is able to express the N gene, while $\lambda\, v_3$ can express the O gene. $\lambda\, v_2v_3$ is not able to grow on a lysogen because v_3 is apparently not sufficiently constitutive. The addition of the v_1 mutation, located very close to v_3, overcomes the remaining repressor control. $\lambda\, v_3v_1$ can express both the right- and left-hand genes in a lysogen, presumably because it is able to replicate extensively resulting in more DNA copies than can be controlled by the repressor present. PTASHNE and HOPKINS showed that these mutations lower the affinity of the operators for the λ repressor, proving that they are in fact operator mutations rather than, for example, transcription restart signals.

Similar sets of mutations have been isolated by HORIUCHI et al. (1969) and KOGA et al. (1970). Starting from the assumption that repressor made by λ *c*Its is less active than the wild type, they isolated a weak virulent mutant able to grow on a λ*c*Its lysogen but not on a normal lysogen. The weak virulent contains two mutations, *vir*L in O_L and *vir*R in O_R. As in the classical virulent, an additional mutation *vir*C, also in O_R, is required to convert the weak virulent into a full virulent. KOGA et al., however, have shown that full virulence can also be obtained from a single mutation in each operator (i.e., $\lambda\, v_2\, virC$) if they confer a sufficient degree of constitutivity.

Additional mutations in O_R have been isolated by selecting for virulents able to grow on bacteria carrying $\lambda\, dv$ plasmids (ORDAL, 1971; ORDAL and KAISER, 1973). These mutations, called v_s, map on either side of promoter mutations in P_R, suggesting that the promoter and the operator may overlap. The possibility was supported by the finding that operator mutations can also affect the rate of transcription in the absence of repressor. Thus, λ *virC virR*

susN makes 2.5 times more RNA from P_R than λ *c*I⁻ *susN* (SAKAKIBARA et al., 1972). A similar observation was made with λ v_1v_3 (KUMAR et al., 1970) suggesting that these operator mutations also affect the promoter.

C. Control of Repressor Synthesis: The *cro* Gene

In the lysogenic state only two λ genes are expressed. These are the *c*I and the *rex* genes, both located in the immunity region and constituting one operon. The function of the *rex* gene product in the physiology of λ is not known but it is able to block the growth of a superinfecting T4 *rII* phage (BENZER, 1955; HOWARD, 1967). If lysogenic cells carrying a temperature-sensitive mutation in the *c*I gene are grown at high temperature, they rapidly lose immunity and, more slowly, also the *rex* function (TOMIZAWA and OGAWA, 1967). This observation, which was at one time thought to indicate that the *rex* gene was a part of the *c*I gene, now appears to be due to the fact that the *c*I-*rex* operon is itself subject to a regulatory mechanism. If the λ *c*Its prophage carries in addition mutations in the *N* and *O* genes to prevent cell death, the lysogenic cells grown at high temperature fail to recover immunity or *rex* function when they are returned to growth at low temperature (EISEN et al., 1968; CALEF and NEUBAUER, 1968). Instead they continue to multiply in a nonimmune lysogenic state. This condition depends on the activity of the *cro* gene which becomes derepressed after heat inactivation of the repressor. If the prophage carries mutations in the right-hand promoter P_R or in the *cro* gene, the lysogens gradually recover immunity and *rex* function when returned to growth at low temperature (EISEN et al., 1970).

The *cro* gene product appears to act as a repressor in many ways similar to the *c*I repressor, regulating the expression of the same three operons: the left-hand and right-hand operon and the *c*I-*rex* operon. In a normal lytic cycle, the expression of the left-hand operon is turned off about 15 minutes after infection in the presence of a functional *cro* gene (PERO, 1970; EISEN et al., 1970). REICHARDT (1975a) and ECHOLS et al. (1973) found that *cro* has a similar effect on the expression of the right-hand operon. Not only the function but also the sites of action of the *cro* product are similar to those of the *c*I repressor. SLY et al. (1971) found that the v_2 mutation in O_L also renders the left-hand operon less sensitive to shut off by the *cro* gene product, suggesting that *cro* product has the same site of action as the repressor. Operator mutations in O_R also appear to render the right-hand operon less sensitive to *cro* regulation (ORDAL, 1971; BERG, 1974).

In at least two situations the *cro* product is able to control the expression of a superinfecting phage and result in a sort of immunity. KOGA and HORIUCHI (1973) report that it is possible to isolate "lysogens" of λ *virCvirR* N^- O^- P^-, where *virC* and *virR* are operator-constitutive mutations in O_R. In such lysogens the prophage is integrated in the bacterial chromosome, makes no *c*I repressor, but nevertheless is able to prevent lytic growth of a superinfecting λ, but not λ imm^{434}, because of the accumulation of *cro* product.

A similar situation was shown by BERG (1974) to exist in bacteria carrying λ *dv* plasmids. λ *dv* is a small, circular fragment of λ DNA, carrying the replicative genes and maintained in the plasmid state with as many as 100 copies per cell (MATSUBARA and KAISER, 1968). BERG found that essential to the maintenance of λ *dv* is the expression of genes *O*, *P*, and *cro* but not *c*I. By accumulating *cro* product, which acts as a leaky inhibitor of the expression of the right-hand operator, the λ *dv* plasmid is able to control its own replication and to prevent the growth of a superinfecting λ. λ *dv* isolated from $\lambda\ v_2v_3v_1$ can also prevent the growth of a superinfecting $\lambda\ v_2v_3v_1$. Supervirulent phage, carrying a v_s mutation, a stronger operator constitutive mutation in O_R, are able to grow on bacteria containing λ *dv* v_3v_1 because the levels of *cro* product present in such cells are not sufficient to repress them. Supervirulent phage are not able to grow on bacteria carrying λ *dv* v_3v_s because the level of *cro* product required to maintain the λ *dv* v_3v_s is also sufficient to repress the λ supervirulent.

D. Promoters of the *c*I Gene

Two other genes are required for the establishment of the lysogenic state: the *c*II and *c*III genes, located in the right and left operons respectively. The products of these genes activate a promoter P_{re} located to the right of the *cro* gene (EISEN et al., 1970; REICHARDT and KAISER, 1971). Transcription initiated at this promoter proceeds leftward through the *cro* gene and includes the *c*I and *rex* genes. The *cro* gene is therefore transcribed in both directions, from left to right in the "sense" direction and from right to left in the "antisense" direction. This was demonstrated by SPIEGELMAN et al. (1972) who isolated P_{re} mRNA and showed that it was complementary to P_R mRNA. Transcription from P_{re} is very active, may involve cyclic AMP and bacterial proteins in addition to *c*II and *c*III products (GRODZICKER et al., 1972) and is shut off about 15 minutes after infection by the action of *cro* product on the transcription of *c*II and *c*III genes. Thus *cro* product regulates indirectly the P_{re} promoter and is effective in doing so because the *c*II and *c*III proteins are unstable (REICHARDT, 1975a).

A different promoter, P_{rm}, is responsible for the transcription of the *c*I-*rex* operon in the lysogenic state, when no *c*II or *c*III products are available (REICHARDT and KAISER, 1971). This transcription occurs at a level almost 10 times lower than the initiating at P_{re} but is sufficient for maintaining the levels of repressor in a lysogen. The P_{rm} promoter is located to the left of the *cro* gene, since no *cro* antisense mRNA is produced in a lysogen (SPIEGELMAN et al., 1972) and since a prophage in which all the genes to the right of *cro*, including P_{re}, are deleted, makes normal levels of repressor (REICHARDT and KAISER, 1971; CASTELLAZZI et al., 1972). A mutation in this promoter maps between *c*I and O_R (YEN and GUSSIN, 1973) and renders the phage unable to synthesize repressor in a lysogen while still producing normal levels of repressor upon infection of a sensitive cell (GUSSIN et al., 1975).

The P_{rm} promoter is both negatively and positively controlled. It is negatively controlled by *cro* product, permitting the establisment of the nonimmune lysogenic state discussed above (EISEN et al., 1970; REICHARDT and KAISER, 1971). In addition, P_{rm} transcription appears to require the presence of repressor acting as an activator. Inactivation of heat-sensitive repressor in a lysogen causes a shutoff of the synthesis of repressor (HEINEMANN and SPIEGELMAN, 1970; KOURILSKY et al., 1970; REICHARDT and KAISER, 1971) and the immediate shutoff of *c*I transcription even if the phage carries a cro^- mutation. The positive control by repressor depends on its ability to bind to O_R (REICHARDT, 1975a; EISEN, 1975). HAYES (1972) and HAYES and SZYBALSKI (1973), however, do not observe the shutoff of *c*I transcription.

The repressor contained in a lysogenic cell is in excess of the amount required to maintain the prophage in the repressed state. At least ten superinfecting λ *c*I phages can be repressed without phage production. WIESMEYER (1966) showed that appreciable breakdown of immunity requires a multiplicity of infection of around 20 phages. Repressor levels were measured directly by CHADWICK (personal communication) who estimated 25 repressor oligomers/cell using the filter binding assay. REICHARDT and KAISER (1971), using a radioimmune assay, found around 200 repressor monomers/cell.

Many attempts have been made to isolate λ mutants which overproduce repressor in the lysogenic state. In the case of the *lac* repressor, such mutants which raise the frequency of transcription of the repressor gene by more than a factor of ten have been isolated using simple selection schemes (MÜLLER-HILL et al., 1968). In the case of λ, a variety of approaches have proved unsuccessful, raising the possibility that high levels of repressor might be harmful to *E. coli*.

III. The λ Repressor

A. Isolation of the Repressor

The genetic data indicated that the λ repressor is the product of the *c*I gene, a diffusible substance, subject to mutations of the missense, nonsense, or temperature-sensitive varieties, indicating that it is in part or entirely a protein. Its isolation, however, was made difficult by the low amounts in which it is present in a lysogenic cell and by the lack of a specific assay for its activity, at the time these experiments were undertaken. To overcome these difficulties PTASHNE (1967a) devised a technique of differential radiolabeling. His approach was to compare radioactively labeled proteins synthesized by cells infected with λ cI^+ with proteins synthesized by cells infected with λ bearing an amber mutation in the *c*I gene. Since the repressor constitutes a very small proportion (0.01 %) of the total protein, in order to detect it as a differentially labeled protein it was necessary to lower the background synthesis by two or

three orders of magnitude. PTASHNE accomplished this by irradiating the host cells with heavy doses of ultraviolet light, thereby damaging the bacterial DNA to the extent that it could not direct the synthesis of bacterial proteins. Furthermore he used as hosts bacteria lysogenic for λ, in order to inhibit the expression of most of the genes of the superinfecting phages. He then labeled the λ $c\mathrm{I}^+$ infected cells with ^{3}H-amino acids and the λ $c\mathrm{I}^-$-infected cells with ^{14}C-amino acids, mixed the two cultures and was able to detect a protein labeled with ^{3}H but not with ^{14}C upon chromatography of the mixed extract. This protein, constituting about 5 % of the incorporated radioactivity, was therefore presumed to be the λ repressor. Subsequent experiments showed that a variety of mutations which altered the behavior of the repressor in vivo also affected the chromatographic properties of this protein and confirmed its identification. SDS-acrylamide gel electrophoresis of the labeled repressor showed that it consisted of a single polypeptide of MW 28000. This polypeptide is the sole product of the *c*I gene, since a variety of amber mutations mapping at the extremities of the *c*I gene fail to produce it in the radiolabeling experiment.

The repressor isolated by this method binds tightly and specifically to DNA containing the λ operators (PTASHNE, 1967b). Labeled repressor cosediments with λ DNA (32 s) through a glycerol gradient, while unbound repressor sediments much more slowly (2.4 s). The repressor does not co-sediment with λ imm^{434} DNA even though this DNA differs from that of λ only in a region comprising 3 % of the total length, indicating that the binding is specific for the region containing the operators of λ.

The demonstration that repressor binds to DNA confirmed the simplest model of repressor action: repressor blocks transcription directly by binding to the operator region of DNA. In addition it made possible the development of a simple and rapid assay for detecting and quantitating repressor activity. This assay, developed by RIGGS and BOURGEOIS (1968) for the *lac* repressor, is based on the properties of nitrocellulose membrane filters. Most proteins, including the λ and *lac* repressors, bind to such filters, while DNA is not retained. Proteins which bind tightly to DNA can retain the DNA on the filter. Using radioactively labeled DNA it is possible to determine the DNA binding activity of proteins present in a bacterial extract.

This assay made possible the large-scale purification of repressor and the isolation of milligram amounts of more than 95 % pure material (PIRROTTA et al., 1971).

In the most commoly used method, sensitive cells are infected at a multiplicity of 5–15 with phage bearing a mutation in the *S* gene to prevent cell lysis. After 1–2 hours growth, the cells are harvested, opened by freezing and thawing and mild homogenization, and the extracts are fractionated by ammonium sulfate precipitation, DEAE cellulose, and phosphocellulose chromatography. An alternative purification step utilizes the low but detectable affinity of repressor for nonspecific DNA to fractionate the partially purified material on a DNA-cellulose column (PIRROTTA, unpublished).

B. The Repressor Protein

The molecular weight of the repressor polypeptide is slightly over 28000 as measured by SDS-acrylamide gel electrophoresis. Unlike the *lac* repressor which exists as a stable tetramer, the λ repressor oligomeric state depends on its concentration. The sedimentation value of repressor increases progressively with its concentration from 2.4 s for extremely dilute repressor (10^{-10} M) to 6.2 s at a concentration of 10^{-5} M (CHADWICK et al., 1970). In all cases, velocity sedimentation produces a single monodisperse peak. Dilute, radioactive, 2.4 s repressor is converted to a faster sedimenting species when mixed with concentrated unlabeled repressor, indicating a concentration-dependent association (PIRROTTA et al., 1970). Evidence that the repressor exists as an oligomer in vivo comes from genetic experiments by LIEB (1966) who showed the existence of intracistronic complementation between *c*I ts mutants. More recently OPPENHEIM and SALOMON (1972) have found negatively complementing *c*I mutants, which are able to render wild type repressor inactive. While it is apparent from the sedimentation results that the repressor subunits associate, it is not clear by what mechanism or to what oligomeric form. The fact that distinct intermediate species cannot be separated by sedimentation indicates that the association equilibrium is rapid and reversible. Some evidence suggests that the repressor oligomer, like many other oligomeric structures, tends to dissociate at the high pressures which develop during high-speed centrifugation and give an underestimate of the actual molecular weight species present (PIRROTTA, unpublished). In vivo it has been observed that lysogens subjected to high pressures become induced with kinetics consistent with direct repressor inactivation (L. RUTBERG, personal communication). Equilibrium sedimentation experiments show the presence of several interacting species ranging in molecular weight from the monomer to something even higher than a tetramer of 4×28000 daltons. The distribution of molecular sizes depends on the initial concentration and is displaced towards lower oligomeric species at lower pH values.

Electron microscopic studies of the repressor show a particle of characteristic structure (Fig. 2) which was interpreted as a tetrameric complex (BRACK and PIRROTTA, 1975). The dimensions of this particle, about 90×110 Å, appear to change somewhat with preparation conditions, probably reflecting different degrees of flattening. The particle appears to consist of two elongated subunits side by side, separated by a deep groove, and two apparently smaller subunits located at the apices. Based on the examination of a large number of particles in a variety of different orientations, BRACK and PIRROTTA proposed a tentative model for the structure of the tetramer. According to the model (Fig. 3) the tetramer is composed of two dimers in which the subunits are arranged in the shape of a V. Two dimers assemble to form a tetramer by placing one subunit from each dimer side by side, with the remaining subunits projecting out of the plane of the first two.

Information about the protein chemistry of the λ repressor is still scarce, partly due to the difficulty of isolating sufficiently large quantities of material.

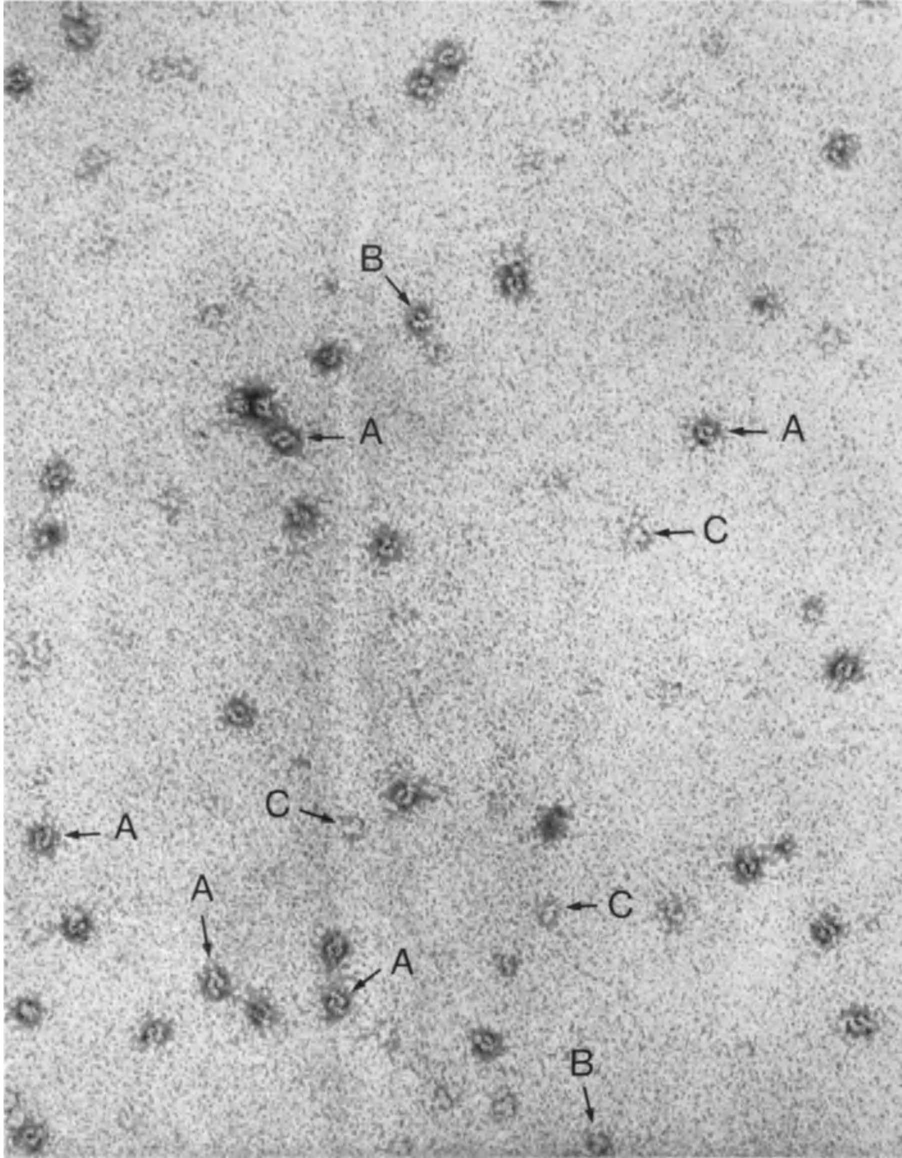


Fig. 2. λ repressor at a concentration of 1 μg/ml, negatively stained with 0.5% uranyl formate. Most molecules show characteristic tetrameric elongated structure (→*A*), sometimes only three subunits are visible (>*B*). Dimers are probably also present (→*C*) but difficult to distinguish from irregularly stained background. ×270000. (From Brack and Pirrotta, 1975)

The repressor is a slightly acid protein, with isoelectric point near 6.2 (Pirrotta and Ptashne, 1969). In spite of this, the λ repressor, like many other proteins which interact with nucleic acids, binds strongly to phosphocellulose, indicating that it contains readily accessible regions of positive charge even at neutral pH.

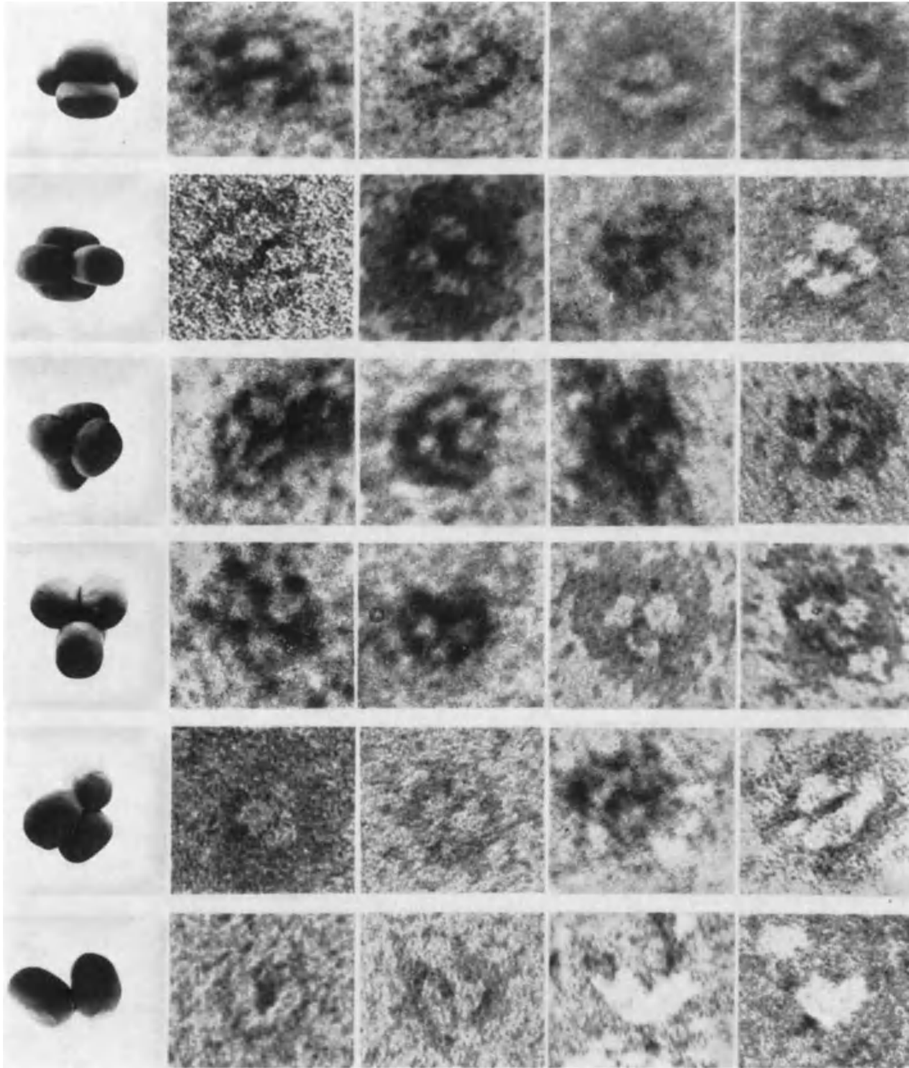


Fig. 3. Selection of micrographs of repressor molecules stained with uranyl formate. Different orientations of tetramer are compared with a plasticine model photographed at various corresponding angles. × approximately 900000. (From BRACK and PIRROTTA, 1975)

IV. Repressor Effect on in vitro Transcription

In vitro, λ DNA can be transcribed by RNA polymerase to produce a variety of RNA species. Only two of these initiate at P_L and P_R. To detect the effect of repressor on P_L and P_R transcription, WU et al. (1972) subjected the total RNA synthesized to a hybridization with DNA extracted from a $\lambda - \phi 80$ hybrid phage in which most of the genes come from $\phi 80$ except for the DNA coming from the λ immunity region and the genes adjacent to it.

When transcription occurred in the presence of increasing amounts of λ repressor, they were able to show increasing repression of RNA synthesis transcribed from the vicinity of the λ immunity region, while RNA originating from the immunity region of λimm^{434} was not affected. Repression could be demonstrated more effectively if rifampicin was added to the DNA-RNA polymerase complex, to decrease the backgrond due to "improper" initiations.

A more specific identification of the mRNA initiated at P_L and P_R was provided by ROBERTS (1969). He found that using (1) λb_2 DNA and (2) termination factor ϱ, the P_L and P_R mRNA's constitute a major fraction of the total RNA synthesized and can be identified respectively as a 12 s and a 7 s component upon gradient sedimentation. Using this system, CHADWICK et al. (1970) and STEINBERG and PTASHNE (1971) could demonstrate a specific effect of repressor on the transcription of these two RNA species. When the repressor is present in a 10–15-fold excess over the operator, both RNA species are completely repressed. At lower concentrations the 12 s species is more easily repressed than the 7 s, suggesting a slight difference in the repressor affinity for the O_L and O_R operators. Mutations in the O_L or O_R operators reduce the effect of repressor and allow transcription of the corresponding operon. However, repression could still be demonstrated by increasing sufficiently the concentration of repressor. The λ repressor had no effect on the transcription of the corresponding RNA species from phage λimm^{434}.

How does the repressor block transcription? It is clear from these experiments performed with highly purified components, that the repressor protein suffices to effect transcriptional repression, without the participation of additional cofactors. The simplest model for repressor action involves inhibition of RNA polymerase activity by simple steric hindrance by repressor bound at the operator. The repressor might thus prevent the polymerase from progressing through the operator to transcribe the genes lying beyond or it might interfere with polymerase binding at the promoter site. The repressor does not affect the elongation of already initiated RNA chains (STEINBERG and PTASHNE, 1971). Nor does it alter the ability of DNA to act as template for transcription. In support of the idea that repressor binding to operator is incompatible with polymerase binding to the promoter, CHADWICK et al. (1970) found that polymerase bound to λ DNA in large excess inhibits the binding of radioactively labeled repressor to the operator. As a control, they showed that this inhibition is partially relieved if the λ DNA carries a mutation in the P_L promoter which decreases the affinity of RNA polymerase for that site.

In agreement with this view, HAYWARD and GREEN (1969) and WU et al. (1972) fond that λ repressor inhibits the binding of RNA polymerase to λ DNA. But while STEINBERG and PTASHNE (1971) observe no repression if the repressor is added after the polymerase is allowd to react with the DNA, WU et al. (1972) find at least partial inhibition even when the polymerase-DNA complex is "preinitiated" by the addition of ATP and GTP. This disagreement might be accounted for in part by multiple binding of RNA polymerase to the promoter. WILLMUND and KNESER (1973) report that in the presence of excess

polymerase, some 3–5 polymerase molecules can bind to each λ promoter in a heparin-resistant form. We might suppose that one of these can no longer be inhibited by the repressor, while the others can be blocked by the repressor from proceeding to the transcription initiation site. In contrast, in the wild type *lac* promoter, repressor does not inhibit polymerase binding. UV-5, a mutation in the promoter, which increases the transcription rate and renders it independent of cyclic AMP activation, alters the site in such a way that repressor and polymerase bind competitively (CHEN et al., 1971). However, J. MAJORS (cited in GILBERT et al., 1975), using a fragment of the *lac* operon produced by restriction endonucleases, found that in the wild type *lac* promoter as well as in the UV-5 mutant, repressor binding and formation of the RNA polymerase initiation complex are mutually exclusive events.

The differences between *lac* and λ, if they exist, probably reflect the differences in the topology of the promoter and operator sites evidenced by sequence studies (see Section VI c).

V. Repressor Interaction with DNA

A. The Active Form

As PTASHNE (1967b) first showed using the cosedimentation method, the λ repressor binds very tightly and specifically to λ DNA. This specific binding requires double stranded DNA containing at least one λ operator. No magnesium or other cofactors are necessary. The double-stranded requirement may indicate that the repressor recognizes features in both strands, or that it requires the three-dimensional structure assumed by double helical DNA.

The quantitative aspects of the repressor-operator interaction have been studied using the filter binding method. Equilibrium binding curves show the fraction of ^{32}P-λDNA bound by increasing amounts of repressor increases until it reaches a plateau when nearly all the DNA is retained on the filter. When the concentration of DNA is low, such curves have a sigmoid shape indicating that the repressor does not bind efficiently at low concentrations (PIRROTTA et al., 1970). Furthermore, when repressor is added to a reaction mixture containing λ DNA, the formation of the filter-binding complex is extremely rapid. but if repressor is diluted into a reaction mixture and the λ DNA is added shortly after, only a very small fraction of the binding activity is detected. If the binding mixture is incubated for longer periods of time, the full binding activity is gradually recovered. The conclusion suggested by these experiments was that repressor, which at high concentrations exists as an oligomer, dissociates upon dilution to a form which is unable to bind to DNA except by slow reassociation reconstituting the active form. If λ DNA is present in the dilution volume, the binding reaction proceeds much faster than the dissociation. On the basis of the kinetics of DNA binding by diluted repressor, PIRROTTA et al. (1970) argued that the active form of the repressor is the dimer. CHADWICK et al. (1970) analyzed equilibrium binding curves to come to the

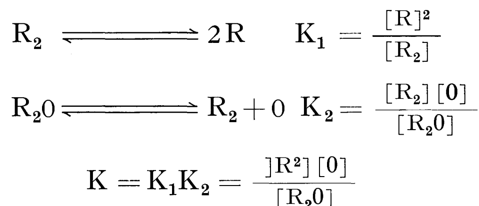
Table 1. Constants regulating repressor-operator interaction. $\tau^1/_2$ is dissociation half-life of repressor-operator complex. (Compiled from PTASHNE, M., 1971, and unpublised data of P. CHADWICK)

K_1	7×10^{-9} mole liter^{-1}
K_2	2×10^{22} mole^2 liter^{-2}
K	3×10^{-14} mole liter^{-1}
$k_{forward}$	3×10^{10} mole^{-1} liter sec^{-1}
$\tau^1/_2$	
at 0°	175 min
at 20°	7 min

same conclusion. In both cases the analysis shows a dependence on the square of repressor concentration. These conclusions, however, could equally well apply to a dimer-tetramer equilibrium.

B. The Binding Reaction

The filter binding technique has been successfully employed by RIGGS and BOURGEOIS (1968) to study the parameters of the *lac* repressor-operator interaction. The same methods were used by CHADWICK et al. (1970) to study the binding of λ repressor to DNA. Assuming that the active binding species is a dimer, they formulated the reaction at the low concentrations used to study the binding equilibrium as follows:



$$R_2 \rightleftharpoons 2R \qquad K_1 = \frac{[R]^2}{[R_2]}$$

$$R_2 0 \rightleftharpoons R_2 + 0 \quad K_2 = \frac{[R_2][0]}{[R_2 0]}$$

$$K = K_1 K_2 = \frac{]R^2][0]}{[R_2 0]}$$

where R is the repressor and O is the operator. The values obtained for these constants are summarized in Table 1.

The repressor clearly binds extremely tightly to the operator. The binding is sensitive to ionic strength. CHADWICK et al. calculate that K increases by a factor of 10 in going from 0.05 M KCl to 0.15 M KCl. WU et al. (1972) find that incubation of the repressor-operator complex in 0.2 M KCl suffices to dissociate 95 % of the complexes. Increasing temperature and pH also weaken the binding. K increases by a factor of 5 with each 10° increase in temperature from 0–30° C and by a factor of 10 for each 0.5 pH units between 6.5 and 8.5. The temperature effect occurs at the level of interaction with the operator rather than on the monomer-oligomer equilibrium, since it is due to an increase in the rate of dissociation of the repressor-operator complex. At 0° C and 0.05 M KCl the half-life of the complex is 175 minutes, while at 20° C it becomes

7 minutes, decreasing by a factor of 5 for every 10° C in crease in temperature. In contrast the half-life of the lac repressor operator complex is about 20 minutes under similar conditions and does not change appreciably between 0° and 30° C.

The forward rate constant, calculated from K_2 and the dissociation rate, or by direct measurement at very low DNA concentrations, is 3×10^{10} M^{-1} sec^{-1} (CHADWICK, unpublished). This value, even higher than that of the *lac* repressor (7×10^9 M^{-1} sec^{-1}), is much too fast even for a diffusion-controlled interaction between the repressor and a target the size of the operator (30–100 base pairs) which is furthermore fixed to a large DNA molecule.

To explain these surprisingly high rate constants, several models have been proposed which would increase the size of the target with which the repressor needs to interact. A particularly attractive model is that based on unidimensional diffusion. According to this model, the repressor after colliding with a DNA molecule, is retained in its vicinity by some nonspecific interaction with the exterior of the double helix and proceeds to scan the molecule by random unidimensional diffusion until it finds the operator site. RICHTER and EIGEN (1975) calculated that this model could account for the kinetic results obtained with the *lac* repressor if the effective target size is assumed to be about 10 times larger than the operator, or of the order of 300 base pairs (1000 Å). Repressor interacting with a DNA region within 300 base pairs of the operator will be able to find the operator before falling off the DNA. This model predicts that above 300 base pairs, the size of the DNA should not affect the rate constant for binding. BOURGEOIS and RIGGS (1970) found that DNA fragments containing the *lac* operator, sonicated to a size of 1500 base pairs, compete for binding to *lac* repressor as effectively as intact λ-φ80 *dlac* DNA (50000 base pairs). This indicates that the equilibrium constant was not affected. However, kinetic measurements were not made.

An alternative version of the model has been proposed by VON HIPPEL et al. (1975). They calculate that at the low concentration of DNA used in the filter-binding experiments, the DNA molecules in solution will separate into "domains" with a characteristic radius equal to the radius of gyration. For a λ DNA molecule under these conditions this radius is about 5000 Å or 10 times the effective target radius required to account for the rate constant of the binding reaction. The DNA concentration within such a domain will be very high, such that the nonspecific affinity of repressor for DNA will cause a repressor molecule entering such a domain to be trapped within it with a high probability. The experimental results can then be accounted for by assuming a 0.1 probability that once inside such a domain the repressor will find the operator by internal transfers before being lost into the solution or exchanged by collision between two domains. VON HIPPEL et al. proposed that the search for the operator proceeds by a direct transfer from one DNA site to another, which would require the repressor molecule to possess two DNA binding sites simultaneously available. The *lac* repressor does in fact have considerable affinity for nonoperator DNA. Competition assays show that this binding is

several orders of magnitude weaker than the specific binding to the operator (LIN and RIGGS, 1972) but, given the large amounts of nonoperator DNA present in a bacterial cell, it plays an important part in governing the supply of free repressor (VON HIPPEL et al., 1974). The fact that the non-specific binding is not released by *lac* inducers also suggest that it might involve a different site in the repressor molecule than that which interacts with the operator.

Like the *lac* repressor, the λ repressor has a low but detectable affinity for nonoperator DNA. Under normal assay conditions the affinity of λ repressor for λ *imm*[434] DNA is about 50 times lower than for λ DNA, indicating an equilibrium constant for nonspecific binding several orders of magnitude lower. That this affinity is nonetheless significant is shown by the fact that nonoperator DNA can prevent the dissociation of the repressor to the inactive form upon dilution in the absence of λ DNA (PIRROTTA, unpublished). This observation supports the model of VON HIPPEL et al. which requires that repressor be found preferentially associated with a DNA "domain," escaping from one only to be captured by another with a rate constant limited only by diffusion.

The nonspecific affinity for DNA increases with decreasing pH, probably as a consequence of increased attraction for the phosphate backbone. At pH values below its isoelectric point, the repressor shows appreciable affinity for any polynucleotide, including RNA and oligonucleotides (PIRROTTA, unpublished). Some evidence suggests that this interaction occurs at a different site on the repressor than the interaction with operator DNA, since RNA in large excess does not compete appreciably with binding to λ DNA.

C. Specificity Considerations

The finding that the operator is DNA raised the question of how the repressor protein might distinguish the operator sequence among thousands of others present in the cell. In an *E. coli* cell in order for at least 50% of the repressor to be available for binding to the operator, the binding constant to the correct sequence must be at least 10^5 times greater than that to any other random sequence in the cell. GIERER (1966) has proposed that at least part of the specificity of the interaction might be due to the formation of intrastrand self-complementary loops. Such loops would confer to the operator region a characteristic cruciform structure and furthermore open a number of hydrogen bonds and make them available for repressor interaction. However attractive this suggestion and all others based on extensive strand separation, they appear now definitely laid to rest, at least in the case of the λ and *lac* repressors. Any strand separation must necessarily involve unwinding of the helix. Such unwinding would cause large effects in the binding affinity of repressor for closed circular, superhelical DNA (DAVIDSON, 1972). In fact no such effects are observed. In the case of λ, superhelical and nicked circular DNA bind repressor equally well (MANIATIS and PTASHNE, 1973a). In the *lac* case, a small unwinding effect of as much as a quarter of a turn has been detected (WANG

et al., 1974). Therefore, while some distortions of the helix might result from repressor binding, it is clear that the operator remains double-stranded.

