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Biochemical and Biophysical Analysis of Cell-to-Cell Channels and Regulation of Gap Junctional Permeability

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1 Introduction

Intercellular communication is necessary in complex and differentiated organisms to coordinate the cellular activities of differentiation and growth. Two modes of cell-cell communication contribute to this coordination in metazoan animals. One form involves the secretion of signal substances like neurotransmitters, hormones and growth factors into the extracellular fluid. Target cells expressing receptors to these substances respond appropriately upon encountering the respective chemical modulators. The other form of communication operates within the limiting plasma membrane of groups of cells which are coupled into integrated units by specialized structures that permit free diffusion of molecules of low molecular weight.

Direct intercellular communication independent of the extracellular transfer of signals was first described by Furshpan and Potter (1959) as electrical coupling between two neurons in the crayfish nerve cord. Ultrastructural investigations have enabled the correlation of the structure responsible for effective cytoplasmic bridges and metabolic or electrical coupling with the typical electron microscopical image of a pentalaminar cross sectional area (Revel and Karnovsky 1967; Gilula et al. 1972). This pattern of alternating light and dark bands represents the region of membrane contact between functionally connected cells. The name gap junction derives from the 2–4 nm gap between the two apposing membranes connected by the channels (Revel and Karnovsky 1967). Gap junctions have been identified in a wide variety of tissues and animal species, as early in the evolutionary tree as hydrozoa (Fraser and Bode 1981). The size of an individual gap junction can vary from very small, composed of just a few cell-to-cell channels, to several square micrometers, containing many thousands. In liver, each hepatocyte forms more than a hundred thousand cell-to-cell channels with its nearest neighbor. The reason why these channels cluster together into plaques is unknown, but it would seem likely that such an arrangement is energetically favorable as it reduces the area of close apposition between adjacent cells to a minimum.

The polygonal structure of the gap junctional channel has been the subject of numerous investigations, originally employing electron microscopical (Makowski et al. 1977) and X-ray diffraction (Unwin and Zamphigi 1980) methods. Analysis of data from murine liver gap junctions (Caspar et al. 1977; Makowski et al. 1977) led to the proposal of a model for the morphological unit of gap junction structures, the gap junctional channel or connexon. Six symmetrically arranged connexins (smallest protein subunits: Goodenough 1974; for review see Beyer et al.

1987) form the wall of a protein tube with an outer radius of roughly 2.6 nm and an inner radius of approximately 1 nm. An aqueous channel extends all the way through this hexamer. This hemichannel is connected across the gap to a second hexamer by head-to-head association, the pair forming the complete gap junctional channel.

This model was extended by the study of Unwin and Zampighi (1980) on rat hepatocyte gap junctions. Their data show that the six subunits of the connexon cylinder are tilted around its axis. A change of the subunit inclinations by rotation and sliding could determine the diameter of the channel pore. This is suggested by the comparison of two subunit configurations of small and large diameter, corresponding to the presence and absence of 0.05m*M* Ca^{2+}, respectively (Unwin and Ennis 1984).

The main question which is still unsolved concerns the role of gap junctions in the concerted action of intercellular communication. But as this review will indicate, the physiological function of gap junctions is probably specific for the corresponding tissue and correlated to its inherent structural and modulatory properties. The review will be focused on the biochemical analysis of the connexins, the regulation of the gap junctional permeability by phosphorylation-dependent mechanisms and the single cell-to-cell channel properties measured by the double whole-cell patch-clamp technique. [For earlier reviews see Loewenstein (1979, 1981, 1987) and the volumes edited by Bennett and Spray (1985), Hertzberg and Johnson (1987), De Mello (1990) and by Robards et al. (1990).]

2 Biochemical Analysis of Gap Junction Subunits

2.1 Protein Composition

The gap junction channel is presently being investigated using protein chemical, molecular biological, and immunological techniques. Bulk isolation methods for gap junctions and increasingly detailed analysis of their constituents have made it evident that there is a multiplicity of gap junction proteins (for review see Revel et al. 1985; Revel et al. 1986). Gap junction protein subunit molecular masses of 16 kDa, 21 kDa, 26–28 kDa, 47 kDa and 70 kDa have been determined by SDS polyacrylamide gel electrophoresis (see Table 1 and Table 2).

Analysis of gap junction proteins in mouse liver shows two major polypeptides of molecular mass 26 kDa for the more abundant species and 21 kDa for the minor component (Henderson et al. 1979). The heart gap junction contains a single subunit of 44–47 kDa, a cytoplasmic surface

component of 14.5–17.5 kDa accounting for the difference between the heart and liver subunits (Manjunath et al. 1984). Whereas disulfide bonds link the subunits of the same connexon or different connexons to each other in rat heart, no disulfide linkages could be detected between rat liver connexins (Manjunath and Page 1986). Yet sequence comparison of the amino-terminal 18 residues of liver and heart subunits show 30% overall homology (Nicholson et al. 1987).

In lens fiber cells, at least two proteins have been discussed as part of the gap junction. The lens main intrinsic protein (MIP), a polypeptide of 26 kDa (Goodenough 1979), shows no homology to the liver 26 kDa polypeptide in the amino-terminal amino acid sequence (Nicholson et al. 1983). According to Gruijters (1989), its role may not actually be that of a channel-forming protein. MIP only temporarily associates with gap junctions, and on average only with less than 5% of all gap junctions at any one time. Since it predominantly appears during the assembly of gap junctions, it is speculated to act as a membrane 'zipper' protein, aiding in the formation of membrane domains requiring close apposition. This function would be required during events such as gap junction formation and fiber differentiation in the lens. The other lens gap protein species of 70 kDa (Kistler et al. 1985), on the other hand, is about 40% identical to heart CX 43 (see Table 1) and 50% homologous to liver CX 32 in the amino-terminal residues (Kistler et al. 1988). The 70 kDa protein is therefore more likely to be a structural protein of the gap junction channel.

There is a controversy over the nature of the 16 kDa gap junction subunit protein identified originally in mouse liver and cultured Buffalo red liver (BRL) cells (Finbow et al. 1983). Tryptic peptide mapping shows no significant differences between the 16 kDa protein of murine liver, heart, brain and kidney (Buultjens et al. 1988). Surprisingly, none of the other subunits appears during the preparation of the 16 kDa subunit (Buultjens et al. 1988). It has been proposed that the 21 kDa and 27 kDa subunits are separated from the 16 kDa polypeptide in the first preparative centrifugation step (Buultjens et al. 1988). An alternative explan-

Table 1. Nomenclature of gap junction protein subunits

"Old" name	16 kDa	21 kDa	26 kDa (MIP)	27 kDa	47 kDa	70 kDa
"New" name	–	CX 26	–	CX 32	CX 43	–

Prior to the determination of the connexin nucleotide sequence, subunits were classified according to their molecular weight as determined by electrophoresis. These values are referred to by their molecular mass in kDa. If the molecular mass was calculated from the amino acid composition determined from the nucleotide sequence, the value is referred to as CX, which stands for connexin (Beyer et al. 1987, 1990)

ation is that the low molecular weight protein results from actions of peptidases released during the preparation, in which whole tissue homogenates instead of membrane fractions are dispersed by detergent (Revel et al. 1985). This seems unlikely though, since recently obtained data suggests that the 16 kDa protein belongs to a family of proton transporting ATPases, bearing no relationship to the other junctional protein species (Dermietzel et al. 1989).

The isolation of gap junction subunit proteins made it possible to immunize animals with connexin to raise antibodies, which can, in turn, be used to locate and purify the junction polypeptide with high specificity. Antibodies against the liver 27 kDa subunit enabled the immunocytochemical localization of structures related to the 27 kDa protein in rat pancreas, heart, brain, kidney, stomach and adrenal gland, in fallopian tube, endometrium and myometrium of delivering rats, and in liver of mammalian, fish, and avian species (Dermietzel et al. 1984; Hertzberg and Skibbens 1984). An antibody prepared against the 21 kDa subunit, which does not react with the 27 kDa peptide (Traub et al. 1982), led to the identification of both subunits in the same gap junction plaque (Nicholson et al. 1987), suggesting that rat hepatic gap junctions are composed of different subunits. The amino-terminal 18 residues of the rat liver 21 kDa and 26 kDa subunits show 50% homology, demonstrating their relatedness (Nicholson et al. 1987). An antibody against a synthetic peptide fragment of the amino terminal region of the 43 kDa connexin of heart was used in (a) immunostaining of whole heart fractions of trout, frog, chicken, guinea pig, mouse and rat, and (b) immunoblotting of whole organ fractions of rat and mouse liver, rat cerebellum, uterus, stomach and kidney (Dupont et al. 1988). The results suggest that the stained proteins share epitopes with the synthetic peptide and the 43 kDa subunit.

In summary, the molecular species of 21 kDa, 26 kDa, 43 kDa and 70 kDa appear to form a family of related proteins. The 26 kDa (MIP) subunit of lens fiber gap junctions, and the more widespread 16 kDa polypeptide probably derive from separate classes of genes. Though the studies mentioned above demonstrate the diversity of gap junction protein components, more thorough investigations into the physiology and appearance are needed. Only then can it be established whether variations in subunit composition are related to functional differences affecting the mechanisms of intercellular communication. But, at present it is not possible to correlate the modulatory properties of gap junctional channels like e.g. voltage-, pH-, Ca-sensitivity to the appearance of specific subunits. Furthermore, molecular genetic studies are required to determine the exact nucleotide and amino acid sequences necessary to compare these protein families quantitatively and establish their genetic origins.

2.2 Nucleotide Sequence

Molecular genetic techniques have enabled the identification and sequencing of DNA coding gap junction polypeptides. This is an important step for the characterization of the connexon structure and determination of its evolutionary origins. The first cDNA clone was identified for the bovine lens 26 kDa peptide (Gorin et al. 1984). It consists of 968 base pairs, with an open reading frame of 789 bases. The molecular mass is predicted to be ~28 kDa. Hydropathicity analysis of the deduced amino acid sequence predicted a structure of six hydrophobic transmembrane segments, the carboxy- and amino-terminal segments facing the cytoplasmic side. Aqueous channel-forming ability is suggested by the presence of an amphiphilic transmembrane region. In accord with protein biochemical studies (see Sect. 2.1), the lens MIP clone does not seem to appear in liver, though hybridization to the rat genome could be detected.

A partial (Heynkes et al. 1986) and complete (Paul 1986; Kumar and Gilula 1986; Miller et al. 1988) nucleotide sequence of rat liver gap junction protein has been obtained. The cDNA clone of Paul (1986) contains 1494 base pairs, corresponding to a calculated molecular mass of 32 kDa for the protein. Four major hydrophobic regions, potentially spanning the membrane, could be detected in hydropathicity profiles. These regions correspond to a predicted β-sheet conformation, unusual for membrane proteins, but nevertheless in accord with similar observations by X-ray diffraction (Makowski et al. 1982). The gap junctional nature of this clone has been demonstrated by the expression of electrical coupling between paired *Xenopus* oocytes injected with liver gap junction RNA homologous to the cDNA clone (Dahl et al. 1987, 1988). Swenson et al. 1989 utilized this expression system to test the ability of the RNAs coding for CX32, CX43 and of MIP26 to induce cell-to-cell coupling. Oocyte pairs form homomolecular CX32/CX32 and CX43/CX43 as well as heteromolecular CX43/CX32 cell-to-cell channels, while for MIP26 channel formation not be shown.

A molecular mass of the liver gap junction protein of 32 kDa is also predicted by Kumar and Gilula (1986). In this study, a human cDNA clone (1574 base pairs) was first obtained and then used to isolate a clone in rat liver (1127 base pairs). The rat liver clone is strongly homologous to the human cDNA in the protein coding region. In view of regulation of gap junctions by phosphorylation mechanisms, sequence analysis demonstrated potential sites for tyrosine kinase and PKA-dependent phosphorylation in the hydrophilic carboxy terminal region (also see Miller et al. 1988). The discrepancy of the predicted liver subunit molecular mass of 32 kDa and the value of 26–28 kDa found for isolated

proteins has not been completely clarified yet. Green et al. (1988) attributed these variations to inherent inaccuracies in assessing molecular masses from polyacrylamide gel profiles due to differences in gel compositions and proteolytic breakdown. Southern and northern blot analyses show the presence of related sequences in human, baboon, rat chicken and *Xenopus laevis* livers (Kumar and Gilula 1986), as well as in the rat brain, kidney and stomach (Paul 1986). These results are in accord with previously attained results on subunit distribution (see Sect. 2.1 and Table 2). A detailed analysis of the rat liver connexon gene has been presented by Miller et al. (1988).

Comparison of the total polypeptide sequence tomicrosequences of proteolytically derived fragments from isolated liver gap junctions enables the formulation of a two-dimensional connexin model (Zimmer et al. 1987; Hertzberg et al. 1988). In agreement with the hydropathicity data obtained by Paul (1986), the amino acid chain crosses the membrane four times, the amino and carboxy terminals located in the cytoplasm (Zimmer et al. 1987). A calmodulin binding region is proposed for the cytoplasmic segment close to the carboxy terminal (Zimmer et al. 1987). The orientation of the amino and carboxy terminals are confirmed by localizations of antibodies against synthetic peptides corresponding to human liver connexon sequences (Milks et al. 1988).

A liver sequence probe was used to identify a cDNA clone for heart connexin (Beyer et al. 1987). The open reading frame of 1146 base pairs codes for a predicted polypeptide of 43 kDa. A model similar to the liver polypeptide is derived from the interpretation of hydropathicity profiles. Northern blotting shows hybridization in RNA from heart, ovary, uterus, kidney and lens. Sequence homologies with the liver clone (~50%) are predominantly found in regions expected to lie in the membrane or extracellular space, while the cytoplasmic segments diverge. Homologies to mammalian gap junction sequences are seen in a comparison with a cDNA for *Xenopus* connexin (Gimlich et al. 1988). Transcripts of the *Xenopus* clone, coding for a 30 kDa protein, are found in lung, kidney and alimentary tract organs, but not in brain, heart, skeletal muscle, spleen and ovary.

2.3 Phosphorylation

Protein phosphorylation is a central mechanism by which changes in cellular activity are regulated (for review see Cohen 1982; Nishizuka 1984). Factors governing phosphorylation have also been associated with the control of intercellular communication via gap junctions (see Sect.

3.3). It therefore seems plausible that phosphorylation of the junctional peptide could take place, possibly resembling the final step in a regulatory sequence.

The 27 kDa subunit of liver gap junctions, for instance, is known to be a phosphoprotein. The extent of phosphorylation is increased by stimulation of cells with cAMP, or incubation of membrane fractions with the catalytic subunit of cAMP-dependent protein kinase, PKA (Saez et al. 1986; Traub et al. 1987). The phosphorylated amino acid residues are 98% serine and ~2% tyrosine (Traub et al. 1987). Similar findings also support the role of PKA in phosphorylation of lens MIP. MIP is phosphorylated at serine residues (90%–95%), threonine (5%–10%), but not at tyrosine. Phosphorylation takes place in vivo in a cAMP-dependent fashion, and in vitro after incubation of membranes with isolated PKA catalytic subunit (Johnson et al. 1986).

Phosphorylation of connexins by phospholipid/Ca^{2+}-dependent protein kinase, PKC (see Sects. 3.3.2, 4.1–4.3) has been the subject of recent investigations. Experiments performed in vitro using isolated PKC show a virtually serine-specific increase of phosphorylation of the 27 kDa liver gap junction protein. The extent of PKC-dependent phosphorylation is about ten times that dependent on the PKA catalytic subunit (Takeda et al. 1987). PKC-dependent phosphorylation of the liver 27 kDa subunit can furthermore be stimulated in vivo by norepinephrine, and the PKC activators 1-oleoyl-2-acetyl-*sn*-glycerol and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (Takeda et al. 1989). This report also shows that phosphorylation is confined to the carboxy terminal domain. Different results were obtained from studies using the lens 26 kDa subunit. Lampe et al. (1986) demonstrated in an in vitro procedure, using isolated brain or lens PKC, that the 26 kDa protein is phosphorylated to 85% on serine and 15% on threonine, while no tyrosine phosphorylation is detectable. This protein is also phosphorylated in vivo, maximal phosphorylation being detected in the presence of TPA with the same ratio of phosphoserine to phosphothreonine (Lampe and Johnson 1989).

3 Regulation of Gap Junctional Permeability

3.1 Gating by Voltage, pH, and pCa

Channel gating refers to the regulation of the permeability as opposed to the number of functional channels in a gap junction. Since their discovery, gap junction channels have been known not to be passive pores in a

permanent open state, but to be sensitive to physicochemical parameters such as transjunctional voltage, the intracellular pH and the cytoplasmic free Ca^{2+} concentration. These properties have been reviewed in detail by Spray and Bennett (1985), and will be given short consideration in the following paragraphs. Most of the corresponding data are summarized in Table 2.

The first gap junction examined, the rectifying synapse of the crayfish, is found to be strongly asymetrically voltage dependent (Furshpan and Potter 1959). Voltage dependence has been most extensively characterized in early embryonic amphibian cells, in which the junctional conductance (g_j) decreases with symmetrical increases of the junctional voltage (V_j) (Spray et al. 1979). A model of two symmetrical voltage sensitive gates at each end of the channel has been proposed. Either gate will close depending on the magnitude and polarity of V_j. This creates a region of high g_j close to zero V_j, flanked by regions of low g_j on the V_j axis (Spray et al. 1981a). A striking similarity in the symmetrical voltage sensitivity of channel gating has been observed for a weakly anion selective channel (Schwarze and Kolb 1984). This anion channel with large single channel conductance (300–400 pS) has been proposed to be hemichannel of cell-to-cell channels. Voltage-dependent gating of gap junctional coupling has furthermore been demonstrated in embryonic cells and rectifying synapses, while mammalian tissues investigated seem largely insensitive to changes in V_j (see Table 2; Spray and Bennett 1985).

A dramatic decrease of coupling is observed in the amphibian embryo after exposure to CO_2 (Turin and Warner 1978). This event is correlated to lowering of the intracellular pH (pH_i) (Turin and Warner 1980). Simultaneous measurement of g_j and pH_i enabled the quantitative characterization of pH-dependence of electrical coupling in fish and amphibian blastomeres in the range of pH 6.8 to pH 7.6 (Spray et al. 1981b). Lowering of pH_i also leads to a reduction of g_j in various mammalian tissues (Spray and Bennett 1985), including heart (Noma and Tsuboi 1987) and pancreas (Iwatsuki and Petersen 1979).

Ca^{2+}-dependent reduction of the junctional permeability has been demonstrated in a variety of preparations, among them *Chironomus* salivary gland (Rose and Loewenstein 1975), heart (DeMello 1975) and electrotonic synapses (Baux et al. 1978). The codetermination of electrical coupling and the cytoplasmic free Ca^{2+} concentration (pCa_i) with aequorin (a Ca^{2+}-sensitive dye) in *Chironomus* salivary gland led to the conclusion that the actual pCa_i is responsible for uncoupling (Rose and Loewenstein 1975; Rose and Loewenstein 1976). In guinea pig heart, pCa_i affects g_j only at levels lower than pCa 6 (Noma and Tsuboi 1987).