A certain amount of specificity can be imagined to derive from local variations in the double helix structure, themselves determinded by the sequence. BRAM (1970) has shown that AT-rich DNA double helices in solution deviate from the classical B structure in a way consistent with a 10% increase in pitch. Other local differences in the tilt of the bases, the interphosphate distances, etc., might be exploited by the repressor protein to increase discrimination. However, these effects are at most auxiliary. We know that single base pair changes can effect a 20-fold change in the affinity of the operator for repressor. Clearly the repressor must interact with the bases and to account for its specificity, the interaction must be based at least in part on stereospecific hydrogen bonding.

This might be accomplished by separating the strands as proposed by CRICK (1971) or by probing into the double helix. The former is ruled out in the case of the λ and lac repressors by the fact that no unwinding occurs. For the lac repressor, ADLER et al. (1972) have proposed that a protrusion formed by some 50 amino acids residues in the amino terminal of the protein could penetrate into a groove of the double helix and interact with the bases. In both the large and small groove there are sufficient functional groups to enable a protein of the appropriate charge and configuration to discriminate between all four possible base pairs. In addition, the subunits of the repressor might form a fold, such as can be seen in the electron micrographs of the lac and λ repressors, into which the DNA might fit. This might stabilize the DNA in a position which facilitates the interaction of the probing elements of the repressor.

The functional groups in the repressor which interact with the base sequence need to all lie in a single probelike arm. It would be difficult for such an arm to follow the helical structure of the grooves for a distance long enough to recognize a sufficient number of bases. Instead, several small probes might interact with bases which need not all be adjacent in the DNA sequence.

Recently, CRICK and KLUG (1975) have proposed a model for discontinuous bending of a DNA double helix. This model, originally devised to account for the compact folding of DNA in structures such as chromatin, has some features which would make it attractive to explain repressor-operator recognition. The authors found that by folding the double helix towards the side of the minor groove, it is possible to introduce a sharp kink of about 90° in the axis of the helix, without breaking base pairs or assuming stereochemically unacceptable bond angles. CRICK and KLUG point out that this fold exposes the major groove for a distance of a few base pairs on either side of the kink and would make the stereospecific hydrogen bonding determinants present in that groove more available for interaction with repressor. The kink might occur spontaneously or be facilitated by certain base sequences or it might be induced by the interaction of the operator with the repressor. Since each kink would unwind the double helix by 15–25°, up to four kinks could be accommodated in the *lac* operator but presumably fewer in the λ operator.

An *E. coli* cell contains approximately 3×10^6 base pairs. If these nucleotides were arranged in a random sequence, the minimum length required for a unique sequence would be 11–12 base pairs (Gilbert and Müller-Hill, 1967). According to this argument then, the specificity or binding requires that the repressor recognize a minimum of 11–12 base pairs or 6 base pairs per monomer if two subunits are involved in the binding. The oligomeric structure of the repressor would therefore decrease the number of bases which must be recognized by a single repressor subunit, and decrease the constraints placed on the geometry of the protein.

VI. Operators and Promoters

A. Isolation of the Operator

The tight complex formed by repressors with the operators has made possible the isolation of the operator region by digesting the complex with DNase (Gilbert, 1972; Pirrotta, 1973). The repressor then protects the DNA region to which it is bound against degradation. Maniatis and Ptashne (1973a) found that the protected region increases in discrete steps as the amount of repressor present during the digestion in increased. At low repressor to operator ratios (R/0) the DNA fragment protected is 30–35 base pairs in length. As R/0 increases, the fragment size increases by steps of approximately 15 nucleotides to a maximum of about 105 nucleotides. In comparison, the *lac* repressor, (a tetramer of 150000 daltons) protects a fragment about 27 base pairs long (Gilbert, 1972).

On the basis of the different sizes of DNA protected, Maniatis and Ptashne concluded that the λ operators are multiple structures, containing up to six repressor binding sites each. Of these sites, labeled S_1–S_6, S_1 is the one with greatest affinity for repressor and requires binding by a repressor oligomer in order to be isolated as a DNase-resistant complex. The remaining sites may be occupied by successive binding of lower oligomeric forms of repressor.

Electron microscopic studies have made it possible to visualize such multiple binding complexes. Brack and Pirrotta (1975) observed an oligomer of tetrameric structure binding to DNA. The tetramer has dimensions in the vicinity of 100 Å which agrees well with a protected fragment of about 30 base pairs. Increasing amounts of repressor result in the appearance of double, triple, and quadruple binding (Fig. 4). The additional repressors also appear to be tetramers and to be bound in a row with no gaps between them. Dimer binding, which would be responsible for half-step increases in the size of the protected fragment, was not observed, possibly because the concentrations of repressor and operator in this experiment were much higher than those used by Maniatis and Ptashne.

The analysis of the operator structure was enormously helped by the finding that both O_L and O_R are cleaved by the restriction endonuclease *Hin*d II, (Maniatis and Ptashne, 1973b). In either case, the endo R *Hin*d II cut separates the primary binding site S_1 from the remaining sites, yielding two

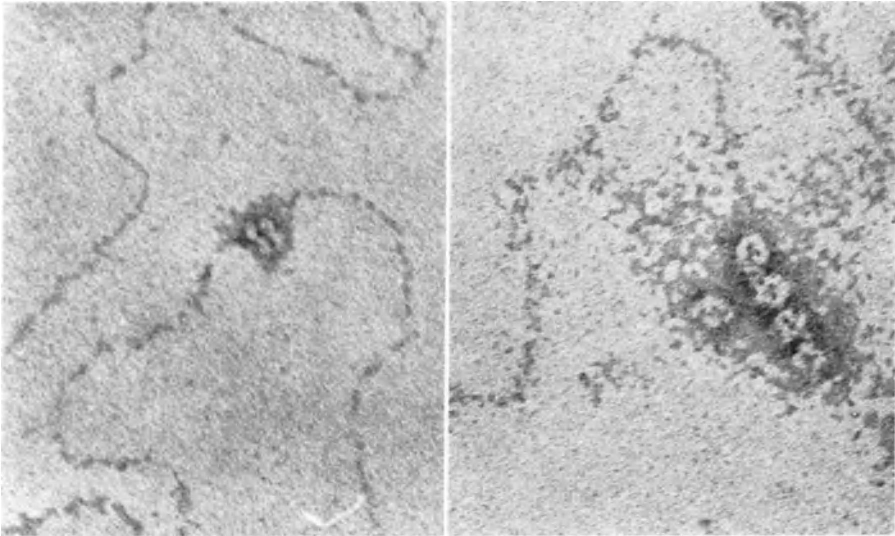


Fig. 4. Repressor binding to DNA fragments containing operator. On the left, a single tetramer. On the right, four tetramers bound in a row. × 450000. (From BRACK and PIRROTTA, 1975)

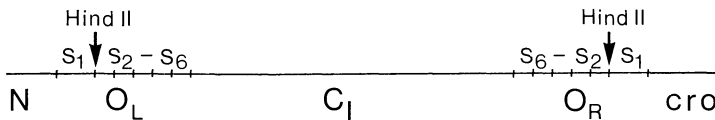


Fig. 5. Multiple operator binding sites, according to MANIATIS and PTASHNE (1973b). Arrows indicate the endoR Hind II cleavage sites inside the operators

fragments from each operator, which have residual affinity for the repressor. In O_L, the S_1 fragment retains 0.66 of the affinity of the intact operator, while the S_2–S_6 fragment retains only 0.15. In O_R the residual affinities of the primary and secondary sites are 0.3 and 0.03 respectively (S. FLASHMAN and M. PTASHNE, personal communication). These values suggest either that some minor determinant of S_1 is cut off by the *Hind* II enzyme, or that binding of the repressor at one end of a DNA fragment is less stable than binding to a site far from the DNA ends. The latter is indicated also by the finding that the half-life of the complex formed by repressor with the protected operator fragment is several times shorter than with the entire lambda DNA. Using a combination of *Hind* II and *Hpa* II restriction endonucleases, MANIATIS and PTASHNE were able to show that in both operators, the S_1 site is the one closest to the genes of the operon under control (Fig. 5).

As expected, S_1 is also the site of most operator constitutive mutations. In O_L all but one of the mutations studied so far are located in S_1. In O_R, however, some important operator mutations seem to be located in the S_2–S_6 fragment and in one case, as yet unexplained, a single mutation affects both S_1 and S_2–S_6, (S. FLASHMAN and M. PTASHNE, personal communication).

The function of the multiple repressor binding site is not clear. In O_L, since mutations in S_1 suffice to confer constitutivity, the remaining sites cannot have more than a secondary role in strengthening repression. In O_R, the role of the secondary sites may be more important. Since $O_R S_1$, has a lower affinity for repressor than $O_L S_1$, the additional binding sites might be required to ensure complete repression. Of the classical virulent mutations, v_3 is in $O_R S_1$, while v_1 is in the S_2–S_6 region (S. FLASHMAN and M. PTASHNE, personal communication). The deletion *spi-274*, which leaves $O_R S_1$–S_2 intact but removes part of S_3 and all of S_4–S_6, results in partial virulence, while deletion *spi-113*, which retains an intact S_3 but not S_4–S_6, allows lysogenization (G. SMITH et al., 1975). Furthermore, as discussed in section VI d, the remaining sites might be involved in the regulation of *c*I transcription by the repressor itself or by the *cro* gene product.

B. Sequence of the Operators

The two λ operators have been partially sequenced using a variety of different methods. From O_L, the 33 nucleotide sequence of the primary binding site was obtained by elongating with DNA polymerase the *Hin*d II fragment reannealed to an intact strand of λ DNA (MANIATIS et al., 1974). This sequence was recently extended by 28 nucleotides (MANIATIS et al., 1975 b) and 14 additional nucleotides (PIRROTTA, unpublished). From O_R, a sequence of 77 base pairs including the primary binding site was obtained by primed transcription of the isolated operator fragments (PIRROTTA, 1975). The primed transcription technique exploits the fact that at low nucleotide triphosphate concentrations, the *E. coli* RNA polymerase is unable to initiate transcription but, given a suitable primer, it can elongate it. The enzyme can therefore be forced to initiate at a unique site (DOWNEY and SO, 1970; GILBERT and MAXAM, 1973). The same region of O_R was also sequenced independently by MANIATIS et al. (1975 a), using a combination of restriction endonucleases to generate small DNA fragments which were then directly sequenced by kinase labeling of the 5' ends and partial degradation with venom phosphodiesterase. The sequences obtained represent at least three-fourths of the entire O_L and O_R operators and therefore contain at least three of the expected four binding sites in each operator.

As expected from the multiple operator model, the O_L and O_R sequences contain near identical repeats corresponding to the repressor binding sites. In each operator sequence MANIATIS et al. (1975 b) distinguished three blocks of 17 base pairs each, which they believe to be the sites recognized by the repressor (Fig. 6). The sites with the greatest similarity are of course the two primary binding sites which differ by only one out of 17 base pairs. This difference may account for the 3–5-fold difference in their affinity for repressor. The remaining sites contain substitutions in 3–7 positions. Each site contains an axis of partial 2-fold rotational symmetry passing through the ninth

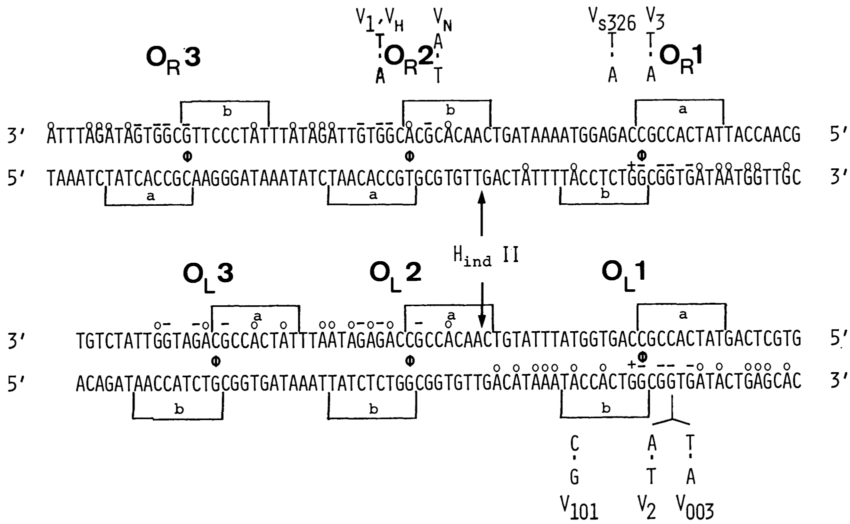


Fig. 6. Sequences of O_R and O_R operators. Brackets indicate blocks of 17 base pairs thought to be repressor binding sites. In each site, an axis of 2-fold symmetry passes through the ninth base pair and divides it into half-sites a and b (see Fig. 7). The location of some operator constitutive mutations is indicated. Effect of repressor binding on methylation of purines is shown by: —, methylation blocked by repressor; +, methylation enhanced by repressor; ○, methylation unaffected by repressor. Effect on unmarked purines has not yet been determined. Data compiled from PIRROTTA (1975), MANIATIS et al. (1975a, b), and KLEID and PTASHNE (personal communication)

base pair and is separated from the neighboring site by 3–7 base pairs of very high AT content. In the O_L^1 sequence, MANIATIS et al. (1974) had earlier pointed out the presence of two additional symmetry axes involving as many as 16 out of 25 base pairs. These additional symmetries are not always present in the other repressor binding sites and their significance remains obscure. They might be involved in the recognition of the *cro* gene product or of the RNA polymerase which are known to interact with the operator region.

A high degree of symmetry was also observed by GILBERT and MAXAM (1973) in the sequence of the *lac* operator and is found on a much reduced scale in the recognition sites of a variety of restriction endonucleases. While this kind of symmetry would permit the formation of self-complementary loops as proposed by GIERER (1966), the experiments with superhelical DNA discussed in section Vc make it unlikely that such structures are involved in repressor binding. It seems more likely instead that the symmetry of the recognition site simply reflects the symmetry of the protein interacting with it. Both the *lac* and the λ repressors are oligomers of identical subunits and may be reasonably expected to possess an axis of 2-fold symmetry. Each half of the oligomer would then be able to interact with an identical sequence in a symmetrical operator. By binding in the form of an oligomer, the repressor multiplies the affinity and the specificity of binding at a minimum cost in terms of information. Each subunit needs to recognize a sequence shorter than the total

	HALF-SITE A 1 2 3 4 5 6 7 8 9	HALF-SITE B 1 2 3 4 5 6 7 8 9
O_L1	T A T C A C C G C	T A C C A C T G G
O_R1	T A T C A C C G C	T A C C T C T G G
O_L2	C A A C A C C G C	T A T C T C T G G
O_R2	T A A C A C C G T	C A A C A C G C A
O_L3	T A T C A C C G C	A A C C A T C T G
O_R3	T A T C A C C G C	T A T C C C T T G
	$T_5 A_6 T_4 C_6 A_6 C_6 C_6 G_6 C_5$	$T_4 A_6 C_3 C_6 A_3 C_5 T_4 G_3 G_5$
	C_1 (1), A_2 (3), T_1 (9)	C_1 (1), T_2 (3), $T_2 T_1 G_1 T_2 A_1$ (5–9)
		A_1 (1), A_1 (3), C_1 (5), $C_1 C_1$ (7–8)

Fig. 7. Comparison of repressor binding sites. Sequences of repressor binding sites, marked by brackets in Fig. 6, are compared by reorienting them to emphasise homologies. The sequences, divided into half-sites a and b, are written in the 5′–3′ direction and base frequency at each position is shown below

operator, with no loss of specificity, while the affinity of the oligomer for the operator will be approximately the product of that of each interacting subunit.

As the 2-fold rotational symmetry implies, each recognition site can be divided into two half-sites which bear a strong resemblance to one another (Fig. 7). However, a closer examination of the six recognition sequences (or twelve half-sites) brings out certain consistent differences between the two halves of each site. In all cases, one-half of the recognition sequence, which I will call half-site a, is very strongly preserved. Its sequence, found in four of six sites is TATCACCGC. Half-site b is considerably different in sequence and more variable from site to site. Its prototype can be taken to be TACCACTGG, found in the O_L1 site. If the repressor recognized a perfectly symmetrical sequence, the two halves would be identical (except for the central nucleotide) and the perfect operator would be presumably TATCACCG$^{G}_{C}$CGGTGATA. The consistent asymmetry in the recognition sequence raises the possibility that the repressor does not interact with the DNA in a perfectly symmetric fashion. A similar conclusion was arrived at by GILBERT et al. (1975) for the *lac* repressor-operator interaction. They found that the effect of mutations in the operator did not display the symmetry expected if both halves of the sequence interacted equally with the repressor. Furthermore, some operator constitutive mutations affected nucleotides not involved in the symmetry. Two possible explanations were suggested: (1) the repressor does interact symmetrically, but the operator contains a "deliberate asymmetry" which causes half of the site to interact less well with the repressor. This might be required for physiologic reasons to permit a basal level of expression of the *lac* operon or to allow effective derepression in the presence of the inducer. (2) The repressor does

not interact perfectly symmetrically with the operator. Good interaction with one-half of the operator might induce alterations in the structure of the DNA or of the protein which result in poorer or different contacts with the other half.

Either of these explanations could be applied to the λ case. However, the consistent deviation of one-half of the site makes the second alternative more convincing.

Which bases are important for the recognition process? It can be argued that bases which are very strongly conserved are likely to be more important for recognition. On this basis the CACCG sequence of the "good" half-site can be supposed to be important. The A at position 2 and the C's at positions 4 and 6, which are strongly conserved in both half-sites, should also be good candidates. More direct information on the involvement of specific bases in the recognition process comes from two other sources: sequence analysis of operator constitutive mutations and the effect of repressor binding on the sensitivity to methylation of purines in the operator sequence.

Eight operator mutations have been sequenced so far, affecting six sites, two in O_R^1, two in O_R^2, and two in O_L^1 (MANIATIS et al., 1975b, and personal communication). Four of these sites involve the CG pair at position 6 in either recognition half-site, the remaining ones are in positions 2 and 8, in half-sites b and a, respectively.

The methylation data were obtained using dimethyl sulfate, a reagent which methylates adenine at position 3, which faces the major groove of the double helix, and guanine at position 7, facing the minor groove. The results available so far (D. KLEID and M. PTASHNE, personal communication) show that methylation of the adenines is not affected by repressor binding, while methylation of guanine residues located in the recognition sequence is generally depressed except for the G at position 8 in which case it is enhanced (Fig. 6). Guanines outside the recognition sequences are not affected. These results suggest that the repressor occupies the minor groove and/or that guanine residues enter in close contact with the repressor. The latter possibility is supported by the fact that all but one of the operator mutations identified so far are in fact conversions of GC pairs to AT pairs. Repressor binding depresses methylation of the G at position 8 of half-site a while it apparently enhances methylation of the G at position 8 of half-site b confirming the idea that the repressor does not interact symmetrically with the two halves of the operator.

Whether or not it is involved in direct contact with the repressor, any one nucleotide might be important in contributing to the properties of the operator. For example, the high AT content of the intersite regions and the high GC content of the central part of the recognition sequence might affect the local structure of the double helix in a way recognized by the repressor.

Another approach to the study of repressor operator interaction is opened by recent advances which make possible the synthesis of oligonucleotides containing any desired variation of the recognition sequence. With the operator sequences currently being synthesized in several laboratories it should soon be possible to answer many of the questions raised above.

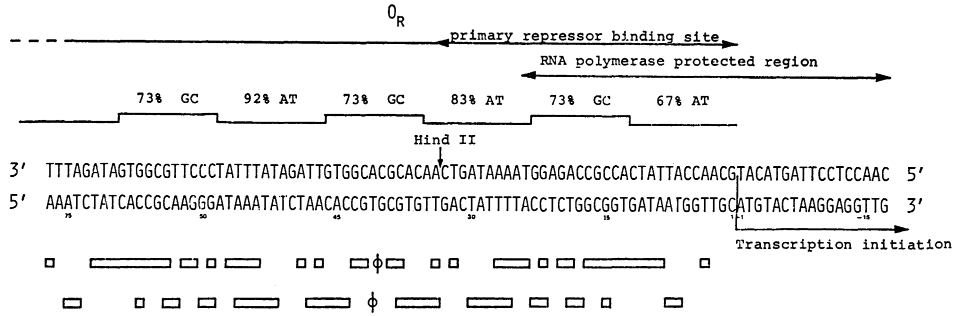


Fig. 8. Promoter-operator sequence from O_R region. Regions of high GC and high AT content are marked above the sequence. Bars below the sequence indicate complementary nucleotides disposed symmetrically with respect to two possible axes of symmetry at or adjacent to nucleotide 41. (From WALZ and PIRROTTA, 1975)

C. Promoter

Several lines of evidence have suggested that the lambda operator and promoter might overlap. The fact that the binding of RNA polymerase and repressor to the operator-promoter region appears to be mutually exclusive indicates at least very close proximity. ORDAL and KAISER (1973) and ORDAL (1971) found that genetic mapping places certain promoter mutations in P_R, between operator mutations in O_R. ALLET et al. (1974) and MAURER et al. (1974) have implicated the endoR Hind II cleavage site within the operators, in the recognition of the promoter by RNA polymerase. Polymerase bound to λ DNA, specifically protects these sites against cleavage by the Hind II enzyme without affecting the action of the restriction enzyme on the remaining 32 sites. This effect could be due to the fact that the promoter site includes the Hind II site, or to the accumulation of several polymerase molecules in the vicinity of the promoter. To demonstrate this effect a large excess (30-fold) of polymerases over DNA molecules is required. However, ALLET et al. (1974) and MAURER et al. (1974) found in addition that certain promoter mutations in P_L or in P_R, which are known to decrease the affinity of polymerase for these sites, also abolish the Hind II cleavage site in the corresponding operator. Revertants which have recovered promoter activity also recover the restriction site. Clearly the two sites must have elements in common.

WALZ and PIRROTTA (1975) have isolated and sequenced the DNA fragment originating from the O_R region, which the RNA polymerase can protect against nuclease digestion. This fragment is 40–43 base pairs in length, is double-stranded, and contains the transcription initiation sequence for the P_R mRNA. Its sequence (Fig. 8) shows that it contains 24 nucleotides of the primary binding site of O_R, plus 17 nucleotides which lie outside the operator and are transcribed both in vivo and in vitro (BLATTNER and DAHLBERG, 1972). It is clear that binding of polymerase to this site is incompatible with repressor binding to S_1. It is also clear why the polymerase does not bind to the isolated operator fragment nor repressor to the polymerase protected fragment, since neither

contains a complete site for the other. It is more difficult to explain how promoter mutations could be located in the *Hind* II recognition sequence which lies several nucleotides outside the polymerase binding site. One explanation might be that the polymerase requires some additional sequence for recognition which is not contained in the protected fragment. WALZ and PIRROTTA found that although the polymerase-DNA complex is very stable once formed, and can be exposed for long periods to DNase without loss, the purified DNA fragment does not rebind to RNA polymerase. Similar observations have been made by others who isolated polymerase protected fragments from other DNA sources (SCHALLER et al., 1975; PRIBNOW, 1975). The polymerase must then recognize some sequence which includes at least 12 nucleotides beyond the region it can protect against DNase and the establishment of the tight complex must involve a shift of some 10–20 nucleotides from the recognition site to the tight binding site. A similar conclusion has been reached by DICKSON et al. (1975) who sequenced the *lac* promoter-operator region. In the *lac* case the promoter sequence precedes the operator and the *lac* mRNA includes the operator sequence. Two promoter mutations are located about 35 nucleotides away from the transcription initiation site and therefore outside the region covered by a polymerase molecule bound to the initiation site. In both the *lac* and the λ sequences, the region supposed to interact with the polymerase is characterized by blocks of high GC content flanking a region of high AT content. These have been proposed as elements regulating the entry of the polymerase into the DNA. Studies on the initiation of RNA transcription (for a review see CHAMBERLIN, 1974) have distinguished four different phases: (1) recognition of the promoter sequence and formation of a loose complex; (2) "entry" of polymerase to form an "open" complex in which 6–10 base pairs are opened; (3) the entry of the polymerase results in a tight complex which is presumably that isolated by the protection experiments; (4) initiation of transcription, which occurs from the tight binding site. These steps need not occur at the same site and may involve a drift or migration from one site to another. In λ, for example, the recognition might occur at a site which includes the *Hind* II sequence. Polymerase binding at this site, in which a 12 base pair AT–rich region is flanked by two 12 base pair GC–rich regions, might open the helix at the AT-rich block. This "entry" into the DNA would then be followed by a shift of some 20 base pairs to the tight binding site from which transcription initiates. The role of the GC blocks might be to stabilize the AT block or to prevent "opening" until a certain activated state is achieved by the polymerase, in other words to differentiate it from other accidentally AT-rich regions.

The sequences of several polymerase binding sites are now available (Fig. 9) and in some cases the sequence upstream of the binding site is also known. The GC-AT-GC block feature noted in the λ P_R and P_L occurs in the *lac* promoter in the same relationship to the transcription initiation site. However, the SV40 sequence recognized by the *E. coli* polymerase lacks GC-rich regions altogether and is instead particularly rich in AT throughout. The block feature

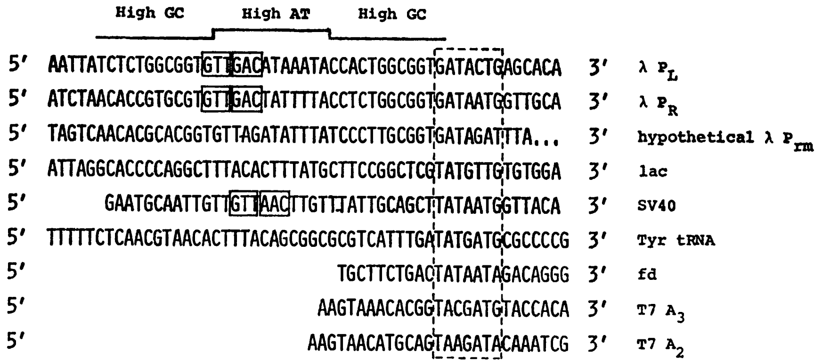


Fig. 9. Promoter sequences. Nucleotide at 3′ end is beginning of the mRNA. Note correspondence of high GC and high AT blocks in top four sequences. Hypothetical λ P_{rm} sequence is obtained from Fig. 8, using axis of symmetry between nucleotides 40 and 41. Solid boxes indicate the *Hin*d II cleavage sites. Dotted lines indicate a heptanucleotide which is nearly homologous to all promoter sequences. Data are compiled from WALZ and PIRROTTA (1975), MANIATIS et al. (1974), DICKSON et al. (1975), DHAR et al. (1974), SEKIYA and KHORANA (1974), SCHALLER et al. (1975), and PRIBNOW (1975)

is definitely present in the *tyr* tRNA promoter where it specifies in addition a region of extensive symmetry. However, the blocks occur immediately before the transcriptional startpoint, possibly suggesting that in this case the entry site and initiation site are very close to one another.

Sequence correspondences can be noticed between the λ P_{R} and P_{L} and the *lac* promoter, on the one hand, and the SV40 promoter on the other, but their significance is difficult to assess. ALLET et al. (1974) found that cleavage sites for the *Hin*d II restriction endonuclease appear to be frequently associated with promoters. Binding of RNA polymerase prevents the *Hin*d II enzyme from cutting at sites in the λ P_{R} and P_{L} promoters, in the *E. coli* RNA polymerase promoters in SV40 and adenovirus-2, and in two sites in phage T7. It is possible therefore that the sequence GTpypuAC (KELLY and SMITH, 1970) recognized by the *Hin*d II enzyme, contains some elements recognized by the polymerase.

Finally, PRIBNOW (1975) has pointed out a block of seven nucleotides which occurs with minor variations in all seven binding site sequences shown in Figure 9.

Clearly, the RNA polymerase, a much more complex protein than the λ or *lac* repressors, has considerable flexibility in the sequences it can recognize and much more work, particularly with promoter mutations, will be necessary to identify the elements important in the recognition.

D. The P_{rm} Promoter

Genetic evidence previously discussed indicated the presence of the P_{rm} promoter for the transcription of the *c*I-*rex* operon, within or very close to O_{R}. In

the O_R sequence there are in fact additional AT-rich blocks alternating with GC-rich blocks which suggested the presence of an additional polymerase recognition site. Moreover, the sequence of this region is related to that of the presumed P_R recognition site by a high degree of symmetry centered around nucleotide 41 in Figure 8. If this sequence represents a second promoter site, it would therefore be oriented in the opposite direction to P_R and have a transcription initiation site located just three or four nucleotides to the left of position 78. This symmetry might of course be coincidental, or due to the disposition of repressor binding sites. It is due to the inversion of the second repressor binding site in O_R and it is not present in O_L where only one promoter is known to exist. G. SMITH et al. (1975) have found that a P_{rm} mutation is located in just this region. They obtained a partial sequence for P_{re} mRNA which hybridizes to this region of DNA. Using appropriate deletions and comparing the P_{re} mRNA with the O_R sequence, it was possible to locate the P_{rm} 116 mutation between nucleotides 48 and 53 shown in Figure 8. On the basis of these findings, WALZ and PIRROTTA (1975) proposed a model for the autoregulation of repressor synthesis based on the overlap of the P_R and P_{rm} recognition regions. P_R is a strong promoter while P_{rm} is a weak one according to the RNA hybridization data. Therefore when both sites are available, RNA polymerase will bind preferentially to P_R. Since the P_R and P_{rm} recognition sites overlap partially, this will interfere with binding at P_{rm} and therefore block transcription of the *c*I-*rex* region. When repressor is present, it binds to the primary site (nucleotides 1–33, Fig. 8) thereby precluding polymerase binding to P_R, but leaving an intact P_{rm} site available. Repressor would therefore act as an activator of P_{rm}. In addition, the repressor might function more directly as an activator by inducing a slight conformational change in the DNA adjacent to the primary binding site, which might make it more attractive to the polymerase in a manner analogous to the activation of promoters by the CAP factor (DE CROMBRUGGHE et al., 1971). Evidence for such "teleactivating" effects has been provided by BURD et al. (1975) who studied the properties of the duplex $d(C_{15}A_{15}) \cdot d(T_{15}G_{15})$ and found that the binding of actinomycin to the GC sequences has a strong effect on the properties of the AT sequences.

The model further predicts that when sufficient repressor has been synthesized, it will begin to occupy the secondary binding sites, thus precluding polymerase binding to P_{rm} and therefore regulating its own synthesis by negative feedback. This effect has now been reported (MEYER et al., 1975).

Another prediction generated by the model is that mutations which lower the affinity of RNA polymerase for P_R should favor the binding at P_{rm} even in the absence of repressor. To observe this effect, however, the P_R mutation must affect the recognition site rather than the initiation site of P_R and it must not alter the P_{rm} recognition site.

It is not clear where in this scheme the *cro* product would intervene. According to this model, in order for *cro* product to control both P_R and P_{rm}, it would be sufficient for it to bind in the operator region immediately adjacent

to the primary site, where the presumed P_R and P_{rm} recognition sites overlap. Gussin et al. (1975) report in fact that phage carrying a mutation in P_{rm} are less sensitive to *cro* shutoff or repressor synthesis from P_{re}. Since this shutoff is effected by controlling the expression of the *c*II gene, their observation suggests that the P_{rm} mutation affects the *cro* control of P_R.

VII. Prophage Induction

The λ prophage in lysogenic bacteria becomes derepressed spontaneously with a frequency of 10^{-4}/cell division. Prophage derepression can also be induced with high efficiency by a variety of treatments which have in common the fact that they block the replication of the bacterial genome. These treatments induce a chain of responses, which require protein synthesis and the expression of certain bacterial genes and leads to the inactivation of repressor after about 20 minutes (Tomizawa and Ogawa, 1967).

Repressor detectable in cell extracts by the DNA binding assays disappears after induction and its activity cannot be restored by dialysis (Shinagawa and Itoh, 1973; Chadwick, personal communication). This suggested that λ repressor inactivation may involve a mechanism basically different from the reversible allosteric transition effected by a small molecule and exemplified by the induction of the *lac* repressor. Roberts and Roberts (1975) made use of anti-λ repressor antibody to follow the fate of the repressor after induction. They found that the repressor antigen suffers a cleavage producing at least one fragment approximately half the original size. This breakdown follows kinetics which mirror the disappearance of repressor activity. Repressor is neither inactivated nor cleaved if it bears an *ind*$^-$ mutation or if the bacteria lack an active *rec*A gene. It remains to be determined whether the proteolytic cleavage is the primary or a secondary event in derepression. The repressor might by inactivated in the classical pattern by a mechanism which lowers its affinity for the operator and at the same time makes the repressor sensitive to proteolytic attack.

The inactivation of repressor is linked by a complicated phenomenology to a number of disparate cell functions. In brief, the evidence is based on two kinds of observations: (1) treatments which cause induction, and (2) bacterial mutations which affect induction.

1. Inactivation of repressor and induction of the prophage result from treatments which interrupt the replication of bacterial or plasmid DNA. Such treatments are thymine starvation (Sicard and Devoret, 1962), exposure to ultraviolet light (Lwoff et al., 1950), mitomycin (Shinagawa and Itoh, 1973), nalidixic acid (Cowlishaw and Ginoza, 1970), and a number of other drugs which inhibit DNA replication. Indirect induction is observed when a replicon from a cell subjected to these treatments is transferred to a lysogen by mating or episome transfer (Borek and Ryan, 1973). The arrest of DNA replication by heat treatment of *dnaB ts* mutant has the same inducing effect (Noack and

KLAUS, 1972). In addition, these treatments result in the premature reinitiation of a new round of replication when DNA synthesis is allowed to resume. Another bacterial mutation, *tif*-1, which renders the bacteria unable to divide at high temperature, also results in temperature-sensitive induction of the prophage (GOLDTHWAIT and JACOB, 1964; CASTELLAZZI et al., 1972a).

2. A number of bacterial mutations prevent the inducing effect of the above-mentioned treatments. These mutations are *recA* (BROOKS and CLARK, 1967), *lexA* (DONCH et al., 1970), *lexB* (BLANCO et al., 1975), *zab* (CASTELLAZZI et al., 1972b), *uvrF* (STORM and ZAUNBRECHER, 1972), *recF* (HORII and CLARK, 1973) and *inf* (BAILONE et al., 1975).

In view of the relationship between prophage induction and DNA replication, it has been suggested that induction shares a common effector with the initiation of rounds of DNA replication (WORCEL, 1970). WORSEY and WILKINS (1975) have shown in synchronous populations of lysogens exposed to low doses of ultraviolet light, induction is most efficient when a round of replication has terminated. Bacterial mutants unable to repair the radiation damage are equally inducible throughout the cell cycle. However, the complex interrelationship between DNA replication, cell division and DNA repair makes it difficult to draw simple conclusions.

Many of the mutations affecting prophage induction are clustered in the same region of the bacterial chromosome and are to different degrees pleiotropic.

In addition to prophage induction they may be defective in genetic recombination and in a complex of responses normally triggered by inducing treatments: mutagenesis, repair of UV damage, initiation of a new round of DNA synthesis, arrest of cell division and, apparently, of aerobic respiration (CASTELLAZZI et al., 1972b; DEFAIS et al., 1971; HOWARD-FLANDERS, 1968; WITKIN, 1969). It has been suggested that these processes might share common steps or are regulated by a common mechanism (WITKIN, 1974). Certain kinds of cell damage, particularly damage affecting the DNA, would induce a kind of emergency syndrome or what has been called the SOS repair system (RADMAN, 1975), whose expression is normally inhibited. It is possible that the λ repressor bears some similarity to the cellular repressors governing these functions and responds to a common derepression mechanism possibly involving proteolytic cleavage.

From the point of view of λ physiology, the induction of these emergency functions would signal to the prophage that the host finds itself in unhealthy circumstances. Inactivation of the λ repressor would then enable the phage to escape the imperilled bacterium and seek a healthier host.