Table 2. Some properties of vertebrate gap junctions

Tissue	Species	Connexin[a]	Single channel conductance [pS]	Sensitivity to pCa[b]	pH	Voltage
Lacrimal gland cells	Rat	?	70–180[c]	?	?	?
Pancreatic acinar cells	Mouse	CX 32[d] CX 26[h]	30, 130[e] 45, 90[i] 60[j]	Yes[f]	Yes[g]	No[e]
Ovary cells	Chinese hamster	?	20, 40, 50, 70, 120[e]	?	?	No[f]
Heart myocyte	Rat	CX 43[k] 16 kDa[p]	43, 18[l] 60[q]	Yes[m]	Yes[n]	+/—[o]
	Human	CX 42[r]	?	?	?	?
	Embryonic chick	?	166, 74[s]	Yes[s]	?	No[s]
Heart fibroblast	Rat	?	22[t]	?	?	?
Hepatocyte	Murine and rat	CX 32[u] CX 26[n] 16 kDa[w]	?	?	Yes[v]	
Lens cells	Sheep and cattle	70 kDa[x] 38 kDa[x]	?	?	?	?
Kidney cells	Rat	16 kDa[p]	?	?	?	?
Brain tissue	Rat	16 kDa[p]	?	?	?	?
Blastomere cells	Fish	?	?	?	Yes[y]	Yes[y]
	Amphibian	?	?	?	Yes[y]	Yes[y]

Connexins identified by immunostaining of various tissues have only been selectively included in this table because of potential errors due to cross-reactions.

[a]See Table 1 for connexin nomenclature.
[b]Ca^{2+} dependence refers to the direct interaction of Ca^{2+} with the channel here. This should be treated with caution, though, because in the cases where Ca^{2+} sensitivity has been demonstrated, it is not always clear whether Ca^{2+} acts on a regulatory protein, i.e. PKC and calmodulin, or whether it binds to the channel directly.
[c]Neyton and Trautmann (1985).
[d]Dermietzel et al. (1984).
[e]Somogyi and Kolb (1988a).
[f]Ngezahayo A et al., submitted.
[g]Iwatsuki and Petersen (1979).
[h]Traub et al. (1989).

[i]Somogyi and Kolb (1988b).
[j]Somogyi et al. (1989).
[k]Manjunath and Page (1986); Beyer et al. (1987).
[l]Rook et al. (1988).
[m]Maurer and Weingart (1987).
[n]Guinea pig (Noma and Tsuboi, 1987).
[o]Voltage dependence could be observed if few channels were active (Rook et al. 1988).
[p]Buultjens et al. (1988).
[q]Burt and Spray (1988).
[r]Manjunath et al. (1987).
[s]Veenstra and DeHaan (1988).
[t]Rook et al. (1989).
[u]Henderson et al. (1979); Nicholson et al. (1987).
[v]Spray et al. (1986b).
[w]Finbow et al. (1983).
[x]Kistler et al. (1985, 1988).
[y]Spray et al. (1984).

Do the cations H^+ and Ca^{2+} influence the junctional permeability by a comparable mechanism, as suggested by Loewenstein (1981)? In the case of the voltage independent junctional conductance of cell pairs of the guinea pig heart, uncoupling by Ca^{2+} is augmented by increased pH, i.e. an upward shift of normalized g_j by one pCa unit on the pCa sensitivity relation is caused by lowering the pH from 7.0 to 6.5 (Noma and Tsuboi 1987). This is in accord with a model of competition for shared H^+ and Ca^{2+} binding sites on the connexon. A detailed gating model has been presented for the cell-to-cell channel of *Chironomus* salivary gland (Obaid et al. 1983). The junctional conductance of this preparation shows a dependence on pCa_i, pH_i, and on the membrane potential (as opposed to the transjunctional potential). The model proposes one gate at each side of the channel. The position on the g_j versus V_j curve of the gate is determined by pCa and pH.

3.2 Gating by Calmodulin

Calmodulin (Mr 17 kDa) is an intracellular Ca^{2+} receptor, acting as a transducer of the Ca^{2+} signal in the regulation of a multitude of processes. Some of these functions are mediated by the Ca^{2+}/calmodulin-dependent activation of protein kinases (for reviews see Cheung 1982; Blackshear et al. 1988). The first indication for the involvement of calmodulin in the regulation of intercellular communication was reported by Peracchia et al. (1983). They showed that uncoupling of *Xenopus* embryonic cells due to acidification can be reduced by a calmodulin inhibitor. Direct evidence for functional interaction between calmodulin and a putative gap junction protein was obtained in a study using a reconstituted system of lens 28 kDa protein and calmodulin. It was demonstrated that the presence of calmodulin is required for the Ca^{2+}-induced reduction of channel permeability (Girsch and Peracchia 1985; Peracchia and Girsch

1985). Similar to the effects observed in *Xenopus*, a calmodulin inhibitor counteracts the uncoupling of crayfish septate axons to acidification (Peracchia 1987). Using another approach in the same preparation, it was shown that perfusion of axons with calmodulin leads to an increase of the junctional resistance only in the presence of elevated Ca^{2+} (Arellano et al. 1988).

3.3 Gating by Phosphorylation-Dependent Mechanisms

Though pCa and pH have been shown to exert a modulatory influence gap junctional permeability in many preparations (see Table 2), these mechanisms may be of limited physiological significance. Variations of these tightly regulated parameters are generally assumed to not enter the concentration range associated with uncoupling under nonpathological conditions (Neyton and Trautmann 1986b; Campos de Carvalho 1988). As advances in the understanding of signal transduction and biochemical regulation mechanisms enable the discrimination between various regulatory pathways, interactions of these systems with gap junctional communication are also becoming evident. As an example, protein phosphorylation by different kinases has been established as the terminal step in the regulation of enzyme activity by important intracellular signalling sequences (Krebs and Beavo 1979; Cohen 1982; Nishizuka 1984; Berridge 1986). Phosphorylation is now being considered in its role as a modulator of the properties of gap junctions.

3.3.1 cAMP-Dependent Protein Kinase

Though phosphorylation of gap junction proteins in vivo after addition of cAMP, or in vitro by isolated catalytic subunit of PKA has been demonstrated (see below), it remains to be shown whether this mechanism is a physiologically necessary step in the regulation of gap junction channels. This possibility is supported by the finding of cAMP-dependent modulation of the junctional permeability on the level of protein synthesis and channel permeability.

Since the report of Hax et al. (1974), stimulation by cAMP has generally been associated with an increase in the junctional permeability. Their original study using *Drosophila* salivary glands demonstrated an increase in electrical coupling within 30 min after incubation with a solution containing 2 m*M* cAMP. One possible explanation for the effect of cAMP is an increase in the number of gap junction channels. This hypothesis is supported by a series of studies using mammalian cell

cultures in which (a) the rise in junctional permeability 4 h after stimulation by cAMP is correlated to an increase of gap junctional membrane particles, a process which is also sensitive to a protein synthesis inhibitor (Flagg-Newton et al. 1981), (b) junctional transfer of fluorescent dyes diminishes as the cAMP concentration inside the cells falls with increasing cell density (Flagg-Newton and Loewenstein 1981), and (c) addition of cAMP to a gap junction deficient mouse cancer cell culture induces junctional communication, as assessed by electrical and dye coupling, and the promotion of gap junctional membrane particles (Azarnia et al. 1981).

To ascertain whether these observations are relevant to effects exerted by physiological stimuli, two non-communicating cell lines, expressing receptors associated with cAMP formation were examined. Rat glioma C-6 cells, expressing β-adrenergic receptors, and human lung WI-38 cells, expressing prostaglandin receptors, were stimulated with isoproterenol or prostaglandin E1, respectively. Peak cAMP levels could be recorded after ~30 min, followed by an increase in the incidence of dye transfer and gap junctional membrane particles after a lag of several hours (Radu et al. 1982). The time course of the effect and actual measurement of the increase of junctional elements support a model of cAMP-dependent induction of gap junction channel expression.

The model proposing that cAMP affects junctional permeability via a PKA-mediated mechanism is supported by the study of Wiener and Loewenstein (1983). They observed an increase in gap junctional communication several hours after addition of PKA catalytic subunit to a gap junction deficient cell culture. In't Veld (1985) detected an induction of gap junction particles between rat pancreatic B cells after raising the cAMP level. A correlation between a cAMP-induced decrease in proliferation and increase in the fractional membrane area occupied by gap junctions was demonstrated for an adenocarcinoma cell line (Murray and Taylor 1988).

Clear evidence of cAMP-dependent induction of gap junctions can be supplied by biochemical studies on the level of direct measurement of gap junction protein expression. Traub et al. (1987) demonstrated increased biosynthesis and phosphorylation of the liver 26 kDa connexin in embryonic mouse hepatocyte cultures stimulated with cAMP. Subunit phosphorylation, almost maximal 15 min after cAMP addition, preceded the augmentation of 26 kDa subunit expression, which increased 2.5 fold after 3 h of incubation.

The studies mentioned above support a model in which cAMP regulates gap junctional permeability by controlling the biosynthesis of gap junction protein subunits. Can short-term modulation of the junc-

tional permeability by cAMP also take place through influencing either the channel open probability or pore size? Evidence for this type of mechanism was presented in the study by DeMello (1984), in which cAMP injection leads to a 50% increase in the coupling coefficient between canine Purkinje fibers. The crucial aspect of this experiment lies in the time course of the cAMP effect, which is completed within 60–90 s after injection of nucleotide. This time interval is too short for new protein to be synthesized, leaving only modulation of existing channels as an explanation. Compatible results were obtained from experiments in which electrical coupling between hepatocytes was found to reach an elevated plateau 5–10 min after stimulation with membrane permeable cAMP analogues (Saez et al. 1986). The rise in the junctional conductance (factor of ~1.5), is paralleled by an increase in 27 kDa protein phosphorylation (factor of ~1.6).

A role for cAMP in conjunction with ATP as a modulator of junctional permeability has been determined for exocrine cells. A typical observation in experiments using the double whole cell technique is spontaneous uncoupling (Neyton and Trautmann 1985). This phenomenon is unaffected by buffering $[Ca^{2+}]_i$ between pCa 6–8 (Neyton and Trautmann 1986a). Isolated pancreatic acinar cell pairs also exhibit a spontaneous decrease in the junctional conductance in the double whole cell configuration if pH- and Ca^{2+}-buffered electrolyte solutions are used. The whole-cell recording mode inevitably leads to the washout of endogenous substances. It is therefore reasonable to assume that spontaneously occurring electrical uncoupling could be the result of the disruption of cellular metabolism due to the washout of intermediary metabolites such as ATP and cyclic nucleotides. Addition of ATP and cAMP to the pipette solution suppressed spontaneous uncoupling in pancreatic acinar cell pairs (Somogyi and Kolb 1988a,b). Yet, a significant cAMP mediated increase in electrical coupling could not be observed within a time scale of minutes. Correlative evidence suggests that the open channel conformation is stabilized by cAMP and ATP, presumably by protein kinase A dependent phosphorylation of the connexons in pairs of acinar cells.

In horizontal cells of fish and reptilian retina, cAMP induces a decrease in junctional communication. This was demonstrated by Teranishi et al. (1983) for carp retina, in which a 30 min application of cAMP inhibits fluorescent dye transfer between contacting cells. Treatment of turtle retina cells for 10 min with forskolin (an activator of adenylate cyclase) and IBMX (an inhibitor of phosphodiesterase), agents which increase the cAMP concentration, resulted in a reduction in electrical coupling and intercellular dye transfer (Piccolino et al. 1984). The direct involvement of PKA in this mechanism is demonstrated by introduction

of isolated catalytic subunit via a patch pipette into bass horizontal cells (Lasater 1987). Total electrical uncoupling occurs between 0.5 and 4.5 min after PKA injection, while coupling is reestablished after removal of the patch pipettes for 30 min. The time course and reversibility of the short-term PKA-dependent effects suggest that modulation of the gap junctional permeability in horizontal cells is not a result of the adjustment of the total number of connexons. Instead, PKA probably regulates the permeability of the existing channels.

3.3.2 Protein Kinase C

Next to the cAMP-dependent second messenger pathway, the most widespread and best characterized receptor-effector coupling system is agonist-induced inositol lipid hydrolysis. Phospholipase C catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate, termed PI breakdown, into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (Michell 1975; Berridge 1983, 1984, 1986). IP_3 is the second messenger responsible for a transient elevation of the intracellular Ca^{2+} concentration. Protein kinase C (PKC) modulates the function of target proteins by phosphorylating seryl and threonyl residues at specific sites.

The first indications that PKC is involved in the regulation of gap junctional permeability appeared in the studies of Murray and Fitzgerald (1979) and Yotti et al. (1979). They investigated the effects of the phorbol ester tumor promoter TPA on metabolic cooperation between epidermal and 3T3 cells, and metabolically interdependent Chinese hamster cells. In both cases, metabolic cooperation is inhibited by incubation in TPA. As it was later discovered that TPA is a potent activator of PKC (Yamamashi et al. 1983; Ashendel et al. 1983; Ashendel 1985), it is plausible that PKC is involved in the reduction of intercellular communication.

Subsequent investigations using assays of electrical coupling (Enomoto et al. 1981, 1984), relative membrane area occupied by gap junctions (Yancey et al. 1982), and dye coupling (Fitzgerald et al. 1983), confirmed the hypothesis that tumor promoters cause a reduction in gap junctional permeability in cultured cells. Yancey et al. (1982) observed a decrease in the relative presence of gap junctions after treatment with the tumor promoter TPA. Furthermore, reestablishment of coupling after exclusion of TPA from the culture medium does not occur in the presence of a protein synthesis inhibitor, puromycin (Fitzgerald et al. 1983). Taking these observations into account, it appears that TPA treatment actually leads to the elimination of junctional channels under these conditions.

Direct correlation of PKC activation to uncoupling by TPA or DAG in cultured epidermal cells serves as a further step in linking PKC to the

modulation of the gap junctional permeability. Gainer and Murray (1985) showed that treatment of cultured epithelial rat liver cells with a DAG analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), causes a transient inhibition of fluorescent dye transfer. Uncoupling peaks following 20 min of incubation with OAG. This effect is accompanied by translocation of PKC from the soluble to the particulate fraction of cell homogenates after 10 min of OAG or TPA treatment, serving as an indication of activation of PKC. In addition, the extent of dye coupling is refractory to TPA or OAG treatment following putative down-regulation of PKC by extended stimulation with TPA.

Two approaches were used to investigate the involvement of PKC as a regulator of junctional communication between exocrine cells. Beside the activation of endogenous PKC by chemical stimulants, exogenous PKC was introduced into the cells to raise PKC activity. A synergistic effect of Ca^{2+} on PKC activation by OAG or TPA was observed by Yada et al. (1985). Treatment of cultured epithelial rat liver cells with OAG (25–50 μg/ml) or TPA (40 ng/ml) leads to a reduced junctional permeability to lucifer yellow within 3–20 min. The effect of OAG and TPA are impeded by addition of TMB-8 (Yada et al. 1985), an inhibitor of intracellular Ca^{2+} mobilization (Chiou and Malagodi 1975).

Dialysis of an acinar cell pair by a solution containing purified PKC isolated from rat brain caused electrical uncoupling. The uncoupling effect of PKC is not counteracted by the cAMP-associated pathway (Somogyi and Kolb 1989). This evidence for the involvement of PKC in the regulation of electrical coupling is supported by the finding that exposure of an acinar cell pair to the permeant diacylglycerol analogue OAG also evokes a significant decrease in the junctional conductance. The efficiency of uncoupling by OAG is variable between different experiments, but in any case the uncoupling action can be suppressed by addition of a PKC inhibitor, polymyxin B (Hausmann and Caig 1954), to the bath. Polymyxin B, a polycationic peptide antibiotic, competitively inhibits PKC with respect to phosphatidylserine, while not affecting cAMP or cAMP-dependent protein kinases (Mazzei et al. 1982). These results are comparable to previous reports, showing that intracellular communication could be inhibited when PKC was activated by either diacylglycerol or phorbol esters in freshly isolated rat lacrimal glands (Randriamampita et al. 1988a,b) or pancreatic acinar cell pairs (Somogyi et al. 1989). Still a significant change in electrical coupling after treatment of cell pairs with tumor promoters in acinar cell pairs could not be observed by Chanson et al. (1988).

As to whether PKC-dependent phosphorylation of the junctional protein or a regulatory component mediates the uncoupling response is

unclear. Evidence for in vitro phosphorylation of lens intrinsic membrane protein as well as the 27 kDa gap junction subunit of rat liver by PKC is available (Takeda et al. 1987; Dermietzel et al. 1984). Since the subunit of rat liver gap junctions is broadly homologous to the pancreatic junctional peptide, based on the subunit antigenic properties (Meda et al. 1987), it seems plausible to assume an analogus effect of PKC on gap junctions of pancreatic acinar cells. Whether phosphorylation then leads to channel closure remains another issue. At this point, intermediate biochemical steps should also be considered in a model of PKC-induced uncoupling.

3.3.3 Cross-Talk Between PKC and cAMP in the Modulation of Coupling

Are there interactions between the PKC- and cAMP-associated regulatory pathways in the control of the gap junctional permeability? According to the investigation of Kanno et al. (1984), cAMP inhibits the uncoupling effect of TPA if cells are coincubated with both agents from the start. Cells previously uncoupled by TPA treatment do not reestablish electrical contact if cAMP is added to the TPA-containing medium. The effect of cAMP is probably due to the inhibition of a TPA-dependent mechanism, as opposed to a competitive synthesis of new gap junction components. Different results are described in the study of Enomoto et al. (1984). On the one hand, the protein synthesis inhibitor cycloheximide eliminates the protective effect of cAMP from TPA-induced inhibition of dye coupling. On the other hand, reestablishment of coupling after cessation of TPA treatment is insensitive to cycloheximide. The effect of cAMP is possibly due to induction of a regulatory component and not the junctional protein itself in this case. In conclusion, cAMP can counteract the down-regulatory effect of TPA in cell cultures, but no definite mechanistic explanation for these effects is available as of yet.

3.3.4 Tyrosine Kinase

Several growth factor receptors express tyrosine kinase activity. This suggests that tyrosine phosphorylation of specific enzymes is involved in the initiation of cell growth (Taylor 1986; Ramachandran and Ullrich 1987). In light of Loewenstein's hypothesis on the role of gap junctional communication in cell proliferation (Loewenstein 1968), the relationship between gap junctional permeability and tyrosine kinase activity was investigated in the studies below.