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Molecular Aspects of DNA Replication in *Escherichia coli* Systems

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With 4 Figures

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Abbreviations: ColE1 = colicinogenic factor E1 (plasmid coding for colicin E1); EcoRI = restriction enzyme from *E. coli* bearing resistance transfer factor I (plasmid); FV = F-pili specific, filamentous virus (bacteriophages fd, f1, M13); HpaII = restriction enzyme from *Haemophilus parainfluenzae*; NAD = nicotinamide adenine dinucleotide; NEM = N-ethyl maleimide; NMN = nicotinamide mononucleotide; pol = polymerase; RF = replicative form DNA of small phages (II: a discontinuity in one strand; I: doubly closed); SDS = sodium dodecyl sulfate; SS = single strand.

I. Introduction

Since the discovery of the double helix by WATSON and CRICK, and the intensive studies of the *Escherichia coli* DNA polymerase I by KORNBERG and coworkers, the understanding of DNA replication has been controversial in many aspects. Some basic ideas have been generally accepted: (1) the DNA is a helical double strand with antiparallel chains; (2) the immediate precursors for DNA synthesis are 5′-deoxynucleoside triphosphates; (3) the replication is semiconservative; and (4) all DNA is synthesized exclusively in 5′→3′ direction. The synthesis of larger DNA appears to be discontinuous in one or often both strands, and no DNA polymerase can start synthesis on a template without a primer.

Current attempts to understand in vitro DNA replication of *E. coli* and its phages are a transition from the studies of isolated DNA polymerases. These systems are more complex and include a more or less complicated initiation

step as well as the interaction of DNA polymerases with other proteins to extend the DNA chain. The mechanism by which the double helix unwinds during replication and the signals which regulate the whole process remain to be elucidated.

Aspects of DNA synthesis have been reviewed on various occasions. An excellent, very comprehensive publication on the subject is ARTHUR KORNBERG'S book *DNA Synthesis* (KORNBERG, 1974); other recent but shorter reviews have been published by GEFTER (1975), GROSS (1972), PATO (1972), GOULIAN (1971), GOULIAN (1972), KLEIN and BONHOEFER (1972) and BECKER and HURWITZ (1971).

This review will summarize the latest developments of DNA replication in vitro and integrate them into the general understanding of DNA replication. The selection of topics has been a matter of personal, and often arbitrary evaluation of the literature and is, for the length of this review, more or less concentrated on biochemical questions or genetic aspects leading to a biochemical approach.

II. A Current View on DNA Replication in vivo

A. Propagation of the *E. coli* Chromosome

1. Involvement of Membranes

Aside from the importance for fixation and organization of the chromosome in the *E. coli* cell an active part in initiation, elongation, and termination of the replicating DNA has been suggested for the cellular membrane. The chromosomes of *E. coli* can be released as fast-sedimenting (up to 4000 S), folded structures after gentle lysis of spheroplasts at low temperatures with mild detergents in salt. The particles contain membranes, proteins, phospholipids, and RNA besides the chromosomal DNA. Treatment of the fast-sedimenting structure with RNAase or detergents like SDS causes the DNA to unfold (STONINGTON and PETTIJOHN, 1971; WORCEL and BURGI, 1974), while polyamines stabilize the DNA folds (FLINK and PETTIJOHN, 1974). The complexed DNA can be visualized in the electron microscope as intact molecules attached to the membrane without detectable single-stranded nicks (DELIUS and WORCEL, 1974).

An association of membrane and DNA has been shown not only for the origin of DNA replication (FIELDING and FOX, 1970) but also for the growing points of the chromosome (FUCHS and HANAWALT, 1970). Adhesion at the origin and at growing points are sensitive to lipase treatment (PARKER and GLASER, 1974). The replicating forks were also enriched in the membrane fraction after phage T4 lysozyme treatment of the cells (SILBERSTEIN and INOYE, 1974). The advantage of the phage lysozyme is that it lyses *E. coli* cells at a much lower enzyme/cell ratio than egg white lysozyme. The latter causes an artificial DNA-membrane association at high enzyme concentrations. Therefore, the demonstration of DNA-membrane complexes may not always reflect their occurrence in the cell.

A mutant, resistant to phenylethyl alcohol (*dna*P), is thermosensitive for DNA synthesis and has an alteration in the membrane structure (WADA and YURA, 1974). Inhibition of DNA synthesis by amino acid or thymine starvation, or by drugs like fluorodeoxyuridine, nalidixic acid, or hydroxyurea, or by mutants such as *dna*A or *dna*B (Table 1) at nonpermissive temperatures, causes a change in two membrane-bound proteins with a molecular weight of 60000 and 30000 daltons (SICCARDI et al., 1972). These findings may reflect a secondary effect caused by an altered cell physiology.

2. Initiation and Termination of Chromosomal Replication

Replication on the *E. coli* chromosome starts at a defined site of the genome. For the initiation event transcription is required, as the RNA polymerase inhibitors rifampicin and streptolydigin turn off DNA synthesis in synchronized or thymine-starved cells at an early stage of the cell cycle (MESSER, 1972; LARK, 1972b). The protein synthesis inhibitor chloramphenicol affects DNA synthesis to a much smaller extent. An RNA chain at the origin may serve as an initiator, and be regulated through repression by the *dna*A protein (MESSER et al., 1975).

DNA synthesis in unsynchronized cells slows down at nonpermissive temperatures in mutants defective for initiation, as unfinished rounds are completed, before replication halts near or at the origin. Besides in *dna*A, mutants in *dna*C, H, and I (Table 1) show a progressive reduction in the rate of DNA synthesis after temperature shift. An induced block during chromosomal replication interrupts further growth of the fork, and when brought to permissive conditions, the chromosome restores initiation at the origin (WORCEL, 1970; BHAUMIK et al., 1973). The initiation site for chromosomal replication is located at 74 min on the genetic map near *ilv* and the growing forks move bidirectionally meeting opposite the origin at 25 min on the genetic map near *trp* (BIRD et al., 1972; HOHLFELD and VIELMETTER, 1973). In the absence of protein synthesis a terminal segment of 0.5 % of the chromosomal length remains unreplicated and its completion gives a signal for cell division (MARUNOUCHI and MESSER, 1973; JONES and DONACHIE, 1973).

Integration of an episome into the *E. coli* chromosome does not influence the location of the origin (MASTERS and BRODA, 1971). On the other hand initiation-negative strains like *dna*A mutants can be suppressed under certain growth conditions after the insertion of an F episome (TRESGUERRES et al., 1975) indicating that the episome can use its own origin for chromosomal initiation.

3. Chain Elongation

In a rapidly growing strain new rounds of replication are initiated before the previous replication fork has reached the terminus. This multiple, bidirectional fork system exhibits a constant fork speed of 41 min per round independent of the growth conditions (COOPER and HELMSTETTER, 1968). The

Table 1. *E. coli* genes involved in DNA replication

Locus	Alternate gene symbols	Function in DNA synthesis	Location on standard chromosome Map	Cotransducible gene	Reference
*dna*A		Initiation at origin	73 min	*ilv*	WECHSLER and GROSS (1971)
*dna*B		Chain growth	81 min	*mal*B	WECHSLER and GROSS (1971)
*dna*C	*dna*D	Initiation at origin	89 min	*dra*, *ser*B	WECHSLER and GROSS (1971)
*dna*D	same cistron as *dna*C				WECHSLER (1973)
*dna*E	see *pol*C				
*dna*F	see *nrd*A				
*dna*G		Chain growth	60 min	*tol*C, *uxa*C	CHEN and CARL (1975)
*dna*H		Initiation at origin	64 min	*thy*A	SAKAI et al. (1974)
*dna*I		Initiation at origin	36 min	—	BEYERSMANN et al. (1974)
*dna*P		Membrane defect	75 min	*ilv*	WADA and YURA (1974)
*dna*S		(Accumulation of very short DNA)	72 min	*pyr*E	KONRAD and LEHMAN (1975)
*dna*Z	(*dna*H revised)	Chain growth	approx. 11 min	*pur*E	FILIP et al. (1974); TRUITT and WALKER (1974)
lig		DNA ligase	46 min	*pts*, *trz*A	GOTTESMAN et al. (1973)
*nrd*A	*dna*F	Subunit B1 of ribonucleotide diphosphate reductase	42 min	*aro*C, *pur*F	WECHSLER and GROSS (1971); FUCHS et al. (1972)
*nrd*B		Subunit B2 of ribonucleotide reductase	42 min	see *nrd*A	FUCHS et al. (1972)
*pol*A		DNA pol I	76 min	*met*E, *rha*	GROSS and GROSS (1969)
*pol*B		DNA pol II	2 min	*leu*, *azi*	HIROTA et al. (1972)
*pol*C	*dna*E	DNA pol III	4 min	*ton*A	WECHSLER and GROSS (1971); GEFTER et al. (1971)
rif		β subunit of RNA pol, sensitive for rifampicin	77 min		TAYLOR and TROTTER (1972); HEIL and ZILLIG (1970)

size of 2.5×10^9 daltons for the bacterial genome yields a rate of 800 deoxynucleotides polymerized per second in one strand of a fork. That is in agreement with the time required for adding the next deoxyribonucleotide to the end of a nascent DNA chain. This steptime is of the order of 1 msec at 37° (MANOR et al., 1971).

The DNA synthesis occurs discontinuously. Initiation and growth of short DNA may not be strictly coordinated since the introduction of pyrimidine dimers by UV irradiation of a UV-repair deficient strain leads to an accumulation of replicative segments (HOWARD-FLANDERS et al., 1968). In a normally growing *E. coli* cell the major part of the newly synthesized DNA is found in pieces sedimenting at 10 S (OKAZAKI et al., 1968). The initiation of this DNA appears to occur via the formation of RNA (SUGINO et al., 1972). The RNA-linked DNA fragments accumulate in *pol*Aexl mutants (OKAZAKI et al., 1975).

Even shorter DNA is preserved in *dna*S mutants (KONRAD and LEHMAN, 1975) or in *dna*B mutants (LARK and WECHSLER, 1975) at nonpermissive conditions. Longer DNA chains than the size of Okazaki pieces were assigned to one strand of the *E. coli* chromosome as replicative intermediates in *pol*A mutants (LOUARN and BIRD, 1974). The other strand replicated in 10 S segments. Different size classes of DNA (30 S and 10 S) were also observed for the unidirectional growth of phage P2 DNA, each hybridizing to only one strand of the phage (KAINUMA-KURODA and OKAZAKI, 1975). As all intermediate chains are subsequently sealed to larger DNA, it is an open question, if replicative intermediates larger than 10 S are incomplete sealing products, and if DNA shorter than 10 S is a precursor or a degradation product of Okazaki pieces. Extreme conditions for labeling the intermediates as used by WERNER and coworkers (DIAZ et al., 1975), but also severe genetic defects, may create replicative artefacts, which are not normal intermediates of *E. coli* DNA replication.

Gene *dna*B belongs to a second class of *dna* mutants (GROSS, 1972) which cease DNA replication immediately upon a shift to elevated temperature and are thus similar to mutations in *dna*E and G (WECHSLER et al., 1973) and possibly also the recently isolated mutant *dna*Z (FILIP et al., 1974). These gene products are thought to maintain replication after initiation has occurred at the origin. As gene *dna*E codes for DNA pol III (GEFTER et al., 1971; NÜSSLEIN et al., 1971) the gene was renamed *pol*C (TAYLOR and TROTTER, 1972). Recently the *pol*C+ region of the chromosome was attached to phage *λdv* DNA, but no increase of cellular DNA pol III activity could be measured (SHIZUYA et al., 1974). On the other hand, a temperature-sensitive *pol*C mutant was rendered phenotypically wild type after infection with phage λ carrying the *pol*C+ gene.

The mutation *dna*F can be attributed to effects on the synthesis of precursor deoxyribonucleotides. Specifically, the B_1 subunit of ribonucleoside diphosphate reductase is mutated in *dna*F strains, and thus this gene has been renamed *nrd*A; *nrd*B codes for the B_2 subunit of the same enzyme (FUCHS et al., 1972).

Curiously, many of the *dna*B mutants are phenotypically reversed during growth in high salt (GROSS, 1972). Some of them reactivate their thermosensitive gene products after a shift back to a permissive temperature (WORCEL, 1970). This was also shown for *dna*C (SCHUBACH et al., 1973) and *dna*A (HANNA and CARL, 1975) mutants. The block of the DNA synthesis at nonpermissive temperatures caused by *dna*B mutants can be transiently overcome by phage P1 infection (LANKA and SCHUSTER, 1970). The gene for a *dna*B analog protein (*ban*), carried by phage P1, can be constitutively expressed (D'ARI et al., 1975), which makes *dna*B cells phenotypically wild type.

4. Mutations affecting DNA Polymerase I and II

Mutations in the genes *pol*A and *pol*B (Table 1) do not affect DNA synthesis as drastically as do mutations in *pol*C. Up to now almost all features of *E. coli* DNA replication have been found to be normal in *pol*B mutants (CAMPBELL et al., 1972; HIROTA et al., 1972), whereas *pol*A mutants have distinguishing properties. The function of *pol*A is strictly required for the propagation of small plasmids. Phenotypically, *pol*A mutants are sensitive to UV and methanesulfonic acid methylester (MMS) indicating a defect in repair synthesis (DE LUCIA and CAIRNS, 1969). They generate deletions spontaneously at high frequency, and single-strand breaks in the DNA are not efficiently repaired (COUKELL and YANOFSKY, 1970). Although the *pol*A1 mutants have less than 1 % of normal DNA pol I activity, residual polymerase I could be isolated from various *pol*A1 strains, and the mutants shown to have wild type levels of the fragment containing the 5'→3' exonuclease activity of DNA pol I (LEHMAN and CHIEN, 1973). This exonuclease activity is essential for the *E. coli* cell, since mutations in the *pol*A gene affecting the 5'→3' exonuclease part of DNA pol I are conditionally lethal (KONRAD and LEHMAN, 1974). This *pol*Aex mutation was also found in mutants with an additional defect in the polymerase activity (OLIVERA and BONHOEFFER, 1974). There are no known mutants where only the DNA polymerase function is impaired and the cells are conditionally lethal. A temperature-sensitive DNA pol I is produced in *pol*A12 mutants without an effect on cell growth at elevated temperature (MONK and KINROSS, 1972). The amount of short DNA is increased in all *pol*A mutants (OKAZAKI et al., 1971) indicating the helper function of DNA pol I in gap filling.

5. Other *E. coli* Genes for DNA Replication

The adjacent DNA ends are sealed by the action of DNA ligase. Mutations in the gene for this enzyme cause accumulation of short DNA, especially when thermosensitive defects in both DNA pol I and ligase are manifested on the same chromosome (KONRAD et al., 1974). One mutant, *lig* 321, has not only a sealing defect, but shows also degradation of the parental chromosome (HORIUCHI et al., 1975). The UV-sensitive, conditionally lethal mutant *lig*ts7 (PAULING and HAMM, 1968) cannot integrate any pulse label in fast sedimenting

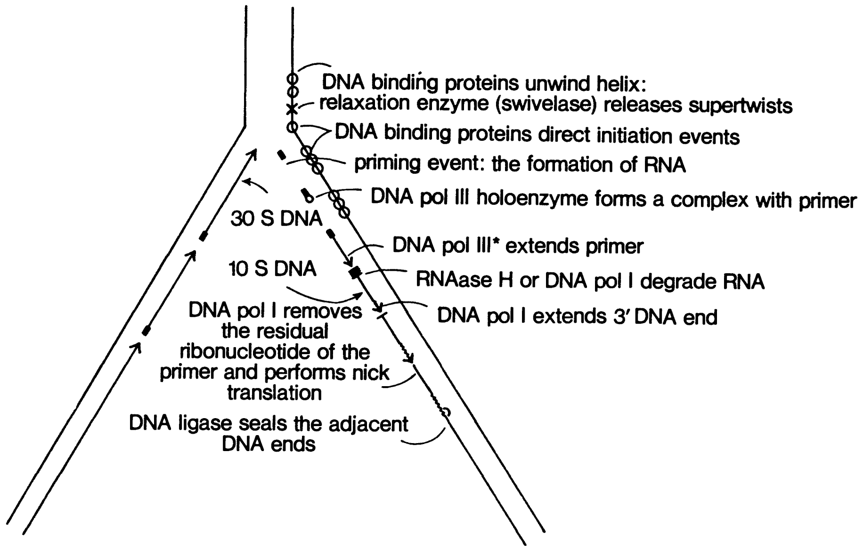

Fig. 1. Model for replication fork of *E. coli* chromosome

DNA at restrictive temperature (KONRAD et al., 1973). This result is consistent with a temperature-sensitive ligase activity, which is reduced under permissive conditions, too. Enzymatic deficiency is also expressed in the *lig*4 mutant which is not UV-sensitive and not conditionally lethal (GOTTESMAN et al., 1973).

There are certainly more proteins involved in *E. coli* DNA duplication than expressed by mutations related to replication (Table 1). DNA unwinding protein has been shown to be indispensable for DNA replication of small phages in vitro (GEIDER and KORNBERG, 1974), but a gene coding for the protein has not been characterized. The in vitro initiation of phage ϕX174 SS→RF conversion requires at least two more proteins without a known genetic locus (WICKNER, S., and HURWITZ, 1974; SCHEKMAN et al., 1974).

6. Events at the Replicating Fork

The current thoughts on replication in a fork of the *E. coli* chromosome are outlined in Figure 1. A double-stranded DNA is ready for replication after the opening of the duplex by DNA binding proteins. The resulting positive supertwists are released by a supertwist relaxing enzyme. Selected parts of the single-stranded areas can attract a system for initiation of DNA synthesis, like proteins coded by *dna*C, *dna*H, *dna*I or RNA polymerase for the chromosomal origin and proteins coded by *dna*B, *dna*G, *dna*Z for replication of short segments. The 3′ end of the initiator RNA is complexed by DNA pol III holoenzyme in the presence of ATP and extended by the polymerase as DNA. The RNA is removed by RNAase H to the last but one ribonucleotide. DNA polymerase I extends the 3′ end of DNA segments, removes the ribonucleotide,

and makes the apposed 5′ phosphoryl and 3′-hydroxyl termini available for DNA ligase action. Further details supporting that scheme will be discussed.

B. Replication of *E. coli* Phages with Double-Stranded DNA and Plasmids

The propagation of bacteriophages requires gene products coded for by both the phage and the host. A membrane attachment of phage DNA has been assumed for most stages of viral DNA replication (see SIEGEL and SCHAECHTER, 1973).

1. Phage T4

After injection of phage T4 DNA into the host cell, the viral DNA replicates as a loop structure with occasional reinitiation in the middle of the loop indicating bidirectional growth (DELIUS et al., 1971). The forks often display single-stranded whiskers which can be degraded by exonuclease I. This DNA may reflect collapsed single-stranded regions in the fork, where the newly synthesized strand is displaced by reannealing of the old DNA. After more than 4 min infection, the DNA forms a fast sedimenting complex (up to 3000 S) with RNA, protein, and cell membrane fragments (ALTMAN and LERMAN, 1970; MILLER and KOZINSKI, 1970a). This cell membrane-DNA replication complex is finally converted into mature 63 S T4 DNA. Mutants in gene 59 show a premature release of 63 S DNA via 200 S intermediates, before DNA synthesis arrests after less than one round of replication (WU and YEH, 1974).

The origin of phage T4 DNA replication has been localized between gene 43 and gene 42 by a gradient of allele frequencies in crosses between normal and small T4 particles (MOSIG, 1970). In contrast to the more or less accepted bidirectional loop formation seen in EM pictures, this analysis reveals unidirectional growth clockwise from gene 43. The DNA is generated in small pieces along the two template strands (OKAZAKI and OKAZAKI, 1969). Chain growth occurs exclusively in 5′→3′ direction as shown by enzymatic degradation of pulse-labeled fragments.

T4 DNA replication requires the function of more than 25 phages genes (WOOD, 1974). Some gene products are involved in precursor synthesis or modification, others in degradation of host DNA. The residual genes specify proteins necessary during replication. For example, gene 43 codes for a DNA pol; gene 32 for a DNA unwinding protein; gene 30 for a DNA ligase. The role of genes causing a delay of DNA synthesis remains unclear (YEGIAN et al., 1971).

2. Phage T7

The rapid growth and the relatively short genome of 25×10^6 daltons (STUDIER, 1965) have made phage T7 an attractive system for studies on double-stranded DNA replication. Phage T7 DNA is associated with the bacterial membrane (STRÄTLING et al., 1973; PACUMBABA and CENTER, 1973) at

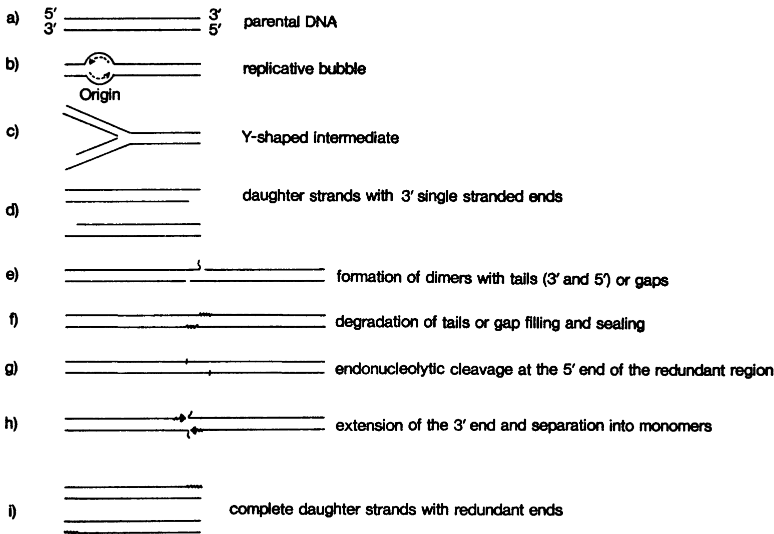

Fig. 2. A scheme for replication of phage T7 DNA [modified according to SCHLEGEL and THOMAS (1972) and WATSON (1972)]

selected gene regions (HELLAND and NYGAARD, 1975). Phage-induced lysozyme, coded by gene 3.5, releases host and T7 DNA from its membrane complex and may therefore play a role in T7 DNA replication (SILBERSTEIN et al., 1975). A membrane-attached T7-induced endonuclease distinct from the gene 3- or gene 6-coded nucleases, is thought to act in the formation of mature phage DNA (PACUMBABA and CENTER, 1974).

The linear genome with terminal repetitions replicates in the cell bidirectionally with the origin at 17% from the genetic left end of the DNA (DRESSLER et al., 1972). The first replicative intermediate is a bubble which is converted into a Y-shaped rod, when it reaches the left end. The other growing point moves to the rightward end (Fig. 2a–c). During that time a second round of replication has been initiated at the origin. That the DNA is made in short pieces, can be demonstrated in a ligase-deficient T7 mutant, infecting an *E. coli* strain thermosensitive for the bacterial ligase at a nonpermissive temperature (MASAMUNE et al., 1971). The short chains may be initiated by an RNA primer, but they cannot fill the duplex to the very 5′ end, as they can be elongated only in 5′→3′ direction even after removal of the RNA. This would result in a reduction of genome size for replication in linear molecules which do not produce circular replicative intermediates (WATSON, 1972). The formation of dimers at the single-stranded end can subsequently allow gap filling and completion of the nonreplicated region (Fig. 2d–f). That molecules with gaps (Fig. 2e) are replicative intermediates is supported by the findings of SCHLEGEL and THOMAS (1972): Newly synthesized T7-DNA has 1, 2 and 3 times the length of the mature molecule; alkaline denaturation of dimers and trimers produces unit length single chains; and digestion with single strand specific endonuclease produces double-stranded monomers.

Sealing by DNA ligase and cleavage in the redundant region allows extension of the 3′ end and separation of the dimer (Fig. 2f–i). The cleaved molecules are completed and represent mature T7 DNA with terminal repetitions.

Phage T7 codes for more than 25 proteins (STUDIER and MAIZEL, 1969) and the early genes 1–6 (located at the left end of the map) are essential for DNA synthesis (STUDIER, 1969). T7 RNA polymerase, which probably has no initiator function in DNA synthesis, is specified by gene 1 (CHAMBERLIN et al., 1970); T7-DNA polymerase by gene 5 (GRIPPO and RICHARDSON, 1971); endonuclease by gene 3 (CENTER et al., 1970; SADOWSKI and KERR, 1970); and an exonuclease degrading double-stranded DNA in 5′→3′ direction by gene 6 (KERR and SADOWSKI, 1972a and b). Both nucleases are involved in host cell DNA degradation, and at least gene 6 is required during phage replication for the formation of concatemers (FRÖHLICH et al., 1975) as described in Figure 2.

A DNA ligase requiring ATP as a cofactor is also induced by phage T7. It is a nonessential enzyme, since a genetic defect located between gene 1 and 2 at 1.3 (STUDIER, 1972) is only apparent in a ligase-deficient *E. coli* strain (MASAMUNE et al., 1971). That means host ligase can substitute for the phage enzyme. The same holds for a phage T7-coded DNA binding protein. The lack of T7 mutants for this protein indicates that the *E. coli* unwinding protein (REUBEN and GEFTER, 1973) or some other host DNA binding proteins such as the protein required for phage ϕX174 SS→RF conversion (SCHEKMAN et al., 1975) can substitute for the phage-binding protein. CHAMBERLIN (1974b) isolated host mutants in *tsn*C affecting early phage DNA synthesis; the class *tsn*B affected a late phase. The gene product of *tsn*C could be identified as thioredoxin acting as subunit of T7 DNA polymerase (MARK and MODRICH, 1975; MODRICH and RICHARDSON, 1975a, b).

3. Phage λ

The life cycle of phage λ can be either lytic, with virulent phage production, or lysogenic with the DNA integrated in the host chromosome near the *gal* operon. The excision of the λ genome can lead to phages carrying parts of the host chromosome already mentioned for λd*pol*C. Recently, viable hybrids of λ and bacterial or eukaryotic DNA have been formed enzymatically and propagated (THOMAS et al., 1974).

The mature λ DNA is packaged into the phage as a linear molecule and circularized after injection into the cell. Replication starts at a site, *ori*, which lies between the genes cII and P, and the forks move bidirectionally (SCHNÖS and INMAN, 1970) as a theta structure. Later in replication the product of gene *gam* is required to allow a rolling circle stage (MCCLURE et al., 1973; TAKAHASHI, 1974) in the presence of the functional *rec*BC genes of the host cell (ENQUIST and SKALKA, 1973). A specific RNA, called *oop*, is formed at *ori*. Its synthesis is dependent on the function of the phages genes O and P, as well as the products of the host genes *dna*B and *dna*G. Rifampicin blocks the *oop* RNA formation (HAYES and SZYBALSKI, 1973). The extensive genetic aspects of phage λ are collected in the book *The Bacteriophage Lambda*, edited by HERSHEY (1971).

4. Plasmids

Plasmids replicate autonomously in *E. coli* strains. RNA priming of their replicating DNA has been deduced from the rifampicin inhibition of the synthesis of the F-episome (BAZZICALUPO et al., 1972; KLINE, 1973), the plasmid ColE1 (CLEWELL et al., 1972), and the minicircular DNA in *E. coli* 15 (MESSING et al., 1972). In the presence of chloramphenicol ColE1 DNA continues to replicate. A large portion of the covalently closed, supercoiled DNA is converted into the open circular DNA form after treatment with RNAase H, pancreatic RNAase or alkali, indicating a single RNA fragment enclosed in the supercoiled DNA in either strand (BLAIR et al., 1972). This structure was not detected in ColE1 DNA isolated from normally growing cells. It may represent a primer residue which is conserved in one of the complementary strands.

That the plasmid initiates replication at a site 18% from the EcoRI end (INSELBURG, 1974; LOVETT et al., 1974) was found by restriction cleavage and electron microscopy of replicative intermediates. The branches rise at 18% from one cleavage end and vary in the distance to the other end, as expected for unidirectional replication. ColE1 replication depends primarily on DNA polymerase I and to a lesser extent on DNA polymerase III. The plasmid can be propagated to some extent in *pol*C mutants under restrictive conditions (GOEBEL, 1972), although at a reduced rate (COLLINS et al., 1975), but not at all in *pol*A strains (KINGSBURY and HELINSKY, 1970). The F-episome, R-factors, or ColV and ColI are not affected by the *pol*A mutation. Other mutations in the host chromosome like *dna*C or *dna*G diminish ColE1 DNA synthesis. The results with *dna*A mutants are controversial. GOEBEL (1974) found a dependency which could be eliminated with low doses of chloramphenicol, whereas COLLINS et al. (1975) reported ColE1 replication to be independent from the *dna*A function. Some host mutations affect plasmid but not host DNA synthesis, and mutations in the ColE1 DNA can be temperature-sensitive for plasmid propagation itself (KINGSBURY and HELINSKI, 1973). Plasmids like minicircular DNA of *E. coli* 15 replicate through short pieces (4–6 S) with a symmetric distribution of the label in both strands (MESSING et al., 1974).

They are also a potent vehicle for cloning and amplification of DNA (HERSHFIELD, V., et al., (1974) as mentioned for phage λ. A hybrid plasmid constructed from two DNAs (pSC101 and ColE1) with distinct requirements for replication uses one replicative mode in *pol*A mutants and the other one in the presence of chloramphenicol (TIMMIS et al., 1974).

C. Growth of Single-Stranded *E. coli* Phages

This chapter will mainly consider the in vivo DNA replication of both the male specific filamentous *E. coli* viruses (FV) like fd, f1, and M 13, and the icosahedral phages like φX174 or S 13. Both types of bacteriophages inject circular single-stranded DNA of about 6000 nucleotides into the host cell where a double stranded replicative form (RF) is synthesized and then duplicated. In late stages of phage development the replication of RF is restricted to the

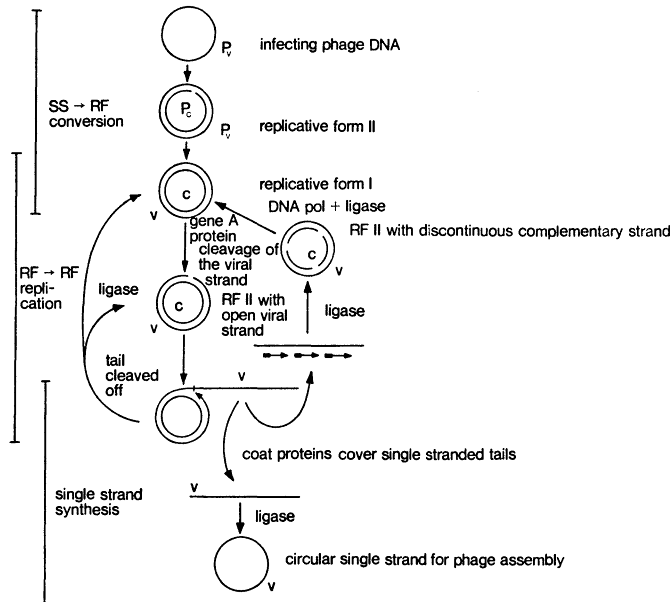

Fig. 3. Intermediates of phage ϕX174 replication in vivo (modified according to SCHRÖDER et al. [1973]). Analog steps for filamentous viruses are the cleavage of RF I by gene 2 protein and coating of the DNA for single strand synthesis by gene 5 protein. The latter prevents subsequent replication of the complementary strand to replicative form DNA. P = parental DNA; v = viral strand; c = complementary strand. Arrowheads indicate 3′ direction of replication. Ligase = sealing by DNA ligase; DNA pol = extension of 3′ end by DNA polymerase

synthesis of viral single-stranded DNA (Fig. 3). The biology of FV has been reviewed by MARVIN and HOHN (1969) and of ϕX174 by SINSHEIMER (1968). A genetic map of ϕX174 has been published by BENBOW et al. (1971), and the Hpa II restriction fragments of FV-DNA have been correlated to the viral genes by SEEBURG and SCHALLER (1974). An extensive summary of the properties of single-stranded phages can be found in KORNBERG'S book *DNA Synthesis* (KORNBERG, 1974) and in a review by DENHARDT (1975).

1. Early Replicative Steps

Phage adsorption to the host cell receptor site and attachment of the DNA to the replication site may require a pilot protein, as in the case of FV A-protein, coded by gene 3 (JAZWINSKI et al., 1973), or for ϕX174 gene H spike protein (JAZWINSKI et al., 1975). The initiation of DNA synthesis via the formation of a short RNA transcript was first observed for FV-DNA replication. The synthesis of parental replicative form is unchanged in the presence of chloramphenicol, but strongly suppressed by rifampicin, a specific inhibitor of the bacterial RNA polymerase (BRUTLAG et al., 1971). On the other hand, ϕX174 parental RF formation is not sensitive to the drug (SILVERSTEIN and

Billen, 1972), indicating an initiation system distinct from transcription by *E. coli* RNA pol. Both the replicating FV-DNA (Staudenbauer and Hofschneider, 1971) and the ϕX174 DNA (Knippers and Sinsheimer, 1968) appear to be membrane attached. Initiation in each of the three stages of ϕX174 replication (see Fig. 3) probably occurs in gene A (Johnson and Sinsheimer, 1974; Godson, 1974). Isolated RF II was nicktranslated by DNA pol I and cleaved into fragments by *H. influenzae* endonuclease where fragment R 3 was predominantly labeled. This fragment maps in the gene A region (Hutchison et al., cited in Johnson and Sinsheimer, 1974). Similar conclusions were drawn from heteroduplex analysis in single burst experiments of spheroplasts (Baas and Janz, 1972). The gradient of recombination events was formed from a point in gene A via the genes B, C. D, E, F, G, and H to the other side in gene A.

2. Phage Proteins in Replication

After infection by the phage DNA, the formation of the first replicative form (Fig. 3) relies only on host proteins, whereas RF replication and single-strand synthesis require the action of both phage- and host-coded proteins. When the parental RF I has been synthesized, a specific single-stranded break has to be introduced into the viral strand of the closed duplex in order to initiate RF synthesis. FV expresses gene 2 for this step (Lin and Pratt, 1972) and ϕX174 gene A (Francke and Ray, 1971). Recently both proteins have been partially characterized (see Chapter V, C3). A small peptide, also coded in cistron A, is responsible for the shutoff of host cell DNA replication after ϕX174 infection (Martin and Godson, 1975).

Amber mutants in gene 2 sustain the synthesis of all other phage-specific proteins at normal rates, which suggests that the parental RF in the doubly closed form can serve as an efficient template for phage messenger RNA synthesis (Henry and Pratt, 1969). Temperature sensitive mutants in gene 2 of FV circumvent RF→RF replication when grown at nonpermissive temperature and then shifted to permissive conditions, since gene 5 protein has been accumulated (Mazur and Model, 1973). Gene 5 protein has negative control in preventing the replication of the complementary strand of RF, and still allows the synthesis of the viral strand (Salstrom and Pratt, 1971). ϕX174-SS-DNA complexes with gene D protein in addition to the coat proteins specified by genes F and G, a process mediated by gene B and C proteins and thereby prevents the replication of the complementary strand (Weisbeek and Sinsheimer, 1974).