It has been determined that the gene product of the cellular (c-*src*) and viral (v-*src*) *src* genes code for a 60 kDa protein, $pp60^{src}$, expressing

tyrosine kinase activity (Hunter and Sefton 1980). Transfection and expression of the *src* gene were used to induce a tyrosine kinase and investigate correlations between transformation and intercellular communication. A preliminary investigation employing cells transformed with a temperature-sensitive Rous sarcoma virus mutant demonstrated inhibition of dye coupling at permissive temperatures (Atkinson et al. 1981). It was shown in another study that activation of v-*src* induces an increase in tyrosine phosphorylation, accompanied by a reduction in dye transfer. The time range of uncoupling (~30 min), and reversal after switching to a nonpermissive temperature (~30 min), speaks for a regulation of the individual channel permeability rather than channel number by way of protein synthesis (Azarnia and Loewenstein 1984). Unlike the case of TPA-induced uncoupling (see Sect. 3.3.2), cAMP does not eliminate the *src* effect. A correlation between transfection of fibroblasts with c-*src*, expression of pp 60^{src}, tyrosine phosphorylation, and inhibition of intercellular dye transfer was shown by Azarnia et al. (1988).

Another approach, used by Maldonado et al. (1988), is stimulation of epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptors, both exhibiting tyrosine kinase activity (Hunter and Cooper 1985). Application of EGF and PDGF to cultures of normal rat kidney (NRK) and BalbC 3T3 cells results in a reduction of electrical and dye coupling for 2–10 min. As in the studies with *src*, the time interval between stimulation and uncoupling is probably too short for changes in protein synthesis to become apparent. This suggests a mechanism of tyrosine kinase action on the single channel permeability, through phosphorylation either of a regulatory protein, or of the channel itself. The latter possibility is supported by the finding of a segment favorable to tyrosine phosphorylation of the 27 kDa liver gap junction subunit cDNA (Kumar and Gilula 1986).

4 Secretagogues and Gap Junctional Uncoupling in Exocrine Cells

4.1 Acetylcholine

The first demonstration of electrical coupling between pancreatic acinar cells was performed by Petersen and Ueda (1976). After impaling different cells within an isolated acinus with two potential measuring microelectrodes, they found that injection of a current pulse into one cell causes a voltage change in both cells. The ratio of both voltage differences, or coupling coefficient (Socolar 1977), is close to one within an acinus. The

electrically coupled unit of pancreatic acinar tissue consists of ~500 cells (Iwatsuki and Petersen 1978a).

Early investigations implied the involvement of junctional communication in the signal transduction pathways associated with secretion. Application of the secretagogue acetylcholine (ACh) increases the junctional resistance, accompanied by a stimulation of amylase release (Petersen and Ueda 1976). A comparison of different secretagogues concluded that the uncoupling response is not limited to stimulation by ACh and that it takes place at agonist levels below those required for a maximal secretory response. The minimal concentrations required to evoke uncoupling are 1 μM for ACh, 0.14 nM for caerulein, and 3 nM for bombesin. The coupling coefficient undergoes changes from unity to as low as 0.008 during stimulation (Iwatsuki and Petersen 1978a).

Further studies concerning secretagogue-induced uncoupling focused on the involvement of the possible junctional modulators, pCa and pH (see Sect. 5). Comparable effects of addition of extracellular ACh and iontophoretic injection of Ca^{2+} into pancreatic acinar cells were found by Iwatsuki and Petersen (1977). Ca^{2+} is only effective in uncoupling after sustained repetitive injections. Assuming that Ca^{2+} is the messenger mediating ACh-induced uncoupling, Iwatsuki and Petersen (1978b) suggested that the Ca^{2+} source for temporary uncoupling lies within the cell, while sustained uncoupling is dependent on extracellular Ca^{2+}. As to the involvement of intracellular pH, acidification by exposure to CO_2 results in uncoupling, reversible after normalization of CO_2 (Iwatsuki and Petersen 1979). Whether this is related to ACh-induced uncoupling was examined in the same study by alkalinization of cells by NH_4Cl. This rise of pH counteracts the ACh-stimulated reduction of the coupling coefficient.

Ca^{2+} does not appear to be the sole intracellular messenger of ACh-induced uncoupling. Using the double whole-cell technique Neyton and Trautmann (1986a) demonstrated that ACh application causes gap junction channel closure in lacrimal gland preparations. ACh induces a reduction of the junctional conductance irrespective of pCa and pH, which were strongly buffered at pCa 8 (20 mM EGTA) and pH 7.2 (30 mM HEPES), respectively. If pCa is weakly buffered, agonist stimulation elicits Ca^{2+}activated membrane currents. This current response is eliminated by buffering Ca^{2+} with 20 mM EGTA, demonstrating the effectiveness of the Ca^{2+} buffer.

In light of the previously mentioned effects of cholinergic agents on junctional permeability and PKC activity, the question arises as to whether PKC mediates agonist-induced uncoupling. Several lines of evidence, presented in the investigation of Randriamampita et al. (1988a),

suggest that this is the case. Lacrimal gland cell pairs in the double whole-cell configuration were subjected to treatment with the PKC activators OAG, dioleoylglycerol (DOG), and TPA. All three modulators lead to uncoupling of acinar cells, although with some variability. To exclude the involvement of arachidonic acid (AA), possibly generated by DAG-activated phospholipase A_2, an inhibitor of PLA_2, 4-bromophenacylbromide (Volwerk et al. 1974), was shown not to abolish DAG-induced uncoupling. If PKC does mediate the uncoupling effect of ACh, long-term stimulation of PKC should result in PKC down-regulation and abolition of the ACh-induced effect, as previously shown by Gainer and Murray (1985). Accordingly, preincubation of cells with OAG for 1.5–3 h eliminates OAG-and ACh-induced uncoupling.

Another investigation by Chanson et al. (1988) dealt with the effects of TPA on cell pairs obtained from rat pancreas or the liver-derived WB cell culture. Treatment of both cell types with 0.1 μM TPA causes translocation of PKC from the soluble to the particulate fraction within 5–10 min. While the cells are in the double whole-cell configuration, addition of TPA significantly reduces the junctional conductance in WB cells, but not in acinar cells. Considering that cytosolic washout of acinar cells may diminish the effect of TPA, cells were preincubated in TPA for up to 30 min before coupling was determined. Only a weak reduction of g_j takes place in acinar cells following this procedure. On the basis of this evidence, it was suggested that PKC is not the primary modulator of coupling in acinar cells, since only weak uncoupling occurs after PKC translocation.

Modulation of gap junction channels by PKC may be subject to more complex regulation than previously assumed. ACh-induced uncoupling of exocrine cells possibly entails additional steps besides PKC activation. An alternative mechanism could involve the release of AA. Dixon and Hokin (1984) demonstrated that caerulein stimulates the release of AA and stearic acid (SA) from mouse pancreatic minilobules, most likely the result of sequential actions of phospholipase C and diglyceride lipase. Moreover, TPA stimulation of PKC has been implied in AA release in MDCK cells, a kidney cell line (Parker et al. 1987).

Giaume et al. (1989) investigated this possibility They demonstrated that extracellular application of 50–100 μM AA to lacrimal gland cell pairs is followed by gap junction channel closure after several minutes. Similar effects are obtained with linoleic, myristic, and lauric acid, the latter being the most potent uncoupling agent. Also taking into account that inhibitors of AA metabolism to prostaglandins and leukotrienes did not inhibit AA-induced uncoupling, it was suggested that the tested fatty acids modulate the gap junction channel itself or its lipid environment. To find

out whether AA generated during cholinergic stimulation affects the junctional conductance, inhibitors of the available AA liberating enzymes PLA_2 and DGL were tested in combination with ACh. These inhibitors did not affect the uncoupling efficiency of ACh. Although extracellular application of AA causes a reduction of g_j, AA probably does not play a primary role in ACh-induced uncoupling of lacrimal gland cells.

4.2 Cholecystokinin

The basolateral membrane of pancreatic acinar cells from all species examined has been shown to interact specifically with cholecystokinin (CCK). The CCK-liganded receptor (Gardner and Jensen 1981, 1988; reviewed by Jensen et al. 1989) stimulates amylase release, causes mobilization of cellular Ca^{2+} and increase of IP_3. The Ca_i^{2+} and IP_3 dose response curves are monophasic. This behavior fits into the concept of agonist-liganded receptors which catalyze, by activation of phospholipase C, the generation of IP_3 and liberation of Ca_i^{2+}. In contrast to this monophasic behavior the amylase release shows a biphasic dependence on CCK concentration with an optimum at about $5 \times 10^{-11} M$ (Sato et al. 1989).

In double whole-cell experiments on pancreatic acinar cell pairs cholecystokinin-octapeptide (CCK-OP) causes a decrease in junctional conductance (Fig. 1; Ngezahayo A et al., submitted). As the figure indicates the time course of uncoupling becomes monotonically accelerated with increase of CCK-OP (see also Fig. 2a). Since it has been suggested that PKC may be involved with CCK-dependent stimulation of secretion (Sung et al. 1988; Bruzzone et al. 1988), it is reasonable to assume that this monophasic behavior reflects the generation of IP_3 and DAG, an activator of PKC. Correlative evidence had been presented by Somogyi et al. (1989) that PKC, in turn, closes gap junctional channels (see Sect. 3.3.2).

Surprisingly, the lag time after which a significant decrease of the gap junctional conductance occurs shows a biphasic behavior as a function of increasing CCK-OP concentration (Fig. 2b). The CCK-OP concentration for a minimal lag time is in the range of 1 n*M*. The striking similarity in the biphasic CCK response of amylase secretion and the lag phase for CCK-induced gap junctional uncoupling gives evidence that the underlying mechanisms might simultaneously be catalyzed by the CCK-liganded receptor. It has been proposed that the biphasic behavior of amylase release reflects the presence of two receptor populations with high and low affinity for CCK binding (reviewed by Jensen et al. 1989). On

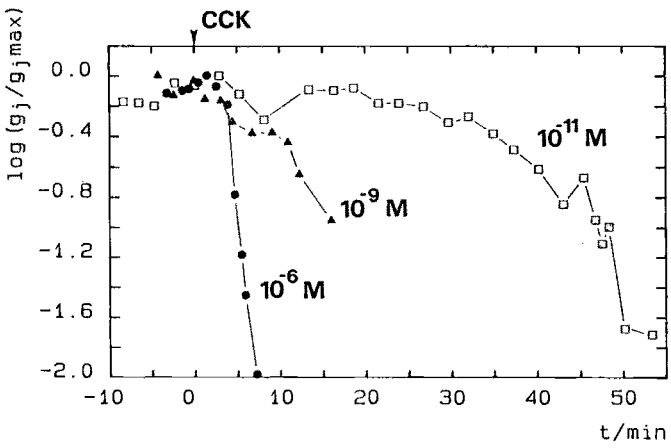

Fig. 1. Gap junctional uncoupling induced by cholecystokinin-octapeptide. The time course of the gap junctional conductance g_j was measured by the double whole-cell patch-clamp technique on pancreatic acinar cell pairs of mouse. g_j is shown at different CCK concentrations as indicated. For clearer presentation the quotients of the individual g_j values over the maximal starting value g_{jmax} are presented, respectively. The time axis origin was calibrated to the addition of CCK to the physiological NaCl-bath medium. The high KCl pipette solution (135 KCl) contained 5 m*M* MgATP and 0.1 m*M* cAMP to suppress spontaneously occurring uncoupling (see Sect. 3.3.1) (Somogyi and Kolb 1988a,b, 1989)

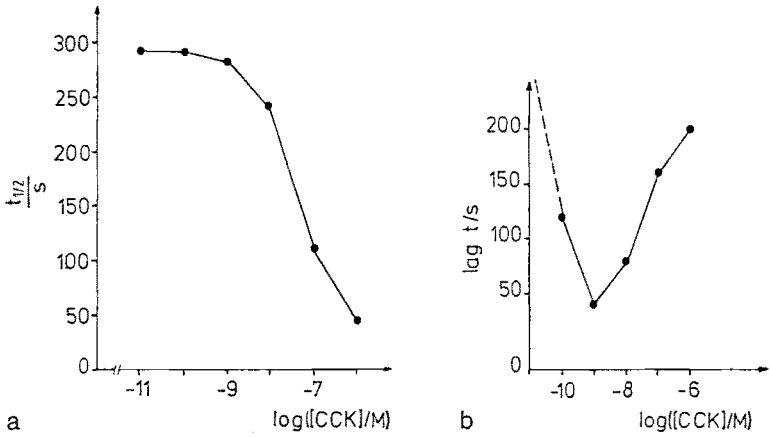

Fig. 2. Kinetic parameters describing the cholecystokinin-octapeptide induced time dependent gap junctional uncoupling. **a** $t_{1/2}$ is the time required for g_j to drop by one half in the decreasing phase which was approximated by a single exponential behavior. **b** Lag time versus the log of CCK concentration. The lag time was determined as the time span after addition of CCK for which a significant decrease of g_j could be observed. The experimental conditions were as described for Fig. 1. Each *point* represents the mean of three independently performed experiments. The value of $t_{1/2}$ at 10^{-11} M CCK is about 20 min (see Fig. 1) and was not included in the figure (see *dashed line*)

the other hand it has been shown that the CCK-liganded receptor acts as a formal catalyst of GDP/GTP exchange on different G-protein (guanine nucleotide-binding protein) molecules (Merritt et al. 1986; Lambert et al. 1985; Schnefel et al. 1988). This means that a single agonist-liganded receptor can interact sequentially with multiple G-protein molecules in a process involving lateral diffusion in the plasma membrane (see also Brandt et al. 1986). Therefore it cannot be ruled out that multiple populations of G-proteins become activated at CCK concentrations below and above the transition point. The G-proteins might activate the involved key enzyme, phospholipase C (Cockroft 1987, 1988; Cockroft and Stutchfield 1988), differently. Further double whole-cell studies are necessary to correlate the biochemical pathways with the detailed kinetics of CCK-dependent gap junctional uncoupling.

4.3 The role of G-Proteins

Involvement of G-proteins in carbamylcholine-induced uncoupling was investigated by Somogyi and Kolb (1989a,b). Carbamylcholine (carbachol, CCh) at concentrations larger than 1 μM induces uncoupling of a fraction of pancreatic acinar cell pairs. Addition of the weakly hydrolyzable analogue GTP[S] (10 μM to 500 μM) to the pipette solution leads to potentiation and reproducibility of CCh-induced uncoupling, suggesting that G-proteins interact with the CCh-liganded muscarinic receptor (see also Evans and Marty 1986; Fleming et al. 1989). This amplification is characterized by a significant reduction of the lag phase between agonist addition and uncoupling, accompanied by an increase in the rate of uncoupling (Fig. 3). The G-proteins involved in the stimulation of the effector, phospholipase C, appear to be different from the case of cholecystokinin-stimulated pancreatic acinar cells (Schnefel et al. 1988; see also Sect. 4.2).

An interesting observation is that GTP[S] does not lead to uncoupling in the absence of an agonist (Somogyi and Kolb 1989a,b). Analogous results have been reported for a G-protein coupling muscarinic receptors to a K^+ channel in heart (Breitwieser and Szabo 1985; for review see Brown and Birnbaumer 1988; Dunlap et al. 1987). This indicates that the basal exchange rate of GTP for bound GDP of this G-protein coupling to cholinergic receptors is slow in the absence of the stimulated receptor (Gilman 1987).

Although it is known that GTP[S] activates phospholipase C in many systems (Gomberts 1983; Haslam and Davidson 1984; Litosch et al. 1985; Wallace and Fain 1985; Uhing et al. 1986) which leads to hydrolysis of

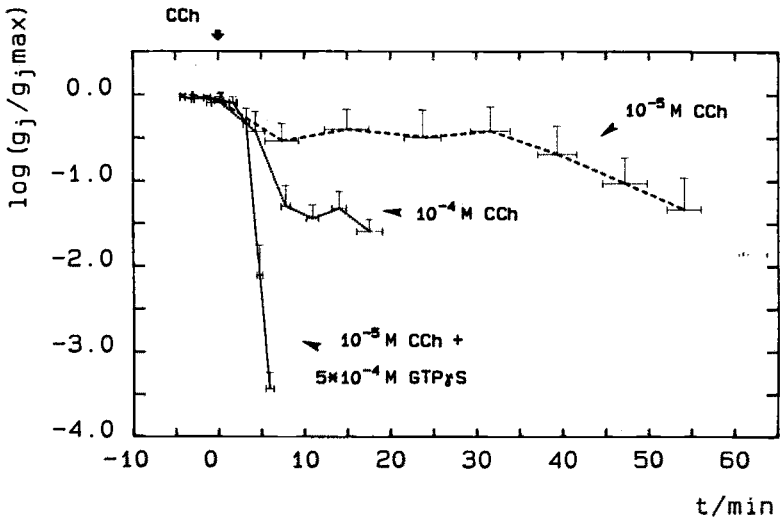

Fig. 3. Effects of different intracellular concentrations of GTP[S] on carbamylcholine (CCh)-induced change of the gap junctional conductance g_j between pancreatic acinar cell pairs. All experiments were performed using the double whole-cell patch-clamp. 1 μM CCh was added to the bath throughout the experiments. GTP[S] in the pipette solution is lowered from 100 μM (*dotted line*, mean of two experiments) to 50 μM (*solid line*, mean of four experiments) and 10 μM (*broken line*, mean of two experiments). A control experiment in the absence of CCh and GTP[S] is shown for comparison (*line interrupted by dots*) (see Somogyi and Kolb 1989b)

phosphatidylinositol-4,5-bisphosphate (Cockroft and Gomberts 1985), also in pancreatic acinar cells (Putney et al. 1986), and redistribution of PKC (Ishizuka et al. 1987; Machado-de Domenech and Söling 1987), other possibilities of G-protein action in the modulation of junctional channels will be discussed. Taking into account that muscarinic cholinergic receptors are involved in the regulation of cAMP metabolism (Harden et al. 1985), what role could be attributed to G-protein modulation of adenylate cyclase in CCh/GTP[S]-induced uncoupling? Since cAMP was present in the cell dialysis medium in a high concentration (0.1 mM), the intracellular cAMP level is expected to be clamped at this value (Somogyi and Kolb 1989b). Effects associated with G_i and G_s are therefore unlikely to have played a major role in this investigation.

Liberation of AA is another plausible uncoupling mechanism in pancreatic exocrine cells, as AA can lead to uncoupling of lacrimal acinar cells in the double whole-cell configuration (Giaume et al. 1989; see above). The role of AA was investigated by Somogyi and Kolb (1989b) using inhibitors acting within this signal transduction system. One source of AA is phospholipase A_2 (PLA_2)-catalyzed breakdown of membrane

phospholipids. To examine this possibility, an inhibitor of PLA_2, quinacrine, was included in the pipette medium at concentrations equal or greater to those established to inhibit AA-dependent physiological functions (Canonico et al. 1985; Cronin et al. 1985). Quinacrine was ineffective in even partially reducing the CCh/GTP[S]-stimulated uncoupling response. This suggests that PLA_2-dependent AA liberation is unlikely to play a significant role in CCh/GTP[S]-stimulated uncoupling, as is also the case for lacrimal gland cells (Giaume et al. 1989).

Arachidonic acid may alternatively be produced during PI breakdown by the sequential actions of phospholipase C and diglyceride lipase (DGL). If DGL-generated AA should play a role in CCh/GTP[S]-stimulated uncoupling, one would expect a reduction of this effect by RHC 80267, a DGL inhibitor in pancreatic acinar cells (Dixon and Hokin 1984; Sekar and Hokin 1987). Adding RHC 80267 to the pipette solution did not inhibit CCh/GTP[S]-induced uncoupling, but, on the contrary, led to further potentiation (Fig. 4). The explanation for this phenomenon is probably an RHC 80267-induced accumulation of DAG, resulting in increased PKC activation.

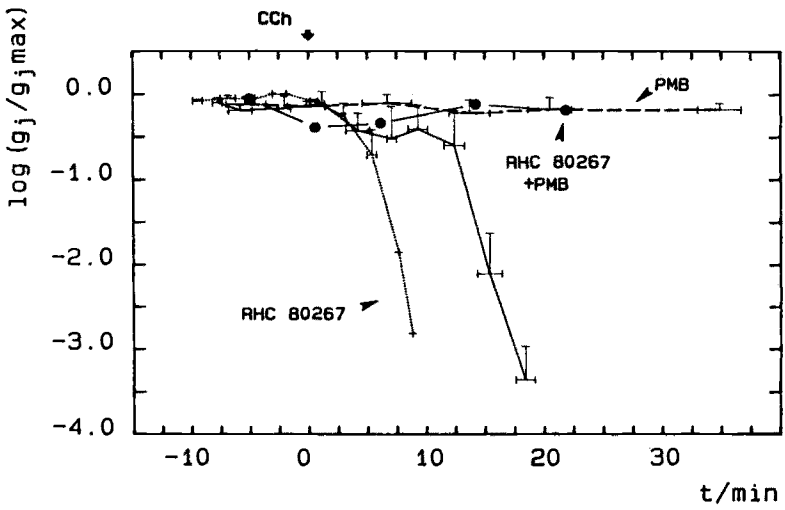

Fig. 4. Effects of the diglyceride inhibitor RHC 80267 and polymyxin B(PMB) on CCh/GTP[S]-induced gap junctional uncoupling of pancreatic acinar cell pairs. 50 μM GTP/[S] was included in pipette solution and 1 μM CCh was added to the bath. *Dotted line* (means of two experiments): Addition of 50 μM or 100 μM RHC 80267 to the pipette solution. *Solid line* (same as in Fig. 3): Supplementation by 50 μM GTP[S] alone. *Broken line* (mean of three experiments): 100 μM to 200 μM polymyxin B (PMB) were included in pipette solution. RHC 80267 was not effective in counteracting the suppression by PMB (*line interrupted by dots*, 50 μM RHC 80267 and 200 μM PMB added to the pipette solution) (see Somogyi and Kolb 1989b)

The link between DAG production and uncoupling suggests the direct involvement of PKC. It was therefore investigated whether the established PKC inhibitor of this system, polymyxin B (see Sect. 3.3.2), is able to block agonist-induced uncoupling. As Fig. 4 shows, addition of polymyxin B to the pipette solution completely suppressed CCh/GTP[S]-induced uncoupling, which could not be reversed by RHC 80267 (Somogyi and Kolb 1989b). Recently it could be demonstrated that exposure of a pair of pancreatic acinar cells to a hypotonic shock activates the signal-transduction chain G-protein → Phospholipase C → DAG → PKC which finally yields to cell-to-cell channel closure (Ngezahayo and Kolb 1990). These results are in clear agreement with the predictions made by the PKC hypothesis of cell-to-cell channel regulation.

5 Biophysical Analysis of the Junctional Channel

5.1 Permeability

To gain a better understanding of the mechanisms of direct cell-to-cell communication and its physiological relevance, it is necessary to determine which type of ions and molecules can pass through the gap junction channels. It can be measured in two ways: a small current is injected into a cell by a microelectrode and the resulting voltage change is monitored in neighbouring cells or, alternatively, dye coupling is measured by injecting small molecules of fluorescent dyes such as fluorescein or lucifer yellow (M_r 443 Da), and observing its diffusion into adjoining cells. Thus, channel permeability can be probed according to the size and three-dimensional structure of substances able to pass through gap junctions. Using this approach, a permeability limit for *Chironomus* salivary gland gap junctions of 1200–1900 Da is estimated, as measured with flourescent dye labelled peptides of different molecular weights. The estimated spatial parameters of permeable molecules allow an approximation of 1.0–1.4 nm for the pore diameter (Simpson et al. 1977). Slightly higher values of 2–3 nm for *Chironomus* salivary gland and an insect cell culture were determined by Schwarzmann et al. (1981), using neutral fluorescent tracer molecules. Experiments in the same study demonstrated a smaller pore diameter of 1.6–2.0 nm for mammalian cell cultures. Based on these data the expected equivalent conductance of a channel is of the order of 100 pS (Loewenstein 1975).

Variations in channel diameter between different mammalian cell cultures (Flagg-Newton et al. 1979) suggested an experiment in which the permeability of gap junctions between heterologous cell pairs in coculture

is compared to determine differences in channel structure. One such study found that asymmetrically permeable junctions are formed between heterologous cells of cultures exhibiting different permeabilities. Larger tracers pass more readily from the side associated with the hemichannel of greater diameter to the side of smaller expected pore size (Flagg-Newton and Loewenstein 1980).

How do permeability studies using synthetic fluorescent tracers compare to data obtained on the transfer of physiologically active molecules, e.g. metabolites and second messengers? Pitts and Simms (1977) demonstrated selectivity of the junctional transfer for nucleotides over DNA and protein macromolecules in various cell cultures. An elegant approach was used by Lawrence et al. (1978) in the demonstration of the intercellular transfer of a soluble messenger. Rat ovarian granulosa cells and mouse myocardial cells respond to hormones using cAMP as a second messenger – the former to FSH (follicle stimulating hormone), which raises the production of plasminogen activator, the latter to noradrenaline, leading to increased action potential frequency. These cells were exposed to either one of these signals in coculture. Stimulation with a hormone specific for one cell type elicits the physiological response from both members of a heterologous culture, implying the intercellular transfer of cAMP. Following up on this study, cells in an analogous coculture system were stimulated with a cAMP-dependent agonist specific to one constituent cell type. The dissociation of cAMP-dependent protein kinase in both cell types was shown to be dependent on contacts between heterologous cells (Murray and Fletcher 1984). Aside from cAMP, two other important second messengers, IP_3 and Ca^{2+}, also permeate gap junctions (Saez et al. 1989).

A central issue in the modulation of gap junctional communication is whether channel closure is an all-or-none process, or whether the individual channel can be subject to gradual closure. Evidence for the latter alternative was obtained in experiments characterizing the junctional transfer of fluorescent tracers of various sizes between *Chironomus* salivary gland cells (Rose et al. 1977). It was demonstrated that Ca^{2+}-induced uncoupling leads to the selective retardation of larger molecules as opposed to smaller ones, the cutoff point being at Mr ~700 Da. This effect suggests the Ca^{2+}-dependent control of either pore size or distribution of channels of different inner diameters. However, differential sequestration of dye might also lead to a reduction in the transfer of injected dye. This would become apparent especially at lowered junctional permeabilities (Zimmermann and Rose 1985).

Also using *Chironomus* salivary gland, the intercellular diffusion of small and large tracers was shown to be equally affected by uncoupling dependent on membrane voltage. The time resolution of these exper-

iments was in the second range, allowing an exact determination of diffusion coefficients. Membrane voltage apparently regulates the number of open channels rather than the individual channel permeability in this cell type (Zimmermann and Rose 1985). The same conclusion was drawn from experiments with *Rana pipiens* blastomere cell pairs, also with a temporal sensitivity in the order of seconds. A linear relationship between the intercellular permeability to tetraethylammonium ions and potassium ions was determined during uncoupling induced by changing the transjunctional voltage (Verselis et al. 1986).

Though the evidence obtained from the voltage-dependent reduction of junctional permeability speaks for a mechanism involving the single channel open probability, Ca^{2+}-induced uncoupling may involve changes in the distribution of the single channel pore size in gap junctions. This could be due to the adjustment of the pore diameter of an individual channel, or the selective modulation of channels a priori differing in size. It has become apparent that a large variety of different modulators induce a reduction of the junctional permeability. Thus, the issue of the control of the distribution of pore sizes is far from resolved, as each modulator may act by a different mechanism. Methods now available to measure the single channel conductance may help to clarify aspects of this problem.

5.2 Single Cell-to-Cell Channel Properties

The identification of single gap junctional currents is only possible if the background noise in the frequency range of channel opening and closing events is lower than the single channel current amplitude. If many channels are active, the sum of the current noise of the channel's open state, and the flickering pattern created by closely spaced opening and closing of these channels create a noisy current pattern that does not meet the requirements for single channel analysis. Therefore, the channel activity should be lowered by either decreasing the number of channels altogether, or by slowing down the channel kinetics to a point at which stronger filtering will allow the identification of current steps. This may be accomplished by either decreasing the number of open channels in cells already coupled, or by recording the formation of coupling through the step by step connexon assembly from hemichannels. The first investigation dealing with this issue employed the latter approach. Loewenstein et al. (1978) were able to observe quantal changes in the transcellular resistance after manipulating cells of *Chironomus* salivary gland together. This supplied important evidence for the model of gap

junction formation by interlocking of functional hemichannels. The resolution of the experimental set-up, however, did not allow for a quantitative evaluation of the single channel conductance. The latter issue was dealt with the approach taken by Chow and Young (1987) and Rook et al. (1988). The mechanism of cell-to-cell channel formation and the consecutive arrangement into clusters is still unsolved. For the latter, two mechanisms are possible: direct insertion of the hemichannels into the membrane junction or random matching of lateral moving hemichannels in the two opposing membranes. The lateral movement model was analyzed quantitatively by Monte Carlo simulation. The model produced channel clusters fast enough even at low initial density of hemichannels (Loewenstein 1981).

5.3 Double Whole-Cell Patch-Clamp Data of Single Channel Conductance

The advent of the patch-clamp technique enabled the high resolution recording of membrane channels on the single channel level (Hamill et al. 1981). How could this method be employed to analyze channels between cells? Neyton and Trautmann (1985) demonstrated that the double whole-cell patch-clamp configuration, characterized by a high signal to noise ratio due to low access resistance and leak, is an excellent means for resolving single gap junctional channels. During the simultaneous voltage clamp of each member of an isolated pair of rat lacrimal gland cells, voltage changes applied to one side led to current responses in both systems. These are attributable to gap junctional currents.

Single channel conductance steps can only be analyzed in the final phase of electrical uncoupling. Therefore, the time span of observation and the associated number of occurring conductance steps is limited. The reported values of single cell-to-cell channel conductances are included in Table 2. Furthermore, the conductance fluctuations appear in a nonstationary state, which does not allow an analysis of channel kinetics in terms of chemical reaction kinetics. The typically observed spontaneous loss of the junctional conductance during an experiment enables the recording of quantal conductance changes, corresponding to fluctuations of single junctional channels (Neyton and Trautmann 1985). The single junctional conductance is scattered between 70 and 180 pS, with randomly distributed intermediate levels occurring between the peak conductances. The voltage insensitive channel kinetics were slow, with an estimated mean open time of 2 s. Transition events between states are not always instantaneous, but take several milliseconds to develop. Comparable to

reconstituted junctional channels (see below), the channel discriminates poorly between ions of opposite charge. But precise data for the ion selectivity of cell-to-cell channels are not available as yet.

Using the same method, Veenstra and DeHaan (1986) characterized single gap junctional currents between embryonic chick heart cell pairs. Since the total junctional conductance is low in this cell system, single channel fluctuations can be recorded without having to lower the initial junctional conductance. The quantal conductance appears in the range of 50–190 pS, the mean value being 164 pS. The junctional conductance, g_j, is independent of voltage, but sensitive to long chain alcohols (see e.g. Bernardini et al. 1984) such as octanol. Octanol treatment causes a reversible disappearance of 188 pS steps. The frequency of transitions between states is about ten times faster than those observed by Neyton and Trautmann (1985). In a further study, a clear discrimination between the 166 pS and a 60–80 pS conductance state was presented (Veenstra and DeHaan 1988). Octanol or Ca^{2+}-induced uncoupling only affects the 166 pS, not the 60–80 pS conductance. In the case of neonatal rat heart cell pairs, only single channel conductances of ~60 pS with little scatter were characterized (Burt and Spray 1988). During uncoupling induced by octanol or acidification, all the channels disappear in a stepwise fashion.

Analogous to the approach of Loewenstein et al. (1978), Chow and Young (1987) characterized the events during formation of coupling between *Xenopus* embryonic muscle cells, using a combined whole cell and current injection technique. Within minutes after manipulating two cells together, quantal changes of the intercellular conductance, corresponding to steps of ~100 pS, can be recorded. A detailed analysis of junctional channels immediately after formation between neonatal rat heart cells is presented in the study of Rook et al. (1988). After manipulating two cells together in the whole-cell configuration, a stepwise increase of g_j occurs at a rate of ~10 channels per minute. In contrast to Burt and Spray (1988), two main conductance states of ~60 pS and 30 pS predominate, accompanied by various other levels. Comparable to the observation of Neyton and Trautmann (1985) and Somogyi and Kolb (1988a,b), transitions between the main states are not always instantaneous, and seem to pass through intermediate conducting states. Though g_j was demonstrated to be independent of voltage after many channels had already formed, a marked voltage sensitivity to gating appears when only few channels are open.

The action of ACh on pairs of rat lacrimal gland cells was investigated in the double whole-cell configuration (Neyton and Trautmann 1986a). Though ACh-induced uncoupling is not always complete, single channel events can be analyzed in some experiments. The single channel conductance adopts various levels, with a maximum of 80–100 pS. Transi-

tions between discrete levels do not always take place in a quantal fashion. However, a progression to conductances lower than 80–100 pS does not occur during uncoupling. It was therefore concluded that ACh equally acts on all channel open states, instead of blocking g_j by increasing the frequency of occurrence of lower conductance states.

This interpretation is supported by a related series of experiments, comparing uncoupling of lacrimal gland cells by AA to channel closure induced by ACh (Giaume et al. 1989). A main single channel conductance of ~110 pS appears during uncoupling induced by AA, again accompained by various substates. These observations are independent of whether ACh or AA is employed to induce channel closure. Nevertheless, the mechanisms of uncoupling by ACh and AA seem to differ. While ACh-stimulated uncoupling is probably the result of the activation of PKC (Randriamampita et al. 1988a), AA has been proposed to act directly on the gap junction channel or its lipid environment (Giaume et al. 1989).

For isolated acinar cell pairs of the mouse pancreas several procedures have been applied to reduce the initially high junctional conductance from the nanosiemens to the picosiemens range:

1. Dialysis of the cell pair with pure electrolytes results in a spontaneous loss of electrical coupling.
2. Infusion of purified PKC or superfusion of a cell pair by media containing DAG analogues (OAG) may elicit a progressive decrease of the junctional conductance.
3. Binding of CCh to muscarinic receptors uncouples cell pairs. The presence of GTP[S] in the pipette solution potentiates CCh-induced electrical uncoupling significantly.
4. Extracellular application of long chain alcohols, such as octanol, results in a rapid reduction of the junctional conductance.
5. Increase of intracellular Ca^{2+} to concentrations larger than 1 m*M* leads to rapid uncoupling.

An analysis of the identifiable quantal steps shows a heterogeneity of conductance values. For each of the first three of the described uncoupling procedures, conductance levels could be identified in the range of approximately 20–130 pS. Within a single experiment, the observed conductance levels occur randomly, i.e. several discrete conductances appear side by side. For the maximal quantal conductance change, values in the range of 90–130 pS are observed. Besides this, values in the range of 20–30 pS and/or 50–70 pS could be recorded in parallel (Somogyi and Kolb 1988a,b; Somogyi et al. 1989).

Three different mechanisms could account for the observation of single junctional channel conductances down to and less than one order of magnitude below the observed maximal value of 130 pS. As a first

interpretation, it may be postulated that gap junctions are composed of single cell-to-cell channels of different single channel conductances. It is generally assumed that the cell-to-cell channel is a transport protein which is formed of six subunits. The existence of varying connexon subunits of 21 and 27 kDa in liver (Traub et al. 1989) suggests the existence of pores composed of different subunits, yielding homomeric or heteromeric structures (Finbow et al. 1983; Nicholson et al. 1987). Such a model could explain the occurrence of different discrete conductance levels during uncoupling by a reduction of the open probability of initially mixed channel populations. Additionally, several observations are reported suggesting that individual cell-to-cell channels adopt multiple conductances, analogous to observations of subconductance states in a variety of other channels (for review see Fox 1987).

Veenstra and DeHaan (1986) and Rooke et al. (1988) proposed subconductance states as an explanation for their observations of equal and opposite steps of lower conductance (50 pS) than the maximum level (165 pS). Subconductance states have been suggested for lens junctional protein (Zampighi et al. 1985) and the junctional channel of hepatocytes (Young et al. 1987), reconstituted in bilayers.

Finally, it should be taken into account that the junctional current records are subject to modulation by the filtering characteristics of the record/replay instruments. The simultaneous observation of different current or conductance levels within one record at constant filtering indicates the steps of different sizes do occur in parallel. The apparent junctional current amplitude decrease at a constant junctional potential could be interpreted as a gradual increase of rapid channel flickering to frequencies beyond the bandwidth of the record/replay system. But flickering of a channel characterized by a single kinetic state could not produce apparent multiple conductance states (Läuger 1985).

5.4 Reconstitution of Gap Junction Channels

Considering that some of the putative gap junction channel proteins have been isolated, it should be possible to measure the single channel properties of the connexon in vitro using this preparation. Though methods for the reconstitution of ion channels in artificial lipid bilayers are available (Latorre 1986; Miller 1986), there is the inherent problem of the channel spanning two bilayers instead of one. Even if one is not able to clearly distinguish between hemichannels and complete connexons from such experiments, it appears reasonable to conduct such investigations to at least obtain preliminary data. In the first of these studies,

solubilized lens gap junction protein, consisting mainly of the 26 kDa main intrinsic protein (MIP) was incorporated into bilayers (Zampighi et al. 1985). Quantal conductance fluctuations corresponding to ~200 pS in 100 m*M* KCl demonstrate channel-forming ability. Besides the main state, at least two putative subconductances can be identified. In accord with the properties of some gap junctions in situ, the channel is sensitive to voltage and octanol. Ca^{2+} in the millimolar range does not affect the channel, this conceivably being attributable to a lack of calmodulin.

In light of the 26 kDa MIP not being representative of gap junctions in general, investigations using isolated liver gap junctions or liver 27 kDa protein should aid in completing the picture. Spray et al. (1986a) used an approach in which a patch pipette is consecutively dipped into three baths, respectively containing a mixed lipid cocktail, isolated liver gap junctions, and unmodified electrolyte. After passing the second bath, channel opening and closing events can be recorded. The quantal conductance is ~150 pS in 150 m*M* KCl, while various lower states are observable. Though gating is not voltage dependent, lowering the pH to 6.0, or adding 0.01 m*M* octanol to the bath, leads to a reduction of the total conductance, comparable to the situation in situ (Spray et al. 1986b). Addition of an anti-27 kDa antibody appears to block the channel, implying that the junctional channel conformation serves as the basis for the observed fluctuations.