3. Host Functions in Phage DNA Synthesis

Of the three stages of phage DNA replication (Fig. 3), host functions are required for initiation and chain elongation. Most of the genes involved in host DNA replication have also been checked for DNA synthesis of small bacteriophages. The results are summarized in Table 2. The *polC* product, DNA poly-

Table 2. Host genes and DNA replication of small bacteriophages

	Host functions required for phage growth	*pol*C function substituted by gene	Host genes not required for phage growth
I. SS→RF conversion			
a) Filamentous virus (FV)	*pol*C [21] *rif* [1]	*pol*A [21] *pol*B [12]	*dna*A [2] *dna*B [13] *nal* [19] *pol*A [3] *pol*B [3]
b) Phage ϕX174	*dna*B [7, 23] *dna*C [4] *dna*G [11] *pol*C [9]	*pol*A [6, 9]	*dna*A [24] *dna*C [10] *dna*H [17] *nal* [19] *pol*A [3] *pol*B [3] *pol*C [6] *rep* [5] *rif* [20]
II. RF→RF replication			
a) Filamentous virus (FV)	*dna*B [13, 22] *dna*G [16] *nal* [19] *pol*C [21] *rep* [5] *rif* [1]		*dna*A [2] *pol*A [3] *pol*B [3]
b) Phage ϕX174	*dna*B [7] *dna*C [10] *dna*G [11] *dna*H [17] *pol*C [6, 9] *rep* [5]		*dna*A [24] *pol*A [3] *pol*B [3]
III. RF→SS synthesis		Circularization of linear SS-intermediates	
a) Filamentous virus (FV)	*nal* [19] *pol*C [21]	*dna*A [2]	*dna*A [2] *dna*B [14, 22] *pol*A [3] *pol*B [3] *rif* [8]
b) Phage ϕX174	*dna*G [11] *pol*C [6, 9]	*lig* [18]	*dna*A [24] *dna*B [7, 15] *dna*C [10] *pol*A [3] *pol*B [3]

References: 1. Brutlag et al. (1971), 2. Bouvier and Zinder (1974), 3. Campbell et al. (1972), Hirota et al. (1972), 4. Hess et al. (1975), 5. Denhardt et al. (1967), 6. Dumas and Miller (1973), 7. Dumas and Miller (1974), 8. Fidanián and Ray (1974), 9. Greenlee (1973), 10. Kranias and Dumas (1974), 11. McFadden and Denhardt (1974), 12. Molineux and Gefter (1974), 13. Olsen et al. (1972), 14. Primrose et al. (1968), 15. Primrose et al. (1971), 16. Ray et al. (1975), 17. Sakai and Komano (1975), 18. Schekman and Ray (1971), 19. Schneck et al. (1973), 20. Silverstein and Billen (1971), 21. Staudenbauer (1974b), 22. Staudenbauer and Hofschneider (1972), 23. Steinberg and Denhardt (1968), 24. Taketo (1973).

lig = DNA ligase; *nal* = inhibition by nalidixic acid; *rep* = gene product participating in RF replication of small phages; *rif* = inhibition by rifampicin, i.e., *E. coli* RNA polymerase involved.

merase III, is probably required for all three stages of FV and ϕX174 replication. Mutants of *pol*B, resulting in a deficiency of DNA pol II, do not alter phage production (CAMPBELL et al., 1972; HIROTA et al., 1972). DNA pol I has been proposed as a substitute for the DNA pol III requirement in phage SS→RF conversion (STAUDENBAUER, 1974b; GREENLEE, 1973). Of the listed genes, *dna*G is also necessary for all stages of ϕX174 DNA synthesis (McFADDEN and DENHARDT, 1974), but only for RF replication of FV (RAY et al., 1975). After onset single strand synthesis of FV is not affected by *dna*B (STAUDENBAUER and HOFSCHNEIDER, 1972), and *dna*A can be omitted from the whole ϕX174 replication (TAKETO, 1973). The block for ϕX174 and FV in *dnaZ* mutants (TRUITT and WALKER, 1974) under nonpermissive conditions has not yet been manifested in distinct stages of phage DNA replication. Controversial results concern the requirement of *dna*C in ϕX174 SS→RF conversion (KRANIAS and DUMAS, 1974; HESS et al., 1975). As already mentioned for salt effects on *dna*B mutants, in vivo systems are sensitive to experimental conditions as well as to properties of the mutants and their deficient gene products. A small amount of the particular protein may suffice for a stage in phage replication, or a defect in host replication may make the cell physiology unfavorable for phage DNA synthesis.

The *rep* gene affects only the RF replication of small bacteriophages, but chromosomal synthesis of the host is almost normal (DENHARDT et al., 1967). Nalidixic acid turns off RF replication and single strand synthesis (SCHNECK et al., 1973). This drug inhibits m-RNA synthesis of the icosahedral phage S 13 (PUGA and TESSMAN, 1973). For FV the interference with the synthesis of the complementary strand is much higher than with the viral strand (FIDANIÁN and RAY, 1974). Since rifampicin has a similar effect at this stage, both drugs may counteract the initiation events of the complementary strand. As predicted from the rolling circle model (Fig. 3), progeny viral strand synthesis is only indirectly impaired by rifampicin, which affects expression of the gene 2 and gene 5 products (FIDANIÁN and RAY, 1974).

4. Replicative Intermediates

DNA synthesis during RF replication and SS-formation is envisioned as a rolling circle of the template DNA (DRESSLER and WOLFSON, 1970). ϕX174 generates a tailed structure (Fig. 3) with the viral strand of double unit length (SCHRÖDER and KAERNER, 1972). For RF synthesis the tail is converted into double-stranded DNA after some delay (SCHRÖDER et al., 1973). Before the new negative strand is completed, the replicative intermediate splits yielding a gapped RF II molecule (SCHEKMAN et al., 1971) containing the old complementary strand with the new viral strand and the partially double-stranded tail with segments of the complementary strand (Fig. 3). This DNA will be circularized and may contain a random gap in the complementary strand (EISENBERG and DENHARDT, 1974a and b) as shown by nicktranslation and random distribution of the label after restriction of the DNA. FV replicates as

rolling circle during SS-synthesis, but may also show, as visualized in the electron-microscope, theta-structures for RF replication. This latter observation was supported by the finding of small DNA fragments in pulse-labeled DNA which hybridize to both strands of the RF (ALLISON et al., 1974).

5. Circularization of Single-Stranded Linears

In the last step before assembling the phage particles, the linear single strands of RF→SS synthesis have to be circularized. In the DNA ligase mutant ts7, ϕX174 packages linear DNA molecules producing an infective mature phage (SCHEKMAN and RAY, 1971). Infectivity of defined ϕX174 linear single strands has also been shown by SCHRÖDER and KAERNER (1971) and GEIDER et al. (1972), which contrasts with randomly nicked ϕX174 DNA (FIERS and SINSHEIMER, 1967). Derived from ϕX174 RF II, linears were nicked in a region of cistron A (MILLER and SINSHEIMER, 1974) and their infectivity was explained by annealing and circularization with contaminating linears of the complementary strand, since linears from phage particles were found by these authors not to be infective in the spheroplast assay. For in vitro circularization, linears isolated from phages required DNA polymerase action before sealing with T4 DNA ligase (IWAYA et al., 1973).

A defect in gene *dna*A prevents the formation of circles of FV under nonpermissive conditions (BOUVIER and ZINDER, 1974). Circularization of open viral DNA may occur after the formation of a hairpin in the linear intermediate (see Fig. 4) (SCHALLER et al., 1969), where the adjacent base-paired regions are subsequently joined by the action of *E. coli* DNA ligase.

III. In vitro Systems for DNA Replication

The goal of the biochemist is not only to understand isolated cellular components as such but also their interaction with other structures or compounds to better comprehend the complexity of the living cell.

Whole cells are very selective in the uptake of molecules and can convert them in their metabolism. Genetics can restrict cellular pathways and point out the requirement and effects of certain gene products. An in vitro system is a more direct tool to look at cellular events like DNA replication. Organic solvents such as diethylether or toluene disrupt the cell wall and make the membrane permeable to smaller molecules. This allows the addition of precursors of DNA synthesis and low molecular weight inhibitors. Lysis of cells on a solid support results in a system which can be further complemented with proteins defective in mutant strains.

The reconstitution of a multicomponent system is only possible via solubilization of the required proteins. Their purification and characterisation gives further elucidation in molecular steps, but may also lead to artificial reactions. Thus, genetics and biochemistry supplement each other.

A. Nucleotide Permeable Cells

1. Properties of Ether- and Toluene-Treated Cells

Cells which have lost their ability to grow after a biochemical treatment shall be defined as a cellular in vitro system. Although they may have maintained some properties of growing cells, the nonviability separates them strictly from living organisms. A sudden transfer of the in vivo to the in vitro situation can be achieved by exposing *E. coli* cells to diethylether for a few seconds. Among 10^{10} cells there are no cells surviving the treatment. Nucleotide permeable cells pour smaller cytoplasmic molecules into their environment and depend on exogenous components like deoxynucleoside triphosphates and ATP for synthetic events (VOSBERG and HOFFMANN-BERLING, 1971).

Shaking *E. coli* cells with toluene for a couple of minutes makes them also permeable to molecules of low molecular weight (MOSES and RICHARDSON, 1970b). This system has properties very similar to those of ether-treated cells. In contrast to repair synthesis, DNA replication in toluene-treated cells requires ATP and is suppressed in temperature-sensitive *dna* mutants under nonpermissive conditions (MOSES and RICHARDSON, 1970b). Fast-stop mutants, like *dna*B, cease DNA replication immediately, whereas slow-stop mutants like *dna*A continue for some time, if the cells are not kept at high temperature before harvesting (MORDOH et al., 1970). This is consistent with the finding that nucleotide permeable cells do not reinitiate at the chromosomal origin.

They show a limited breakdown of cellular DNA under the assay conditions used (VOSBERG and HOFFMANN-BERLING, 1971). Interconversion of nucleotides, especially between deoxyribonucleoside di- and triphosphates, is fast in nucleotide permeable cells (GEIDER, 1972). However, since deoxynucleosides are barely phosphorylated, they are unavailable for use in DNA synthesis (VOSBERG and HOFFMANN-BERLING, 1971; MORDOH et al., 1970). The cells suffer a relatively small loss of large proteins and added pancreatic DNAase does not interfere with DNA replication in *pol*A1 cells. This observation indicates a barrier for big molecules in nucleotide permeable cells. However, addition of pancreatic endonuclease can induce repair synthesis and thus must enter at least some of the most heavily damaged cells. This entry of DNAase I seems to be facilitated in T4-infected cells. DNA pol I cannot enter even this latter class of cells.

The replication of the *E. coli* chromosome is semiconservative, where short DNA segments (Okazaki pieces) are extended, new ones are generated, and the short chains integrated into larger DNA (GEIDER and HOFFMANN-BERLING, 1971). This step can be prevented by nicotinamide mononucleotide (GEIDER, 1972), an inhibitor of DNA ligase. The same effect can also be observed in toluene-treated cells (CAMPBELL et al., 1971) and in the cellophane disk system (OLIVERA and BONHOEFFER, 1972b). Nalidixic acid allows joining of Okazaki pieces to 30–40 S material, but not to fast sedimenting DNA (PISETSKY et al., 1972). Very short DNA (5 S) as a replicative intermediate in ether-treated cells has been found after short pulses or at low concentrations of deoxyribonucleoside triphosphates (GEIDER and HOFFMANN-BERLING, 1971).

2. Phage T4-Infected Cells

Toluene-treated cells which were infected with phage T4 before treatment showed that phage DNA replication was controlled by gene 43 (T4 DNA polymerase) and proceeded by a semiconservative mechanism (MILLER, JR., R. C. et al., 1973). When toluene-treatment was performed 6 min or less after phage infection, T4 specific DNA synthesis was not dependent on gene 43 product at nonpermissive temperature. Presumably host DNA polymerase has not been shut off early after infection. In addition to gene 43 mutants, T4 DNA replication in the cellular in vitro system was also reduced in mutants in genes 1 (deoxyribonucleoside kinase), 30 (DNA ligase), 32 (DNA unwinding protein), 41, 42 (dCMP hydroxymethylase), 44, 45, 46, and 62 (DICOU and COZZARELLI, 1973). T4 DNA synthesis proceeded in a discontinuous manner, generating and joining small (10 S) DNA fragments.

3. Phage ϕX174 DNA Synthesis

Mitomycin treatments shuts off cellular replication and facilitates studies on phage DNA synthesis in infected, nucleotide permeable cells. Infection of *E. coli* with ϕX174 in the presence of caffeine allows the phage to eclipse, but prevents the formation of the parental replicative form (HESS et al., 1973). Extensive incubation leads predominantly to RFI; preincubation and a pulse for a few seconds or low deoxyribonucleoside triphosphate concentrations cause the formation of duplex DNA with multiple short segments (5 S) attached to the viral template strand. In the presence of NMN, an inhibitor of *E. coli* DNA ligase, the segments can be sealed, not to RFI, but to RFII with an open complementary strand of almost unit length, providing a hint of a DNA ligase activity distinct from the known DNA ligase (HESS et al., 1973). The segments can also be extended by DNA pol II to 11 S and sealed by DNA ligase to 14 S complementary strands (HESS et al., 1975). Short DNA replication intermediates were also found for RF→RF replication (DÜRWALD and HOFFMANN-BERLIN, 1971), by applying short pulses of label or low concentrations of nucleotides. ϕX174 RF replication in nucleotide permeable cells results in two classes of hybrid RF after incorporation of bromodeoxythymidine triphosphate: either a heavy complementary or a heavy viral strand and the other strand of light density (MÜLLER-WECKER et al., 1972). The chain growth was estimated as 400 nucleotides per second at 37° or about one-half of the in vivo rate of *E. coli* DNA. RF with the heavy complementary strand is generated first, indicating the replication of the tail in an intermediate (Fig. 3). The unwinding of the duplex structure in the core would be the slow reaction and yields RF with the heavy viral strand (GEIDER et al., 1972). RFI appears with a lag of 4 min after the formation of RFII, which is found as a hybrid duplex within the first minute of incubation.

B. Partially Lysed Cells

Although nucleotide permeable cells are freely diffusible for highly charged ions like nucleoside triphosphates, they cannot be complemented by the addi-

tion of proteins. For that purpose the cell membrane must be heavily damaged, and this can be achieved by detergents, osmotic shock, or digestion with lysozyme.

1. Detergent-Treated Toluenized Cells

DNA pol I or lactate dehydrogenase diffuse from toluene-treated cells in 1 % Triton X100, and DNA pol I antibodies prevent repair synthesis (Moses, 1972). The cell population still exhibits the properties of nucleotide permeable cells, such as replication deficiency in *dna*B mutants at high temperature, requirement for ATP, deoxynucleoside triphosphates, and sensitivity to sulfhydryl-blocking agents.

2. Plasmolyzed Cells

A comparable cell preparation is obtained by disrupting log-phase *E. coli* cells osmotically in 2M sucrose (Wickner, R.B. and Hurwitz, 1972). Plasmolyzed cells also require the addition of nucleotides for DNA synthesis. The incorporation is temperature-sensitive in *dna*B, *dna*G, and *pol*C mutants, and Okazaki fragments which can be chased into larger material are produced. DNA replication is inhibited by pancreatic DNAase, trypsin, or exonuclease III in addition to nalidixic acid or sulfhydryl-blocking agents. Phage T4 DNA synthesis is reduced in plasmolyzed preparations from cells infected by amber mutants defective in phage DNA replication, namely genes 1, 32, 41, 42, 43, 44, 45 (Wovcha et al., 1973). It is worth noting that Collinsworth and Mathews (1974) found genes 41 and 62 not to affect DNA synthesis in the cellular in vitro system. Filamentous virus in the stage of RF replication was found to produce the two hybrid classes of RF (see above: ϕX RF in nucleotide permeable cells), and some material tentatively explained as fully heavy RF and mature viral single strands (Staudenbauer and Hofschneider, 1973; Staudenbauer, 1974a).

3. Agar-Embedded Cells and Cellophane Disks

After rigorous destruction of the cell by penicillin or lysozyme together with the detergent "Brij 58," a matrix is required to prevent the disorganization of the cell and the chromosome. Embedding *E. coli* in agar (Smith et al., 1970) or lysing them on cellophane disks (Schaller et al., 1972) creates a system open to the addition of macromolecules in addition to the properties of nucleotide permeable cells. The cellophane disk system keeps macromolecules of the cells in in vivo concentrations, while the small molecules diffuse through the cellophane and can be replaced by precursors for DNA synthesis. The *E. coli* chromosome replicates in a semiconservative and discontinuous manner. Two size classes of fragments, 10 S and 30 S, are generated (Olivera and Bonhoeffer, 1972a). These classes exhibit cross hybridization, but do not hybridize among themselves (Herrmann et al., 1972), indicating that each class is derived from one of the two chromosomal strands (Fig. 1).

Mutants in *dna*G are impaired for initiation of the 10 S segments (Okazaki pieces) at elevated temperature (LARK, 1972a). They produce larger DNA with diminishing initiation events, and DNA synthesis ceases after 2 min in vivo and 20 min on cellophane disks.

Phage φX174 single-stranded DNA was added to the cells before lysis on the cellophane disks, and could be converted to RF II and, after a lag, to RF I (OLIVERA and BONHOEFFER, 1972b). The sealing to the doubly closed form was inhibited by NMN suggesting an involvement of DNA ligase.

Complementation of mutant cells lysed on cellophane disks led to the purification of the *pol*C product DNA polymerase III (OTTO et al., 1973) as well as the *dna*G product (KLEIN et al., 1973). Under these conditions the polymerase III was less salt sensitive than in an assay with DNA. Phage λ-infected cells replicate phage DNA on the disk system (KLEIN and POWLING, 1972). Mixed lysates with mutants in genes O and P complement the deficient products for DNA synthesis.

4. Membrane Enriched Lysates

As will be discussed in the next chapters, many features of cellular DNA replication are not membrane bound. But the attachment of replicating DNA to a fast-sedimenting cell structure made those subcellular fractions attractive for studies on DNA synthesis. T4-infected lysed cells showed DNA synthesis on cell structures which were retained on glass fiber filters (MILLER and KOZINSKI, 1970b), or sedimented through sucrose (OKAZAKI et al., 1970). Complementation of partial lysates from cells infected with phage T4 mutants has led to the purification of the T4 specific proteins coded by genes 44 and 62 (BARRY and ALBERT, 1972).

Lysed, phage φX174-infected cells, in the stage of RF replication, were sedimented and incubated with nucleotides for DNA synthesis (KNIPPERS and STRÄTLING, 1970). The bromouracil-substituted RF was separated into the two classes of hybrid density, but the assumption of fully heavy RF for the band of highest density—presumably RF with a heavy viral strand—seems to be a misinterpretation (see MÜLLER-WECKER et al., 1972).

C. Soluble Cell Extracts

1. Filamentous Virus and Phage φX174 SS→RF Conversion

Gentle lysis of cells and sedimentation of the debris produce a DNA-free cell extract capable of converting single-stranded phage DNA to double-stranded RF (WICKNER, W. et al., 1972). DNA synthesis is dependent on the ribo- and deoxynucleoside triphosphates and the addition of isolated phage DNA. As in vivo, replication of filamentous virus DNA is inhibited by rifampicin, whereas φX174 DNA is not. The extracts convert both viral DNAs to RF II with an open complementary strand. FV-DNA requires the *pol*C gene

product (GEIDER and KORNBERG, 1974), but not those of *dna*B, C, and G (WICKNER, R.B., et al., 1972b), all of which are required for ϕX174 SS to RF conversion (SCHEKMAN et al., 1972; WICKNER, R.B., et al., 1972b). In the latter reaction a *dna*B analog protein, produced by prophage P1 in *dna*B mutants, can replace defective *dna*B protein in mutant extracts (SCHUSTER et al., 1975), a situation similar to the in vivo findings. Mutant extracts can be complemented by wild type protein fractions, resulting in the isolation of the unknown gene products (see Chapter VI, A2). The RNA, priming both the *E. coli* RNA pol-dependent FV reaction and the RNA pol-independent ϕX174 DNA synthesis, was demonstrated by label transfer and by sealing the open strand to alkali labile RF I with T4 ligase (WESTERGAARD et al., 1973). The FV-RF II has a gap at a specific site on the genome, indicating specific initiation and termination for the DNA synthesis (TABAK et al., 1974). This was shown by analysis with restriction enzymes, with fingerprints and annealing of the DNA after S_1 nuclease treatment. The fragments in the gap region and the 5′ end of the complementary strand have been assigned to genes 4 and 2 (SEEBURG and SCHALLER, 1975).

A specific location of the gap in RF II was also found for phage G4 SS→RF conversion in soluble cell extracts (ZECHEL et al., 1975). This phage is similar to ϕX174 (GODSON, 1974b) and shows also rifampicin-resistant initiation of DNA replication in vitro. No specificity could be demonstrated for ϕX174 RF II synthesized in soluble extracts (TABAK et al., 1974; LAUPPE, 1976).

2. Synthesis on Isolated *E. coli* Chromosomes and on Plasmid DNA

Cell extracts are also useful for studies on the replication of duplex DNA. The isolated, folded *E. coli* chromosome synthesizes DNA which sediments as short chains in alkali (KORNBERG, T., et al., 1974). Folded chromosomal DNA from mutants with an immediate shutoff of DNA replication at restrictive temperature (*dna*B, G, and *pol*C) display a thermolabile DNA synthesis in the presence of the mutant extract, and can be complemented by wild type proteins (NÜSSLEIN et al., 1975).

The DNA of the colicin E1 plasmid has been successfully used for replication in soluble cell extracts (SAKAHIBARA and TOMIZAWA, 1974a and b; TOMIZAWA et al., 1975). Replication of the doubly closed circular template depends on the four ribonucleoside triphosphates and RNA polymerase for initiation, and short chains (6 S) are synthesized as intermediates. They finally appear in super-coiled, completely replicated molecules, which are not found in the presence of glycerol or spermidine even though these two compounds increase the initial replication rate of ColE1 DNA added to cell extracts (TOMIZAWA et al., 1975). When examined by electron microscopy, the replicated plasmid DNA had loops with one end 17% from the EcoRI restriction site (TOMIZAWA et al., 1974) in agreement with in vivo results (LOVETT et al., 1974; INSELBURG, 1974). The resolution of the system into purified components is still in progress.

3. Replication of Phage λ and Phage T7 DNA

DNA synthesis with exogenous λ DNA requires the O and P gene products of an extract prepared from phage-infected cells (SHIZUYA and RICHARDSON, 1974), but the DNA synthesized is not more than 1 % of the added template. In that respect T7 DNA is much more efficient, as DNA up to 3-times the amount of template DNA is synthesized (HINKLE and RICHARDSON, 1974). In the T7 in vitro systems the generation of short DNA (11 S) is dependent on the phage T7 genes 4 and 5 besides other proteins and the four ribonucleoside triphosphates. Complementation with protein fractions from wild type T7-infected cells has given some purification of the gene 4 product (STRÄTLING and KNIPPERS, 1973; HINKLE and RICHARDSON, 1974 and 1975).

The preparation and properties of in vitro systems like nucleotide permeable cells, partially lysed cells and soluble extracts have been reviewed by various authors in the collective volume *DNA Replication*, edited by R.B. WICKNER (1974).

IV. RNA as Initiator for DNA Replication

The inability of the known DNA polymerases to start synthesis on an unprimed DNA template was a serious limitation in understanding DNA replication. The discovery that the synthesis of an RNA primer on the DNA template could be the missing inititation event for DNA synthesis, led to new emphasis in that direction. The initial finding was that the filamentous viruses could not form their parental RF upon infection of male *E. coli* in the presence of rifampicin (BRUTLAG et al., 1971), a specific inhibitor of *E. coli* RNA polymerase. From that time on, numerous publications dealing with RNA involvement in DNA synthesis appeared in rapid succession.

A. Methods to Demonstrate Initiator RNA

Even if RNA priming of DNA replication is a general phenomenon, not all experiments published to date prove it. The methods used to show covalent linkage of RNA and DNA include:

1. Label transfer (from DNA to a ribonucleotide after incorporation of [α-^{32}P] deoxynucleoside triphosphates into the RNA-primed DNA followed by hydrolysis in alkali): This procedure demonstrated the use of an RNA primer by filamentous virus and phage φX174 SS→RF conversion in soluble cell extracts (SCHEKMAN et al., 1972); by the FV reaction with DNA unwinding protein, RNA pol and DNA pol II (MOLINEUX et al., 1974); and by *E. coli* short segments synthesized in toluenized cells (SUGINO and OKAZAKI, 1973). FV RF II (phage M13) synthesized in soluble extracts showed a significant release of 3′ (2′) adenosinemonophosphate after label transfer, whereas the other systems did not give any specificity.

2. Cosedimentation (of labeled RNA and DNA under conditions when noncovalent RNA-DNA hybrids would be denaturated: formaldehyde, formamide,

dimethylsulfoxide, urea, heat): This method demonstrated the RNA primer attached to G4 DNA after replication with *dna*G protein, DNA unwinding protein, and DNA polymerase III holoenzyme (Bouché et al., 1975); and, in a model experiment, synthesis on a multiply primed ϕX174 DNA (Keller, 1972).

3. *Density shift* (of the DNA in Cs-salt gradients before or after denaturation and demonstrated by alkali or RNAase sensitivity of the RNA-DNA linkage): This technique showed the covalent nature of the short RNA-DNA chains isolated from *E. coli* in vivo (Sugino et al., 1972; Hirose et al., 1973), for those chains in a synthetic system using ϕX174 DNA as template (Keller, 1972), and for early T7 (Miller, 1972) and those of T4 (Buckley et al., 1972) replicative intermediates.

4. *Sealing* (of RNA into a DNA chain and lability of this structure to alkali or RNAase): Using this method RNA was joined with T4 DNA ligase into FV and phage ϕX174 RF (Westergaard et al., 1973); and RNA was found in ColE1 plasmid DNA when grown in the presence of chloramphenicol (Blair et al., 1972).

5. *Nature of the 5'-OH terminus:* Covalently linked RNA protects DNA ends from phosphorylation with polynucleotide kinase. The label introduced in RNA ends is labile to alkali or RNAase, and this treatment creates new 5'-OH ends upon hydrolysis. This method has demonstrated RNA primed short-chain intermediates in vivo (Hirose et al., 1973; Okazaki et al., 1975).

6. *Hybridization* (of a distinguished RNA to one strand of a duplex DNA): This procedure has been used with phage λ to demonstrate the minor leftward RNA transcribed from the l-strand (Blattner and Dahlberg, 1972), which depends on the functions of phage genes O and P, and the host genes *dna*B and G (Hayes and Szybalski, 1973); with phage T7 to demonstrate that an RNA made during initiation of replication will anneal to the right strand of T7 DNA (Miller, 1972); and with phage T4 to demonstrate that an RNA detected shortly after phage infection will hybridize to the left strand of T4 (Buckley et al., 1972).

The following arguments oppose the experimental data for RNA-DNA linkage:

to 1. The label transfer has a low probability, i.e., one transfer occurs in a long DNA chain, as compared to one per 6000 nucleotides for the RF of small phages. The transfer experiment might pick up contaminations yielding 3'-ribonucleotides. This could occur after misincorporation of a ribonucleotide into the DNA chain and nucleolytic cleavage at the 5'-ribo-junction before isolation of the DNA. Another explanation may be some cobanding of a mixed polymer generated by ribo- and deoxynucleotide phosphorylase action, or by attachment of one or a few deoxynucleotides to 3' RNA termini.

to 2. The rapid annealing of RNA to DNA may not be strictly excluded for residual RNA even under denaturing conditions.

to 3. Pancreatic ribonuclease also degrades hybrid RNA at moderate or high enzyme concentrations, low salt and elevated temperature (Tabak and

BORST, 1971). Therefore to prevent the degradation of RNA-DNA hybrids the conditions have to be carefully checked.

to 4. RNA from other transcriptional events could be sealed into DNA or a misincorporation of a ribonucleotide into DNA, which was being excised by an endonucleolytic cut at the 5′ side, could be integrated by the ligase action.

to 5. Phosphate exchange between [γ-^{32}P] ATP and DNA with a 5′-P terminus can occur in the kinase step. This side reaction is reduced at low temperature (OKAZAKI et al., 1975). RNA-linked, non-replicative intermediates, such as DNA with a misincorporated ribonucleotide, would also produce 5′-OH DNA ends upon alkaline hydrolysis.

to 6. The RNA annealing specifically to one phage strand could serve other purposes than priming DNA replication.

Improved techniques such as sequencing nucleotides in the RNA-DNA junction or collection of evidence in favor of RNA priming a given replicative event, will provide more convincing arguments one way or the other. Such arguments have been outlined for FV SS to RF conversion: inhibition by rifampicin in cells (BRUTLAG et al., 1971), or in soluble extracts (WICKNER, W., et al., 1971); direct requirement for functional RNA polymerase (GEIDER and KORNBERG, 1974); label transfer (SCHEKMAN et al., 1972), and alkaline labile sealing with T4 ligase (WESTERGAARD et al., 1973). RNA in crude or even partially purified *E. coli* systems is susceptible to the abundant RNAase activities; however, RNA is more stable in higher organisms, where the RNA-DNA linkage has been demonstrated by label-transfer in 4 S segments from polyoma virus infection (PIGIET et al., 1974; REICHARD et al., 1974). The unspecific RNA-DNA junction suggests that the switch from the primer to DNA synthesis is determined by the length of the RNA rather than the nucleotide sequence. The primer was found ten nucleotides long with an ATP or GTP attached to the 5′ end.

B. Sequencing of Initiator RNA

The RNA segments in ColE1 DNA (WILLIAMS et al., 1973), rescued in the presence of chloramphenicol, consist of 38 ribonucleotides with a base composition of 17 G, 5 A, 8 C, and 8 U when derived from the light strand and 15 ribonucleotides with 5 G, 2 A, 4 C, and 4 U found in the heavy strand (HELINSKI et al., 1975). Partial 5′-terminal sequences of the RNA segments and the DNA junctions were determined in the light strand to be: 5′...p(dC)p(rG)p(rG)p(rG)p(rG)p(rA)p(rC)... 3′ and in the heavy strand to be: 5′...p(dA)p(rG)p(rA)p(rA)p(rU)p(rG)... 3′. Unlike the RNA segment in the light strand, the heavy strand RNA segment appears to be distributed randomly or at multiple sites. This can be expected for the residual RNA priming Okazaki pieces which are intermediates of ColE1 synthesis (HELINSKI et al., 1975).

The junction of the 4 S primer RNA in Rous sarcoma virus with DNA after reverse transcription was determined as an adenylate end of the primer linked

to the deoxysequence d(AATGAAGC . . .) (TAYLOR et al., 1974), This initiator RNA is a t-RNA-like molecule and appears to be ubiquitous in higher eukaryotic cells (SAWYER et al., 1974). The minor leftward RNA, synthesized by purified RNA polymerase on phage λ DNA, has been entirely sequenced (DAHLBERG and BLATTNER, 1973). This 81-nucleotide-long RNA has been suspected as a primer for λ DNA synthesis and starts with pppGUU and is terminated by . . . $(U)_6$ A-OH. Aside from the finding that the formation of the *oop* RNA depends on genes required in DNA replication there is no evidence for a direct initiator function of this species of λ RNA.

C. Enzymatic Synthesis and Removal of Initiator RNA

The only well-characterized, template-dependent RNA synthesizing activity in the *E. coli* cell is RNA polymerase (recently reviewed by CHAMBERLIN, 1974a). Its involvement in DNA replication has been shown via inhibition by rifampicin for *E. coli* (MESSER, 1972; LARK, 1972b), for plasmids like ColE1 (CLEWELL et al., 1972), for the F-episome (BAZZICALUPO et al., 1972), for minicircular DNA in coli 15 (MESSING et al., 1972), for phage λ DNA synthesis (KLEIN and POWLING, 1972), for filamentous virus single strand SS to RF conversion and for RF replication (BRUTLAG et al., 1971); but not for phage φX174 SS to RF conversion (SILVERSTEIN and BILLEN, 1971) or for the formation of Okazaki pieces during *E. coli* replication. Since soluble extracts from *E. coli* cells can discriminate the RNA polymerase requiring FV reaction from the rifampicin-resistent φX174 DNA synthesis (WICKNER, W., et al., 1971), WICKNER and KORNBERG (1974a) were able to isolate a small factor which renders RNA polymerase inert for φX174 single-stranded DNA in the presence of DNA unwinding protein. The factor-RNA pol complex, named RNA pol III, can also poorly transcribe duplex DNA.

RNAase H with a molecular weight of 40000 daltons specifically degrades RNA-DNA hybrids (HENRY et al., 1973; MILLER, H. I., et al., 1973). A role for this enzyme in the removal of the initiator RNA has been proposed. This reaction stimulates RNA-primed DNA synthesis with DNA pol II (BERKOWER et al., 1973). RNAase H degrades only RNA-DNA hybrids and acts as an endonuclease, which cannot cleave the phosphodiester bond covalently linking ribonucleotides to the 5′ end of the DNA. The 5′→3′ exonuclease activity in DNA pol I can also degrade hybrid RNA from the 5′-end (ROYCHOUDHURY and KÖSSEL, 1973; BERTSCH and KORNBERG, cited in WESTERGAARD et al., 1973). This function may be essential for the cell, since a mutation in the 5′ exonuclease part of DNA pol I is conditionally lethal (KONRAD and LEHMAN, 1974).

T-phages code for proteins which complex with the host RNA polymerase or modify subunits of the enzyme. Neither the phage-modified host RNA polymerase nor phage T3- and phage T7-induced RNA polymerase appear to be involved in priming T-phage DNA synthesis.

Phage ϕX174 single-stranded DNA serves as an efficient template for transcription with T7-induced RNA polymerase (CHAMBERLIN and RING, 1973). With ATP and ϕX174 DNA present the T7 enzyme catalyze the formation of polyadenylic acid, a primer for DNA synthesis with T7 DNA polymerase on the single-stranded template (SCHERZINGER and LITFIN, 1974a). This model reaction reveals that poly(A) may also serve as a suitable primer for DNA synthesis.

Novel proteins, initiating phage DNA synthesis probably in a physiologic manner have been partially characterized: Phage-induced gene 4 protein for T7 replication (SCHERZINGER and LITFIN, 1974b; HINKLE and RICHARDSON, 1975) and *E. coli dna*G protein for phage G4 SS→RF conversion (ZECHEL et al., 1975; BOUCHÉ et al., 1975). Gene 4 product may be involved in the synthesis of H-strand DNA of phage T7, since the reduced rate of DNA synthesis in gene 4 mutant extracts led to fragments hybridizing to the H-strand. The addition of isolated gene 4 protein reconstituted the annealing of the newly synthesized DNA to both strands (STRÄTLING and KNIPPERS, 1973).

The physiologic initiation of ϕX174 DNA requires many host gene products including those coded by *dna*B, C, and G as well as proteins named i and n (SCHEKMAN et al., 1975) or X, Y, and Z (WICKNER, S., and HURWITZ, 1974). The reactions of priming and DNA synthesis on ϕX174 DNA were separated into two stages, and the priming event shown to be dependent on the presence of the four ribonucleoside triphosphates (SCHEKMAN et al., 1974); however, DNA synthesis of the two stage incubation was only 20% of the simultaneous reaction.

D. Initiation Different from RNA Priming

In contrast to SCHEKMAN et al. (1974), WICKNER and HURWITZ (1974) only found a requirement for ATP and an insignificant stimulation by CTP, GTP, and UTP for phageϕX174 DNA replication with purified proteins. In addition, the incorporation of less than 0.02 pmol A per 0.06 pmol complementary strands synthesized, led them to conclude that a protein-AMP-DNA complex may prime the ϕX174 SS to RF conversion without concomitant RNA synthesis. These results could also be explained by poly(A) priming and subsequent degradation of a primer RNA by the 5'→3' exonuclease function of DNA pol III (LIVINGSTONE and RICHARDSON, 1975) or by contaminating RNAase. Thus, an accumulation of facts does support the idea of the initiator RNA as a prerequisite for DNA synthesis.

DNA can also serve as a primer for further DNA synthesis, as in RF→RF replication and RF→SS synthesis for small bacteriophages, and in the late stage of phage λ replication. A rolling circle (see Fig. 3) extends one strand at the 3' end, while the other strand is formed via RNA priming. Other models involving the extension of existing DNA have been discussed (see KLEIN and BONHOEFFER, 1972), but now appear less likely since RNA is connected with many replicative events.

V. Proteins Involved in DNA Synthesis

A. Bacterial and Phage-Induced DNA Polymerases

DNA replication relies on the action of DNA polymerases. Their catalytic property is the stepwise addition of 5′ deoxynucleotides to the 3′ OH end of primers. The *E. coli* DNA polymerases have different roles in DNA metabolism which is expressed in their turnover numbers, their nuclease activities, their number of copies in the cell and their interaction with other proteins. For reviews on bacterial DNA polymerases see KORNBERG (1974), GEFTER (1974), or KORNBERG and KORNBERG (1974)

1. DNA Polymerase I

Many features of DNA polymerase I are representative for bacterial or eukaryotic DNA polymerases and will therefore be briefly summarized. Multiple sites enable the enzyme to simultaneously bind the template (single-stranded DNA) (ENGLUND et al., 1969b), the 3′-OH primer terminus (RNA or DNA) (GOULIAN, 1968), and a nucleoside triphosphate (ENGLUND et al., 1969a), with the enzyme recognizing the deox-configuration and matching the nucleotide with the template. After a correctly base-paired deoxynucleoside triphosphate is activated for a nucleophilic attack on the primer terminus, the pyrophosphate group is cleaved off and the deoxyribonucleoside monophosphate attached to the primer. Since this chain elongation probably interferes with the binding of the polymerase to the template, the enzyme diffuses from the DNA (MCCLURE and JOVIN, 1975; CHANG, 1975) and enters mostly the same site for catalyzing the next incorporation step (UYEMURA et al., 1975). The primer end is always checked for proper base pairing. Mismatching of one or more bases will be recognized and those ends subsequently degraded by the 3′→5′ exonuclease activity of the enzyme (BRUTLAG and KORNBERG, 1972). This proofreading function of DNA pol I reduces copying errors. The 3′→5′ exonuclease activity is directed against linear single-stranded DNA and acts on nicked duplex DNA in the absence of deoxynucleoside triphosphates. As alkaline pH and high temperature enhance the hydrolysis, temporarily frayed ends of the duplex serve as the single-stranded DNA substrate.

The primer must have an exposed 3′-OH group in the ribo-configuration (KORNBERG, 1969). Other groups like 3′-phosphate, 3′-deoxy, 3′-fluoro or 3′-arabinosyl residues are unproductive. A mismatched ribonucleotide will also be removed by the 3′→5′ exonuclease (KÖSSEL and ROYCHOUDHURY, 1974).

A second exonuclease activity is associated with DNA pol I. This activity initiates attack on double-stranded DNA from the phosphorylated or hydroxyl 5′ terminus (KLETT et al., 1968; DEUTSCHER and KORNBERG, 1969) releasing mono- or oligonucleotides. The weak incorporation of dUTP in ether-treated cells (GEIDER, 1972) could indicate an endonucleolytic degradation of uracil-containing DNA by the 5′→3′ exonuclease activity of DNA pol I (WOVCHA and WARNER, 1973). The 5′→3′ exonuclease in DNA pol I is enhanced by concomitant polymerization. This activity can be proteolytically cleaved from a

peptide with the DNA polymerizing activity (KLENOW and HENNINGSEN, 1970; BRUTLAG et al., 1969), and then these fragments can be reconstituted to yield the features of DNA pol I (SETLOW and KORNBERG, 1972). Sequencing the N-terminal amino acids of the two fragments and the DNA polymerase I has shown that the small fragment with the 5'→3' exonuclease is located at the N-terminus and that the large fragment is located at the side of the C-terminus of DNA pol I (JACOBSEN et al., 1974):

DNA pol I: Met-Val-Glx-Ile-Pro-Glx-Leu-Asx ...	109000 dalton, 993 amino acids
Small fragment: Met-Val-Glx-Ile ...	35000 dalton, 326 amino acids
Large fragment: Val-Ile-Met ...	75000 dalton, 675 amino acids

DNA polymerase contains one disulfide bond and a single reactive sulfhydryl group (JOVIN et al., 1969), which can be modified by iodoacetic acid, mercury, or N-ethylmaleimide without affecting the polymerase or exonuclease activities. This is in contrast to the sensitivity of DNA pol II or DNA pol III against sulfhydryl blocking agents. The zinc ion intimately bound to each of the DNA pol I molecules (SPRINGGATE et al., 1973) may facilitate the deprotonation of the primer 3'-OH group and release a counterion from the enzyme upon binding to DNA. The enzyme has been recovered in a large protein complex, presumably associated with *rec*BC endonuclease (HENDLER et al., 1975). DNA pol I does not bind to intact duplex regions of DNA (GRIFFITH et al., 1971), but uses nicked double-stranded DNA or primed single strands for synthesis. Long m-RNA with secondary structure is nicked endonucleolytically by DNA pol I and used as primer template for DNA synthesis at high enzyme-RNA ratios (TRAVAGLINI and LOEB, 1974). This reverse transcription is common for RNA tumor virus DNA polymerases (recently reviewed by TEMIN and MIZUTANI, 1974). DNA pol I can catalyze de novo synthesis of poly (dAT), but undetectable DNA contaminations in the polymerase may prime the reaction (KORNBERG, 1974). Whereas DNA pol I uses all combinations of deoxy- and ribopolymers with primer oligonucleotides (KARKAS, 1973; WELLS et al., 1972), neither DNA pol II nor III can work on single-stranded homopolymers with only a few primers annealed (LEHMAN and CHIEN, 1973).

2. DNA Polymerase II

DNA polymerase II is the stable polymerizing activity found in *pol*A cells (KNIPPERS, 1970; KORNBERG and GEFTER, 1971; MOSES and RICHARDSON, 1970a). The enzyme has a molecular weight of 120000 daltons and an associated 3'→5' exonuclease activity which degrades single-stranded DNA (WICKNER, R.B., et al., 1972a). As the 5'→3' exonuclease is missing, DNA polymerase II cannot perform nick translation like DNA pol I. DNA pol II requires a gapped duplex DNA for DNA synthesis. Longer single-stranded regions inhibit polymerization since DNA pol II cannot act on regions with secondary structure. *E. coli* DNA unwinding protein prevents this effect (SIGAL et al., 1972) and also forms a complex with the polymerase (MOLINEUX and GEFTER, 1974).

3. DNA Polymerase III

DNA polymerase III is very sensitive to salt and is stimulated by a less polar environment like 90% water, 10% ethanol (KORNBERG and GEFTER, 1972). The purified enzyme has a low affinity for deoxynucleoside triphosphates and can only add a few nucleotides to a primer. Its suggested molecular weight of 140000 daltons (KORNBERG and GEFTER, 1972; OTTO et al., 1973) has been refined after the purification of the enzyme to homogeneity (LIVINGSTON et al., 1975). DNA pol III has in addition to the large protein a 40000-dalton subunit, and a 3'→5' exonuclease activity as well as a single strand directed 5'→3' exonuclease activity (LIVINGSTON and RICHARDSON, 1975). Hydrolysis at the 5' single-stranded end releases oligonucleotides and proceeds into the double-stranded region. The strand specificity distinguishes the 5'→3' exonuclease of DNA pol I and DNA pol III.

4. DNA Polymerase III Holoenzyme

A more physiologic form, named DNA polymerase III*, has been isolated, and is, in contrast to DNA pol III described above, capable of synthesizing long DNA chains on a RNA-primed single-stranded template (WICKNER, W., et al., 1973). This form is also physically different from the DNA pol III, described by KORNBERG and GEFTER (1972). The differences include its binding to phosphocellulose and DEAE-cellulose at high pH; its fast migration in biogel; and its ability to complement *pol*C mutant extracts for single strand phage DNA replication (WICKNER, W., et al., 1973) or *E. coli* replication on cellophane disks (OTTO et al., 1973). However, a fresh preparation of DNA pol III prepared according to the procedure of KORNBERG and GEFTER (1972) contains DNA pol III* activity as measured in the complementation assay (GEIDER, unpublished observation). This confirms the complementation assays of OTTO et al. (1973) and MOLINEUX et al. (1974) with DNA pol III. Mild heat treatment, aging, or freezing and thawing converts the DNA pol III* activity into a form of DNA pol III unable to complement *pol*C mutant extracts or to synthesize long stretches of DNA on primed single strands; however, it is still active on extensively gapped duplex DNA (WICKNER, W., et al., 1973). A protein, called copolymerase III*, with a molecular weight of 77000 daltons (WICKNER, W., et al., 1973), forms a complex with DNA pol III* at the 3' end of an RNA primer in the presence of ATP (WICKNER, W. and KORNBERG, 1973). These two proteins can also be isolated as a holoenzyme complex (WICKNER, W. and KORNBERG, 1974b), containing equal molar ratios of copol III* (MW 77000 daltons) and DNA pol III* (MW 90000 daltons in SDS). The native molecular weight of the holoenzyme, around 350000 daltons, suggests a tetramer with two subunits of each protein. DNA pol III* appears highly asymmetric on gel-filtration, but sediments like DNA pol III in glycerol gradients, and may consist of 2 or 4 subunits with molecular weights of 90000 daltons (WICKNER, W., et al., 1973) which contrasts to the molecular weights of the DNA pol III subunits. HURWITZ and WICKNER (1974) have isolated two

Table 3. Forms of DNA polymerase III

					Reference
DNA pol III holoenzyme	⇄	DNA pol III*	(dissociation: →	phosphocellulose	[1, 2]
		+ 2 copol III*	(aggregation:) ←	complex with RNA primer	[3]
DNA pol III*	⇄	DNA pol III	(inactivation:) →	heat, aging, dilution	[1]
			(restoration:) ←	addition of factor II (MW 150 000 daltons)	[4, 5]
DNA pol III	→	subunits	in SDS gel a)	1 large peptide (MW 140 000 daltons)	[5, 6]
			and	1 small peptide (MW 40000 daltons)	[5]
			b)	2 identical peptides (MW 90 000 daltons)	[1]
DNA pol III holoenzyme	→	subunits	in SDS gel:	2 peptides; MW 90 000 daltons (polymerase subunits)	[2]
			and	2 peptides; MW 77 000 daltons (copolymerase subunits)	
Copolymerase III*			in SDS gel and native:	77 000 daltons	[1]
Factor I (comparable with copol III*)			in glycerol gradient:	90 000 daltons	[4]

References: 1. Wickner, W. et al. (1973); 2. Wickner, W. and Kornberg (1974b); 3. Wickner, W. and Kornberg (1973); 4. Wickner, S. and Hurwitz (1974); 5. Livingston et al. (1975); 6. Kornberg and Gefter (1973); Otto et al., 1973.

factors that stimulate DNA pol III in DNA replication. Elongation factor I has been assumed to be identical with copol III*; elongation factor II to be necessary for DNA pol III to replicate long chains. Thus, factor II would restore DNA pol III* activity from a DNA pol III form unable to replicate long DNA segments (Livingston et al., 1975). Possible interconversions of DNA pol III are listed in Table 3.

5. DNA Polymerase Inhibitors

DNA synthesis with *E. coli* DNA polymerases can be inhibited by di-deoxy-analogs of nucleoside triphosphates. DNA pol I attaches ddATP (Toji and Cohen, 1969) or ddTTP (Atkinson et al., 1969) to growing 3′ DNA ends. The latter compound is even more inhibitory for DNA replication in ether-treated cells (Geider, 1972). This agrees with a discriminating effect of ddTTP on the three *E. coli* polymerases (Berthold and Geider, unpublished), where highest inhibition is obtained with the DNA pol III holoenzyme. Inhibition of DNA pol II and possibly of DNA pol III can be achieved by arabinosyl-CTP (Rama Reddy et al., 1971). A 8000 dalton protein inhibits the three DNA polymerases by its strong binding to single- and double-stranded DNA (Berthold and Geider, unpublished) and displaces *E. coli* DNA unwinding protein in the complex with FV-DNA (Zentgraf, Berthold, Geider, unpublished).

Table 4. A comparison of

DNA polymerase	Coded by gene	Conditional lethal mutants	3′–5′ Exonuclease	5′–3′ Exonuclease	Molecules per cell	Molecular weight (daltons)
DNA pol I	*pol*A	+	+	+	400	109 000
DNA pol II	*pol*B	–	+	–	< 17	120 000
DNA pol III	*pol*C	+	+	+	< 10	180 000
DNA pol III holoenzyme	*pol*C	+	+	(+)	(< 10)	~350 000
Phage T7 induced DNA pol	5/*tsn*C[b]	+	+	–		96 000
Phage T4 induced DNA pol	43	+	+	–	600	112 000

6. Phage T4 DNA Polymerase

Phage T4 induces a DNA polymerase, which is distinct from the host DNA polymerases (GOULIAN et al., 1968). The absence of an associated 5′→3′ exonuclease has concentrated the efforts on the 3′→5′ exonuclease. A fragment of the T4 DNA pol was isolated from an amber mutant exposing only the 3′→5′ exonuclease avtivity (NOSSAL and HERSHFIELD, 1971). The molecular weight of the fragment (82000 daltons) is in agreement with the position of the amber mutation on the genetic map. Mutator or antimutator strains of T4 are known to map in gene 43, the gene for DNA polymerase. MUZYCZKA et al. (1972) could link elevated spontaneous mutations to impaired 3′→5′ exonuclease activity, and low spontaneous mutations to an efficient proofreading function of the exonuclease.

T4 polymerase can also synthesize DNA on a linear single-stranded template. A short sequence at the 3′ terminus folds back, forming a loop by base-pairing with an internal nucleotide sequence (ENGLUND, 1971). The 3′→5′ exonuclease degrades the free end up to the base-paired region, where the polymerase avtivity begins incorporating deoxynucleoside triphosphates. Observed misincorporation by T4 DNA polymerase is 10^{-5}–10^{-6} (HALL and LEHMAN, 1968), but the actual incorporation of false nucleotides appears to be much higher, since deoxynucleoside triphosphates not found in the product polymer are also incorporated into the DNA (HERSHFIELD and NOSSAL, 1972) and subsequently removed by the proofreading function of the 3′→5′ exonuclease.

7. Phage T7 DNA Polymerase

T7 DNA polymerase (OEY et al., 1971; GRIPPO and RICHARDSON, 1971) shows only 3′→5′ exonuclease activity, has a molecular weight of 96000 daltons and properties similar to phage T4 induced DNA polymerase. This

various DNA polymerases

Template specificites					Salt optimum in mM	pH optimum	Inhibition by sulfhydryl inhibitors
Primed single strand	Intact duplex	Nicked duplex	Duplex with small gaps	Duplex with large gaps			
+	−	+	+	+	100	7.4	−
−	−	−	+	−	80	7.8	+
−	−	−	+	−	< 20	7.0	+
+[a]	−	−	+	+	< 20	7.5	+
+	−	−	+	+	50	7.7	+
+	−	−	+	+	50	8.5	+

References may be found in the text.
[a] In the presence of spermidine or DNA unwinding protein.
[b] Phage gene 5 and host gene *tsn*C.

includes stimulation of polymerization by a phage-induced DNA binding protein (REUBEN and GEFTER, 1973). The polymerizing activity of T7 DNA polymerase molecule relies on a protein coded by phage gene 5 and another protein coded by the host (MODRICH and RICHARDSON, 1975a). Phage T7-infected *tsn*C mutants do not propagate the phage (CHAMBERLIN, 1974b) and complementation of extracts derived from the infected cells resulted in the purification of the gene product from wild type cells. A 12000-dalton heat-stable protein which restores the phage polymerase activity was thus purified to near homogeneity (MODRICH and RICHARDSON, 1975a). It forms a tight one-to-one complex with the T7 gene 5 protein (84000 daltons) and is dissociated from homogenous T7 DNA pol by heat treatment (MODRICH and RICHARDSON, 1975b). This *tsn*C protein is identical with thioredoxin (MARK and MODRICH, 1975), a cofactor of ribonucleotide reductase, as judged by several criteria including molecular weight, stability, amino acid composition, and enzyme activities.

Some properties of these various DNA polymerases are listed in Table 4. The physiologic importance of *E. coli* DNA polymerases III and I and of the T-phage polymerases has been clearly demonstrated by the isolation of conditional lethal mutants in genes coding for these polymerases.

B. DNA Binding Proteins

Many proteins in a cell extract bind to single-stranded DNA and can be eluted by varying the salt concentration from DNA cellulose or agarose columns. Proteins with a high affinity for denaturated DNA (eluted with 0.4 M

salt or higher concentrations) and with no known enzymatic activity shall be definded as DNA binding proteins.

Their importance in replication may be a regulatory effect such as the binding of these proteins to single-stranded regions in replicative forks, thus protecting some parts from initiation. Distinct sequences like the partially double-stranded region of the origin of replication in single-stranded FV-DNA (SCHALLER et al., 1976) will be spared by binding proteins, but they can still be available for the attachment of proteins initiating DNA synthesis. A comparable regulatory function has been discussed for the histone arrangement in eukaryotic DNA.

SIGAL et al. (1972) have described three proteins with strong DNA binding properties isolated from *E. coli* and having molecular weights of 70000, 20000, and 10000 daltons, respectively. ALBERTS and FREY (1970) isolated such a protein from phage T4-infected cells, and REUBEN and GEFTER (1973) from phage T7-infected cells. The 20000 dalton *E. coli* protein and the T4-induced protein can denature double-stranded DNA at low ionic strength and are therefore called DNA unwinding proteins.

1. *E. coli* DNA Unwinding Protein

E. coli unwinding protein is a tetramer of 76000 daltons with four identical 18500-dalton subunits (WEINER et al., 1975). The heat-stable protein has an isoelectric point of 6.0. It also binds to homopolymers like poly(dC), (dI), (dT), (rI) and (rU), but unefficiently to oligo (dT), poly(dA), (rC), or (rA), and to poly(dAT). The fast binding to single-stranded DNA is followed by a slow release. This cooperative effect leaves excess DNA uncovered, whereas the faster sedimenting protein-DNA complexes contain eight nucleotides bound per monomer DNA unwinding protein (SIGAL et al., 1972). The 800–1200 copies of monomer present per cell are thought to act along single-stranded regions of the replication fork. The DNA unwinding protein stimulates the polymerizing activities of DNA pol II and DNA pol III* but not those of DNA pol I or DNA pol III. The $3' \rightarrow 5'$ exonuclease activites of all three polymerases are inhibited by unwinding protein (MOLINEUX and GEFTER, 1974). At low salt concentration DNA unwinding protein and DNA pol II form a complex which can degrade single-stranded DNA and act on oligo (dT) primed poly(dA). The synthesis of long chains by DNA pol III* in the absence of spermidine is dependent on DNA unwinding protein (GEIDER and KORNBERG, 1974), which also directs the priming RNA polymerase to a unique site of the viral DNA.

2. Phage T4 DNA Unwinding Protein

Gene 32 is essential for phage T4 replication. Its product is a DNA unwinding protein, which is made in 10000 copies per infected cell (ALBERTS and FREY, 1970). Temperature sensitive mutants in gene 32 induce a thermosensitive unwinding protein. The 35000 dalton protein showed both self-association (CARROLL et al., 1972) and cooperative binding to single-stranded DNA as

visualized in the electron microscope (DELIUS et al., 1972). The T4 specific DNA unwinding protein was cleaved in crude extracts to a 27000-dalton protein, which copurified with the larger molecule (HOSODA et al., 1974). The cleavage product appears to denature native T4 DNA even at 0.1 M salt, in contrast to the 32000-dalton protein. Formation of a specific complex between phage T4-induced DNA unwinding protein and the T4 DNA polymerase was concomitant with a stimulation of DNA synthesis (HUBERMAN et al., 1971). In contrast to T4 DNA polymerase alone a mixture of both proteins can synthesize DNA on nicked double strands (NOSSAL, 1974). It has been assumed that the unwinding protein facilitates strand displacement, when the polymerase starts DNA synthesis at the 3′ end of the apposed strand. During early synthesis the products renature rapidly; later on poly(dAT) is predominantly synthesized.

3. Phage T7-Induced DNA Binding Protein

Phage T7 induces a DNA binding protein, which specifically stimulates T7 DNA polymerase (SCHERZINGER et al., 1973; REUBEN and GEFTER, 1973). This protein does not cooperatively bind to single-stranded DNA and cannot denature native T7-DNA, but lowers the melting point of poly(dAT). *E. coli* DNA unwinding protein can substitute for T7-induced DNA binding protein in the stimulation of T7 DNA polymerase.

4. DNA Binding Proteins Coded by Small Bacteriophages

The single-stranded DNA phages induce proteins to shut off RF replication during the synthesis of viral strands for progeny phages (see Fig. 3). Phage ϕX174 uses a complex system including coat proteins (genes F, G) and gene B, C, D proteins (WEISBEEK and SINSHEIMER, 1974). The filamentous viruses produce large amounts of a protein coded by gene 5 which acts in the late stages of phage development (SALSTROM and PRATT, 1971). This protein of 10000 daltons on SDS gels was isolated from infected cells (OEY and KNIPPERS, 1972; ALBERTS et al., 1972) and cooperatively binds to single-stranded DNA at a ratio of four nucleotides per monomer of gene 5 protein. Its synthesis in an in vitro system was demonstrated by KONINGS (1973) and the 87 amino acids of the protein were sequenced by CUYPERS et al. (1974). Gene 5 protein most likely controls phage single-strand DNA synthesis by preventing conversion of the replicative intermediate to RF. A purified protein system for the conversion of single-stranded DNA to replicative form shuts off DNA synthesis after the addition of gene 5 protein sufficient to cooperatively bind to all template DNA (GEIDER and KORNBERG, 1974).

C. Enzymatic Sealing and Nicking of DNA

1. *E. coli* DNA Ligase

The use of short DNA chains as replicative intermediates requires an effective mechanism for their integration into the large chromosomal DNA. *E. coli*

generates a DNA ligase, which has recently been purified to homogeneity (MODRICH et al., 1973). A single polypeptide with a molecular weight of 75000 daltons converts adjacent DNA segments to longer products. The reaction requires NAD to form a ligase-AMP complex, and can be reversed by the addition of the NAD-cleavage product nicotinamide mononucleotide (ZIMMERMAN and OSHINSKY, 1969). The AMP is bound to the enzyme via a phosphoamide bond of the ε-amino group of lysine (GUMPORT and LEHMAN, 1971) and transferred to the 5′-phosphate group of a hybrid DNA terminus (OLIVERA et al., 1968). The formation of the DNA adenylate intermediate is impaired, when the other DNA end is not terminated by 3′ hydroxyl group (LEHMAN, 1974), suggesting an involvement of both DNA ends in intermediate steps of the joining reaction. Unadenylated ligase releases AMP from the activated 5′-DNA terminus while sealing the two DNA ends. Ammonium ions accelerate the overall reaction. The sealing reaction can be reversed at high DNA ligase concentrations in the presence of AMP (MODRICH et al., 1972). In contrast to the ω-protein (WANG, 1971), this system relaxes both negative and positive superhelical DNA.

A possible second DNA sealing activity in *E. coli* cells has been discussed (HESS et al., 1973). The importance of the DNA ligase, described above, for living cells is manifested by the conditional lethal mutant *lig* ts 7 (PAULING and HAMM, 1967). A review on the structure, mechanism, and cellular function of *E. coli* DNA ligase as well as T4-induced DNA ligase has been recently published by LEHMAN (1974).

2. Phage-Induced DNA Ligases

Phage T7 (MASAMUNE et al., 1971) and phage T4 (WEISS and RICHARDSON, 1967) induce a DNA ligase requiring ATP as cofactor. The catalytic steps are similar to the *E. coli* DNA ligase reaction, but AMP and pyrophosphate are the products instead of AMP and NMN. An interesting property of T4 DNA ligase is the intermolecular end-to-end joining reaction of base-paired termini in duplex DNA (SGARAMELLA and KHORANA, 1972). Intramolecular joining or pairing of temporary single-stranded terminal regions of the duplex was ruled out by use of defined substrates preventing side reactions. The linear double-stranded phage P22 can be converted into oligomers with the T4 ligase end-to-end joining reaction which *E. coli* DNA ligase apparently cannot catalyze (SGARAMELLA, 1972).

Whereas the *E. coli* enzyme reacts mainly with DNA segments annealed to a DNA strand, T4-induced DNA ligase has a broad spectrum of joining all combinations of nucleic acids. DNA segments can also be sealed, when hybridized to RNA. RNA chains can be joined, whether they are annealed to either a DNA (FAREED et al., 1971; KLEPPE et al., 1970) or an RNA template (SANO and FEIX, 1974). Furthermore, T4 DNA ligase can join RNA to the 5′ end of DNA or DNA to the 5′ terminus of RNA (NATH and HURWITZ, 1974). Additionally, RNA can be sealed to a 5′ DNA end by *E. coli* DNA ligase. The

rates of these reactions are considerably lower than those for joining DNA in DNA duplexes.

3. Nucleases

Enzymatic cleavage of DNA in the course of replication has been inferred from the growth of bacteriophages. T-phages induce nucleases to degrade the host DNA and reuse the nucleotides for their own DNA synthesis. Some of these enzymes may also be involved in cleavage or degradation of replicative intermediates (STRÄTLING et al., 1973). A likely candidate for such a cleavage activity is a membrane-attached endonuclease induced by phage T7 (PACUMBABA and CENTER, 1974) which may size the concatemeric replicative intermediate into mature phage DNA. No DNA sequence specificity has been demonstrated for either the phage T7 or phage T4 endonucleases.

Gene 6 of phage T7 codes for an exonuclease, which degrades duplex DNA from the 5′ end (KERR and SADOWSKI, 1972b). Its location, next to gene 5 specifying T7 DNA polymerase, and the sum of the respective molecular weights (30000 and 84000 daltons) resemble *E. coli* DNA polymerase I (109000 daltons), as pointed out by KORNBERG (1974). The two proteins may constitute an enzymatic entity like the large and the small fragments of DNA pol I and thus enable the polymerase-exonuclease also to perform nick-translation.

The gene A product of phage ϕX174, necessary for RF replication, was isolated and shown to be a specific endonuclease which only cleaves ϕX174 viral DNA and breaks one phosphodiester bound per circle in the single-stranded or duplex DNA (HENRY and KNIPPERS, 1974). This endonuclease has a molecular weight of 560000 in SDS gels, and about 3000 molecules are present per infected cell. The analogous gene 2 protein of FV is accumulated at high temperature (42° C) in the infected cell (LIN and PRATT, 1974). It has a molecular weight of 40000 and sediments in the particulate cell fraction, from which it can only be extracted by urea-SDS treatment in a denatured form. Similar nucleolytic properties as for phage ϕX174 gene A protein can be expected for the gene 2 product of filamentous phages (LIN and PRATT, 1974; FIDANIÁN and RAY, 1972).

4. Plasmid Relaxation Complexes

The super-coiled ColE1 plasmid exists as a DNA-protein relaxation complex. Treatments destroying the protein structure like pronase or heat convert the complex into a relaxed state (CLEWELL and HELINSKI, 1970). The break is strand-specific and is located 19% of the length from one end of the EcoRI endonuclease cleaved ColE1 DNA (LOVETT et al., 1974). Both the coherence of origin of replication and the location of the complexed protein in ColE1 DNA point to an involvement of the DNA-protein complex in DNA synthesis, as either a swivel mechanism for unwinding of the duplex or a regulator function for initiation. The protein part consists of three major species of molecular

weights of 60000, 16000, and 11000 daltons (LOVETT and HELINSKI, 1975). The 60000-dalton protein is covalently attached to the 5′ terminus of the open plasmid strand (GUINEY and HELINSKI, 1975).

A DNA-protein complex of the minicircular DNA form I exhibits endonuclease activity which introduces one single-stranded break per molecule (MESSING et al., 1973).

5. The Removal of Helical Supertwists

Relaxing enzymes release supertwists from duplex DNA through concomitant nicking and sealing, thus reducing the winding number to the level of a relaxed double-helix. Swivelling, during the movement of the replication fork, should result in an excess of supertwists (more positive turns). On the other hand, phage DNA (λ, ϕX174-RFI, FV-RFI) yields negative supertwists, when isolated from infected cells or from phage particles in the case of phage PM2. Negatively super-coiled DNA may not really occur in the cell, since DNA-bound proteins like histones in eukaryotic chromosomes keep the helix in an ordered configuration. Their removal, however, disturbs the winding number of a closed double-stranded DNA and results in the isolation of a supercoiled structure (GERMOND et al., 1975). Relaxing enzymes from eukaryotic cells can release both types of supertwists (CHAMPOUX and DULBECCO, 1972; KELLER and WENDEL, 1974; VOSBERG et al., 1975), whereas a relaxing enzyme from *E. coli*, called ω-protein (WANG, 1971), can only relax negative turns. The latter protein is not coded by known mutants involved in DNA synthesis (WANG, 1973). The ω-protein may not act in the presence of intercalating ethidium bromide which is used to produce positive helical turns. To achieve this type of relaxation, additional factors may be required together with the ω-protein, or other proteins may act as a swivelase in replication of *E. coli*.

VI. Replication with Isolated DNA and Purified Proteins

A. The Conversion of Single-Stranded DNA to Replicative Forms

Single-stranded, circular DNA of small bacteriophages is a simple and convenient template for the study of DNA synthesis. The conversion to double-stranded replicative form I was first achieved by using a boiled cell extract for priming, DNA pol I for DNA synthesis, and DNA ligase for strand closure (GOULIAN and KORNBERG, 1967). The exact copying of the viral strand information has been demonstrated by spheroplast infection with the separated complementary DNA (GOULIAN et al., 1967). Deoxyoligonucleotides used as primers of DNA synthesis by DNA pol I on FV-DNA (OERTEL and SCHALLER, 1973) result in random starts of the polymerase on the genome. Short RNA is a suitable primer in this reaction for DNA pol I (ROYCHOUDHURY and KÖSSEL, 1973), DNA pol II (MOLINEUX et al., 1974), DNA pol III (HURWITZ and WICKNER, 1974), or DNA pol III* (WICKNER, W., et al., 1973).

1. Filamentous Virus-DNA

DNA unwinding protein prevents multiple initiation and directs the synthesis of the primer RNA to a unique site on the FV-genome (Fig. 4). Supporting evidence includes: the unit length of the complementary strand synthesized with DNA pol III* and copolymerase III*, and the specific location of the gap with respect to the cleavage pattern obtained with an endonuclease of *H. influenzae* (GEIDER and KORNBERG, 1974). Furthermore, the initiation region (see Fig. 4) has been protected against nucleases in a complex with RNA polymerase and isolated as a segment of 120 nucleotides (SCHALLER et al., 1976). The DNA is partially double-stranded and hybridizes to the complementary strand of a single restriction fragment derived from the gene 2 area of the phage genome.

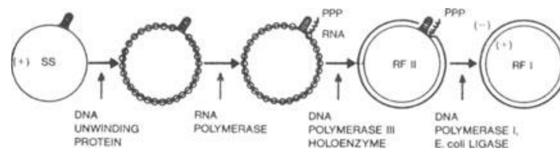

Fig. 4. Scheme for action of proteins involved in filamentous virus single strand to replicative form I conversion (according to GEIDER and KORNBERG, 1974). On phage G4 DNA initiator RNA is formed by action of *dna*G protein instead of RNA polymerase (BOUCHÉ et al., 1975); phage ϕX174 DNA uses for this purpose a protein complex consisting of proteins coded by genes *dnaB*, C, G in addition to factors n and i (SCHEKMAN et al., 1974) or X, Y, and Z (WICKNER, S., and HURWITZ, 1974)

Either DNA pol I or II can replace DNA pol III holoenzyme in the DNA unwinding protein-RNA polymerase-FV DNA system. If the rate of DNA synthesis with pol III is 100, then the rate with pol I is 10 and with pol II is 3 within the first 10 minutes of incubation and the products appear to be less defined in the latter reactions (GEIDER, unpublished results). This may reflect the physiologic involvement of the *pol*C gene product in the formation of the parental RF upon phage infection and indicate alternative functions of *pol*A or *pol*B under restrictive conditions for *pol*C. Figure 4 shows the enzymatic steps involved for the conversion of FV single-stranded DNA to doubly closed, but not supertwisted RFI.

2. Phage ϕX174-DNA

Conversion of ϕX174 SS to duplex RF II in crude extracts is resistant to rifampicin and does not use RNA polymerase (SCHEKMAN et al., 1972). Replication of ϕX174 DNA requires the products of genes *dna*B, C, G, and *pol*C as well as other proteins for which mutants have not yet been isolated (SCHEKMAN et al., 1974; SCHEKMAN et al., 1975; WICKNER, S., and HURWITZ, 1974). Complementation assays of mutant extracts, as well as direct fractionation, have led to the purification of the required components and reconstitution of the reaction.

The product of the *dna*B gene has a high native molecular weight (250000 daltons) and is temperature-sensitive when isolated from *dna*B mutants (Wright et al., 1973; Schekman et al., 1975). A single-strand DNA dependent ATPase activity is associated with the purified *dna*B protein (Wickner, S., et al., 1974). The *dna*C product has been isolated from wild-type cells and is thermosensitive from a *dna*C strain (Wickner, S., et al., 1973a; Schekman et al., 1975). The *dna*C and *dna*B proteins interact to form a complex. In the complex, ATPase activity is inhibited and the *dna*C protein is converted from an NEM-sensitive to an NEM-resistant state (Wickner, S., and Hurwitz, 1975a).

The *dna*G gene product was isolated by complementation of *E. coli* DNA synthesis on cellophane disks (Klein et al., 1973) and *φ*X174 DNA synthesis in soluble extracts (Wickner, S., et al., 1973b; Bouché et al., 1975). The 60000 dalton protein is insensitive to NEM and thermolabile from *dna*G mutants.

Schekman et al. (1974, 1975) have identified two additional proteins required for *φ*X174 RF synthesis called protein i and protein n. Protein n is an 82000-dalton polypeptide which cooperatively binds to single-stranded DNA, constricting the contour length about 6-fold (Weiner and Kornberg, unpublished). Protein i is 40000 daltons. Wickner and Hurwitz (1975b) have identified three factors required for DNA synthesis: protein X, apparently identical to protein i; protein Y, approximately 60000 daltons; and protein Z, approximately 30000 daltons. The relation of factors Y and Z to protein n is unclear at this time. A fourth factor, named W, and factors X and Z have no known enzymatic activity, but a *φ*X174 DNA dependent ATPase copurifies with factor Y (Wickner, S., and Hurwitz, 1975c). Wickner and Hurwitz (1974) propose that initiation on a *φ*X174 DNA template requires *dna*B and *dna*C, proteins X, Y, and Z, and ATP and that *dna*G protein acts in a subsequent step.

3. Phage G4-DNA

Using phage G4 (Godson, 1974b), which is closely related to *φ*X174, Zechel et al. (1975) showed that replication of this DNA in vitro only requires DNA polymerase III holoenzyme, DNA unwinding protein, and a third protein subsequently identified as *dna*G protein (Bouché et al., 1975). *Dna*G protein is a rifampicin-resistant RNA polymerase which synthesizes short RNA primers on G4 templates covered with unwinding protein. This primer, about 20 nucleotides long, is covalently bound to the 5′ end of the complementary strand.

4. Extension of the Primer and Ring Closure to RFI

Artificially RNA-primed *φ*X174 is ready for DNA synthesis after the formation of a complex of DNA pol III*, copolymerase III*, and ATP at the 3′ primer terminus (Wickner, W., and Kornberg, 1973). This complex may also be built up in physiologically primed phage *φ*X174, phage G4, or filamentous virus SS→RF conversion. After the onset of DNA synthesis, copolymerase III*

is no longer required, because DNA replication is unaffected by copol III*-antiserum after complex formation. The resulting replicative form II is converted into RFI after extension of the complementary strand by DNA pol I and sealing by DNA ligase (Fig. 4). As DNA pol III* in the presence of DNA ligase is not efficient for closing the gap in the RFII formed, DNA pol I appears having the capacity to overcome a special structure in the gap region and to cleave off primer RNA left from chain initiation (GEIDER and KORNBERG, 1974). The peculiar structure may be the hairpinlike region demonstrated for the origin of replication of FV SS→RF conversion (SCHALLER et al., 1976), or for other systems sequences with a comparable secondary structure which cannot be covered by DNA unwinding protein, required for DNA pol III* action. Moreover, the partially double-stranded character in the template would also be unfavorable for the proceeding of DNA pol III*.

One can assume that Okazaki pieces are preserved by a similar mechanism, where initiation occurs at a region with secondary structure and a gap is left at this site until DNA pol I or—in *pol*A mutants—a less efficient substitutive process slowly replicates this region and DNA ligase seals the strands.

B. Replication of Double-Stranded DNAs by Phage T4 and Phage T7 Specific Proteins

Complementation of extracts from phage T4 mutant-infected cells has resulted in a purification of wild-type proteins induced by genes for phage replication (ALBERTS et al., 1975; MORRIS et al., 1975). In order to get primer-independent DNA synthesis, T4 DNA polymerase (gene 43) and the phage DNA unwinding protein, previously characterized (see chapter V, A and B), were supplemented by gene 41 protein (58000 daltons), gene 45-protein (2×27000 daltons), and a complex between gene 44 and gene 62 proteins (4×34000 and 2×20000 daltons, respectively). This tight complex complements both mutant extracts (BARRY and ALBERTS, 1972) and displays DNA-dependent ATPase activity.

The hydrolysis of ATP can be further stimulated by adding gene 45 protein to the gene 44–62 protein complex. These three proteins bring DNA synthesis with T4 DNA polymerase on primed single-stranded templates to almost in vivo rates, and they seem also to increase the distance of phage DNA polymerase for sticking to the primer-template. Therefore, one might speculate that the cleavage of ATP provides the energy for driving the replicating complex along the template without diffusion of the polymerase from the DNA. This role of ATP in replication was suggested by Dr H. HOFFMANN-BERLING (personal communication) and appears to be realized for enzyme movement of ATPases separating duplex DNA or DNA/RNA hybrids without (ABDEL-MONEM et al., 1976) or with (FRIEDMAN and SMITH, 1973) concomitant nicking of the DNA.