Incorporation of either isolated liver gap junctions or the purified 27 kDa peptide into bilayers demonstrated functional channel-forming ability (Young et al. 1987). This indicates that the 27 kDa protein is an actual channel-forming component of gap junctions. The channels adopt a broad range of quantal conductances, the main histogram peaks lying at ~140 pS and its multiples. In addition, at least three subconductance states below 140 pS exist. From the permeability analysis of various ions, it was inferred that the channel is relatively unselective in respect to ion charge, and that it has a pore diameter of 0.8–1.2 nm, smaller than the values obtained by Schwarzmann et al. (1981). As opposed to the in situ hepatocyte gap junctions (Spray et al. 1986b), and the results of Spray et al. (1986a), gating is strongly voltage dependent, while pH and Ca^{2+} have a relatively minor influence on the kinetics. The normalized channel conductance begins to drop at voltages >25 mV. Lowering the pH from 7.0 to 5.0 increases the slope of the voltage dependence, without affecting V_0 (the voltage at which half the channels are open). Nominal elimination of Ca^{2+} by addition of 1.5 m*M* EGTA causes an upward shift of V_0 by 30 mV, compared to the presence of 1 m*M* Ca^{2+}.

Reconstitution of putative gap junction channels in artificial lipid environments demonstrated the inherent channel—forming properties of

connexins, limited conservation of gating mechanisms, and a relative lack of selectivity for different ions. To what extent the reconstituted channel resembles a hemichannel or complete connexon, and how closely it approximates the in situ conformation, remains elusive in these investigations.

6 Physiological Functions

The identification of gap junctions in a particular cell type or tissue is not always accompanied by a detailed characterization of their specific physiological role. One of the earliest proposed functions for gap junctions is the control of growth and carcinogenesis. It has become clear that the modulation of junctional communication is interwoven with the physiological control mechanisms of cellular activity. Gap junctions may transmit messengers involved in these systems. Signalling involved in cellular growth control is also part of this system. Hence, one would assume the involvement of gap junctions in cell proliferation at some point. A detailed model and review of junctional growth control has been presented by Loewenstein (1979). The pathological case of carcinogenesis has been treated by Kanno (1985).

Gap junctional communication plays a crucial role during development (for review see Guthrie and Gilula 1989). Embryonic cells are electrically coupled and have gap junctions (Potter et al. 1966). Disruption of intercellular communication by antibodies against gap junction proteins can drastically change development. This has been demonstrated on *Xenopus* embryos (Warner et al. 1984). Correlative evidence suggests that the change in temporal and spatial pattern of gap junctions parallels the progressive determination of cell fate. The question arises as to the nature of the signal which becomes exchanged. For *Hydra*, a hydrophilic non-peptide with a molecular mass less than 0.5 kDa has been proposed as morphogen (Blennerhassett and Caveney 1984). The expression and pattern of gap junctions would thus determine and modulate the three-dimensional morphogenetic gradient.

An important role for direct intercellular communication may be found in hormonal signal transduction. This appears plausible since important messenger molecules (cAMP, Ca^{2+}, IP_3) are able to pass through gap junctions (see Sect. 5.1). Although correlations of several physiological functions with changes of junctional permeability have been demonstrated, the mechanism by which cellular activity is influenced through junctional communication has rarely been found.

Probably the most obvious function of gap junctions is transduction of impulses between electrically excitable cells. The first demonstration of direct junctional communication was performed on the junction between the lateral axon of the crayfish nerve cord and the giant motor axon (Furshpan and Potter 1959). Due to the rectifying properties of this junction, action potentials traverse from the lateral axons to the giant motor axon. Rectifying electrical synapses have been reviewed in detail by Ramon and Rivera (1986).

Electrical signal transduction in a non-nervous tissue is best exemplified by myocardium. The role of gap junctions in the synchronization and control of contractility by hormones has been the subject of numerous investigations (DeHaan et al. 1981; Page and Shibata 1981; DeMello 1987). Ca^{2+} and pH are implied in the regulation of the internal longitudinal resistance (Pressler 1987), but it remains to be demonstrated whether they do so under conditions other than pathological cases (Ramon and Rivera 1986), e.g. healing over after injury (DeMello et al. 1969) or acidification during ischemia (Spray et al. 1985). An important demonstration with respect to cardiac signal transduction is that action potentials are conducted through junctions between isolated ventricle cells (Rook et al. 1988; Weingart and Maurer 1988). The modulation of junctional permeability may also be important during the stimulation of contractility by epinephrine. It has been shown that elevated levels of cAMP, the second messenger of the β-adrenergic receptor, leads to an increase of the junctional conductance. This facilitates the transmission of electrical impulses between cardiac cells (DeMello 1984).

A role of gap junctions in the mammalian female reproductive tract is suggested in various studies (for review see Ramon and Rivera 1986). Since gap junctions between cells of the ovarian follicle have been identified (Albertini and Anderson 1974), the regulation of junctional communication within the follicle has been investigated in detail. The in vitro system of Dekel and Beers (1980) permits the characterization of interactions within the rat cumulus-oocyte complex. It could be shown that oocyte maturation, induced by luteinizing hormone (LH), is dependent on the presence of the surrounding follicle cells. Oocyte maturation is also inhibited by cAMP. A complete model is presented in the study of Dekel et al. (1981), proposing that follicle cells transmit the maturation inhibitor cAMP to the oocyte via gap junctions. Once junctional blockage is induced by LH, the oocyte cAMP level drops and normal development ensues.

Reminiscent of coupling in myocardium, junctional communication is apparently involved in coordinating the contractile activity of uterine smooth muscle during parturition (see Sims et al. 1982). The presence of

junctional communication is tightly controlled during pregnancy. Gap junctions only appear immediately prior to and shortly after delivery, coincident with a decrease in progesterone and an increase in estradiol and prostaglandin F2α levels (Garfield et al. 1977; Puri and Garfield 1982). This is in accord with a model proposing that gap junctions are only present while needed for the intercellular passage of messages controlling smooth muscle contraction during birth.

The exocrine and endocrine pancreas are the best-investigated model systems concerning the role of gap junctional intercellular communication in secretion (for review see Bruzzone et al. 1988; Trautmann et al. 1990). Studies on the islet of Langerhans (reviewed by Sheridan and Atkinson 1985) led to the formulation of the "pacemaker hypothesis" (Meissner 1976). Glucose in a concentration which stimulates insulin release induces bursting electrical activity in B cells. Due to electrical coupling, this activity is synchronized within a high percentage of closely located cells. It was suggested that glucose may only stimulate a limited number of "pacemaker cells", from which the induced activity spreads electrotonically and leads to secretion in coupled cells. Further facilitation of the transmission of the insulin-releasing signal may occur with a glucose-dependent increase of coupling (Kohen et al. 1979).

Heterologous electrical coupling between islet A and B cells raises the possibility of coordinated secretion of glucagon and insulin (Michaels and Sheridan 1981). Such a possibility was discussed by Raskin and Unger (1978). They proposed that an intracellular messenger mediating insulin release is produced in B cells as a result of glucose stimulation. Inhibition of glucagon release from A cells may then result from the passage of this messenger from B cells to A cells through gap junctions. This hypothesis is also supported by the finding that A cells lacking direct contact with B cells are unresponsive to glucose in juvenile diabetics (Orci et al. 1976).

Considering that uncoupling coincides with secretion elicited by certain classes of secretagogues, is there a role for junctional communication in this process? The evidence available so far shows that lowered junctional permeability increases both basal and agonist-stimulated secretion in acinar cells, provided the secretagogue does not lead to uncoupling on its own (Meda et al. 1986; Bruzzone et al. 1988; Trautmann et al. 1990). A model claiming that reduced gap junctional communication generally is a positive modulator of secretion would nevertheless be an oversimplification, since pancreatic endocrine secretion is severely hampered in uncoupled B cells. Even though the mechanism of the involvement of coupling in secretion has not yet been elucidated, it appears that the control of junctional permeability does play a physiologically significant role in stimulus-secretion coupling of exocrine and endocrine cells.

7 Conclusion

Gap junctional communication is a universal property of metazoan animals. Biochemical, immunological, molecular biological, ultrastructural, biophysical and physiological studies of gap junctions have permitted increasingly detailed modelling of gap junctional structure and function. In spite of this progress the questions to be addressed are whether the channel is a mixed oligomer and the stoichiometry for each tissue is fixed. Also the extent of homology among gap junction proteins in different tissues and their possible regulatory function have to be clarified. As long as the different channels are not cloned and expressed, the ultrastructural correlates of the physiological concepts such as channel gating, selectivity and regulation, as well as assembly and disassembly cannot be determined. The genetic approach is in full progress. The observed differences between gap junction proteins from different tissues and the multiplicity of subunits in even one channel implies a functional specialization for gap junctions. Correlative studies on the molecular and cellular level should help to clarify the physiological meaning of intercellular communication by gap junctions.

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References

Albertini DF, Anderson E (1974) The appearance and structure of inter-cellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions. J Cell Biol 63:234–250

Arellano RO, Ramon F, Rivera A, Zampighi (1988) Calmodulin acts as an intermediary for the effects of calcium on gap junctions from crayfish lateral axons. J Membr Biol 101:119–131

Ashendel CL (1985) The phorbol ester receptor: a phospholipid-regulated protein kinase. Biochim Biophys Acta 822:219–242

Ashendel CL, Staller JM, Boutwell RK (1983) Protein kinase activity associated with a phorbol ester receptor purified from mouse brain. Cancer Res 43:4333–4337

Atkinson MM, Menko AS, Johnson RG, Sheppard JR, Sheridan JD (1981) Rapid and reversible reduction of junctional permeability in cells infected with a temperature-sensitive mutant of avian sarcoma virus. J Cell Biol 91:573–578

Azarnia R, Loewenstein WR (1984) Intercellular communication and the control of growth. X. Alteration of junctional permeability by the src gene. A study with temperature-sensitive mutant Rous sarcoma virus. J Membr Biol 82:191–205

Azarnia R, Dahl G, Loewenstein WRL (1981) Cell junction and cyclic AMP. III. promotion of junctional membrane permeability and junctional membrane particles in a junction-deficient cell type. J Membr Biol 63:133–146

Azarnia R, Reddy S, Kmiecik TE, Shalloway D, Loewenstein WR (1988) The cellular src gene product regulates junctional cell-to-cell communication. Science 239:398–401

Baux GM, Simmoneau M, Tauc L, Segundo JP (1978) Uncoupling of electrotonic synapses by calcium. Proc Natl Acad Sci USA 75:4577–4581

Bennett MVL, Spray DC (eds) (1985) Gap Junctions. Cold Spring Harbor Laboratory, Cold Spring Harbor NY, pp 1–222

Bernardini F, Peracchia C, Peracchia LL (1984) Reversible effects of heptanol on gap junction structure and cell-to-cell electrical coupling. Eur J Cell Biol 34:307–312

Berridge MJ (1983) Ca^{2+} and protein kinase C: two synergistic cellular signals. Trends Biochem Sci 8:263–265

Berridge MJ (1986) Agonist-dependent phosphoinositide metabolism. A bifurcating signal pathway. In: Poste G, Crooke ST (eds) New insights into cell and membrane transport processes. Plenum, New York

Berridge MJ, Irvine RF (1984) Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature 312:315–321

Beyer EC, Paul DL, Goodenough DA (1987) Connexin 43: a protein from rat heart homologous to gap junction protein from liver. J Cell Biol 105:2621–2629

Beyer EC, Goodenough DA, Paul DL (1988) The connexins, a family of related gap junction proteins. In: Hertzberg EL, Johnson RG (eds) Gap junctions. Liss, New York (Modern cell biology, vol 7)

Beyer EC, Paul DL, Goodenough DA (1990) Connexin family of gap junction proteins. J Membr Biol 116:187–194

Blackshear PJ, Nairn AC, Kuo JF (1988) Protein kinases 1988: a current perspective. FASEB J 2:2957–2969

Blennerhassett MG, Caveney S (1984) Separation of developmental compartments by a cell type with reduced junctional permeability. Nature 309:361–364

Brandt DR, Ross EM (1986) Catecholamine-stimulated GTPase cycle: multiple sites of regulation by β-adrenergic receptor and Mg^{2+} studied in reconstituted receptor-G_s vesicles. J Biol Chem 261:1656–1664

Breitwieser GE, Szabo G (1985) Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. Nature 317:538–540

Brown AM, Birnbaumer L (1988) Direct G protein gating of ion channels. Am J Physiol 254:H401–H410

Bruzzone R, Trimble ER, Gjinovci A, Traub O, Willecke K, Meda P (1987) Regulation of pancreatic exocrine function: a role for cell-to-cell communication? Pancreas 3:262–271

Bruzzone R, Regazzi R, Wollheim CB (1988) Caerulein causes translocation of protein kinase C in rat acini without increasing cytosolic free Ca^{2+}. Am J Physiol G33–G39

Burt JM, Spray DC (1988) Single-channel events and gating behavior of the cardiac gap junction channel. Proc Natl Acad Sci USA 85:3431–3434

Buultjens TEJ, Finbow ME, Lane NJ, Pitts JD (1988) Tissue and species conservation of the vertebrate and arthropod forms of the low molecular weight (16–18 000) proteins of gap junctions. Cell Tissue Res 251:571–580

Campos de Carvalho AC (1988) Regulation of gap junctional channels. Braz J Med Biol Res 21:177–188

Canonico PL, Speciale C, Sortino MA, Scapagnini U (1985) Involvement of arachidonate metabolism in neurotensin-induced prolactin release in vitro. Am J Physiol 249:E257–E263

Caspar ELD, Goodenough DA, Makowski L, Phillips WC (1977) Gap junction structures I. Correlated electron microscopy and X-ray diffraction. J Cell Biol 74:605–628

Chanson M, Bruzzone R, Spray DC, Regazzi R, Meda P (1988) Cell uncoupling and protein kinase C: correlation in a cell line but not in a differentiated tissue. Am J Physiol 255:C699–C704

Cheung WY (1982) Calmodulin: an overview. Fed Proc 41:2253–2257

Chiou CY, Malagodi MH (1975) Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscle. Br J Pharmacol 53:279–285

Chow I, Young SH (1987) Opening of single gap junction channels during formation of electrical coupling between embryonic muscle cells. Dev Biol 122:332–337
Cockroft S (1987) Polyphosphoinositide phosphodiesterase: regulation by a novel guanine nucleotide binding protein, Gp. Trends Biochem Sci 12:75–78
Cockroft S (1988) Inositol lipids, G-proteins and signal transduction. In: Gerday C, Gilles R, Bolis L (eds) Calcium and calcium binding proteins. Springer, Berlin Heidelberg New York
Cockroft S, Gomberts BD (1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature 314:534–536
Cockroft S, Stutchfield J (1988) G-proteins, the inositol lipid signalling pathway, and secretion. Philos Trans R Soc Lond [Biol] 320:247–265
Cohen P (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature 296:613–620
Cronin MJ, MacLeod RM, Canonico PL (1985) Modification of basal and GRF-stimulated cAMP levels and growth hormone release by phospholipid metabolic enzyme inhibitors. Neuroendocrinology 40:332–338
Dahl G, Miller T, Paul D, Voellmy R, Werner R (1987) Expression of functional cell-cell channels from cloned rat liver gap junction complementary DNA. Science 236:1290–1293
Dahl G, Werner R, Levine E (1988) Paired oocytes: An expression system for cell-cell channels. In: Hertzberg EL, Johnson RG (eds) Gap Junctions. New York: Alan R. Liss, pp 183–197
DeHaan RL, Williams EH, Ypey EL, Clapham DE (1981) Intercellular coupling of embryonic heart cells. In: Pexieder T (ed) Mechanism of Cardiac Morphogenesis and Teratogenesis. Raven, New York (Perspective in Cardiovascular Research, vol 5)
Dekel N, Beers WH (1980) Development of the rat oocyte in vitro: inhibition and induction of maturation in the presence or absence of the cumulus oophorus. Dev Biol 75:247–254
Dekel N, Lawrence TS, Gilula NB, Beers WH (1981) Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH. Dev Biol 86:356–362
DeMello WC (1975) Effects of intracellular injection of calcium and strontium on cell communication in heart. J Physiol 250:231–245
DeMello WC (1984) Effect of intracellular injection of cAMP on the electrical coupling of mammalian cardiac cells. Biochem Biophys Res Comm 119:1001–1007
DeMello WC (1987) Cell-to-cell coupling assayed by means of electrical measurements. Experientia 43:1075–1079
DeMello WC (ed) (1990) Cell intercommunication. CRC Press, Boca Raton
DeMello WC, Motta GE, Chapeau M (1969) A study on the healing-over of myocardial cells of toads. Circ Res 24:475–487
Dermietzel R, Leibstein A, Frixen U, Janssen-Timmen U, Traub O, Willecke K (1984) Gap junctions in several tissues share antigenic determinants with liver gap junctions. EMBO J 3:2261–2270
Dermietzel R, Völker M, Hwang T-K, Berzborn R, Meyer H (1989) A 16 kDa protein co-isolating with gap junctions from brain tissue belonging to the class of proteolipids of the vacuolar H^+-ATPases. FEBS Lett 253:1–5
Dixon JF, Hokin LE (1984) Secretagogue-stimulated phosphatidylinositol breakdown in the exocrine pancreas liberates arachidonic acid, stearic acid, and glycerol by sequential actions of phospholipase C and diglyceride lipase. J Biol Chem 14418–14425
Dunlap K, Holz GG, Rane SG (1987) G proteins as regulators of ion channel function. Trends Neurosci 10:241–244
Dupont E, El Aoumari A, Roustiau-Sévère S, Briand JP, Gros D (1988) Immunological characterization of rat cardiac gap junctions: presence of common antigenic determinants in heart of other vertebrate species and in various organs. J Membr Biol 104:119–128

Enomoto T, Sasaki Y, Shiba Y, Kanno Y, Yamasaki H (1981) Tumor promoters cause a rapid and reversible inhibition of the formation and maintenance of electrical cell coupling in culture. Proc Natl Acad Sci USA 78:5628–5632

Enomoto T, Martel N, Kanno Y, Yamasaki H (1984) Inhibition of cell communication between Balb/c 3T3 cells by tumor promoters and protection by cAMP. J Cell Physiol 121:323–333

Evans MG, Marty A (1986) Potentiation of muscarinic and α-adrenergic responses by an analogue of guanosine 5′-triphosphate. Proc Natl Acad Sci USA 83:4099–4103

Finbow ME, Shuttleworth J, Hamilton AE, Pitts JD (1983) Analysis of vertebrate gap junction protein. EMBO J 2:1479–1486

Fitzgerald DJ, Knowles SE, Ballard FJ, Murray AW (1983) Rapid and reversible inhibition of junctional communication by tumor promoters in a mouse cell line. Cancer Res 43:3614–3618

Flagg-Newton JL, Loewenstein WR (1980) Asymmetrically permeable membrane channels in cell junction. Science 207:771–773

Flagg-Newton JL, Loewenstein WRL (1981) Cell junction and cyclic AMP. II. modulations of junctional membrane permeability, dependent on serum and cell density. J Membr Biol 63:123–131

Flagg-Newton JL, Simpson I, Loewenstein WR (1979) Permeability of the cell-to-cell membrane channels in mammalian cell junction. Science 205:404–407

Flagg-Newton JL, Dahl G, Loewenstein WRL (1981) Cell junction and cyclic AMP: I. upregulation of junctional membrane permeability and junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. J Membr Biol 63:105–121

Fleming N, Sliwinski-Lis E, Burke DN (1989) G regulatory proteins and muscarinic receptor signal transduction in mucous acini or rat submandibular gland. Life Sci 44:1027–1035

Fox JA (1987) Ion channel subconductance states. J Membr Biol 53:1–8

Fraser SE, Bode HR (1981) Epithelial cells of Hydra are dye-coupled. Nature 294:356

Furshpan EJ, Potter DD (1959) Transmission at the giant motor synapses of the crayfish. J Physiol 145:289–325

Gainer HStC, Murray AW (1985) Diacylglycerol inhibits gap junctional communication in cultured epidermal cells: evidence for a role of protein kinase C. Biochem Biophys Res Comm 126:1109–1113

Gardner JD, Jensen RT (1981) Regulation of pancreatic exocrine secretion in vitro: the action of secretagogues. Philos Trans R Soc Lond 296:17–26

Gardner JD, Jensen RT (1988) Cell membrane receptors for secretagogues on pancreatic acinar cells. Adv Metabol Disord 11:113–141

Garfield RE, Sims S, Daniel EE (1977) Gap junctions: their presence and necessity in myometrium during parturition. Science 198:958–960

Giaume C, Randriamampita C, Trautmann A (1989) Arachidonic acid closes gap junction channels in rat lacrimal glands. Pflügers Arch 413:273–279

Gilman AG (1987) G Proteins: transducers of receptor-generated signals. Annu Rev Biochem 56:615–649

Gilula NB, Reeves RO, Steinbach A (1972) Metabolic coupling, ionic coupling and cell contacts. Nature 235:262–265

Gimlich RL, Kumar NM, Gilula NB (1988) Sequence and developmental expression of mRNA coding for a gap junction protein in Xenopus. J Cell Biol 107:1065–1073

Girsch SJ, Peracchia C (1985) Lens cell-to-cell channel protein. I. self-assembly into liposomes and permeability regulation by calmodulin. J Membr Biol 83:217–225

Gomberts BD (1983) Involvement of guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors. Nature 306:64–66

Goodenough DA (1974) Bulk isolation of mouse hepotocyte gap junctions. Characterization of the principal protein, connexin. J Cell Biol 61:557–563

Goodenough DA (1979) Lens gap junctions: a structural hypothesis for nonregulated low-resistance intercellular pathways. Invest Ophthalmol 18:1104–1122
Gorin MB, Yancey SB, Cline J, Revel JP, Horwitz J (1984) The major intrinsic protein (MIP) of the bovine lens fiber membrane: characterization and structure based on cDNA cloning. Cell 89:49–59
Green CR, Harfst E, Gourdie RG, Severs NJ (1988) Analysis of the rat liver gap junction protein: clarification of anomalies in its molecular size. Proc R Soc Lond [Biol] 233:165–174
Gruijters WTM (1989) A non-connexon protein (MIP) is involved in eye lens gap-junction formation. J Cell Science 93:509–513
Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
Guthrie SC, Gilula NB (1989) Gap junctional communication and development. Trends Neurosci 12:12–16
Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp technique for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100
Harden TK, Evans T, Hepler JR, Hughes AR, Martin MW, Meeker RB, Smith MM, Tanner LI (1985) Regulation of cyclic AMP metabolism by muscarinic cholinergic receptors. Adv Cyclic Nucleotide Protein Phosphorylation Res 19:207–220
Haslam RJ, Davidson MML (1984) Receptor-induced diacylglycerol formation in permeabilized platelets: possible role for a GTP-binding protein. J Receptor Res 4:605–629
Hausmann W, Caig LC (1954) Polymyxin B1. Fractionation, molecular weight determination, amino acid and fatty acid composition. J Am Chem Soc 76:4892–4896
Hax WMA, van Venrooji GEPM, Vossenberg JBJ (1974) Cell communication: a cyclic-AMP mediated phenomenon. J Membr Biol 19:253–266
Henderson D, Eibl H, Weber K (1979) Structure and biochemistry of mouse hepatic gap junctions. J Mol Biol 132:193–218
Hertzberg EL, Johnson RG (eds) (1987) Gap Junctions. Liss, New York (Modern cell biology, vol 7)
Hertzberg EL, Skibbens RV (1984) A protein homologous to the 27 000 dalton liver gap junction protein is present in a wide variety of species and tissues. Cell 39:61–69
Hertzberg EL, Disher RM, Tiller AA, Zhou Y, Cook RG (1988) Topology of the M_r 27 000 liver gap junction protein. J Biol Mem 263:19105–19111
Heynkes R, Kozjek G, Traub O, Willecke K (1986) Identification of a rat liver cDNA and mRNA coding for the 28 kD gap junction protein. FEBS Lett 205:56–60
Hunter T, Cooper J (1985) Protein tyrosine kinases. Annu Rev Biochem 54:897–930
Hunter T, Sefton BM (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci USA 77:1311–1315
Ishizuka T, Ito Y, Kajita K, Miura K, Nagao S, Nagata K, Nozawa Y (1987) Redistribution of protein kinase C in pancreatic acinar cells stimulated with caerulein or carbachol. Biochem Biophys Res Comm 144:551–559
Iwatsuki N, Petersen OH (1977) Acetylcholine-like effects of intracellular calcium application in pancreatic acinar cells. Nature 268:147–149
Iwatsuki N, Petersen OH (1978a) Electrical coupling and uncoupling of exocrine acinar cells. J Cell Biol 79:533–545
Iwatsuki N, Petersen OH (1978b) Pancreatic acinar cells: acetylcholine-evoked electrical uncoupling and its ionic dependency. J Physiol 274:81–96
Iwatsuki N, Petersen OH (1979) Pancreatic acinar cells: the effect of carbon dioxide, ammonium chloride and acetylcholine on intercellular communication. J Physiol 291:317–326
Jensen RT, Wank SA, Rowley WH, Sato S, Gaardner JD (1989) Trends Physiol Sci 10:418–423

Johnson KR, Lampe PD, Hur KC, Louis CF, Johnson RG (1986) A lens intercellular junction protein, MP26, is a phosphoprotein. J Cell Biol 102:1334–1343
Kanno Y (1985) Modulation of cell communication and carcinogenesis. Jpn J Physiol 35:693–707
Kanno Y, Enomoto T, Shiba Y, Yamasaki H (1984) Protective effect of cAMP on tumor promoter-mediated inhibition of cell-cell communication. Exp Cell Res 152:31–37
Kistler J, Kirkland B, Bullivant S (1985) Identification of a 70 000-D protein in lens membrane junctional domains. J Cell Biol 101:28–35
Kistler J, Christie D, Bullivant S (1988) Homologies between gap junction proteins in lens, heart and liver. Nature 331:721–723
Kohen E, Kohen C, Thorell B, Mintz DH, Rabinovitch A (1979) Intercellular communication in pancreatic islet monolayer cultures: a microfluorometric study. Science 204:862–865
Krebs EG, Beavo JA (1979) Phosphorylation-dephosphorylation of enzymes. Annu Rev Biochem 48:923–959
Kumar NK, Gilula NB (1986) Cloning and characterization of human and rat liver cDNAs coding for a gap junction protein. J Cell Biol 103:767–776
Lambert M, Svoboda M, Furnelle J, Christophe J (1985) Solubilization form rat pancreatic plasma membranes of a cholecystokinin (CCK) agonist-receptor complex interacting with guanine nucleotide regulatory proteins coexisting in the same macromolecular system. Eur J Biochem 147:611–617
Lampe PD, Johnson RG (1989) Phosphorylation of MP26, a lens junction protein, is enhanced by activators of protein kinase C. J Membr Biol 107:145–155
Lampe PD, Bazzi D, Nelsestuen GL, Johnson RG (1986) Phosphorylation of lens intrinsic membrane proteins by protein kinase C. Eur J Biochem 156:351–357
Lasater EM (1987) Retinal horizontal cell gap junctional conductance is modulated by dopamine through a cyclic AMP-dependent protein kinase. Proc Natl Acad Sci USA 84:7319–7323
Latorre R (1986) Ionic channels in cells and model systems. Plenum, New York
Läuger P (1985) Ionic channels with conformationalsubstates. Biophys J 47:581–591
Lawrence TS, Beers WH, Gilula NB (1978) Transmission of hormonal stimulation by cell-to-cell communication. Nature 272:501–506
Litosch I, Wallis C, Fain JN (1985) 5-hydroxytryptamine stimulates inositol phosphate production in a cell-free system from blowfly salivary glands. Evidence for a role of GTP in coupling receptor activation to phosphoinositide breakdown. J Biol Chem 260:5464–5471
Loewenstein WR (1968) Some reflections on growth and differentiation. Perspect Biol Med 11:260–272
Loewenstein WR (1975) Permeable junctions. Cold Spring Harbor Symp Quant Biol 40:49–63
Loewenstein WR (1979) Junctional intercellular communication and the control of growth. Biochim Biophys Acta 560:1–65
Loewenstein WR (1981) Junctional intercellular communication: the cell to cell membrane channel. Physiol Rev 61:829–913
Loewenstein WR (1987) The cell-to-cell channel of gap junctions. Cell 48:725–726
Loewenstein WR, Kanno Y, Socolar SJ (1978) Quantum jumps of conductance during formation of membrane channels at cell-cell junction. Nature 274:133–136
Machado-de Domenech E, Söling H-D (1987) Effects of stimulation of muscarinic and of β-catecholamine receptors on the intracellular distribution of protein kinase C in guinea pig exocrine glands. Biochem J 242:749–754
Makowski L, Caspar DLD, Phillips WC, Goodenough DA (1977) Gap junction structures. II. Analysis of the X-ray diffraction data. J Cell Biol 74:629–645
Makowski L, Caspar DLD, Goodenough DA, Phillips WC (1982) Gap junction structures. III. The effects of variations in the isolation procedure. Biophys J 37:189–191

Maldonado PE, Rose B, Loewenstein WR (1988) Growth factors modulate junctional cell-to-cell communication. J Membr Biol 106:203–210
Manjunath CK, Page E (1986) Rat heart gap junctions as disulfide-bonded connexon multimers: their depolymerization and solubilization in deoxycholate. J Membr Biol 90:43–57
Manjunath CK, Goings GE, Page E (1984) Cytoplasmic surface and intramembrane components of rat heart gap junctional proteins. Am J Physiol 246:H865–H875
Manjunath CK, Goings GE, Page E (1987) Human cardiac gap junctions: isolation, ultrastructure, and protein composition. J Mol Cell Cardiol 19:131–134
Maurer P, Weingart R (1987) Cell pairs isolated form adult guinea pig and rat hearts: effects of $[Ca^{2+}]_i$ on nexal membrane resistance. Pflugers Arch 409:394–402
Mazzei GJ, Katoh N, Kuo JF (1982) Polymyxin B is a more selective inhibitor for phospholipid-sensitive Ca^{2+}-dependent protein kinase than for calmodulin-sensitive Ca^{2+} dependent protein kinase. Biochem Biophys Res Comm 109:1129–1133
Meda P, Bruzzone R, Knodel S, Orci L (1986) Blockage of cell-to-cell communication within pancreatic acini is associated with increased basal release of amylase. J Cell Biol 103:475–483
Meda P, Bruzzone R, Chanson M, Bosco D, Orci L (1987) Gap junctional coupling modulates secretion of exocrine pancreas. Proc Natl Acad Sci USA 84:4901–4904
Meissner HP (1976) Electrophysiological evidence for coupling between β cells of pancreatic islets. Nature 262:502–504
Merritt JE, Rubin RP (1985) Pancreatic amylase secretion and cytoplasmic free calcium. Biochem J 230:151–159
Merritt JE, Taylor CW, Rubin RP, Putney JW Jr (1986) Evidence suggesting that a novel guanine nucleotide regulatory protein couples receptors to phospholipase C in exocrine pancreas. Biochem J 236:337–343
Michaels RL, Sheridan JD (1981) Islets of Langerhans: dye coupling among immunocytochemically distinct cell types. Science 214:801–803
Michell RH (1975) Inositol phospholipids and cell surface receptor function. Biochim Biophys Acta 415:81–147
Milks LC, Kumar NM, Houghten R, Unwin N, Gilula NB (1988) Topology of the 32-kD liver gap junction protein determined by site-directed antibody localizations. EMBO J 7:2967–2975
Miller C (1986) Ion channel reconstitution. Plenum, New York
Miller T, Dahl G, Werner R (1988) Structure of a gap junction gene: rat connexin-32. Biosci Rep 8:455–464
Murray AW, Fitzgerald DJ (1979) Tumor promoters inhibit metabolic cooperation in cocultures of epidermal and 3T3 cells. Biochem Biophys Res Comm 91:395–401
Murray SA, Fletcher WH (1984) Hormone-induced intercellular signal transfer dissociates cyclic AMP-dependent protein kinase. J Cell Biol 98:1710–1719
Murray SA, Taylor F (1988) Dibutyryl cyclic AMP modulation of gap junctions in SW-13 human adrenal cortical tumor cells. Am J Anat 181:141–148
Neyton J, Trautmann A (1985) Single-channel currents of an intercellular junction. Nature 317:331–335
Neyton J, Trautmann A (1986a) Acetylcholine modulation of the conductance of intercellular junctions between rat lacrimal cells. J Physiol 377:283–295
Neyton J, Trautmann A (1986b) Physiological modulation of gap junction permeability. J Exp Biol 124:93–114
Ngezahayo A, Kolb HA (1990) Gap junctional permeability is affected by cell volume changes and modulates volume regulation. FEBS Letters 276:6–8
Nicholson BJ, Takemoto LJ, Hunkapiller MW, Hood LE, Revel JE (1983) Differences between liver gap junction protein and lens MIP 26 from rat: implications for tissue specificity of gap junctions. Cell 32:967–978

Nicholson BJ, Dermietzel R, Teplow D, Traub O, Willecke K, Revel JP (1987) Two homologous protein components of hepatic gap junctions. Nature 329:732–734

Nishizuka Y (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308:693–698

Noguchi M, Adachi H, Gardner JD, Jensen RT (1985) Calcium-activated, phospholipid-dependent protein kinase in pancreatic acinar cells. Am J Physiol 248:G692–G701

Noma A, Tsuboi N (1987) Dependence of junctional conductance on proton, calcium and magnesium ions in cardiac paired cells of guinea pig. J Physiol 382:193–211

Obaid AL, Socolar SJ, Rose B (1983) Cell-to-cell channels with two independently regulated gates in series: analysis of junctional conductance modulation by membrane potential, calcium, and pH. J Membr Biol 73:69–89

Orci L, Baetens D, Rufener C, Amherdt M, Ravazzola M, Studer P, Malaisse-Lagae F, Unger RH (1976) Hypertrophy and hyperplasia of somatostatin-containing D-cells in diabetes. Proc Natl Acad Sci USA 73:1338–1342

Page E, Shibata Y (1981) Permeable junctions between cardiac cells. Annu Rev Physiol 43:431–441

Parker J, Daniel LW, Waite M (1987) Evidence of protein kinase C involvement in phorbol diester-stimulated arachidonic acid release and prostaglandin synthesis. J Biol Chem 262:5385–5393

Paul DL (1986) Molecular cloning of cDNA for rat liver gap junction protein. J Cell Biol 103:123–134

Peracchia C (1987) Calmodulin-like proteins and communicating junctions: electrical uncoupling of crayfish septate axons is inhibited by the calmodulin inhibitor W7 and is not affected by cyclic nucleotides. Pflugers Arch 408:379–385

Peracchia C, Girsch SJ (1985) Functional modulation of cell coupling: evidence for a calmodulin-driven channel gate. Am J Physiol 248:H765–H782

Peracchia C, Bernardini G, Peracchia L (1983) Is calmodulin involved in the regulation of gap junction permeability? Pflugers Arch 399:152–154

Petersen OH, Ueda N (1976) Pancreatic acinar cells: the role of calcium in stimulus-secretion coupling. J Physiol 254:583–606

Piccolino M, Neyton J, Gerschenfeld HM (1984) Decrease of gap junction permeability induced by dopamine and cyclic adenosine 3′:5′-monophosphate in horizontal cells of turtle retina. J Neurosci 4:2477–2488

Pitts JD, Simms JW (1977) Permeability of junctions between animal cells. Intercellular transfer of nucleotides but not of macromolecules. Exp Cell Res 104:153–163

Potter DD, Furshpan EJ, Lennox ES (1966) Connections between cells of the developing squid as revealed by electrophysiological methods. Proc Natl Acad Sci USA 55:328–336

Pressler ML (1987) Effects of pCa_i and pH_i on cell-to-cell coupling. Experientia 1084–1090

Puri CP, Garfield RE (1982) Changes in hormone levels and gap junctions in the rat uterus during pregnancy and parturition. Biol Reprod 27:967–975

Putney JW Jr, Aub DL, Taylor CW, Merritt JE (1986) Formation and biological action of inositol 1,4,5-trisphosphate. Fed Proc 45:2634–2638

Radu A, Dahl G, Loewenstein WR (1982) Hormonal regulation of cell junction permeability: upregulation by catecholamine and prostaglandin El. J Membr Biol 70:239–251

Ramachandran J, Ullrich A (1987) Hormonal regulation of protein tyrosine kinase activity. Trends Pharmacol Sci 8:28–31

Ramon F, Rivera A (1986) Gap junction channel modulation – a physiological viewpoint. Prog Biophys Mol Biol 48:127–153

Randriamampita C, Giaume C, Neyton J, Trautmann A (1988a) Acetylcholine-induced closure of gap junction channels in rat lacrimal glands is probably mediated by protein kinase C. Pflugers Arch 412:462–468