Synthesis of an RNA primer has not yet been demonstrated in the system, although all four ribonucleoside triphosphates are required for efficient replica-

tion of the single-stranded template DNA. Any one of the six proteins left out reduces DNA synthesis to 1 % of the complete reaction. Using circular FV-DNA as template the replication product in this system has rolling circle structure (see Fig. 3) with partially double-stranded tails as visualized in the electron microscope. This is consistant with discontinuous synthesis of the (+) strand complement on the single-stranded tail.

For duplex replication gene 32 protein (T4 DNA unwinding protein) may open the strands at a nick and the proteins extend one strand at the 3'-end (leading strand). The displaced complementary strand (lagging strand) is initiated at multiple sites and then replicated in short DNA segments.

The replication system of ALBERTS and coworkers uses templates from various sources: single-stranded DNAs from phages fd and G4 or denaturated T4 DNA, double-stranded DNA not only from phage T4, but also from Pseudomonas phage PM2 or animal virus SV40. On the other hand, the system is selective against phage T7 DNA or many synthetic polymers.

A comparable replicative system has been created from purified proteins of phage T7 infected cells (SCHERZINGER and SEIFFERT, 1975). Among the phage induced proteins DNA binding protein (27000 daltons), DNA polymerase (96000 daltons, including host factor), and DNA priming protein (gene 4 protein, 65000 daltons) are required for replication of the double-stranded phage DNA. In addition to the deoxyribonucleotides the reaction depends on all four ribonucleoside triphosphates, and T7 RNA polymerase cannot replace the DNA priming protein (gene 4 protein) for initiation, although the RNA polymerase enhances DNA synthesis in this system.

Replication is initiated at many sites, the short chains are not linked to the template, and they are sealed by *E. coli* DNA polymerase I and phage T7 DNA ligase into DNA of higher molecular weight. The products of an extensive synthesis, exceeding the amount of template 10-fold, are large, multiply forked molecules (SCHERZINGER and KLOTZ, 1975).

Although these efficient systems rely on proteins coded by genes necessary for phage replication, it might not reflect the complete in vivo situation. As discussed in chapter II, B 1 and 2, the infecting T4 or T7 DNA becomes membrane attached and originates replication at a distinct site with discontinuous growth in both strands. The forks move bidirectionally and growth must be terminated by a complicated chromosomal rearrangement (see GEFTER, 1975, for phage T4 and Fig. 2 for phage T7). Thus, an even more complex multicomponent system appears to be required to achieve those properties, but the described phage T4 and phage T7 coded proteins nevertheless are a promising approach to understanding molecular aspects of replicating double-stranded DNA.

VII. Conclusions

The progress in the biochemistry of DNA replication in *E. coli* during the last 5 years has been marked by several points. (1) The isolation of mutants deficient for DNA polymerase I has led to the characterization of two more

DNA polymerases and to the demonstration of both the physiologic requirement of DNA polymerase III and the 5'→3' exonuclease function of DNA polymerase I. (2) The priming event for DNA synthesis has been shown to be the formation of an RNA segment synthesized by the action of RNA polymerizing activities. (3) Nonenzymatic DNA binding proteins participate in replication. (4) Cellular in vitro systems have been exploited for structural analysis of replicative intermediates. (5) Partially lysed cells and soluble cell extracts have been complemented with proteins or DNA species, thus characterizing their role in more complex replicating systems. (6) Protein systems have been isolated which can replicate phage DNA with physiologic properties. (7) Restriction enzymes have allowed the analysis of larger DNAs to a molecular level. The next steps will comprise a better characterization of multicomponent systems and the research on the regulation of DNA synthesis and its influence on other cellular events. These advanced studies on DNA replication of prokaryotes and eukaryotes will gradually lead to a deeper understanding of the chromosomal organization and will increase our knowledge of cell physiology.

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Teichoic Acids[1]

ROMAN DZIARSKI[2]

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I. Introduction

The teichoic acids, which are found in the cell walls of many gram-positive bacteria, are polymers of phosphates of polyhydroxyalcohols and sugars. Their name is derived from the Greek word *teichos*, "the wall". It was in 1951 [108] when the possibility that such components may exist in bacteria was suggested for the first time by detection of considerable phosphorus excess in the staphylococcal cells, as compared to the nucleic acids, phospholipids, and other phosphorus-containing compounds known at that time. In the next years ARMSTRONG, BADDILEY, ARCHIBALD and their co-workers published a number of papers elucidating the structure of teichoic acids in many bacterial species.

Parallel immunochemical investigations linked known immunologic properties with chemical structures just discovered. For example, the staphylococcal polysaccharide A isolated in 1935 [155] appeared to be a mucopeptide-teichoic acid complex, whose immunologic specificity depends on sugar substituents of teichoic acid [65].

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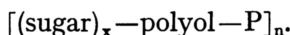
The "intracellular" ("membrane") teichoic acids have been also discovered and isolated together with cell membranes and ribosomes upon centrifugation of disintegrated bacterial cells [95, 34, 44, 68, 124, 137, 158, 141].

II. Structure

The teichoic acids are polymers of polyhydroxyalcohols, usually glycerol or ribitol, bound by phosphodiester linkages. The alcohol hydroxyl groups are most frequently substituted by saccharides and D-alanine.

The simplest teichoic acid containing no substituents, polyglycerophosphate, is present in many species of gram-positive bacteria (*Staphylococcus*, *Streptococcus*, *Bacillus*) [95, 87]. However, usually some free OH groups at position 2 of glycerol are substituted by D-alanine (by ester linkage), whereas the remaining are bound by glycoside linkages to such saccharides as glucose [34, 56, 138], N-acetylglucosamine [124], N-acetylgalactosamine [44], gentibiose [124], and cojibiose or cojitriose [157, 149].

There are many exceptions to the glycerol teichoic acid structure, generally described above. Beside glycerol, saccharide units can be incorporated into the basic chain, e.g., glucose or galactose in the wall teichoic acids of *B. licheniformis* [25], and N-acetylglucosamine with D-alanine bound by ester linkage at position 6 in *S. lactis I3* [7, 26]. The structure of such teichoic acids can be schematically presented as follows:



$$[(\text{sugar})_x\text{—polyol—P}]_n.$$

As an example of the most complex teichoic acids of such type, the capsular polysaccharides of pneumococci can be mentioned. They are built of sequential olygosaccharide-ribitol-phosphate units [92].

The ribitol teichoic acids are not extractable from the membrane fractions, as they are constituents of bacterial cell wall only. They usually contain D-alanine which is attached by an ester linkage at position 2 or 3 of ribitol, and sugar is bound by glycoside linkage at position 4 of ribitol. For example, in *B. subtilis* D-glucose [12, 13], and in *Staph. aureus* N-acetylglucosamine, bound by α- or β-linkages, are always present in the separate chains, although a given strain most frequently contains a mixture of α- and β-chains [14, 15, 16, 131].

In both glycerol and ribitol teichoic acids not all alcohol-OH groups are substituted. The incomplete substitution of the OH groups may sometimes be apparent only. In some bacteria (e.g., *B. subtilis*) two teichoic acids can be isolated, one being completely substituted and the other containing no substituents [23, 28].

The length of teichoic acid chains differs from one bacterial species to another. The ribitol chains are rather short, containing 7–9 ribitol units in *Staph. aureus* [14, 67], 9 units in *B. subtilis* [13], and 8–11 units in *Lactobacillus arabinosus* [67]. However, 12–16 units were found in *Staph. aureus* strain Copenhagen [131] and 22–24 in *Staph. lactis* [8]. The glycerol teichoic acids are usual-

ly composed of a greater number of glycerophosphate units (e.g., 18 in *Staph. albus*) [44], and 28–35 in *Str. faecalis* [149]. In cell membranes they are usually bound to glycolipids through a phosphodiester bond involving the terminal glycerol phosphate residue of the teichoic acid chain and a sugar hydroxyl group of the glycolipid. The lipid moiety was shown to be composed of phosphatidyl diglucosyl diglyceride components [159, 160, 149, 123, 161]. Lipoteichoic acids are amphipathic molecules. Each has a long polar polyglycerol phosphate chain linked to a small hydrophobic lipid portion, enabling them to form micelles in solution. Mild deacylation procedures destroy micellar structure [149, 92, 161].

III. Cellular Localization in the Cell Wall and Membrane

A. Cell Wall Teichoic Acids

In the cell wall, teichoic acids form a complex with the mucopeptide, probably by covalent phosphodiester bonds which are sensitive to acid hydrolysis and link the teichoic acid terminal groups with the mucopeptide muramic acid [26]. One teichoic acid molecule is associated with any glycan chain of peptidoglycan [8]. Presence of these bonds was not demonstrated directly. Nevertheless, their existence is indicated by some indirect evidence. The teichoic acid-mucopeptide complex cannot be separated by physical methods (electrophoresis, chromatography, etc.); no phosphomonoester groups in cell wall teichoic acid were demonstrated, while after extraction these compounds were found to contain one phosphomonoester group per 14 phosphodiester groups [146]. Phosphoramidate linkage between the terminal phosphate group of teichoic acid and one of the amino groups of glucosaminopeptide was also suggested [5, 66].

Spatial arrangement of teichoic acids in the cell wall is not yet sufficiently and unequivocally explained, as is also the case with space structure of the wall itself. Using concanavalin A labeled with mercury, colloidal gold coated with concanavalin A, or indirect concanavalin A-peroxidase technique, a dense and homogenous distribution of teichoic acid in the cell walls of *Str. faecalis* was revealed in nondividing and dividing cells [18]. Electron-microscopic studies of ultrathin sections of *B. megatherium* cells [114] exhibited existence of three layers in the cell wall. Thickness of the wall was reduced by half, following removal of teichoic acid from cells by hot formamide. This resulted in removal of the outer soft layer with the rigid "mucopeptide membrane" intact.

However, teichoic acids are arranged rather inside the mucopeptide network, as suggested by studies on accessibility of these acids for specific antibodies. In the isolated walls of *B. subtilis*, only 19% of the total amount of teichoic acid antigenic determinants were accessible for antibodies. In the intact cells 10% were accessible: a finding also not indicative of the arrangement of teichoic acids on the inner side of the cell wall. The amount of accessible antigenic determinants of teichoic acid was considerably higher after a short

lysozyme digestion of the cell wall preparations [23]. This finding was, however, not confirmed in experiments with *B. licheniformis*. Walls of this microorganism not treated with lysozyme, bound 71 % of the theoretical maximal amount of antibodies against teichoic acid [23].

Accessibility of the wall teichoid acids on the cell surface is also suggested by their participation as the constituent of phage receptors in many gram-positive bacteria [27, 33, 170, 107, 139, 57, 156, 171, 132, 31, 122]. In some phage-ersistant mutants, the wall teichoic acid is modified or is not present at all.

B. Membrane Teichoic Acids

The membrane teichoic acids were found in all investigated gram-positive bacteria. They exhibit a rather uniform structure: polyglycerol phosphate polymers with D-alanine and saccharides as substituents [34, 68, 124, 157, 158, 137]. In cell fractionation procedures, upon centrifugation (100000 *g*), they sediment together with cell membranes [34, 44]. However, as shown by several investigators (cell wall treatment with lysozyme and formation of stable L-forms in *Str. faecalis* [137], protoplast formation in *B. megatherium* cells [68]), membrane teichoic acids seem to be attached neither to the cell wall nor the cell membrane. They were not found in cell wall fractions, and were released into the medium by L-forms or during protoplast formation.

Recent studies indicate, however, that membrane teichoic acids are tied to the cell membrane surface [141]. Direct evidence was provided by electron microscopic investigation of *Lactobacillus fermenti* and *L. casei* protoplasts, treated with ferritin-labeled specific teichoic acid antiserum [152].

The membrane teichoic acid occurs in combination with glycolipidis or phospholipids of the cell membrane, as the so-called lipoteichoic acid. It can be extracted from disintegrated cells with hot phenol (trichloracetic acid disrupts the linkage teichoic acid-lipid [160]). In several bacterial strains (*L. buchneri, L. plantarum, Staph. aureus H* and *A1, Micrococcus sp., B. subtilis*), only lipoteichoic acid was demonstrated, while free membrane teichoic acid was undetectable [32].

Although it seems now that membrane teichoic acids are attached to the cytoplasmic membrane, in some cases antibodies against them can be produced by immunization with intact bacterial cells. Their presence can also be demonstrated by whole-cell agglutination test with specific immune antiserum, as shown with *L. fermenti*. These findings were not confirmed with *L. casei* [152]. Similar difficulties are encountered with *Str. faecalis*, in which membrane teichoic acid is a group D antigen [136, 144]. Electron microscopy with aplication of ferritin-labeled antibodies [152] demonstrated presence of quantitative differences only: in both *L. fermenti* and *L. casei*, the membrane teichoic acid appeared to be accessible for antibodies on the cell sufface. Thin sections of bacteria with preserved antigenic specificity of their lipoteichoic acids were stained with ferritin-labeled antibodies to localize lipoteichoic acid. The label extended from the outer surface of the membrane through the cell wall and in

some cases beyond the outer boundary of the cell into the external environments. Mesosomes also carried the label [161].

As might be inferred from higher effectiveness of agglutination with IgM than IgG antibodies, there is penetration of membrane teichoic acid toward the cell surface rather than penetration of antibodies through the cell wall toward the cytoplasmic membrane [152].

These observations resulted in designation of a model according to which the membrane teichoic acids are attached to cytoplasmic membrane lipids by one end, whereas the other penetrates into the cell wall toward the cell surface. The length of the membrane teichoic acid chains, evaluated by size of the molecule, corresponds with thickness of the cell walls as observed by electron microscopy [152].

This model has not been confirmed in studies performed on *Staph. aureus* and other bacteria [74]. Mesosomes were separated from membrane fraction and where shown to contain almost the whole lipoteichoic acid present in the cell. It comprised 18% of the dry weight of mesosomal vesicles, while purified plasma membranes contained no lipoteichoid acid.

C. Extracellular Teichoic Acids

Teichoid acids which can be found in culture media may be regarded as extracellular. The release from the cell is probably due to the cell wall and membrane turnover or autolysis [92]. Lipoteichoic acid can be removed from whole cells by washing with saline or by "cold shock." Culture fluis of oral streptococci and lactobacilli may contain up to 50 µg/ml of lipoteichoic acid: an amount comparable to the percentage of this acid within the cells. Nonmicellar glycerol teichoic acid with low fatty acid ester content has been found in varying amounts in cultures of *L. fermenti* and *L. casei* [161].

IV. Methods of Isolation

In the bacterial cell, teichoic acids form covalently linked complexes, either with mucopeptide or with lipids. There is a question, therefore, how to define "purity" of teichoic acid preparations. These complexes cannot be separated by physical methods, and in this sense they are homogenous. However, phosphodiester linkages between terminal groups of teichoic acid and mucopeptide or lipids are more prone to hydrolysis than phosphodiester linkages inside the chain. The complexes can thus be disrupted and teichoic acids isolated.

Treatment with 5–10% TCA is most frequently employed [12, 14, 16]. This method yields rather short-chain teichoic acids, as acid from *Staph. aureus* cell walls composed of eight sequential ribitol units [14, 15]. Therefore, objections have arisen as to degradation of chains due to hydrolysis of intramolecular phosphodiester linkages. Teichoic acids referred to as "native" were isolated from *Staph. aureus Copenhagen* cell walls by treatment with acetylmuramidase and acetylmuramyl-α-alanine amidase. Their molecular weight amounted to

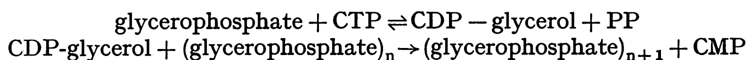
20000, thus corresponding to a chain containing 40–50 units [53]. This problem was again studied with *Staph. aureus*, *B. subtilis*, and *L. arabinosus*, using a method for direct determination of the length of teichoic acid chains without extracting them from the cell walls, as well as two other methods after TCA extraction. The results were rather concordant. Chain length ranged from 7–9 units (*Staph. aureus*) to 7–11 units (*L. arabinosum*), indicating that teichoic acid chains are not degraded by 5–10% TCA [66, 67].

Other methods used for teichoic acid isolation are phenylhydrazine extraction [5, 62] and alkaline extraction with 0.1 N NaOH. The latter results, however, in hydrolysis of alanine-polyol ester linkage or even total degradation of some teichoic acids, as illustrated by separation from *Staph. lactis* cell walls of an acid containing in its basic chain N-acetylglucosamine together with glycerol [9, 11, 77].

The wall teichoic acid can be isolated by the above methods. Cell walls isolated from the disintegrated bacterial cells or purified mucopeptide-teichoic acid complex obtained by protease and nuclease digestion, can be used as the starting material. Membrane-ribosomal fraction is applied as the initial product for isolation of the membrane teichoic acid, but it can also be extracted from intact bacterial cells [34] or after lysozyme digestion of the cell wall [141]. Lipoteichoic acid can be prepared by hot 45% aqueous phenol extraction, similarly to lipopolysaccharides from gram-negative bacteria. After TCA treatment, this preparation yields teichoic acid without the lipid component [159, 160]. An alternative procedure is to treat freeze-dried cells with a mixture of chloroform and methanol to deplete them of lipid and then extract lipoteichoic acid from lyophilized material with hot water [161].

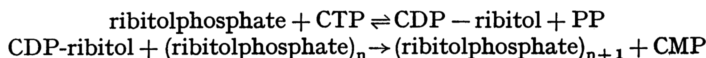
V. Biosynthesis

Cytidinediphosphoglycerol (CDP-glycerol) is the substrate for glycerol teichoic acids synthesis, and cytidinediphosphoribitol (CDP-ribitol) for ribitol teichoic acid [24, 54, 79]. The synthesis runs as follows:



$$\text{glycerophosphate} + \text{CTP} \rightleftharpoons \text{CDP} - \text{glycerol} + \text{PP}$$
$$\text{CDP-glycerol} + (\text{glycerophosphate})_n \rightarrow (\text{glycerophosphate})_{n+1} + \text{CMP}$$

and



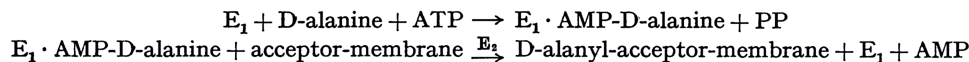
$$\text{ribitolphosphate} + \text{CTP} \rightleftharpoons \text{CDP} - \text{ribitol} + \text{PP}$$
$$\text{CDP-ribitol} + (\text{ribitolphosphate})_n \rightarrow (\text{ribitolphosphate})_{n+1} + \text{CMP}$$

A number of enzyme preparations were isolated from *B. licheniformis* and *B. subtilis* cells which catalyzed the synthesis of polyglycerophosphate [24, 128]. CDP-glycerol pyrophosphorylase was present in the soluble fraction and polyglycerophosphate synthetase was associated with the cell membrane. In *Str. lactis* and *L. buchneri*, however, both enzymes were found to be cell membrane-associated [6]. The presence of Ca^{++} or Mg^{++} ions is necessary for enzyme activation. Length of the chains formed and the rate of their synthesis are in keeping with in vivo findings. Similar observations were also reported for *Staph. aureus* [79] and *L. plantarum* [54].

Recently the polyribitol phosphate polymerase from *Staph. aureus H* has been purified and showed to require an acceptor—the so-called lipoteichoic acid carrier (LTC) [48, 49]. LTC is a polyglycerol phosphate, 12 units long, with glucose and fatty acids attached at its hydrophobic end [48, 58]. The synthesis of polyribitol phosphate proceeds by a single chain mechanism. The enzyme completes a chain of approximately 30 units linked to LTC before starting a new chain. LTC of *Staph. aureus*, *B. subtilis* and several other species are interchangeable. Both polymers (polyribitol and polyglycerol phosphates) appear to be able to bind to the same site of the LTC.

Uridinediphosphates, as for example UDP-glucose in *B. subtilis* [28, 31] and UDP-N-acetylglucosamine in *Staph. aureus* [79, 113], are donors of the saccharide substituents. As an acceptor, a nonglucosylated acid may be utilized, synthesized both in vivo and in vitro [56]. Three steps in glucosylation of teichoic acid in *B. subtilis* were identified with three enzymes involved—(1) phosphoglucomutase, (2) UDP-glucose pyrophosphorylase, and (3) membrane-associated transferase. The first enzyme (1), which was purified and shown to be composed of two distinct forms constituted of dimers, was proposed to play a vital role in regulation of glucosylation of teichoic acid [97, 98]. The activities of transferase and polyribitol phosphate synthetase are probably correlated within cells, since transferase yield is much higher when it acts simultaneously with synthetase [113]. In *Staph. aureus*, which contains wall teichoic acids of two types (with α- and β-N-acetylglucosamine), two specific transferases have been identified. Their specificity was a little lower in vitro than in vivo [113]. They are probably spatially separated within the cell. Two spatially separated synthesizing systems probably occur in *B. subtilis* which contains ribitol teichoic acids of two types: nonglucosylated and completely glucosylated. The extracted nonglucosylated acid can be fully substituted in vitro with D-glucose, using the enzyme preparation from the same microorganism [28].

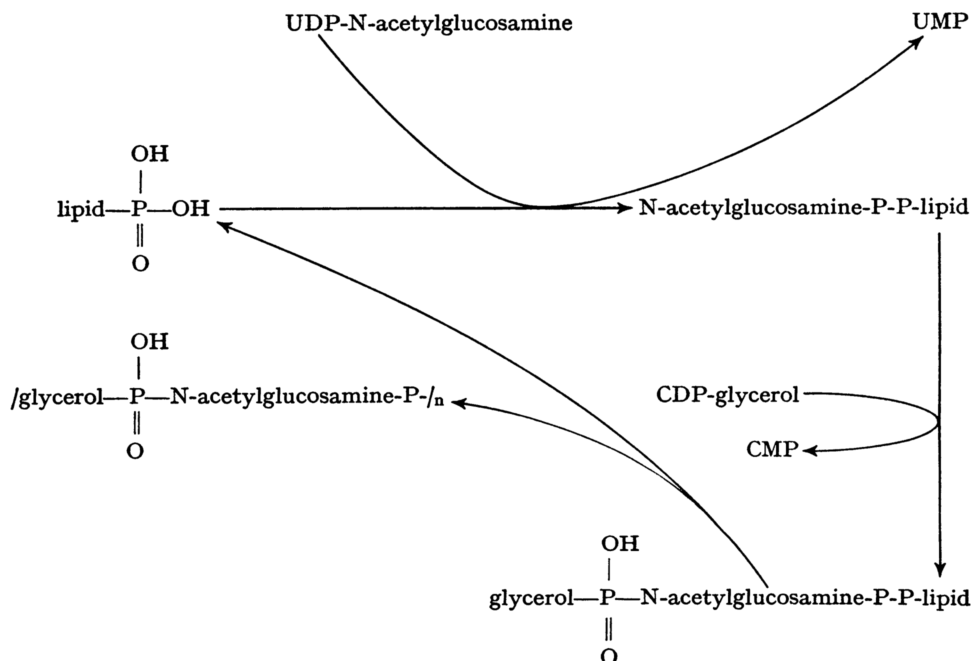
Alanine is bound probably to a complete glucosylated chain, whereas alanine-substituted phosphoribitol was found to be an acceptor by a half less active than the chain without alanine [113]. Alanine binding is a two-step reaction, with at least two enzymes involved: D-alanine-activating enzyme (E_1) and ligase (E_2). E_1 acts as an E_2 stimulator [115]. The presence of ATP and of the acceptor (teichoic acid) associated with cytoplasmic membrane fragments is necessary. All attempts of separation of the acceptor from the membrane failed and the role of membrane fragments is still unclear. The reaction follows this scheme:



$$E_1 + \text{D-alanine} + \text{ATP} \longrightarrow E_1 \cdot \text{AMP-D-alanine} + \text{PP}$$
$$E_1 \cdot \text{AMP-D-alanine} + \text{acceptor-membrane} \xrightarrow{E_2} \text{D-alanyl-acceptor-membrane} + E_1 + \text{AMP}$$

Detailed studies, including isolation and purification of the E_1 and E_2 enzymes, were performed with *L. casei*. General reaction scheme was confirmed with *Str. pyogenes* and *Staph. aureus* [115]. Inactive D-alanine-adenosine 5-phosphate + enzyme complexes have been isolated earlier from several bacterial species [17].

In bacteria containing sugars as components of the basic chain of teichoic acids—N-acetylglucosamine in *Str. lactis* [154, 78] and glucose in *B. licheniformis* [4]—uridine-diphosphates act as donors of these sugars. The produced glycerophosphate-sugar complexes are sequentially linked to the growing chain of teichoic acid. In these bacteria a lipid intermediate (phosphoisoprenoid) was detected, which acts as the acceptor and then transfers sugar and glycerol phosphates on growing teichoic acid chain [4, 63, 78, 154]. The reaction runs according to the following scheme (for *S. lactis I3* [78]):



The same lipid intermediate participates in the mucopeptide synthesis. Teichoic acid and mucopeptide synthesis are thus correlated as was directly demonstrated in *Str. lactis* [78, 154], *B. subtilis*, and *B. licheniformis* [4, 104]. A correlation of the synthesis of both components was observed also in *Staph. aureus* [129]. It is difficult to explain the existence of mutants not synthesizing teichoic acids in vivo and not containing it in cell walls, but possessing enzyme systeme capable of synthesis in vitro [122, 139].

Recent studies with purified enzyme preparations from *B. subtilis* and *Staph. aureus* [50, 49] do not confirm participation of phosphoisoprenoid (or other similar compounds) in teichoic acid synthesis. Correlation with the mucopeptide synthesis is probably based on binding of complete teichoic acid chains to peptidoglycan, prior to the liberation of the latter from the cell membrane. It should be noted that during the biosynthesis, peptidoglycan is combined with the membrane through the isoprene carrier. Teichoic acid cannot be linked to preexisting peptidoglycan chains [55, 148].

In the synthesis of lipoteichoic acid, phosphatidyl glycerol serves as a donor of glycerol, phosphate, and fatty acid, as shown in vivo and in vitro with particulate enzyme from *Str. sanguis* [46].

Cell wall teichoic acids, together with peptidoglycan, undergo turnover during exponential growth of bacterial culture. The rate of turnover differs from one species to another; for example, in *L. acidophilus* it is equivalent to the loss of one-third of peptidoglycan per generation [20] and in *Staph. aureus* of about 15 % [165]. At the moment, turnover of wall polymers seems to be not essential for cell wall growth, as there exist bacteria with very low level [148] or without any detectable wall turnover [20].

The existence of teichoic acid-hydrolasing enzymes was also reported [163] and recently such an enzyme was shown to control N-acetylmuramyl acid L-alanine amidase activity in *B. subtilis* [71].

Antibiotics, which are often used for investigations on cell wall biosynthesis, influence mainly mucopeptide formation and find no extensive application in studies on teichoic acid synthesis. For example, penicillin in a concentration blocking mucopeptide synthesis to 90–100%, only slightly inhibits the teichoic acid formation. Its presence results, however, in formation of the acid with slightly modified properties [129]. Vancomycin and novobiocin inhibit formation of both polyglycerol and polyribitol phosphates [24, 54], as well as glucosylation of the chain [56, 76]. Chloramphenicol inhibits teichoic acid synthesis in *B. licheniformis* and *Micrococcus A1*, by selective blocking of glucose transfer from UDP-glucose to the lipid intermediate [145]. Higher antibiotic concentrations are required for inhibition of teichoic acid synthesis in vitro than for bacterial growth inhibition [6].

VI. Cellular Role

The function of both membrane and wall teichoic acids is to maintain high concentration of cations near the cell membrane, mainly of Mg^{++} which is indispensable for the activity of several membrane-associated enzymes [70, 76]. The isolated cell walls bind large amounts of cations (mainly bivalent) by teichoic acid phosphate groups which contain up to 50% of phosphorus present in the cell. Selective destruction of teichoic acids is followed by a decrease in the cell wall capability to bind Mg^{++}, and bacteria grown under Mg^{++} deficiency contain large amounts of teichnoic acids in their cell walls [70, 147]. During phosphorus starvation, in the cell walls of *B. subtilis* teichuronic acid is formed instead of teichoic acid. The teichuronic acid is composed of glucuronic acid and N-acetylgalactosamine and is also cation-binding. Under these conditions, the membrane teichoic acid is still present, a finding which suggests that it is of greater importance for the cell [43, 70, 166]. That teichoic acids play a role in maintaining adequate concentration of Mg^{++} ions, was proved directly by investigation of enzyme systems associated with the cell membrane: highest activity was observed in the presence of Mg^{++} bound with the endogenous teichoic acid. The system was not sensitive to changes of Mg^{++} concentration

in the medium [75, 76]. A block in the biosynthesis of wall and membrane teichoic acids might thus result in cell death due to Mg^{++} deficiency. Such is probably the mechanism of novobiocin action, which by inhibition of teichoic acid synthesis indirectly affects all processes in which Mg^{++} ions are involved [76].

The wall teichoic acid is not indispensable for the cell, as proved by isolation of several mutants partly or completely deprived of this acid [21, 31, 122, 139]. Beside phage resistance these mutants were in some respects clearly different morphologically and physiologically from standard cells. Additional cellular septa and rather loosely organized cell wall [31], disturbed cell division and arrangement in large clusters (30–100 cells), lower growth rate (by 30%), larger size and mass of cells, and more rough and irregular colonies, were described [122]. As indicated by these data, the wall teichoic acid is also an important structural constituent of the cell wall. Its function, at least in part, can be substituted by teichuronic acids. A *Staph. aureus* mutant, with teichuronic acids predominating in its cell wall, exhibited no such features [122].

Teichoic acids probably exert an effect on electric charge of the cell surface which plays a role in dispersion of bacteria in the medium. In alkaline medium, teichoic acids probably lose alanine and acquire negative charge, and in an acid one the charge is positive due to alanine binding [45, 81, 164]. The amount of alanine linked to teichoic acids does not influence the capability of Mg^{++} ions binding by the cell wall [45].

Pneumococcal teichoic acid was shown to inhibit the induction of competence by a specific substance in a genetically transformable but physiologically incompetent cell culture of *Diplococcus pneumoniae* [127]. The process is reversible and seems not to be very specific, as the concentration of teichoic acid used was rather high (1 mg/ml).

VII. Immunogenicity

A. Cell Wall Teichoic Acids

The injection of intact cells, such as *Staph. aureus* [65, 73, 82, 150], *Staph. epidermidis* [1], *Lactobacillus* [90, 135], and other bacterial species [2], is followed by the formation of specific antibodies against teichoic acids present in the walls of these microorganisms.

Cell wall preparations not digested by enzymes also exhibit good immunogenicity [90], whereas cell walls deprived of proteins by proteolytic enzyme treatment fail to induce the production of teichoic acid antibodies [61, 90].

Purified teichoic acids were found to be not immunogenic in rabbits, even when applied with complete or incomplete Freund's adjuvant [23, 64]. However, immunogenicity can be induced by combining the teichoic acids with methylated albumin [23] or with chromium chloide-treated erythrocytes [22].

Most sera from healthy human subjects contain variable amounts of antibodies against staphylococcal teichoic acid. They are detectable in concen-

trated sera [37] but are seldom found in nonconcentrated samples [36, 102, 103, 150]. Sera of patients with prolonged staphylococcal infections [102, 36, 42] had higher titers of teichoic acid antibodies. In two subjects investigated, an injection of teichoic acid from *Staph. aureus Copenhagen* was followed by a rise of circulating antibodies, while in the third case no such reaction was observed [150]. Significant rise in teichoic acid antibodies is observed in patients with staphylococcal endocarditis. It is utilized in serologic diagnosis of the disease by gel diffusion and counterimmunoelectrophoretic methods [36, 100, 111]. Normal sera of humans and many animal species were found to contain antibodies against bacillary glycerol teichoic acids [39].

In man, the natural antibodies against staphylococcal teichoic acids belong mainly to IgG, to a small extent to IgM, and were not detected in the IgA class [103]. In human and guinea pig sera the natural antibodies against bacillary teichoic acids were mainly IgM and IgG globulins, and in rabbit sera IgM globulins. No natural teichoic acid antibodies were detected in mice [39]. In the immune rabbit and mouse sera, antibodies against staphylococcal teichoic acids belonged to IgM class [169].

B. Membrane Teichoic Acids

The injection of intact bacterial cells is not always followed by production of antibodies against the membrane teichoic acid. Good effects were observed after mechanical disintegration of cells, for example group D streptococci [136] or *L. plantarum* [90].

Purified membrane teichoic acid is immunogenic when combined with methylated albumin [23]. Membrane teichoic acid from *L. fermenti* is also immunogenic, if isolated by phenol extraction and injected into rabbits with complete Freund's adjuvant. High molecular fractions of the acid, obtained by TCA extraction, are weakly immunogenic, and low-molecular fractions are inactive [89]. The phenol fraction probably contains lipoteichoic acid contaminated with protein, since good correlation was found between protein content in lipoteichoic acid preparations and their immunogenicity [159]. Purified lipoteichoic acid was not immunogenic in rabbits when administered in a soluble state or as a suspension with Freund's incomplete adjuvant. However, it was highly immunogenic after combination with methylated bovine albumin [130].

Natural antibodies against purified polyglycerol phosphate were observed in rats [19] and ontogeny of the natural immune response in rats and guinea pigs was then studied [29]. Antibodies of both IgM and IgG (γ_1) class were demonstrated at birth (presumably maternal), then IgG antibodies disappeared by the 6th week, followed by a slow rise in IgM titer. At about the 21st week γ_1 antibodies reappeared in some animals. Neither IgA nor γ_2 antibodies were detectable.

Human antibodies against group A streptococcal teichoic acid studied in healthy persons under the age of 16 years where shown to belong to IgM class

predominantly, while older persons had both IgM and IgA classes. Sera of patients with history of acute rheumatic fever or rheumatic heart disease had elevated titers of teichoic acid antibodies [88]. Majority of human sera had detectable amounts of antibodies against membrane teichoic acids from different lactobacilli [99].

Cell-mediated immunity to purified polyglycerol phosphate was observed in normal experimental animals [51, 19, 29]. It developed in 24–26-weeks-old rats and guinea pigs, concurrent with the appearance of IgG antibodies in the sera of both species described above [29].

VIII. Specificity of Antibodies and Immunochemical Properties

Antibodies are formed usually against the exposed sugar substituents of teichoic acids, which mask the glycerol- or ribitolphosphate core [23, 38, 65, 83, 90, 150, 162]. This has been proved by many experiments with specific inhibitors of the antigen-antibody reaction, and by cross reactions of teichoic acids with identical or related substituents. Not only is the saccharide type important, but also the kind of glycoside linkage (α or β). For example, in *Staph. aureus* the ribitol teichoic acids containing α- or β-N-acetylglucosamine are of different immunologic specificity [150]. The linkage type is sometimes even more important, since α-teichoic acid from *Staph. aureus* cross-reacts with teichoic acid from *Staph. albus*, containing α-N-acetylgalactosamine [65]. On the other hand, however, the reaction of specific antiserum with N-acetylgalactosamine-containing teichoic acid from *Staph. aureus* (phage type 187) is not inhibited by N-acetylglucosamine [83].

However, when the teichoic acid is poorly substituted or unsubstituted, as in the case of lipoteichoic acid of *L. casei*, antibodies are specific against glycerophosphate backbone. Lipoteichoic acids from *Str. lactis*, *Str. faecalis*, and *L. fermenti* with high degree of substitution stimulate antibodies mainly against sugar substituents, although specificity for the glycerol phosphate chain is also detected. This can be explained in terms of antibodies being formed against one "face" of immunogen, because a molecular model of these acids can assume a configuration in which all sugar substituents are on the same side of the chain. Antibodies specific against the glycolipid moiety of lipoteichoic acids could not be detected [161].