Randriamampita C, Chanson M, Trautmann A (1988b) Calcium and secretagogue-induced conductances in rat exocrine pancreas. Pflugers Arch 411:53–57
Raskin P, Unger RH (1978) Effect of insulin therapy on the profiles of plasma immunoreactive glucagon in juvenile-type and adult-type diabetics. Diabetes 27:411–419
Revel JP, Karnovsky MJ (1967) Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. J Cell Biol 33:C7–C12
Revel JP, Nicholson BJ, Yancey SB (1985) Chemistry of gap junctions. Annu Rev Physiol 47:263–279
Revel JP, Yancey SB, Nicholson BJ (1986) The gap junction proteins. Trends Biochem Sci 11:375–377
Robards AW, Lucas WJ, Pitts JD, Jongsma HJ, Spray DC (eds) (1990) Parallels in cell-to-cell junctions in plants and animals. NATO ASI Vol H46, Springer, Berlin
Rook MB, Jongsma HJ, van Ginneken ACG (1988) Properties of single gap junctional channels between isolated neonatal rat heart cells. Am J Physiol 255:H770–H782
Rook MB, Jongsma HJ, de Jonge B (1989) Single channel currents of homo- and heterologous gap junctions between cardiac fibroblasts and myocytes. Pflugers Arch 414:95–98
Rose B, Loewenstein WR (1975) Permeability of cell junctions depends on local cytoplasmic calcium activity. Nature 254:250–252
Rose B, Loewenstein WR (1976) Permeability of cell junctions and the local cytoplasmic free ionized calcium concentration: a study with aequorin. J Membr Biol 28:87–119
Rose B, Simpson I, Loewenstein WR (1977) Calcium ion produces graded changes in permeability of membrane channels in cell junction. Science 267:625–627
Saez JC, Spray DC, Nairn AC, Hertzberg E, Greengard P, Bennett MVL (1986) cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principal gap junction polypeptide. Proc Natl Acad Sci USA 83:2473–2477
Saez JC, Connor JA, Spray DC, Bennett MVL (1989) Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-triphosphate, and to calcium ions. Proc Natl Acad Sci USA 86:2708–2712
Sato S, Stark HA, Martinez J, Beaven MA, Jensen RT, Gardner JD (1989) Receptor occupation, calcium mobilization, and amylase release in pancreatic acini: effect of CCK-JMV-80. Am J Physiol 257:G202–G209
Schnefel S, Banfic H, Eckhardt L, Schultz G, Schulz I (1988) Acetylcholine and cholecystokinin receptors functionally couple by different G-proteins to phospholipase C in pancreatic acinar cells. FEBS Lett 230:125–130
Schwarze W, Kolb HA (1984) Voltage-dependent kinetics of an anionic channel of large unit conductance in macrophages and myotube membranes. Pflugers Arch 402:281–291
Schwarzmann G, Weigandt H, Rose B, Zimmermann A, Ben-Haim D, Loewenstein WR (1981) Diameter of the cell-to-cell junctional membrane channels as probed with neutral molecules. Science 213:551–553
Sekar MC, Hokin LE (1987) Inhibitors of diacylglycerol lipase and diacylglycerol kinase inhibit carbamylcholine-stimulated responses in guinea pig pancreatic minilobules. Arch Biochem Biophys 256:509–514
Sheridan JD, Atkinson MM (1985) Physiological roles of permeable junctions: some possibilities. Annu Rev Physiol 47:337–353
Simpson I, Rose B, Loewenstein WR (1977) Size limit of molecules permeating the junctional membrane channels. Science 195:294–296
Sims SM, Daniel EE, Garfield RE (1982) Improved electrical coupling in uterine smooth muscle is associated with increased numbers of gap junctions at parturition. J Gen Physiol 80:353–375
Socolar SJ (1977) The coupling coefficient as an index of junctional conductance. J Membr Biol 34:29–37
Somogyi R, Kolb HA (1988a) Cell-to-cell channel conductance during loss of gap junctional coupling in pairs of pancreatic acinar and Chinese hamster ovary cells. Pflügers Arch 412:54–65

Somogyi R, Kolb HA (1988b) Modulation of gap junctional coupling in pairs of pancreatic acinar cells by cAMP, OAG and protein kinase C. Ber Bunsenges Phys Chem 92:993–998

Somogyi R, Kolb HA (1989a) Possible involvement of a G-protein in carbamylcholine induced gap junction closure. J Prot Chem 8:434–436

Somogyi R, Kolb HA (1989b) A G-protein mediates secretagogue-induced gap junctional channel closure in pancreatic acinar cells. FEBS Letters 258:216–218

Somogyi R, Batzer A, Kolb HA (1989) Inhibition of electrical coupling in pairs of murine pancreatic acinar cells by OAG and isolated protein kinase C. J Membr Biol 108:273–282

Spray DC, Bennett MVL (1985) Physiology and pharmacology of gap junctions. Annu Rev Physiol 47:281–303

Spray DC, Harris AL, Bennett MVL (1979) Voltage dependence of junctional conductance in early amphibian embryos. Science 204:432–434

Spray DC, Harris AL, Bennett MVL (1981a) Equilibrium properties of a voltage dependent junctional conductance. J Gen Physiol 77:75–94

Spray DC, Harris AL, Bennett MVL (1981b) Gap junctional conductance is a simple and sensitive function of intracellular pH. Science 211:712–715

Spray DC, White RL, Campos de Carvalho A, Harris AL, Bennett MVL (1984) Gating of gap junction channels. Biophys J 45:219–230

Spray DC, White RL, Mazet F, Bennett MVL (1985) Regulation of gap junctional conductance. Am J Physiol 17:H753–H764

Spray DC, Saez JC, Brosius D, Bennett MVL, Hertzberg EL (1986a) Isolated liver gap junctions: gating of transjunctional currents is similar to that in intact pairs of rat hepatocytes. Proc Natl Acad Sci USA 83:5494–5497

Spray DC, Ginzberg RD, Morales EA, Gatmaitan Z, Arias IM (1986b) Electrophysiological properties of gap junctions between dissociated pairs of rat hepatocytes. J Cell Biol 103:135–144

Sung CK, Hootman SR, Stuenkel EL, Kuroiwa C, Williams JA (1988) Downregulation of protein kinase C in guinea pig pancreatic acini: effects of secretion. Am J Physiol 254:G242–248

Swenson KI, Jordan JR, Beyer EC, Paul DL (1989) Formation of gap junctions by expression of connexins in Xenopus oocyte pairs. Cell 57:145–155

Takeda A, Hashimoto E, Yamamura H, Shimazu T (1987) Phosphorylation of liver gap junction protein by protein kinase C. FEBS Lett 210:169–172

Takeda A, Saheki S, Shimazu T, Takeuchi N (1989) Phosphorylation of the 27-kDa gap junction protein by protein kinase C in vitro and in rat hepatocytes. J Biochem 106:723–727

Taylor CW (1986) Growth factors control a network of interacting messengers. Trends Pharmacol Sci 7:467–471

Teranishi T, Negishi K, Kato S (1983) Dopamine modulates S-potential amplitude and dye-coupling between external horizontal cells in carp retina. Nature 301:243–246

Traub O, Janssen-Timmen U, Drüge PM, Dermietzel R, Willecke K (1982) Immunological properties of gap junction protein from mouse liver. J Cell Biochem 19:27–44

Traub O, Look J, Paul D, Willecke K (1987) Cyclic adenosine monophosphate stimulates biosynthesis and phosphorylation of the 26 kDa gap junction protein in cultured mouse hepatocytes. Eur J Cell Biol 43:48–53

Traub O, Look J, Dermietzel R, Brümmer F, Hülser D, Willecke K (1989) Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. J Cell Biol 108:1039–1051

Trautmann A, Randriamampita C, Giaume C (1990) Gap junctions in exocrine glands. In: Young JA, Wong PY (eds) Epithelial secretion of water and electrolytes. Springer, Berlin Heidelberg New York

Turin L, Warner AE (1978) Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo. Nature 270:56–57
Turin L, Warner AE (1980) Intracellular pH in early Xenopus embryos: its effects on current flow between blastomeres. J Physiol 300:489–504
Uhing RJ, Pripc V, Jiang H, Exton JH (1986) Hormone-stimulated polyphosphoinositide breakdown in liver plasma membranes. Roles of guanine nucleotides and calcium. J Biol Chem 261:2140–2146
Unwin PNT, Ennis PD (1984) Two configurations of a channel-forming membrane protein. Nature 307:609–613
Unwin PNT, Zampighi G (1980) Structure of the junction between communicating cells. Nature 283:545–549
Veenstra RD, DeHaan RL (1986) Measurement of single channel currents from cardiac gap junctions. Science 233:972–974
Veenstra RD, DeHaan RL (1988) Cardiac gap junction channel activity in embryonic chick ventricle cells. Am J Physiol 254:H170–H180
in't Veld P (1985) Gap junctions between pancreatic B-cells are modulated by cyclic AMP. Eur J Cell Biol 36:269–276
Verselis V, White RL, Spray DC, Bennett MVL (1986) Gap junctional conductance and permeability are linearly related. Science 234:461–464
Volwerk IJ, Pieterson WA, DeHaas GH (1974) Histidine at the active site of phospholipase A_2. Biochemistry 13:1446–1454
Wallace MA, Fain JN (1985) Guanosine 5′-O-thiotriphosphate stimulates phospholipase C activity in plasma membranes of rat hepatocytes. J Biol Chem 260:9527–9530
Warner AE, Guthrie SC, Gilula NB (1984) Antibodies to gap junctional protein selectively disrupt communication in the early amphibian embryo. Nature 311:127–131
Weingart R, Maurer P (1988) Action potential transfer in cell pairs isolated from adult rat and guinea pig ventricles. Circ Res 63:72–80
Wiener EC, Loewenstein WR (1983) Correction of cell-cell communication defect by introduction of a protein kinase into mutant cells. Nature 305:433–435
Yada T, Rose B, Loewenstein WR (1985) Diacylglycerol downregulates junctional membrane permeability. TMB-8 blocks this effect. J Membr Biol 88:217–232
Yamamashi J, Takai Y, Kaibuchi K, Sano K, Castagna M, Nishizuka Y (1983) Synergistic functions of phorbol ester and calcium in serotonin release from human platelets. Biochem Biophys Res Comm 112:778–786
Yancey SB, Edens JE, Trosko JE, Chang C-C, Revel JP (1982) Decreased incidence of gap junctions between Chinese hamster V-79 cells upon exposure to the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate. Exp Cell Res 139:329–340
Yotti LP, Chang CC, Trosko JE (1979) Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. Science 206:1089–1091
Young JD-E, Cohn ZA, Gilula NB (1987) Functional assembly of gap junction conductance in lipid bilayers: demonstration that the major 27 kD protein forms the junctional channel. Cell 48:733–743
Zampighi GA, Hall JE, Kreman M (1985) Purified lens junctional protein forms channels in planar lipid films. Proc Natl Acad Sci USA 82:8468–8472
Zimmer DB, Green CR, Evans WH, Gilula NB (1987) Topological analysis of the major protein in isolated intact rat liver gap junctions and gap junction-derived single membrane structures. J Biol Chem 262:7751–7763
Zimmermann AL, Rose B (1985) Permeability properties of cell-to-cell channels: kinetics of fluorescent tracer diffusion through a cell junction. J Membr Biol 84:269–283

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Biochemical Events Controlling Initiation and Propagation of the S Phase of the Cell Cycle

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Contents

1 Introduction

Mechanisms which control growth, cell division and the cell cycle have been of primary interest to biologists for many years largely because of their connection with development and oncogenesis. The almost 40-year-old concept of a cell cycle (Howard and Pelc 1953) consisting of four phases, S (for DNA synthesis), M (for mitosis) and G_1 and G_2 (for the two

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gaps between M and S and S and M, respectively) has provided a framework for experimental studies on the cell cycle. To these four phases most biologists add a fifth category, namely G_0, the stage of resting, quiescent cells (Baserga 1985; Pardee 1989). There is much evidence indicating that cells in G_0 differ significantly from those in any one of the four phases of the cell cycle, including G_1, and recent extensive studies have provided evidence for dramatic differences between cells moving from G_0 into the cycle (quiescent cells stimulated to growth) and those cells which pass through the various phases of the cycle during continued cell division. This difference is often acknowledged by distinguishing between proliferation control (transition from quiescence into the cycle) and cell cycle regulation (events occurring in dividing cells moving through the four phases of the cycle).

The former process includes regulatory events which are not manifest in the latter one. The main difference obviously is the absence of growth factors or the absence of a response to growth factors in quiescent cells, both of which have to be present for cells to proliferate continuously. Current knowledge reveals the presence of three major control points in the division cycle of eukaryotic cells (Fig. 1): the passage of quiescent cells into the cell cycle (transition G_0 to G_1), the initiation of the S phase (transition G_1 to S), and the initiation of mitosis (transition G_2 to M). Each one of these events appears to be regulated by a network of regulatory factors, the elucidation of which is at the center of research on the cell cycle.

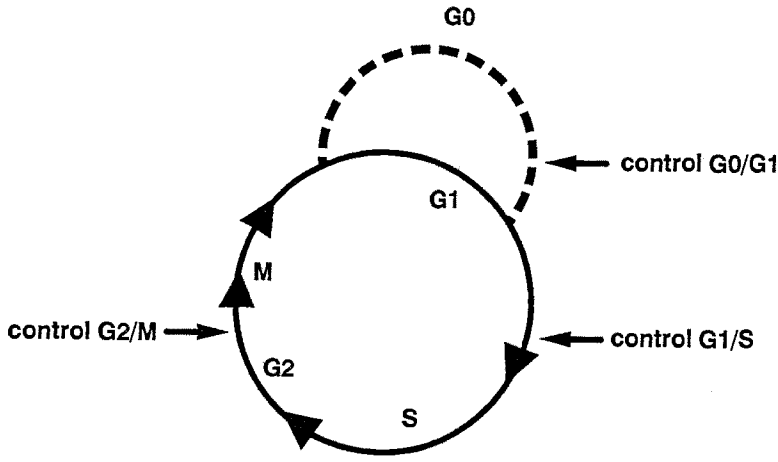

Fig. 1. Schematic drawing of the cell cycle, its four phases and three important control points. The points of exit into G_0 and of re-entry into G_1 were chosen arbitrarily. In particular, it is not known whether there are events taking place in G_1 but not in G_0

Several recent reviews have covered aspects of cell cycle and proliferation control (Pardee 1989; Laskey et al. 1989; Cross et al. 1989; Norbury and Nurse 1989; Murray and Kirschner 1989). Two areas of research have seen major advances in recent years and have received much attention in the literature. These are:

1. The events following the addition of growth factors to quiescent cells grown in culture, in particular the role of so called "immediate early" gene products (expressed immediately following the growth stimulus) among which there are important transcription regulators such as *Fos* and *Jun* (Pardee 1989; Johnson and McKnight 1989; Wintersberger 1990).
2. The regulation of the initiation of mitosis and the role of a protein complex called maturation promoting factor (Lee and Nurse 1988; Norbury and Nurse 1989; Blow 1989a; Murray and Kirschner 1989; Nurse 1990).

This review will focus on an area which was less frequently summarized: regulatory events in G_1 leading to the initiation of S phase; and the ordered propagation and the completion of S phase as a prerequisite for continuation of the cell cycle into G_2 and M.

2 The G_1 Phase

Howard and Pelc (1953) defined the first gap, G_1, as the period between mitosis and S phase, both of which are the two "visible" time periods of the cell cycle. Mitosis is characterized by the appearance in the microscope of mitotic chromosomes, S phase by the ability of cells to incorporate radioactive precursors into DNA, which can be visualized by autoradiography. G_1 is the most variable phase of the cell cycle in terms of duration; it varies not only between cell types (this may also be true for other cell cycle phases, in particular for the S phase), but within individual cells of the same type in a population. G_1 can last for many hours (some authors define G_0 as an indefinitely long G_1 phase) or may be nearly or totally absent, as in early development or in a few cell lines grown in culture (Liskay and Prescott 1978; but see Brooks et al. 1983). It is precisely this fact which created some controversy among scientists as to the role and importance of the G_1 period (see, for instance, Okuda and Cooper 1989). While many investigators define G_1 as the period of preparation for the S phase in which a series of specific events takes place culminating in the onset of DNA synthesis, others cannot accept this view, because

obviously in some cases these events can also take place in other phases of the cell cycle, eliminating the need for a G_1 phase. Several facts seem to be clear:

1. Arresting cells most often accumulate in a long or endless G_1 phase which is biochemically distinguisable from the G_1 phase of dividing cells and therefore is conveniently defined as G_0. This arrest is physiological, in that cells can remain viable in this state for long periods, which is not true for cells blocked in the S or M phase by inhibitors. There may be another physiological arrest point in the cell cycle, late in G_2.

2. Because the lengths of the S, G_2 and M phases vary only slightly for a given cell type under given growth conditions, it is the variable length of G_1 which causes the difference in the cycling time of individual cells in a population and, consequently, asynchrony of growth. This difference is brought about by differences in cytoplasmic components because nuclei in the same cytoplasm, such as those in the plasmodium stage of slime molds or in fertilized eggs, divide synchronously (Cross et al. 1989).

3. It is often assumed that initiation of DNA synthesis requires a threshold level of (a) protein(s) which accumulates gradually during G_1 Coppock and Pardee 1985; see also Pardee 1989). The observation of very short or sometimes even absent G_1 phases must mean that this protein can also be produced in other phases of the cell cycle. In accordance with this view is the finding that cells characterized by the absence of G_1 can adopt a G_1 phase when protein synthesis is slowed down by the addition of inhibitors like cycloheximide (Liskay et al. 1980), and notwithstanding the fact that cells may exhibit a very short G_1 phase, there is compelling evidence for the existence of a non-compressible part of G_1. Experiments with yeast suggest that this part is at the end of the G_1 phase shortly before the transition into the S phase (Johnston and Singer 1983; Singer and Johnston 1983).

In any case, the importance of a G_1 phase as a cell cycle phase in which specific events have to take place is rated differently by different investigators, resulting in quite contrasting models for the regulation of the cell cycle (compare, for instance, Pardee 1989 with Okuda and Cooper 1989). However, it is quite clear that this question becomes less pressing when considering the time of transition of G_1 to S phase. Regardless of when during the cycle the requirements for this transition are met, they must be fulfilled if cells are to start DNA synthesis. It is, therefore, appropriate to consider the transition from G_1 into S phase as a decisive time period in the cell cycle and as a good starting point for unravelling biochemical events leading to the S phase.

3 Initiation of the S Phase

Cell fusion studies have yielded important information with regard to events in G_1 required for the initiation of DNA synthesis (for review see Zelenin and Prudovsky 1989). Fusion of G_1 cells with S phase cells, for instance, showed that both the S phase and the G_1 nuclei carry out DNA synthesis (Rao and Johnson 1970). In other words, the cytoplasm of the S phase cell can provide (a) factor(s) *in trans* which allows the G_1 nucleus to start an S phase. The nucleus in G_1 therefore must be competent to start DNA synthesis provided some *trans*-acting inducer(s) is (are) present. This led to the idea that initiation of DNA synthesis is positively controlled and that a limiting, diffusible inducer is accumulating during G_1. The S phase may then start when the inducer reaches a certain threshold level.