Wall or membrane teichoic acids serve as group antigens in many bacterial species and sugar substituents of these acids are in fact the essential antigenic determinants. For example, in group D streptococci (*Str. faecalis* and *Str. faecium*) lipoteichoic acid with α-glucosyl-1-2-glucose acts as antigenic determinant, in groups A, D, and E of *Lactobacillus* wall teichoic acid with α-linked glucose [135, 161], and in group F membrane acid with α-linked galactose [91]. The major part of *Staph. aureus* strains possess α- or β-ribitol teichoic acid, but a number of strains have been isolated with atypical teichoic acid [42, 47, 52, 83, 84, 126]. A division of staphylococci into four groups has been recently proposed, based on the type of teichoic acid found in their walls [116]. The

glycerol teichoic acid from *Staph. epidermidis* contains α- or β-linked glucose as determinants, enabling differentiation of coagulase-negative strains, among which coagulase-negative *Staph. aureus* strains also may be found. Cell walls of micrococci contain glycerol or ribitol teichoic acid with the same immunologic specificity as that of β-teichoic acid from *Staph. aureus* cells [118]. It is combined with the C component of unknown structure resulting in AβC polysaccharide formation [94].

It was shown that D-alanine, bound to teichoic acid chains by ester linkage, is not an antigenic determinant of these polymers. In only few papers was D-alanine suggested as an antigenic determinant of teichoic acids in group A streptococci [83], or to be responsible for cross-reactions of staphylococcal teichoic acids with specific antisera [83]. However, in these experiments methylated alanine esters were used for inhibition of the serologic reactions and this compound was recently shown to produce unspecific inhibition of teichoic acid precipitation reaction [91].

Precipitation [89, 135] or diffusion in agar gel [38, 73, 150] are most frequently applied in immunological investigations of this type. Only some 30% of the extracted teichoic acid is precipitated, since low-molecular acid remains in solution [89]. Hemagglutination is a more precise method. However, contrary to the initial observations [109], staphylococcal teichoic acid fails to sensitize normal or tanned erythrocytes [60, 169]. Mucopeptide, which forms polysaccharide A with β-teichoic acid [59, 60] and polysaccharide 263 with α-teichoic acid [73], possesses this property. Serologic specificity of polysaccharide A is determined by β-N-acetylglucosamine, and that of polysaccharide 263 by α-N-acetylglucosamine of teichoic acid, though mucopeptide itself also has its specific immunologic properties [2, 69, 85]. Erythrocytes can be sensitized with staphylococcal teichoic acid, when chromium chloride is used as binding agent [22]. Such erythrocytes exhibited good immunogenic activity. Lipoteichoic acid adsorbs on normal sheep erythrocytes by formation of hydrophobic bonds with membrane lipids [72, 130, 161]. Polyglycerol phosphate without the lipid component fails to sensitize erythrocytes (though it inhibits the hemagglutination reaction), or it binds to red cells weakly and reversibly [72, 30]. Despite these difficulties the hemagglutination method is widely used [99, 130, 29, 88]. Erythrocytes can be sensitized by streptococcal teichoic acid, in which alanine is probably responsible for adsorption [80, 110].

The agglutination reaction is rather rarely used although teichoic acids may act as antigenic determinants in agglutination of whole cells [152] or the cell walls [113].

In studies on the configuration of sugar substituents of teichoic acids, concanvalin A is more and more widely applied. It is a plant lecithin which specifically reacts with α-linked glucosyl substituents [10, 125]. The complexes thus formed can be estimated turbidimetrically, by double gel diffusion, or by analysis of the precipitate [40].

A number of agents nonspecifically inhibiting the reactions of teichoic acids with immune antisera, have also been studied. These are mainly inorganic

salts, such as 0.4 N NaCl inhibiting in 40 % the reaction of ribitol teichoic acid from *L. plantarum*. A more marked effect was observed with bivalent cations. Nonspecific inhibition of the precipitation reaction was also produced by hydrochlorides of alanine and lysine or glucosamine esters [91]. An increase in ionic strength of the solution was found to be accompanied by a rise in the intrinsic viscosity of teichoic acid solutions. It suggests a change of the configuration of teichoic acid molecules from a rigid rod into a random coil. In such a way the serologically active glucosyl groups may be masked. This hypothesis is supported by observation that increasing salt concentrations inhibit also the reaction of teichoic acids with concanavalin A [40]. An essential role of stereochemical factors in determining the antigenic specificity of polysaccharides has been demonstrated in many instances [142].

IX. Immunobiologic Properties

The cell walls of various gram-positive bacteria exert toxic effects in higher animals. Characteristic nodular skin lesions in rabbits were observed for at least 10 days following intradermal injection of proteolytic enzyme- and ribonuclease-digested cell walls from *Staph. aureus*, group A, B, C and E streptococci, *L. casei*, *Aactinomyces israeli* and *Odontomyces viscosus* [3, 120, 133].

When injected into the joints of rabbits, the isolated fragments of cell walls from group A streptococci produced a prolonged inflammatory process, with histologic lesions resembling the early stages of rheumatic arthritis. The presence of the injected antigen in the synovia and in macrophages (where it persisted for at least 5 weeks) could be detected by specific mucopeptide antiserum labeled with fluorescein or ^{125}I [134]. The same cell wall preparations, when injected intraperitoneally into mice, produced in the heart an inflammatory process with histologic symptoms of rheumatic fever. The presence of injected antigen, localized around surrounding heart lesions, could be demonstrated by the labeled specific antisera [35, 121]. Similar heart lesions in mice could be produced with the use of soluble products of digestion of the cell walls from group A streptococci by L-11 enzyme from *Flavobacterium* [112].

Skin lesions are produced probably by the mucopeptide component [133]. The molecule size is also essential, as the preparations were less active after partial acid hydrolysis. Similar conclusions can be drawn from experiments in guinea pigs [93]. The animals were sensitized with killed cells of *Staph. aureus 263* or *Copenhagen* and subjected to skin tests with killed or living cells, cell walls, peptideglycan, teichoic acid-peptideglycan complex and peptideglycan fragments. In nonsensitized animals, all antigens used, except teichoic acid, produced an acute inflammatory reaction which began to disappear after 10 hours. In animals sensitized with the Copenhagen strain, all antigens used, except teichoic acid and mucopeptide fragments, produced swelling and erythema which persisted for at least 30 hours, a reaction which was recognized as delayed-type hypersensitivity. Such reactions were observed in only a few animals sensitized with strain 263. Teichoic acid alone was thus inactive, al-

though its complex with mucopeptide was more active than mucopeptide alone. Differences between strains Copenhagen and 263 in animal-sensitizing ability may depend on different cell wall composition in these strains. β-teichoic acids is quantitatively predominant in strain Copenhagen and strain 263 contains α-teichoic acid only. In experiments on immunization of rabbits [117], higher antibody titers were obtained with polysaccharide A (containing β-teichoic acid) than with polysaccharide 263 (containing α-teichoic acid).

Skin hypersensitivity to teichoic acid from *Staph. aureus* cell walls was also observed in humans [101, 102, 168]. In all of the 14 persons tested, an intradermal injection of 10 μg of teichoic acid produced instant inflammatory reaction with swelling and erythema disappearing after 24 hours [102]. Necrosis occurred only in patients with higher teichoic acid antibody level, typical for the past staphylococcal infections. It was also observed [101] that lysozyme from lysosomes of human leukocytes is released, following incubation of these cells with teichoic acid and high-titer antiserum. Antibody titers necessary for this phenomenon are rarely found in healthy subjects, but sera of persons with active staphylococcal infection frequently contain them. Protein A of *Staph. aureus* exhibited no such properties. The appearance of the Arthus reaction is associated with high level of circulating antibodies and the presence of the antigen and granulocytes in blood plasma. In this process liberation of lysosomal enzymes from leukocytes undoubtedly occurs after phagocytosis of the antigen-antibody complex.

The staphylococcal mucopeptide and teichoic acid possess toxic properties. This has been demonstrated by determination of the respiration of mouse liver cells at various time intervals after injection of these antigens [86].

The role of the surface antigens in breakdown of the immunity barrier and in the development of infection remains still unclear. They are certainly essential, as may be inferred from at least partial protective effect of immunization with staphylococcal teichoic acid or aggressin (mucopeptide), or administration of specific immune antisera in experimental staphylococcal infection of rabbits [167]. In experimental infection of mice, when staphylococci were suspended in mucin solution [41], teichoic acid was the main factor in antistaphylococcal immunity, both active and passive. A certain protection against staphylococcal mastitis in goats was produced by immunization with cell walls from various strains of staphylococci. The most effective were preparations from strain 3528, containing α-teichoic acid only, and the least successful walls from strain 845 Glaxo, containing β-teichoic acid only [143]. Immunogenicity of α- and β-teichoic acids is thus probably different in goats than in rabbits [117].

Teichoic acids from the cell walls of *Staph. aureus*, *Staph. epidermidis*, and group A streptococci were also shown to possess immunosuppressive activity [42, 105]. Intraperitoneal injection of 2.5 mg of teichoic acid into mice inhibited by half the immune response to sheep erythrocytes administered 2 days later. However, the large amount of teichoic acid needed and injection of the second antigen by the same route suggest that this reaction is not specific and depends on activation of periteoneal cells, which destroy the second antigen

[42]. Group A streptococcal lipoteichoic acid also exhibits immunosuppressive activity [106, 151]. The reaction seems to be more specific and dependent on the lipid part of the acid. In contrast to the suppression of antibodies to sheep erythrocytes in mice, lipoteichoic acid enhanced antibody production to lipopolysaccharide antigens and stimulated reticuloendothelial system. Also significant inhibition of ^{3}H-thymidine incorporation in concanavalin A-treated mouse spleen cell cultures was caused by 2.5 μg of streptococcal teichoic acid.

After absorption of human serum with *Staph. aureus* cell walls (digested or not digested with trypsin), the rate of phagocytosis and killing of *Staph. aureus* cells by leukocytes is lower, when compared with phagocytosis in nonabsorbed serum. The mucopeptide seems to be a cell wall component responsible for this phenomenon. Teichoic acids, protein A, and products of mucopeptide digestion with different enzymes are inactive [140].

An interesting hypothesis was proposed concerning the role of lipoteichoic acid in the etiology of rheumatic fever and glomerulonephritis. This compound easily adsorbs on erythrocytes. The reaction is reversible and the lipoteichoic acid can subsequently be transmitted to tissues [80, 110]. The process has been proposed as a mechanism of antigen transfer from the infection site to other parts of the host organism. If the reaction is specific, the antigen may accumulate in tissues with a large number of membrane receptors. The prinicipal argument against this hypothesis is that lipoteichoic acids of different streptococcal groups are lacking specificity, as compared with high group specificity in evoking infection with group A streptococci. However, lipoteichoic acids may occur in complexes with proteins, upon which specificity observed in these diseases may depend [110].

Repeated injections of the membrane teichoic acid from *Str. pyogenes* into rabbits, produced distinct kidney calcification and necrosis of renal tubules, without a significant rise of teichoic acid antibodies. Elimination of alanine from the acid molecule resulted in a loss of this activity [153]. It cannot be excluded, however, that the preparation used was lipoteichoic acid and the lipid component eliminated together with alanine.

In contrast to lipopolysaccharides from gram-negative bacteria, lipoteichoid acids are neither pyrogenic for rabbits nor lethal for mice. They do not stimulate B cell transformation. Nevertheless, lipoteichoic acids produce local and generalized Shwartzman reaction in rabbits, with bilateral kidney cortical necrosis, and stimulate bone resorption in tissue cultures. High doses of lipoteichoic acids are necessary for this purpose, when compared with typical lipopolysaccharide dosis. Teichoic acid without the lipid component is inactive [92, 161].

It can be concluded that theichoic acid complexes with mucopeptides or lipids, which are normally found in the cell, are biologically active antigens, rather than teichoic acids alone. The latter may at most determine their immunologic specificity. Teichoic acids lack clear in vivo activity, probably due to low molecular weight. The role of the surface cell antigens in microbial pathogenicity is not yet fully elucidated and the problem needs further research.

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Adenovirus Transcription

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I. Introduction

Adenoviruses have been isolated from diverse mammalian species, including man and some birds, and some 80 serotypes have now been described (see Norrby, 1971, for a review). Viruses of this group were originally isolated from the respiratory tracts of their hosts, hence their name (Rowe et al., 1953; Enders et al., 1956) and, in man at least, are associated with mild disease of these organs. The 33 known human serotypes of adenovirus have been classified on the basis of their hemagglutinating activity (Rosen, 1960) and their ability to induce tumors in newborn rodents (Huebner, 1967; MacAllister et al., 1969). Either scheme defines four subgroups, but there is little correlation between hemagglutination subgroups I–IV and subgroups A–D defined by oncogenicity in vivo. On the other hand, the allocation of human adenoviruses to oncogenic groups A–D correlates well with the G+C content of their DNA genomes (Green, 1970), DNA:DNA and DNA:RNA homology studies (Lacy and Green, 1964, 1965, 1967) and the results of heteroduplex mapping (Garon et al., 1973).

Adenovirus particles are icosahedral structures, about 65–80 nm in diameter, comprising only DNA and protein. The DNA genomes of adenovirus are linear, double-stranded molecules with a molecular weight of $20–25 \times 10^6$ daltons (Green et al., 1967; Van der Eb, 1969) containing an inverted terminal repetition (Garon et al., 1972; Wolfson and Dressler, 1972) 100–120 nucleotides in length (Arrand et al., 1974). The protein capsid of the virion is composed of 252 capsomers which are of two types: those at the 12 vertices of the icosahedron are surrounded by five neighbours and are therefore termed pen-

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tons, while all others are surrounded by six neighbours and have thus been designated hexons (GINSBERG et al., 1966). Each penton unit is formed by a penton base and projecting fiber (VALENTINE and PEREIRA, 1965; NORRBY, 1966), which is probably responsible for the attachment of the virion to its host cell. The individual polypeptides of the adenovirus particle and their structural role have now been studied in great detail (see PHILIPSON and PETTERSSON, 1973; PHILIPSON and LINDBERG, 1974, for recent reviews) but will not be discussed here.

Adenovirus mRNA can be most stringently prepared by releasing viral RNA from polysomes isolated from infected cells. Almost all such viral RNA species contain tracts of polyadenylic acid at their 3′-termini (PHILIPSON et al., 1971) and 5′-termini capped by modified 5′-5′ linked nucleotides (SHATKIN, personal communication). This viral mRNA is assumed to comprise distinct mRNA species, which are defined as covalently linked chains of nucleotides complementary to discrete segments of the viral genome.

Attempts have been made to describe adenovirus mRNA species synthesized during three types of virus-cell interaction, the "early" phase and the "late" phase of the lytic cycle following infection of permissive human cells and in adenovirus-transformed rodent cells. The early phase of infection is that period of the lytic cycle preceding viral DNA synthesis and is best defined operationally by the cultivation of infected cells in the presence of drugs which inhibit DNA synthesis, such as cytosine arabinoside or fluorodeoxyuridine (WIGAND and SCHMEIDER, 1972): events that take place once viral DNA replication has been initiated are taken to constitute the late phase. Undoubtedly, the late phase could be subdivided into several kinetically defined periods in which different activities command the metabolic energies of the cell. Most studies on late events in RNA expression have been carried out between 15 and 18 h following adenovirus infection, because the rate of viral RNA synthesis is then maximal.

All viral messenger RNAs are initially transcribed from an adenovirus template by an RNA polymerase using 5′-ribonucleotide triphosphates, but the details of these events remain obscure. Very little attention, for example, has been paid to the state of the viral template in the infected cell—whether it is circular or linear, protein-covered (like eukaryotic chromatin) or free DNA. Somewhat more effort has been directed toward defining the RNA polymerase responsible for transcribing the adenovirus genome (see later). However, it has not proved possible to use purified adenovirus DNA and any eukaryotic polymerase to devise an in vitro transcription system whose products could be considered a reasonable facsimile of those present in the infected cell.

In this review, we will summarize current data describing the expression of viral mRNA in lytically infected and transformed cells, and will place most emphasis on the adenovirus 2 (Ad2) system. Adenovirus 2 is a very lytic virus, its brief infectious cycle and large burst of physical particles making it attractive to study. Moreover, biochemical work on Ad2 has advanced rapidly with the use of restriction endonucleases, which cleave viral DNA into discrete frag-

ments. Such fragments have been used to map viral mRNA, viral proteins, the sites of temperature-sensitive mutations, to study viral DNA replication and the viral DNA sequences present in Ad2-transformed rodent cells and as oncogenic elements to transform mammalian cells. Hopefully, all this new information will ultimately be drawn into a comprehensive picture of the molecular biology of adenovirus. Presently, however, only the outlines of this panorama are visible.

II. Early Viral mRNA Synthesis

When adenoviruses infect HeLa or KB cells they rapidly usurp the metabolic machinery of the host cell to establish a lytic infection, resulting in the ultimate destruction of the host and the release of a new crop of virus particles. The virus appears to adsorb, by means of its fibers (PHILIPSON et al., 1968), to specific receptors on the cell surface (MORGAN et al., 1969; LONBERG-HOLM and PHILIPSON, 1969). Once within the cell, it is uncoated in several stages until the DNA genome is almost free of protein (see PHILIPSON and LINDBERG, 1973, for details). During the early phase, the virus has relatively little effect on the biosynthetic activity of exponentially growing cells although it can induce both cellular DNA synthesis and certain enzymes, including thymidine kinase, when resting cells are infected (see GREEN, 1970).

The first efforts to detect virus-specific proteins early in adenovirus infection relied on immunologic techniques, and two antigens have been described. The tumor (T) antigen has been defined by reaction in immunofluorescence or complementation fixation tests with sera isolated from rodents bearing tumors induced by adenovirus or adenovirus-transformed cells (HUEBNER, 1967). Antigen(s) reacting against such sera can be detected in infected human cells as soon as 4–6 h after infection and their synthesis is not inhibited by inhibitors of DNA synthesis such as cytosine arabinoside and fluorodeoxyuridine (GILEAD and GINSBERG, 1965; HOGGAN et al., 1965; HOLLINSHEAD and HUEBNER, 1969). Although attemps to purify "T" antigen have been made (TAVITIAN et al., 1967; GILEAD and GINSBERG, 1968a, b; TOCKSTEIN et al., 1968), they have met with little success; consequently, no specific function, or indeed polypeptide, can be designated as "T" antigen. In this respect, rather more progress has been made with another "early" antigen, the "P" antigen. Rabbit kidney cells, infected with Ad5 in the presence of cytosine arabinoside, were used to raise sera in rabbits: using antiserum prepared in this fashion, an antigen(s), "P", whose time course of synthesis is very similar to that of "T", have been detected early after infection (RUSSELL et al., 1967; HAYASHI and RUSSELL, 1968). This antiserum appears to precipitate specifically a polypeptide with a molecular weight of 70000 daltons whose synthesis is first observed very early after infection (RUSSELL and SKEHEL, 1972). Other novel polypeptides can be detected in the infected cell before replication of viral DNA (RUSSELL and SKEHEL, 1972; BABLANIAN and RUSSELL, 1974; WALTER and MAIZEL, 1974), but their significance remains to be established.

Extensive sets of temperature-sensitive mutants of Ad5, Ad12, and Ad2 have now been isolated (WILLIAMS et al., 1971; ENSINGER and GINSBERG, 1972; LUNDHOLM and DOERFLER, 1971; SHIROKI et al., 1972; BEGIN and WEBER, 1975; WEBER et al., 1975). The 80 randomly isolated mutants of Ad5 have been characterized best, and when a complementation index of 10 is used, they fall into 18 complementation groups (WILLIAMS and USTACELEBI, 1971; WILLIAMS et al., 1974). As 12 of these groups are defined by only one mutant, many more complementation groups undoubtedly remain to be identified. Conversely, it may well be that some of the observed complementation resulted from intracistronic complementation, as has been observed with "late" mutants of SV40 (LAI and NATHANS, 1974). Two of these 18 Ad5 complementation groups and 3 of Ad12 have been shown to represent "early" functions, that is, those whose expression is required for viral DNA synthesis (RUSSELL et al., 1972; SHIROKI et al., 1972; WILKIE et al., 1973; RUSSELL et al., 1974; SHIROKI and SHIMOJO, 1974). One of the "early" Ad5 mutants is thought to contain a lesion in a single-stranded DNA-binding protein (VAN DER VLIET et al., 1973; LEVINE et al., 1974), probably similar in function to the gene 32 protein of bacteriophage T_4 (ALBERTS and FREY, 1970). The adenovirus 2 DNA-binding protein has a molecular weight of about 70000 daltons (LEVINE et al., 1974) and presumably corresponds to the "P" antigen described above. The other mutant "early" function remains to be identified, but the viral DNA sequences coding for it have been shown, by physical mapping of recombinants between mutants of the closely related serotypes Ad5 and $Ad2^{+}ND_1$, to lie in the left 30% of the viral genome (GRODZICKER et al., 1974; WILLIAMS et al., 1975; SAMBROOK et al., 1975). The three "early" complementation groups of Ad12 have not been studied in detail, but all three functions seem to be required for initiation of viral DNA synthesis and the formation of replication complexes (SHIMOJO et al., 1974).

During the early phase of the lytic cycle, a fraction only of the genome is expressed as mRNA (FUJINAGA and GREEN, 1970; GREEN et al., 1970). Conventional filter hybridization techniques first detect the synthesis of this cytoplasmic viral RNA about 2 h after infection. When cells are labeled with ^{3}H-uridine from 4–6 h following Ad2 infection, about 4% of the newly synthesized, poly (A)-containing, cytoplasmic RNA will hybridize to viral DNA; however, when the same labeling protocol is followed in the presence of cyclohexamide at concentrations sufficient to inhibit protein synthesis, about 20% of this same RNA fraction is complementary to Ad2 DNA (CRAIG and RASKAS, 1974).

Many attempts have been made to define distinct "early" viral RNA species by resolving RNA, labeled in the presence or absence of drugs by electrophoresis in polyacrylamide gels or by sucrose density gradient centrifugation. A brief summary of these data is given in Table 1: some putative viral RNA species have been described only as a "shoulder" on the predominate peak of RNA hybridizable to Ad2 DNA and such species are indicated in the Table. Each group that has studied labeled RNA extracted from cytoplasm early after infection has described a distinct fraction of viral RNA mi-

Table 1. "Early" Ad2 RNA species

Authors	Labeling conditions	RNA analyzed	Viral RNA species observed	% viral RNA in peak	Comments
PARSONS and GREEN, 1971	2–6 h after infection + cyclohexamide	polysomal RNA	26–28S (shoulder) 22–24S (major) 15–17S	15 57 28	26–28S RNA not seen if DMSO treat RNA
LINDBERG et al., 1972	3–5 h after infection	poly(U)-selected polysomal RNA	22S (major) 15S (minor)	67 33	
BÜTTNER et al., 1974	5–8 h after infection + cyclohexamide	polysomal RNA	24–26S (shoulder) 19–20S (major) 15–18S (shoulder)	15 74 11	19–20S RNA hybridized to both strands of the genome
TAL et al., 1974	2–6 h after infection + cyclohexamide	poly(A)-selected cytoplasmic RNA	27S 24S 19S (major) 12–15S (shoulder)	19 17 43 17	
PHILIPSON et al., 1974	3–7 h after infection + cytosine arabinoside	poly(A)-selected polysomal RNA	20S (major) 15S 13S	60 20 20	20S RNA contains species complementary to both strands of the genome

grating at 20S (PARSONS and GREEN, 1971; LINDBERG et al., 1972; BÜTTNER et al. 1974; PHILIPSON et al., 1974; TAL et al., 1974; CRAIG et al., 1975): BÜTTNER et al. (1974) and PHILIPSON et al. (1974) have shown that this size class of "early" viral RNA contains sequences complementary to both strands of the Ad2 genome. Thus, two RNA chains of molecular weight of about 0.75×10^6 daltons, must be present early after infection. In addition to the "early" viral RNA species migrating at 20S, Ad2 RNA species of 13–15 S and 24–26S have also been generally observed. The proportion of the total viral RNA found in these two size classes varies a great deal (Table 1, column 5). As the 24–26S fraction is usually observed as a slower-migrating shoulder on the 20S peak, it may not represent a distinct RNA species, but rather a collection of species. Indeed, the slower migration could be due to conformation as much as to size, as these analyses were not generally carried out under denaturing conditions. By contrast, the 13–15 S RNA is resolved as a distinct peak during polyacrylamide gel electrophoresis. Because of the heterogeneity of this peak, several authors have suggested that the 13–15S RNA, molecular weight $0.35–0.43 \times 10^6$ daltons, comprises at least 2 different RNA species. Analogy with SV40 "late" RNA, where 19S species has been shown to be the kinetic precursor of 16S "late" SV40 mRNA (WEINBERG et al., 1974; ALONI et al., 1975) suggests the possibility that the 19–20S species may have sequence

homology with the 13–15 S RNA. In summary, then, a major "early" mRNA species, approximately 0.75×10^6 daltons, must be transcribed from each strand of the viral genome while mRNA species of $0.35–0.43 \times 10^6$ daltons also appear to be present.

One physical limitation to the study of "early" Ad2 mRNA is the scarcity of material; this has precluded the isolation of chemically pure mRNA species and their mapping on the viral genome by the techniques developed with bacteriophage DNAs. The recent recognition of the utility of bacterial restriction endonucleases has, however, permitted the development of methods with which small amounts of viral RNA can be mapped. These enzymes recognize, and cut within, short (4–6 base-pairs), palindromic DNA sequences and can thus be used to generate discrete fragments of viral DNA. Methods for mapping viral RNA, first developed with SV40 DNA (KHOURY et al., 1972; SAMBROOK et al., 1972) rely on the annealing of unlabeled RNA extracted from infected cells to ^{32}P-labeled viral DNA of high specific activity prepared by in vivo incorporation of the isotope. The strands of the ^{32}P-labeled DNA probe are first spearated by a variety of methods to eliminate the complication of competing DNA-DNA and DNA-RNA reactions: thus, all of the labeled DNA entering duplex during the hybridization reaction must have annealed with complementary RNA. Hybrid formation can be monitored readily either by chromatography on hydroxylapatite (MIYAZAWA and THOMAS, 1965; BERNARDI, 1965) or by resistance to S_1 nuclease (VOGT, 1973).

When cytoplasmic RNA extracted from Ad2-infected cells during the early phase of the lytic cycle is annealed to strands of the six *Eco* RI fragments of Ad2 RNA, separated by electrophoresis of denatured DNA (SHARP et al., 1974b), only a distinct fraction of each fragment strand enters hybrid. A range of "early" RNA concentrations is added to a series of hybridization mixtures to ensure that the complementary DNA sequences are saturated. Only 40% of the *r* strand of *Eco* RI fragment D, for example, enters hybrid when 0.5×10^{-5} μg of the ^{32}P-labeled DNA is annealed with 0.5, 1.0, or 1.5 mg/ml of "early" cytoplasmic RNA for 24 h: none of the *l* strand of *Eco* RI fragment D enters hybrid with the same series of RNA concentration. Saturation values observed with each strand of each *Eco* RI fragment are given in Table 2. In total, about 20% of one strand equivalent of Ad2 DNA is complementary to "early" viral RNA.

PHILIPSON et al. (1974) have carried out similar experiments using *Eco* RI fragment strands separated by a different method: ^{32}P-labeled fragment DNA was denatured and annealed to excess, unlabeled, separated strands of intact DNA. One strand of the ^{32}P-labeled annealed to its unlabeled complement and was separated from the other ^{32}P-labeled strand by chromatography on hydroxylapatite. These authors (PHILIPSON et al., 1974; TIBBETTS et al., 1974) estimate that over 40% of the Ad2 genome is expressed during the early phase of the lytic cycle using strands separated in this fashion (Table 2). These authors observed higher levels of hybridization to all *Eco* RI fragment strands than did SHARP et al. (1974b), including those reported to be negative by the latter

Table 2. Regions of *Eco* RI fragments of Ad2 DNA complementary to "early" mRNA. The fraction of labeled fragment strand entering hybrid when annealed with excess "early" cytoplasmic RNA is given for *Eco* RI fragment and strand combination (columns 1–12). The values given in lines 1 and 2 are taken from SHARP et al. (1974b) and PHILIPSON et al. (1974a), respectively. The percentage of the genome expressed as "early" mRNA was calculated by summing the products of the fraction of a strand annealing to RNA and the known fractional size of that fragment

	% ^{32}P-Labelled DNA in hybrid												% genome	
Eco RI fragment	A		B		C		D		E		F			
Strand	r	l	r	l	r	l	r	l	r	l	r	l	r	l
SHARP et al. (1974)	15	0	0	30	0	50	40	0	30	0	0	0	13.8	8.7
PHILIPSON et al. (1974)	21	9	7	42	9	60	72	3	40	4	23	13	23.0	17.3

authors. These differences may be attributable to a number of factors, including nuclear leakage (see below).

As discussed above, distinct size-classes of "early" Ad2 mRNA have been described. TAL et al. (1974) have attempted to localize the different RNA size-classes on the viral genome by hybridizing ^{32}P-labeled RNA from different fractions of polyacrylamide gels to a battery of filters each with a different *Eco* RI fragment of Ad2 DNA immobilized on it. Thus, they could determine the size-class to which the RNA complementary to the six *Eco* RI fragments belonged. From such experiments, these authors concluded that a 13 S species and part of an 11 S species were complementary to *Eco* RI fragment A, the remaining part of this 11 S species and a 19 S species were transcribed from *Eco* RI fragment B, no RNA was complementary to *Eco* RI fragment F, 20 S and 13 S species were transcribed from *Eco* RI fragment D, and a 13 S species was also complementary to fragment E, while the RNA transcribed from *Eco* RI fragment C was apparently heterogenous in size. These results agree in part with the data obtained using separated strands of the *Eco* RI fragments: those experiments demonstrate, for example, that "early" mRNA is complementary to *Eco* RI fragments A, B, D, E, and C while none or very little, is transcribed from fragment F. BÜTTNER and GREEN (1975) have recently shown that "early" viral RNA sedimenting at 20 S anneals to *Eco* RI fragments B, D, E, and C. If the regions of these fragments expressed as "early" viral RNA determined by mapping experiments (see Table 2) are arranged as shown in Figure 1 (SHARP et al., 1974b), then the RNA would be expected to sediment at about 20 S. BÜTTNER and GREEN also concluded that 11–13 S "early" mRNA is transcribed from *Eco* RI fragment A.

Because all the mapping experiments described above are very recent and have not yet stood the test of time, too much emphasis should not be placed on them. However, all the available data suggest that a fraction only of the Ad2 genome is expressed during the early phase of the lytic cycle; "early" mRNA

is undoubtedly transcribed from both strands of the viral genome and the coding sequences appear to be distributed over the entire genome. The topographic arrangement of early gene products on the Ad2 genome is in striking contrast to the arrangement of early bacteriophage gene functions. In Figure 1, we show a comparison of the maps of the "early" mRNA of Ad2, T7, and λ, T7 "early" mRNA is transcribed from the left hand 20% of the genome and its transcription is initiated from three adjacent promoter sites at the far left (SUMMERS and SIEGEL, 1969; MILLETTE et al., 1970; MINKLEY and PRIBNOW, 1973). The mRNA coding for the four early gene products is thought to be coordinately transcribed and individual mRNA species cleaved from the initial transcript by the endoribonuclease activity, RNAase III (DUNN and STUDIER, 1973a, b; KRAMER et al., 1974). In the case of phage λ, "early" mRNA transcription is initiated at two sites, one to the left of the *l* strand and one to the right on the *r* strand, resulting in the expression of early gene products (TAYLOR et al., 1967; KOURILSKY et al., 1968). Thus, in both these systems, a number of early gene products are expressed coordinately as a result of initiation of transcription from a few sites on the genome. The map of "early" Ad2 mRNA shown in Figure 1 was suggested as the simplest possible arrangement based on our hybridization data: it is obvious from this map, that there is no contiguous arrangement of the viral sequences coding for "early" gene products. This suggests that "early" Ad2 mRNA is synthesized either by RNA polymerase inititation at multiple sites on each strand of the genome or by extensive processing of a primary transcript, the length of the entire genome, whose synthesis is initiated at one site at the end of each strand.

The second conclusion that can be drawn from the map of Ad2 "early" mRNA shown in Figure 1 is that Ad2 must synthesize at least four "early" gene products. This is only a lower limit, because each region of Ad2 DNA complementary to "early" RNA is of sufficient size to code for more than one polypeptide. So far, only two "early" gene products have been defined by temperature-sensitive mutants of Ad5; hopefully, others will be identified in the near future. However, it may not be possible to isolate temperature-sensitive mutants in some "early" Ad2 genes as they may not be essential for plaquing of the virus on tissue-culture cells. In fact, this situation has been shown to exist for an "early" Ad2 gene mapping around 0.85 on the genome (see Fig. 1), because deletion of this segment of the viral genome does not produce a virus defective for growth in tissue culture (FLINT et al., 1975).

A. "Late" Ad2 mRNA

The late phase of Ad2 infection commences with the onset of replication of viral DNA. The rates of *total* DNA, RNA, and protein synthesis are increased after infection (PĨNA and GREEN, 1969), but cellular functions are progressively inhibited (PĨNA and GREEN, 1969; LEDINKO and FONG, 1969). Cellular DNA synthesis, for example, has almost ceased by 18 h after infection, when the maximal rate of viral RNA synthesis is attained. During the late phase of

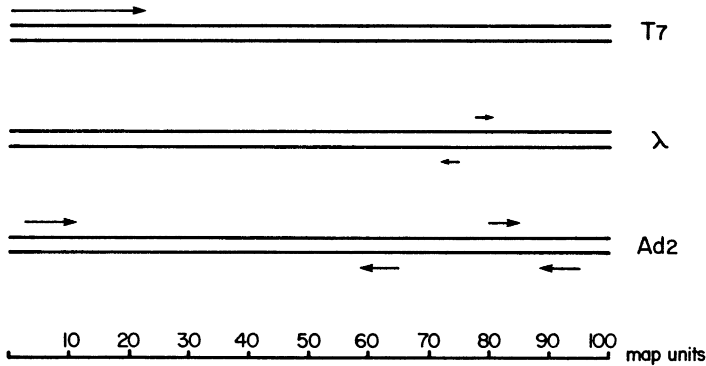

Fig. 1. Early transcription of T7, λ, and Ad2 DNAs. Each viral DNA genome is represented as 2 solid horizontal lines. Lines drawn above and below genomes represent transcribed before onset of viral DNA replication. Arrows indicate direction of transcription. For convenience, the 3 genomes are drawn on same scale, 0–100 units. T7 and λ maps are modified from DUNN and STUDIER (1974) and SZYBALSKI (1970), respectively, while map of Ad2 "early" mRNA is taken from SHARP et al. (1974b)

the lytic cycle, about 50% of all the RNA synthesized is transcribed from a viral template (GREEN et al., 1970) and nearly all the newly synthesized RNA reaching the cells' polyribosomes is viral in origin (THOMAS and GREEN, 1966). Concurrent with this increase in the proportion of cytoplasmic mRNA that is viral, there is an 80–90% inhibition of the appearance of newly synthesized ribosomal RNA in the cytoplasm (RASKAS et al., 1970; LEDINKO, 1972). This inhibition seems to occur at the level of processing of the 45 S ribosomal precursor, because synthesis of this precursor continues (RASKAS et al., 1970; LEDINKO, 1972). A similar inhibition in the production of mature ribosomal RNA is observed in uninfected cells when protein synthesis is blocked (MADEN et al., 1969); cellular protein synthesis is disrupted late during Ad2 infection (BELLO and GINSBERG, 1965; GINSBERG et al., 1967), possibly accounting for the observed inhibition of the production of ribosomal RNA (PHILIPSON et al., 1974a). Although the infected human cell accumulates large amounts of viral viral mRNA late in the lytic cycle, most of the previously synthesized cellular mRNA decays with a half-life similar to that observed in uninfected cell mRNA (PHILIPSON et al., 1974a): thus, cellular mRNA does not appear to be preferentially degraded. Large amounts of a low molecular weight (5.5 S) class of viral-specific RNA, known as virus-associated (VA), are also synthesized late during the lytic phase of Ad2 infection (OHE et al., 1969; OHE, 1972). This type of RNA has been studied in detail: it is 156 nucleotides in length and has been completely sequenced (OHE and WEISSMAN, 1971). It now appears that at least two VA RNA species are present late during Ad2 infection, both of which map between positions 30 and 32 on the viral genome (MATHEWS, 1975; PETTERSSON and PHILIPSON, 1975). Although VA RNA is synthesized in such large amounts at late times (about 20% of the total viral cytoplasmic RNA) and has been studied in some detail, the function of this RNA is totally unknown at present.

Table 3. Fraction of *Eco* RI fragments of Ad2 DNA complementary to 'late' mRNA. The data is expressed in the same way as in Table 2 and is taken from the sources cited in the legend to that Table

	% ^{32}P-Labeled RNA in hybrid												% genome	
Eco RI fragment	A		B		C		D		E		F			
Strand	r	l	r	l	r	l	r	l	r	l	r	l	r	l
SHARP et al. (1974)	80	15	65	35	25	60	100	0	85	10	100	0	75.3	19.9
PHILIPSON et al. (1974)	71	15	52	40	31	50	82	2	82	12	84	11	66.8	21.7

Many attempts have been made to define late viral mRNA species, but the degree of resolution achieved to date has not been great. "Late" RNA is usually extracted about 18 h after Ad2 infection and labeled for the preceding 2–3 h. Sucrose density centrifugation or polyacrylamide gel electrophoresis of "late", polyadenylated Ad2 mRNA resolve major peaks at 26S and 19S and a number of minor peaks and shoulders (PARSONS et al., 1971; BHADURI et al., 1972; LINDBERG et al., 1972; WALL et al., 1972; PHILIPSON et al., 1974a; TAL et al., 1974). Detectable "late", labeled Ad2 mRNA from as small as 12S to a heterogenous class in the 29–40S range has been described, but the significance of these minor species remains to be established.

Two groups of workers have described the annealing of ^{32}P-labeled strands of Ad2 DNA fragments to polyadenylated, "late", cytoplasmic RNA (SHARP et al., 1974b; TIBBETTS and PETTERSSON, 1974): the saturation values observed with the 12 *Eco* RI fragment strands are given in Table 3. Essentially one strand equivalent of each fragment is complementary to "late" mRNA. All "early" mRNA sequences, therefore, seem to be present during the late phase of the lytic cycle. In fact, "early" mRNA transcribed from the *l* strand of Ad2 DNA is highly amplified at this time: while 0.70 mg/ml of "early" cytoplasmic RNA are required to saturate 2×10^{-3} μg of ^{32}P-labeled *Eco* RI–D*r* strand, a "late" cytoplasmic RNA concentration of only 0.1 mg/ml is sufficient (SHARP et al., 1974b).

The data given in Table 3, obtained by annealing the separated strands of *Eco* RI fragments of Ad2 DNA with RNA extracted from the cytoplasm of cells late after infection, can be used to construct a map of the regions of the genome expressed (Fig. 2). These data are in agreement with the results of previous experiments in which complete separated strands of Ad2 DNA were annealed to saturating amounts of labeled RNA (GREEN et al., 1970; TIBBETTS et al., 1974). In both cases, one strand of the viral DNA-formed hybrid with 80% of the total RNA: this strand sedimented to a lower buoyant density in CsCl equilibrium gradients after binding of poly (U:G) to denatured DNA. Ad2 DNA, specifically-labeled at its 3'-termini by limited treatment with exonuclease III followed by replacement of the excised nucleotides using a DNA polymerase and ^{32}P-labeled deoxynucleotide triphosphates, has been used to

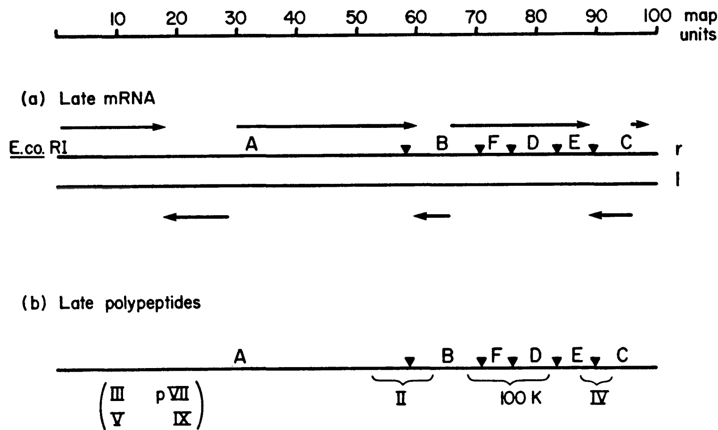

Fig. 2. Regions of the Ad2 genome coding for "late" mRNA and some "late" viral polypeptides. (a) 2 solid, horizontal lines represent 2 strands of Ad2 DNA. *Eco* RI cleavage sites are represented by triangles abov the 2 lines, while fragments are designated A–F. Arrows pointing from left to right above genome show region of Ad2 r strand expressed as "late" mRNA and indicate 5′ to 3′ polarity of RNA transcript. Arrows below genome show corresponding information for "late" mRNA complementary to l strand. Mapping within *Eco* RI fragment A achieved using fragments produced by other restriction endonucleases (SHARP et al., 1974b) and arrangement shown is simplest possible that is consistent with available data. (b) Ad2 DNA represented by horizontal line and fragments produced by cleavage with *Eco* RI are shown as in (a). Late viral polypeptides whose in vitro synthesis is stimulated by "late" RNA selected on particular fragment is listed below that fragment. Polypeptides II, III and IV form virion hexon, penton, and fiber, respectively, V is minor core protein, pVII precursor to major core protein (ANDERSON et al., 1973), IX is a hexon-associated virion protein, while 100k polypeptide is nonstructural and of unknown function. Parentheses indicate unknown order of indicated polypeptides within *Eco* RI fragment A. This data is taken from LEWIS et al. (1975)

show that the 3′-terminus of the strand banding at the lower density in poly (U, G) gradients lies to the left, when the genome is drawn conventionally, as in Figure 2 (SHARP et al., 1974b). Thus, the Ad2 DNA strand, 80% of which is complementary to "late" mRNA, is transcribed from left to right as shown in Figure 2, and is designated the *r* strand, while its complement, the *l* strand, is transcribed toward the left. Most of the viral information that is expressed as mRNA only during the late phase of the lytic cycle is from the *r* strand, with only 10% of the *l* strand being expressed exclusively "late".

Preliminary attempts have been made to map "late" mRNA species of different size to discrete regions of the Ad2 genome. TAL et al. (1974) have reported that labeled, "late" cytoplasmic RNA contains 27S, 24S, 19S, and 11S RNA species transcribed from *Eco* RI A, 24S RNA complementay to both fragments C and E and 23S RNA which hybridized to *Eco* RI fragment F. *Eco* RI fragment A, which codes for most of these "late" viral RNA species comprises nearly 60% of the Ad2 genome (Fig. 2): clearly, then further studies of this type using different sets of restriction endonuclease fragments, will be required to define Ad2 late mRNA in detail.

Ad2 codes for several large polypeptides that are expressed in significant amounts late during the lytic cycle (WHITE et al., 1969; RUSSELL and SKEHEL, 1972; ANDERSON et al., 1974). One of these, polypeptide II, has a molecular weight of about 100000–120000 daltons and forms the subunits of the virion hexon (PETTERSSON et al., 1967; MAIZEL et al., 1968; HORWITZ et al., 1969). The mRNA from which the hexon is translated must be at least 1×10^6 daltons in molecular weight, and thus should sediment more rapidly than 22S: viral proteins of molecular weight 13000 daltons could be translated from 12S mRNA species. Thus the described sizes of the late mRNA vary sufficiently to permit each known viral protein to be translated from a monocistronic mRNA: although different size-classes of "late" viral mRNA have been repeatedly separated following fractionation of polyacrylamide gels or sucrose density gradients, it has not yet been shown that these size-classes actually contain different viral sequences. Thus, 26S "late" viral mRNA could be cleaved into a 19S species and a smaller RNA chain. This type of alteration could conceivably play a regulatory role in the synthesis of a second viral gene product from one mRNA species. ANDERSON et al. (1974) have approached this question by resolving "late" mRNA on sucrose density gradients and translating, in vitro, the RNA from each fraction: little correlation between the size of the mRNA and the size of the protein whose synthesis is stimulated in vitro was observed. It was, however, clear from these data that mRNA of a particular size-class did code for a specific viral polypeptide.

It is now possible to map viral proteins to defined regions of the genome either by translating mRNA selected by annealing to restriction endonuclease fragments of viral DNA (LEWIS et al., 1975) or by generating translatable RNA in vitro from specific fragments using a purified RNA polymerase (CRAWFORD and GESTELAND, 1973; ROBERTS et al., 1975). LEWIS et al. (1975) hybridized Ad2 late cytoplasmic RNA to the 6 *Eco* RI fragments of Ad2 DNA and, after purification, translated the RNA selected in this way in an in vitro system. Figure 2b summarizes the results of these experiments and compares this map of "late" viral proteins with the current map of "late" viral mRNA. Consider, for example, polypeptides II and IV, the constituents of the virion hexon and fiber, respectively. In vitro synthesis of polypeptide II was stimulated by "late" mRNA selected on *Eco* RI fragments a And B: LEWIS et al. (1975) therefore suggest the mRNA coding for this polypeptide maps across the A:B *Eco* RI junction (position 58.5). Their data does not distinguish between the *r* and *l* strands, as double-stranded fragments were used in the RNA selection. However, comparison with the map of "late" mRNA (part a of Fig. 2) suggests that hexon polypeptide mRNA must be transcribed from the *r* strand, as no exclusively late sequences are complementary to the *l* strand in this region. Similarly, polypeptide IV, whose in vitro synthesis was stimulated by RNA selected on *Eco* RI fragments C and E, appears to be transcribed from the *r* strand within the region bounded by positions 85 and 100 (see Fig. 2). The assignements of these genes described above are in agreement with those recently made by the application of both physical mapping techniques (GRODZICKER et

al., 1974; WILLIAMS et al., 1975) and serologic analysis (MAUTNER et al., 1975) to recombinants of Ad5 and the nondefective adenovirus 2: SV40 hybrid virus, Ad2^{+}ND$_1$. These powerful methods in combination should allow the Ad2 genome to be structurally delineated in some detail in the near future, although the role of many of the gene products thus defined may not be apparent for some time.

B. Messenger RNA in Adenovirus 2-Transformed Cells

When rodent cells in tissue culture are infected with a reasonably high multiplicity of Ad2 a few of the cells will become transformed in their morphology and growth properties (FREEMAN et al., 1967a, b; REED, 1967; CASTO, 1968; MACALLISTER et al., 1969a, b; GALLIMORE, 1974). Although all adenovirus serotypes transform mammalian cells inefficiently, the in vitro growth properties of the transformants vary depending on the transforming serotype (FREEMAN et al., 1967a; GALLIMORE, 1974). Cells transformed by the "high oncogenic" serotypes 12 and 18 produce tumors when infected into newborn animals, while cells transformed by nononcogenic serotypes 2 and 5 do not unless the animals are immunosuppressed (FREEMAN et al., 1967c; STROHL et al., 1967; CASTO, 1968; MACALLISTER et al., 1969a, b; GALLIMORE, 1973; WILLIAMS, 1973). Apart from the stable, altered cell morphology, the only indication of continued expression of viral information in the transformed cells is the presence of virus-specific, tumor antigen, T (FREEMAN et al., 1967; HUEBNER, 1967; GILDEN et al., 1968; GALLIMORE, 1974) located in the nucleus and virus-specific transplantation antigen(s) (SJÖGREN et al., 1967; PANTELEAKIS et al., 1978; HOLLINSHEAD and ALFORD, 1969). It has not been possible to rescue infectious virus from adenovirus-transformed cells either by induction with chemical or biological agents (LANDAU et al., 1966; LARSON et al., 1966) or by cultivation with permissive cells (DUNN et al., 1973). In support of the hypothesis that viral gene product(s) is responsible for either induction or maintenance of the altered cell morphology, Ad2, 7, and 12 transformed cells have been shown to contain viral DNA sequences and to express viral-specific RNA in their cytoplasm (FUJINAGA and GREEN, 1966, 1967a, b; GREEN et al., 1970; ORTIN and DOERFLER, 1975).

One virus/cell system that has been studied in detail both biologically and physically is the Ad2-transformed rat cell. Rat cells are semipermissive for infection by Ad2: if the rat cells are derived from embryonic brain, about 90% of the infected cells synthesize viral capsid antigens and viral DNA, and produce virions when infected at 20 PFU/cell (GALLIMORE, 1974). Most of the cells succumb to the infecting virus and produce some 400 PFU/cell. A few cells, however, survive this attenuated lytic passage and a fraction of the survivors ultimately become transformed. Several lines of rat embryo cells independently transformed by Ad2 have been examined for their viral DNA content by the DNA renaturation kinetics technique first described by GELB et al. (1971). Restriction endonuclease fragments of Ad2 DNA, labeled to a high specific

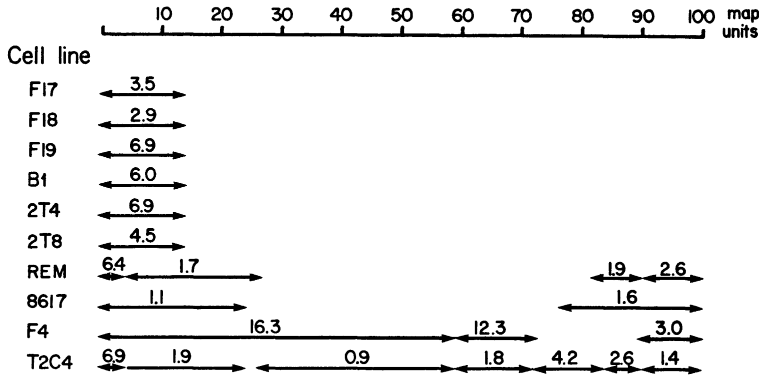

Fig. 3. Viral DNA content of Ad2-transformed rat embryo cell lines. Ad2 genome is represented by solid horizontal line, 0–100 units, at top of figure. Viral DNA sequences present in each of 10 Ad2-transformed rat embryo cell lines listed in column 1 are indicated by arrows, ⟷, below approximate region of genome. Numbers given above each arrow thus, $\overset{X}{\longleftrightarrow}$, show number of copies per diploid quantity of DNA of indicated sequences in that particular cell line. The data on which this figure is based were taken from SHARP et al., 1974a and GALLIMORE et al., 1974

activity with ^{32}P, were used to probe for the presence or absence of homologous DNA in the transformed cell nucleus (SHARP et al., 1974a; GALLIMORE et al., 1974). The result of this analysis is outlined in Figure 3.

It is clear that all Ad2-transformed cell lines examined contain DNA sequences homologous to the left end of the viral genome, indicating that any gene(s) responsible for the transformed phenotype must reside in this region of the genome. Consistent with this hypothesis is the recent report by GRAHAM et al. (1974) that a specific restriction endonuclease fragment of Ad5 DNA comprising the left-hand 8% of the viral genome will transform rat cells in vitro. In addition to the viral DNA from the left end, some transformed cells also contain DNA homologous to other parts of the genome, but no transformed cells retained viral sequences homologues to the entire genome. The fact that Ad2-transformed cells contain a defective viral genome probably reflects the lytic response with subsequent death of most rat cells to an intact genome.

The size of viral, cytoplasmic mRNA has been examined in detail in one Ad2-transformed rat cell line, 8617 (FREEMAN et al., 1967a), which grows well in suspension culture: WALL et al. (1973) resolved polysomal, viral RNA from this cell line into three classes, sedimenting at 26S, 20S, and 16S on 15–30% SDS-sucrose gradients. "Early" mRNA species with similar sedimentation coefficients have also been described (see above). As "T" antigen is expressed both in transformed cells and during the early phase of lytic infection, similarities in transformed and "early" mRNA are expected. In a study of the same cell line, FUJINAGA and GREEN (1970) showed that the hybridization of ^{3}H-early Ad2 mRNA to limiting amounts of viral DNA immobilized on a filter was competed 50% by transformed-cell RNA. They, therefore, concluded that half of the viral information expressed "early" during infection is also ex-

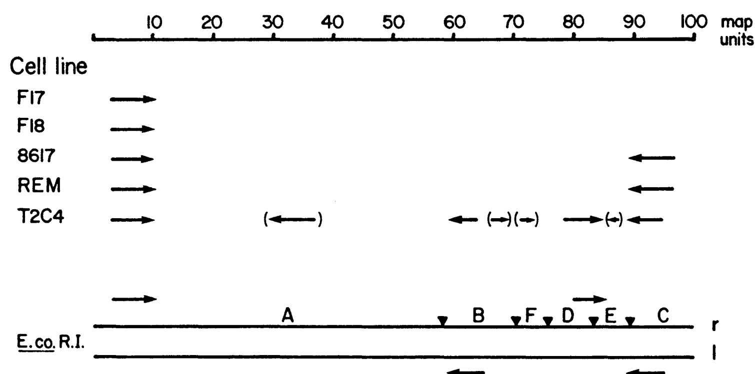

Fig. 4. Viral mRNA sequences in Ad2-transformed rat embryo cell lines. Ad2 genome depicted as in Fig. 3. Regions of genome from which mRNA is transcribed in each of 5 cell lines listed in column 1 indicated by arrows drawn below appropriate region of genome. Arrows show direction of transcription. Parentheses around arrows (→) indicate sequences whose precise location not yet shown. Figure is based on data given in FLINT et al. (1975). Second part of figure shows map of "early" Ad2 RNA. Constructed as map of "late" mRNA (Fig. 2a) from data taken from FLINT et al. (1975)

pressed in this transformed-cell line. Ad12-transformed hamster cells show a pattern of viral mRNA species very similar to that observed in 8617 cells (ORTIN and DOERFLER, 1975). Although few other adenovirus-transformed cells have been examined for the size of viral mRNA, FUJINAGA and GREEN (1966, 1967a, b) have shown that types 3, 5, 6, 7, 12, 14, 16, 18, and 31 transformed cells do contain viral mRNA. It is likely that transformation by all of these serotypes proceeds by the same mechanism and that all adenovirus transformants contain only fragments of the viral genome integrated into their cellular DNA.

When cytoplasmic RNA from five of the ten transformed-cell lines listed in Figure 3 is mapped on the viral genome by hybridization to the separated strands of ^{32}P-labeled restriction endonuclease fragments of Ad2 DNA, it becomes possible to designate each line as one of three classes (FLINT et al., 1975). The first class comprises those lines that synthesize mRNA that is complementary to only 6% of the left end of the viral genome (Fig. 4). This region of the genome is also expressed during the early phase of a lytic infection. These cells are transformed and "T"-antigen positive, indicating that both of these properties must be coded for by these mRNA sequences. The second class of transformed cells contain viral mRNA sequences that are complementary to both the left and right ends of the genome (Fig. 4, lines 3 and 4), both regions that are expressed "early" in infection. The remaining class of transformed cells, expresses mRNA from all of the four "early" coding regions, as well as some additional sequences (Fig. 4, line 5).

Comparison of lines 1–5 with the map of "early" mRNA also shown in Figure 4 reveals that DNA sequences of "early" mRNA are preferentially expressed in transformed cells in comparison to the viral sequences coding for

exclusively "late" mRNA. In fact, when a transformed cell contains viral DNA coding for "early" gene products, it appears that these sequences are transcribed into viral mRNA: the only exception of this generality is the absence of mRNA complementary to the *r* strand of *Eco* RI fragments D and E in 8617 and REM cells.

A major consequence of the presence of several "early" mRNAs in transformed cells is the possibility that they may be translated into the corresponding "early" proteins, which would then be detected as tumor specific antigens. Thus, a single transformed cell could contain several specific antigens and stimulate the production of antibody to each of them in a tumor-bearing animal. If we assume that a viral gene product is active in maintaining the transformed phenotype, then it must be located between map units 3 and 8 on the Ad2 genome: all transformed cells should then express an antigen encoded by this region and in addition may also express other viral antigens. The term "T" or tumor-specific antigen is therefore ambiguous as commonly used and should be redefined as an antigen whose production is stimulated in *all* cells transformed by a particular virus.

An example of the ambiguity presented by the expression of several "early" viral gene functions is illustrated in a recent report by GILEAD et al. (1975). These authors demonstrated that animals bearing tumors induced by injection of Ad2-transformed cells produce antibodies against the "early" viral, 70k, DNA-binding protein (see above). This viral gene product can therefore be considered as a viral specific tumor antigen, "T" antigen. However, the 70k protein has been mapped at the middle of the genome, between 60–70 map units, by both genetic and physical methods (WILLIAMS et al., 1974; GRODZICKER et al., 1974; LEWIS et al., 1975; SAMBROOK et al., 1975; WILLIAMS et al., 1975). Rat cells containing only 14% of the left-hand end of the genome are transformed (GALLIMORE et al., 1974) indicating that the oncogenic information carried by Ad2 must be in this region. Moreover, virus with temperature-sensitive mutations in the 70k DNA-binding protein (VAN DER VLIET et al., 1975) retain the ability to transform rat cells at the nonpermissive temperature (GINSBERG et al., 1974). Thus, the 70k protein cannot play a critical role in cell transformation. It is clear, then, that "early" viral gene products can be expressed in certain Ad2-transformed cells, with the consequence that a tumor-specific viral antigen may have no relation to the expression of viral oncogenic information.

C. Nuclear Viral RNA

In contrast to prokaryotic systems, transcription and translation are not coupled in eukaryotic cells. Consequently, an additional level of gene regulation could be imposed between these two processes, that of selection and transport of only a fraction of the available RNA sequences to the cell's cytoplasm. This concept was supported by the discovery of rapidly labeled, large RNA mole-

cules in eukaryotic cell nuclei (WARNER, 1966; PENMAN, 1966). These large RNA chains, termed heterogenous, nuclear RNA (Hn RNA) seemed to share some sequences with cytoplasmic RNA, but most of their sequences are degraded rapidly within the nucleus (SHEARER and MCCARTHY, 1970; SOEIRO et al., 1968; PENMAN et al., 1968; SOEIRO and DARNELL, 1970; SCHERRER et al., 1970). These observations led to the suggestion that large Hn RNA molecules were the primary transcripts of chromosomal DNA and that a fraction of these sequences were selectively transported to the cytoplasm. Subsequently, it was discovered that both Hn RNA and mRNA contain sequences of poly(A) at their 3'-termini (DARNELL et al., 1971; EDMONDS et al., 1971; LEE et al., 1971), which is added post-transcriptionally in the nucleus (DARNELL et al., 1971; PHILIPSON et al., 1971). This retention of poly(A) during selection of mRNA led to the suggestion that the RNA sequences in an Hn RNA chain adjacent to the 3'-termini are the actual precursors to mRNA (ADESNIK et al., 1972). As discussed above, all adenovirus-transformed cells contain viral DNA sequences integrated into their chromosomes and also translate viral-specific mRNA on their polysomes; the finding of RNA sequences complementary to adenovirus DNA in large Hn RNA species would thus further support the notion that Hn RNA was the precursor to mRNA. Indeed, nuclear RNA, with a sedimentation coefficient of more than 50 S, isolated from the Ad2-transformed cell line 8617, has been reported to contain viral RNA sequences (PARSONS and GREEN, 1971; WALL et al., 1973). Large ^{3}H-labeled RNA, when selected by gently annealing to denatured Ad2 DNA immobilized on a filter, will anneal to filters containing both viral and cellular DNA in a second round of hybridization (WALL et al., 1973; TSUEI et al., 1972) suggesting linkage of viral and cellular sequences. As the selected Hn RNA annealed to cellular DNA at relatively low Cot values, this RNA must be complementary to repetitive sequences in host DNA. In these experiments, it is very difficult to exclude the possibility of nonspecific aggregation during RNA extraction and to show convincingly that all the viral RNA sequences present in the nucleus of a transformed cell are destined to function as mRNA. In fact, GEORGIEFF et al. (1974) have recently demonstrated that when Ad2-transformed cell (8617) Hn RNA, sedimenting faster than 32 S, was treated with denaturing agents, a 20 S viral RNA chain, indistinguishable from the viral mRNA, was released. These authors suggest that the 20 S mRNA may be very rapidly cleaved from the larger nuclear molecules, but remains associated with them by bonds that are disrupted under denaturing conditions. The mechanisms by which viral mRNA in adenovirus-transformed cells, and indeed eukaryotic mRNA are generally synthesized, thus remain obscure; studies on other systems, for example hemoglobin (IMAIZUMI et al., 1972; MAC NAUGHTON et al., 1974) and ovalbumin (MCKNIGHT and SCHIMKE, 1974) mRNA synthesis, have also produced conflicting results. It would seem, then, that the pathway of selection of mRNA in eukaryotic cells is more complex than originally imagined and more subtle methods of analysis will be required to unravel it.

Synthesis of Ad2 RNA during lytic infection also appears to exhibit similarities with mRNA synthesis in normal cells. During the late phase of the lytic cycle, for example, large viral RNA chains can be detected in abundancy in the nucleus. WALL et al. (1972) described viral RNA species sedimenting faster than 80S, the expected sedimentation rate constant for an RNA chain the length of the Ad2 genome, while other authors have reported viral RNA chains of about 52S which would correspond to a molecular weight of 4×10^6 (PARSONS and GREEN, 1971; McGUIRE et al. 1972; PHILIPSON et al., 1974). After a 4-h labeling period, those RNA chains sedimenting faster than 32S account for about half of the viral RNA present in the nucleus. Essentially, no viral RNA of this size can be found in the infected cell's cytoplasm, where the largest species have a sedimentation coefficient of 26S. The role these large Ad2 RNA molecules play in the lytic cycle of the virus remains obscure, partly because only 20% of the nuclear viral RNA labeled during a 4-h period seems to be transported to the cytoplasm (PHILIPSON et al., 1974). Thus a majority of the viral RNA synthesized either accumulates in the nucleus or is degraded.

These same large, nuclear RNA chains contain viral sequences that do not find their way to the cytoplasm: in competition hybridization experiments between unlabeled cytoplasmic RNA and labeled nuclear RNA, some 30% of the nuclear, viral RNA could not be detected in the cytoplasm (LUCAS and GINS/BERG, 1972; McGUIRE et al., 1972; WALL et al., 1972). During the late phase of infection, viral RNA complementary to one strand equivalent of the genome is found in the cytoplasm (see above): if 30% of the viral RNA sequences found in the nucleus are not found in the cytoplasm, then these additional RNA sequences must be complementary to cytoplasmic viral RNA. When nuclear RNA is extracted from cells 18 h after infection, allowed to self-anneal and then treated with ribonuclease to degrade single-stranded RNA, double-stranded RNA can be easily isolated (FLINT and SHARP, 1974; SHARP et al., 1974b; PETTERSON and PHILIPSON, 1974). Viral RNA isolated in this fashion is complementary to all of both strands of Ad2 DNA (FLINT and SHARP, 1974; SHARP et al., 1974). Although both strands of the viral genome are transcribed in the nucleus, transcription from the two strands probably occurs at very different frequencies. PETTERSSON and PHILIPSON (1974) pulse-labeled infected cells for 10 min with ^{3}H-uridine, extracted nuclear RNA, and annealed it with excess, unlabeled cytoplasmic RNA. The maximum amount of labeled RNA that would form duplex under these conditions was 1.3%. This value decreases to 0.5% after a 3-h pulse and to less than 0.1% in the cytoplasm. If complementary transcription is defined as the simultaneous transcription of RNA with equal frequency from all parts of both strands of the template, then the above data are not consistent with complementary transcription of the Ad2 genome (with the reservation that unstable sequences might be degraded during the 10-min labeling period). Therefore, one strand of Ad2 DNA is probably preferentially transcribed by some 10–20 fold, during the late phase of infection (PHILIPSON et al., 1974).

ALONI'S discovery of symmetrical transcription of both strands of mitochondrial DNA, when stable RNA complementary to only one strand equivalent of this DNA could be detected under conditions of uniform labeling (ALONI and ATTARDI, 1971 a, b), has stimulated the search for complementary transcription in other mammalian systems. Complementary viral RNA sequences can be detected during the late phase of infection in cells infected with polyoma, SV40, Ad2, and herpes virus (ALONI and LOCKER, 1973; KAMEN et al., 1974; ALONI, 1972, 1973; SHARP et al., 1974b; PETTERSSON and PHILIPSON, 1974; KOZAK and ROIZMAN, 1975). In the first three instances, 50% only of these sequences are representated in "late" messenger RNA which comprises sequences complementary to part of both strands of the viral genome (KAMEN et al., 1974; SHARP et al., 1974b; TIBBETTS and PETTERSSON, 1974; KHOURY et al., 1972; SAMBROOK et al., 1972). It is unlikely that true symmetrical transcription takes place in any of these systems, as RNA complementary to only one strand is produced in abundance at late times. These nonmessenger, viral sequences found in the nucleus of cells infected by DNA viruses could represent either aberrant transcription products (from integrated viral DNA for example) or noncoding regions of mRNA precursors that are processed by mechanisms as yet unknown. At the moment, there exists no convincing evidence on which to distinguish between these possibilities, although some attempts have been made to investigate precursor-product relationships using drugs that block the normal metabolism of nuclear RNA (PHILIPSON et al., 1972; MCGUIRE et al., 1972). Aberrant transcription in vivo has not been observed in the well-studied phage and bacterial systems, but then extensive processing and selection of RNA sequences are not features of the synthesis of prokaryotic mRNA. The animal viruses mentioned above are currently the subject of study by several groups, and the answers to these questions should become more obvious in the near future.

Viral RNA sequences present in the nucleus during the early phase of infection are difficult to study because of their scarcity. However, it has been possible to demonstrate the presence of exclusively nuclear viral RNA sequences using "early" RNA prepared from large numbers of infected cells: when separated strands of *Eco* RI cleavage fragments were annealed to large amounts of total "early" RNA, one strand equivalent of the Ad2 genome entered hybrid (FLINT and SHARP, 1974; SHARP et al., 1974b). "Early" cytoplasmic RNA hybridizes to only 20% of Ad2 DNA (see above). Thus, RNA sequences complementary to 80% of the genome must be confined to the infected cell's nucleus early in infection. Although large nuclear viral RNA species have also been described early in infection (WALL et al., 1972), no direct evidence implicates such exclusively nuclear RNA sequences in the normal production of "early" mRNA. The finding of adenovirus "early" gene products in transformed cells strongly suggests that these "early" mRNAs are synthesized in the same way as cellular genes: if "early" adenovirus mRNAs are cleaved from a larger primary transcript, then the same process probably occurs in uninfected cells.

D. RNA Polymerases and Adenovirus Transcription

Three classes of DNA-dependent RNA polymerases can commonly be identified in mammalian cells (see Cold Spring Harbor Symposium *35* (1970) for numerous examples). RNA polymerase I (nomenclature of ROEDER and RUTTER, 1969) is located in the nucleolus of the cell (ROEDER and RUTTER, 1970; JACOB et al., 1971; JACOB, 1973) and is thought to transcribe ribosomal RNA (WIDNALL and TATA, 1966; REEDER and ROEDER, 1972; BEEBEE and BUTTERWORTH, 1974). RNA polymerases II and III are both found in the nucleoplasm (ROEDER and RUTTER, 1970) but they can be distinguished from one another by their chromatographic and catalytic properties. α-Amanitine, a toxin extracted from the toadstool *Amanita phalloides* (WIELAND, 1968) inhibits RNA polymerase II in vitro at very low concentrations, about 0.5 μg/ml (KEDINGER et al., 1970; NOVELLO and STIRPE, 1970) while more than 20 μg/ml are required to inhibit RNA polymerase III activity (SCHWARTZ et al., 1974). The differential sensitivities of RNA polymerases to α-amanitine permit the study of their involvement in in vivo RNA synthesis. Using this criterion, several groups have suggested that RNA polymerase II is involved in the synthesis of mRNA in mammalian cells, while RNA polymerase III probably transcribes 5 S and t-RNA genes (PRICE and PENMAN, 1972; WENMAN and ROEDER, 1974).

As "early" viral RNA synthesis readily occurs in infected cells maintained in the presence of cyclohexamide (PARSONS and GREEN, 1971; CRAIG and RASKAS, 1974) the viral DNA must either be transcribed by a pre-existing cellular RNA polymerase or a viral polymerase in the virion. To date, no virion associated-RNA polymerase has been detected. If either RNA polymerase II or III were responsible for the transcription of viral DNA in vivo, viral RNA synthesis should be α-amanitine sensitive. α-Amanitine (5 μg/ml) inhibits the production of adenovirus 12 and 5 when added to the media of infected HeLa cells (LEDINKO, 1971; CHARDONNET et al., 1972) but this inhibition could be due to the reduction in the expression of cellular RNA and not simply to an inhibition of transcription of viral DNA. Because HeLa cells are not permeable to α-aminitine, it is not possible to compare, by radioisotopic labeling, the in vivo sensitivity of viral transcription to the in vitro sensitivity of purified RNA polymerase. However, examination of viral RNA synthesis in crude permeable nuclear preparations removes this uncertainty, but additional problems then arise. Foremost is the need to prove that the RNA synthesis measured in permeable nuclei corresponds to an activity that has significance in vivo.

Sixteen hours after infection, 18% of the large nuclear RNA will hybridize to Ad2 DNA, while about the same percentage of RNA synthesis in permeable nuclei is from a viral template (WALLACE and KATES, 1972; PRICE and PENMAN, 1972b). RNA synthesis in such nuclear preparations may thus well correspond to RNA synthesis in intact cells. PRICE and PENMAN (1972b) and WALLACE and KATES (1972) demonstrated that 0.2 μg/ml of α-amanitine would inhibit over 85% of the viral RNA synthesis from nuclei isolated from cells late

after infection. In semipurified preparations, only RNA polymerase II is inhibited at this concentration of α-amanitine: this enzyme is therefore probably responsible for the transcription of most of the viral mRNA. The viral specific RNA synthesized in the presence of 0.4 μg/ml of α-amanitine is exclusively one class, virus-associated (V.A.) RNA (WEINMANN et al., 1974). When the α-amanitine concentration is further increased to over 20 μg/ml, the synthesis of this small RNA in permeable nuclei (WEINMANN et al., 1974) is inhibited. Purified preparations of eukaryotic RNA polymerase III are inhibited at this concentration of α-amanitine.

In summary, adenovirus DNA is thought to be transcribed by two RNA polymerases in vivo: RNA polymerase III produces a class of small viral RNAs whose cytoplasmic form probably corresponds to the direct transcription product. Adenovirus mRNA is probably synthesized by RNA polymerase II, which is also thought to be responsible for the transcription of cellular mRNA. Most of the viral RNA produced in permeable nuclei is undoubtedly synthesized by this polymerase. Whether this in vitro activity is characteristic of the in vivo situation is not entirely clear. A complete understanding of the process by which viral mRNA is produced will require the development of an in vitro system using purified viral DNA, RNA polymerases, and perhaps other cofactors that allow transcription of adenovirus DNA. Only with such a defined system will it be possible to assay for the viral and cellular components involved in control of adenovirus transcription.

III. Conclusion

Currently, very little is known about the physiology of adenovirus regulation. We do not know, for example, whether a particular set only of viral genes is expressed during the early phase of the lytic cycle as the result of positive regulation at a few promoter sites or of negative regulation in the sense that other RNA sequences constitutively transcribed are degraded. Similarly, the expression of "late" mRNA could reflect one regulation mechanism maintaining a constant pattern of RNA synthesis or represent the outcome of several sequential steps. Whatever the mechanisms of regulation, it is probable that several viral gene products are involved during the lytic cycle.

As techniques for resolving polypeptides and polynucleotides become increasingly sophisticated, structural maps of the adenovirus genome will be considerably refined. This exercise in biochemistry must be completed to provide the basis for studying the physiology of adenovirus infection and transformation. Further developments in two major areas are required if our understanding of adenovirus transcription is to advance beyond the basic, structural levels the current status of which is reviewed here. The first of these is the isolation of regulatory mutants of adenovirus: although manipulation of eukaryotic systems using genetic techniques remains relatively primitive, such mutants may well be constructed in the near future using biochemical methods which have recently advanced rapidly with the use of restriction endonucleases. The

second essential development is the refinement of in vitro systems with which to study interactions between the viral DNA template and RNA polymerase(s) and between viral RNA species and putative processing proteins. Unless adenovirus DNA is transcribed from an integrated state during the lytic cycle, which we consider unlikely, there must be at least two, and possibly many more, promoter sites on the viral genome. Identification of these sites and the proteins involved in their recognition is clearly essential.

Perhaps the most interesting aspect of adenovirus regulation is the control that viral information exerts over the infected cell: this, undoubtedly, will provide the key to our understanding of viral transformation.

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