This need not mean that the inducer is directly involved in DNA synthesis; rather, it may somehow activate proteins necessary for replication. Interesting in this connection is the observation that simian virus 40 (SV40) or polyomavirus small tumor antigens (T-antigens) stimulate replication of viral DNA indirectly (Berger and Wintersberger 1986). Small T-antigens form complexes with two cellular proteins of 36 and 63 kDa, and recent experiments showed that the smaller of these proteins is the catalytic subunit of protein phosphatase 2A while the larger protein is the regulatory subunit of this enzyme (Pallas et al. 1990). Other experiments proved that one of the cellular proteins stimulating SV40 replication in vitro (Virshup and Kelly 1989) is identical with protein phosphatase 2A (Virshup et al. 1989). Hence, it is possible that most proteins required for replication are present in non-limiting amounts in G_1 phase cells, but that their phosphorylation status limits their activity and changes during G_1/S transition. One substrate for protein phosphatase 2A was very recently found to be SV40 large T-antigen (Lawson et al. 1990), a virally coded protein involved in the initiation of viral DNA replication. This observation may, in fact, explain the stimulatory effect of small T-antigen on viral DNA synthesis mentioned above. Another candidate for control by phosphorylation/dephosphorylation is a protein able to activate the SV40 T-antigen-induced unwinding of DNA during the initiation of SV40 DNA replication in vitro (Roberts and d'Urso 1988). This factor increases in activity in cells at the G_1/S border of the cell cycle.

That changes in the phosphorylation status of proteins play an important role in cell cycle controls is evident from experiments on the function of maturation promoting factor (MPF) in the induction of the M phase (for summaries of an extensive literature on this subject see Norbury and Nurse 1989; Murray and Kirschner 1989; Nurse 1990). MPF is a complex of a protein (p34) of 34 kDa (which in the yeast *Schizosac-*

charomyces pombe is the product of the cell division cycle gene *cdc2* with a protein of the family of cyclins). This complex has protein kinase activity at G_2/M transition, one substrate being histone H1. $p34^{cdc2}$ protein kinase isolated from HeLa cells was found to phosphorylate the large T-antigen from SV40, thereby stimulating DNA replication in vitro (McVey et al. 1989). This function correlates with the initiation of the S phase rather than with that of mitosis. In fact, studies on yeast have shown that p34 in addition to its function in mitosis initiation plays a role during G_1/S transition. A suggestion emanating from recent experiments with *Saccharomyces cerevisiae* is that p34 interacts with a member of a protein family resembling cyclins (called G_1 cyclins) to function as protein kinase also during the initiation of the S phase (Richardson et al. 1989). The phosphorylation status of p34 itself changes throughout the cell cycle and is probably decisive for specific interactions of the protein with cyclins and the specificity of protein kinase activity resulting therefrom.

Pressing questions at this time are: Which proteins are the targets for protein kinases or protein phosphatases during G_1/S transition? Is the regulation of the cell cycle totally post translational or does control at the transcriptional or post transcriptional level play an additional role? What differences exist between the processes taking place in cycling cells moving from G_1 to S phase and those in cells stimulated to proliferate from a quiescent state? Whereas proteins controlling the initiation of DNA synthesis are largely unknown, several enzymes involved in DNA replication are well studied, and changes in their activity and expression during G_1/S transition can be examined in the hope that such studies might eventually lead to proteins more directly involved in the control of S phase initiation.

3.1 Enzyme Induction During G_1/S Transition

Several enzymes involved in the nucleotide metabolism and in DNA replication show low activity and expression in quiescent cells but increase in both parameters upon growth stimulation of cells. This is particularly true for dihydrofolate reductase (DHFR), thymidylate synthase (TS), thymidine kinase (TK), ribonucleotide reductase, DNA polymerases, DNA ligase, DNA topoisomerase I, ornithine decarboxylase, DNA and RNA helicases and DNA methylase. The genes (including the regulatory regions) and the cDNAs have been cloned for some of these enzymes, allowing detailed study of their regulation. The low abundance of the mRNAs for most of these enzymes renders such studies difficult, and many investigations were therefore carried out with systems in which the

gene for the enzyme (and consequently the mRNA and the protein) could be amplified by treatment of cells with stepwise increasing concentrations of drugs acting as enzyme inhibitors. Examples are the now widely used cell lines carrying the gene for DHFR in amplified form due to selection for resistance to methotrexate, and cell lines having amplified the gene for TS due to selection for resistance to fluorodesoxyuridine. The regulation of these two enzymes has been studied for a number of years and many interesting results have emerged. Such studies are now extended to enzymes and proteins whose genes resist amplification, TK, DNA polymerase α and proliferating cell nuclear antigen. This chapter mainly deals with the control of these genes; other results are mentioned only in passing. New observations on the regulation of ribonucleotide reductase, primarily of yeast, were summarized by Fernandez-Sarabia and Fantes (1990). Earlier data on this subject were reviewed by Denhardt et al. in 1986. Emphasis will therefore be placed on new findings obtained since then, and articles published up to mid 1990 are considered.

3.1.1 Dihydrofolate Reductase

Dihydrofolate reductase catalyzes the reduction of dihydrofolate to tetrahydrofolate. Dihydrofolate is the byproduct of reactions involved in the formation of thymidine phosphate and of purine nucleotides. The gene coding for DHFR is around 30 kb long, of which only 1.6 kb account for the six exons. It was cloned from a number of organisms, including mouse, hamster and man. This work was facilitated by the creation and the use of methotrexate-resistant cell lines carrying up to several hundred genes for DHFR. Most of the studies on DHFR expression were likewise done with such cell lines. The analysis of transcription products yielded the surprising result of extreme heterogeneity with regard to initiation sites at the 5′ end and termination sites at the 3′ end (Farnham and Schimke 1985, 1986a; Sazer and Schimke 1986; Yen and Kellems 1987). Apart from major initiation and termination (polyadenylation) sites, as many as five alternative cap sites and 11 alternative polyadenylation sites are used with low but varying frequency during expression of the mouse DHFR gene. 5′ and 3′ ends of the mRNAs are chosen independently from each other (Yen and Kellems 1987). Similar heterogeneity of transcripts is obtained by in vitro transcription of DHFR sequences (Farnham and Schimke 1986b). In these experiments it was also shown that three out of the four GC boxes in the promoter region (see below, p. 57) can be removed without interfering with the accuracy or efficiency of in vitro transcription. Even the dependence of transcription activity on the growth state of cells seems to be retained in vitro. Extracts from cells held in S

phase by inhibition of DNA synthesis with aphidocolin exhibited increased transcription activity (Farnham and Schimke 1986b) as did cells whose DNA synthesis was transiently inhibited in vivo (Johnston et al. 1986).

DHFR expression has been known for a long time to be growth-regulated. After Kaufmann and Sharp (1983) obtained results supporting a role of post-transcriptional events at the level of polyadenylation in this control, intensive studies were carried out to determine whether the different species of mRNA vary in growing compared to resting cells and in cells at different stages of the cell cycle as isolated by the mitotic shake-off method. In fact, no differences were found, which led to the conclusion that switches to or selection of mRNAs with particular cap sites or polyadenylation sites do not contribute to the control of DHFR expression (Farnham and Schimke 1986a; Yen and Kellems 1987). Rather, it appears from all these studies (including those on in vitro transcription mentioned above) that the major control over the expression of DHFR is exerted at the transcriptional level. In accordance with this assumption is the observation of Schmidt and Merrill (1989) that mouse DHFR gene transcription is repressed in differentiation-competent mouse muscle cells during muscle cell commitment. Since differentiation was induced by removing serum from the medium, these studies, unfortunately, do not allow distinction between differentiation-driven events and those accompanying the growth arrest of cells due to withdrawal of growth factors. Nevertheless, they are fully in line with the view that growth regulation of DHFR is achieved by activation or deactivation of the promoter.

An earlier conclusion, namely that there is a significant increase in expression of DHFR when cells move from the G_1 into the S phase, was drawn from cell cycle studies using cells synchronized by mitotic selection (Farnham and Schimke 1986a). A recent careful reexamination of the regulation of DHFR in the cell cycle of rodent and human cells using centrifugal elutriation led to a revision of this view (Feder et al. 1989). It was found that DHFR activity or mRNA levels do not change more than cell volume and general protein content. Hence, the DHFR gene and its product are not differentially expressed through a continuous cell cycle. DHFR, therefore, cannot be considered a cell cycle regulated enzyme. It still, of course, remains a growth-regulated enzyme, and this regulation according to present knowledge is primarily, if not exclusively, transcriptional. Additional post-transcriptional regulatory events cannot be completely excluded at this time, particularly as it was recently shown by Urlaub et al. (1989) that mutations in the DHFR gene of Chinese hamster ovary cells can influence splicing and cytoplasmic transport which by themselves seem to be coupled to translation.

The importance of transcriptional regulation of the DHFR gene has caused increased interest in the promoter of the gene. The 5′ flanking region of the DHFR gene, like that of some other "housekeeping" genes and unlike that of most genes coding for differentiation functions, lacks typical TATA and CAAT boxes but it does contain several GC boxes which are known binding sites for transcription factor SP1. The upstream region furthermore functions as a bidirectional promoter which commands transcription not only in the direction of the DHFR gene but also in the opposite orientation (Farnham et al. 1985; Crouse et al. 1985; Mitchell et al. 1986; Schilling and Farnham 1989). The divergently regulated gene appears to be involved in DNA mismatch repair (Linton et al. 1989; Fujii and Shimada 1989). The promoter regions active in opposite directions partially overlap.

More careful dissection of the DHFR promoter has been initiated recently (Means and Farnham 1990), and it was shown that apart from SP1 another transcription factor, called HIP1 (for housekeeping initiation protein 1) binds to the DHFR promoter and determines initiation sites for transcription. Recognition sequences for HIP1 are present in a number of other promoters (some are mentioned by Means and Farnham 1990) including the TK promoter (Seiser et al. 1989). HIP1 may play a role in determining initiation sites for transcription in promoters lacking a TATA box analogous to the TATA-binding protein TFIID in promoters possessing a TATA box. Using the in vitro transcription assay, Farnham and Means (1990) made the interesting observation that sequences downstream of the transcription initiation sites also influence the transcriptional activity of the DHFR promoter. This even includes sequences within exon 1 and intron 1 of the DHFR gene. Proteins binding to these regions were identified by DNase I footprinting analysis. It is not yet known whether any of the transcription signals are responsible for growth dependence of transcription.

3.1.2 Thymidylate Synthase

Thymidylate synthase is a key enzyme involved in the synthesis of thymidine monophosphate. It catalyzes the methylation of desoxyuridine monophosphate to thymidine monophosphate with the help of methylene tetrahydrofolic acid as methyl group donor. A by-product of this reaction is dihydrofolate, which has to be reduced to tetrahydrofolate before it can be used again. The regulation of the expression of TS was studied primarily with cell lines having amplified the gene about 50-fold due to selection for resistance to 5-fluorodesoxyuridine. TS mRNA increases 20- to 40-fold when cells progress from a resting state to the S phase. The

half-life of the mRNA does not change significantly during this transition; mRNA synthesis, on the other hand, was found to increase only by a factor of 3–4. Some post-transcriptional process must therefore play a role in the regulation of TS, and experiments suggest that polyadenylation is important in this regard (Jenh et al. 1985). This was reinforced in an independent study in which TS mRNA was found to rise severalfold when quiescent cells were stimulated to proliferate, whereas no change was observed in the rate of gene transcription (Ayusawa et al. 1986). TS mRNA is unusual in that it does not carry an untranslated region in front of the polyadenylation site. In order to be able to dissect the regulatory mechanisms, Deng et al. (1986) isolated the gene for TS from fluorodesoxyuridine-resistant 3T6 mouse fibroblasts harboring 50 copies of that gene. TS is encoded by 12 kb of DNA, and only 1.1 kb is represented by mRNA. There are six introns of varying length (from 0.6 to 4.1 kb). The regulatory region lacks TATA and CAAT sequences but contains binding sites for transcription factor SP1. Another sequence element named USF resembles a binding site within the major late promoter of adenovirus. Deletion analysis and site-directed mutagenesis revealed the importance of the SP1 binding site for high level activity of the promoter, whereas elimination of USF has little effect. Binding sites for transcription factors other than SP1 must exist, because the GC element, though it appears to function as a positive element, is not absolutely essential for transcription. All of the upstream elements that are essential for promoter activity must reside within a short DNA fragment of 22 bp upstream of the first transcription start site (Deng et al. 1989). Whether any of the transcription elements analyzed so far have to do with growth regulation and/or cell cycle regulation is not yet known.

Cell cycle studies have been carried out at the level of TS mRNA and TS protein. Using suspension cultures of the mouse lymphoblastoid cell line L1210, cells in different stages of the cell cycle were isolated by centrifugal elutriation and analyzed for enzyme amounts (as measured by binding of 5-fluorodesoxyuridylate). A positive correlation was found between the amount of TS protein and the number of cells in S phase; an approximately fourfold lower enzyme level was present in cells in G_1 (Cadman and Heimer 1986). From the same cell line, a variant resistant to the competitive inhibitor of TS, CB3717, and having the TS gene amplified about 50-fold was produced in order to study the fate of TS mRNA during the cell cycle, again using centrifugal elutriation (Imam et al. 1987). Surprisingly, considering the results obtained on enzyme levels described above, no variation of mRNA levels was found in cells at different stages of the cell cycle. This may point to translational and/or post-translational controls of TS expression and resembles a situation

presently revealed in the case of TK. More recent data by Nagarajan and Johnson (1989), however, make things appear even more complex. These authors used mitotic shake-off to synchronize cells and to isolate cell populations in various phases of the cycle. They found that TS mRNA increased by a factor of 5 to 10 when cells progressed from the G_1 phase to the mid-S phase, but despite this the level of enzyme was augmented only by a factor of 2, the enzyme itself being very stable. This situation appears to resemble in its contradistinction that in DHFR. In both cases there is a strong suggestion that the method of preparing cell populations in various stages of the cell cycle greatly influences the results.

While in both examples (DHFR and TS) mitotic selection yields results indicative of cell cycle regulation of mRNA, no differences in mRNA levels are seen in elutriated cell populations. It was furthermore demonstrated by Feder et al. (1989) that addition of fresh medium to cells shortly before elutriation dramatically affected the results obtained in that this treatment caused a strong increase in DHFR mRNA. Since cells collected by mitotic selection are commonly seeded onto fresh medium, this may at least partly explain the different results obtained with the two methods most widely used to obtain cells in various phases of the cycle.

3.1.3 Thymidine Kinase

Thymidine kinase is an enzyme of the salvage pathway of nucleotide synthesis. It catalyzes the formation of thymidine monophosphate from thymidine and ATP. Two genes are present in most cells, one coding for an enzyme present in the cytoplasm and involved in salvaging thymidine for nuclear DNA synthesis, the other one for an enzyme found in mitochondria. It has been known for a long time that cytoplasmic TK activity is low in quiescent cells and increases when cells are stimulated to proliferate (Bello 1974; Wawra et al. 1981; Johnson et al. 1982). The mechanism of this regulation, however, is only starting to be unravelled. Genomic TK sequences were isolated first from the chicken (Perucho et al. 1980; Merrill et al. 1984). More recently TK cDNA and genomic DNA was isolated from human (Bradshaw 1983; Lin et al. 1983; Lau and Kan 1984; Bradshaw and Deininger 1984; Flemington et al. 1987), hamster (Lewis et al. 1983; Lewis 1986) and mouse (Lin et al. 1985; Hofbauer et al. 1987; Seiser et al. 1989) cells. Partial TK cDNA sequences from the rat were also reported (Murphy and Efstratiadis 1987). Using TK cDNA as probe it was observed that, like TK enzyme activity, TK mRNA increases during transition of proliferation-stimulated cells from the G_1 to the S phase of the cell cycle (Stuart et al. 1985; Coppock and Pardee 1987; see also Kasid et al. 1986) and that the control involved is at least in part

post-transcriptional (Groudine and Casimir 1984; Lewis and Matkovich 1986; Hofbauer et al. 1987; Stewart et al. 1987; Gudas et al. 1988; Lieberman et al. 1988).

More recent experiments point out the importance of the promoter in growth regulation of TK expression, which at first sight appeared surprising since the promoter sequences of TK genes of different origin show little resemblance, a fact which sharply contrasts with the high conservation of the amino acid sequence of TK from chicken to man. The human promoter region contains a sequence resembling a TATA box and it carries two CCAAT boxes as well as a series of GC elements, binding sites for transcription factor SP1 (Kreidberg and Kelly 1986; Flemington et al. 1987; Arcot et al. 1989). Results of several independent studies implicate a role of the promoter in the growth regulation of TK gene expression (Kim et al. 1988; Travali et al. 1988; Lipson et al. 1989; Lipson and Baserga 1989; Weichselbraun et al. 1990). It appears that a DNA sequence of less than 100 bp upstream of the transcription start site is sufficient for growth-dependent transcription of human or mouse TK (Lieberman et al. 1988; Lipson et al. 1989; Arcot et al. 1989; Weichselbraun et al. 1990). Within this sequence there is one CCAAT box in the human promoter which seems to be important for the regulation (Knight et al. 1987; Arcot et al. 1989). While the hamster TK promoter does carry a TATA element, it lacks a CCAAT box (Lewis 1986), and the mouse promoter has neither of these characteristic transcription factor binding sites (Lieberman et al. 1988; Seiser et al. 1989). Therefore, if, as suggested (Knight et al. 1987; Arcot et al. 1989), the CCAAT box does indeed play an important role in transcription control of the human TK gene, the absence of such sequences from the TK promoter of the hamster and the mouse must mean that other transcription factors are involved in these cases, and hence that there is no uniformity in the regulation of TK in mammalian cells. This has to be taken into account when possible common elements of the regulation of TK gene expression in mammals are considered.

In this context it is interesting to note that all three mammalian TK promoters share the presence of binding sites for SP1 and a sequence of 18 bp which varies only at two nucleotides between mouse and man (Seiser et al. 1989). This sequence, however, is located at varying distance from the transcription start sites and is not within that part of the promoter which fulfills the minimal requirements for growth-regulated transcription. Its role is so far unknown. DNase I footprinting studies showed the existence of proteins binding to various regions within the promoter, including sequences which are transcribed, but excluding the conserved 18-mer. In the case of the mouse TK gene, proteins were found to bind even to specific regions as far downstream as and intron 2 (Rotheneder

et al., unpublished observations); this is reminiscent of the situation in the murine DHFR gene (Farnham and Means 1990). Proteins binding in transcribed parts of genes have been known for some time in the case of products of RNA polymerases I and III (ribosomal and transfer RNAs). Extending to coding regions they are now increasingly found also for products of RNA polymerase II, that is for messenger RNAs. Such proteins might play a role in *assembly* of transcription complexes rather than being part of the transcription complex itself. Interestingly, results obtained with the human TK gene (Ito and Conrad 1990) do not point to a role of 5′ coding sequences in transcription regulation. It should also be stressed that the finding of important transcriptional elements present in TK promoters has not yet yielded information on their involvement in growth-regulated gene expression. Assays which measure the binding of proteins from nuclear extracts to particular regions of the murine TK promoter strongly suggest that all DNA binding transcription factors observed so far are present in nuclei of quiescent and proliferating cells (Rotheneder et al., unpublished). This could mean that a protein responsible for growth regulation is not directly binding to a DNA sequence in the promoter. Rather such a protein might interact indirectly with constitutively present DNA binding proteins and thereby control their activity.

While many data do point to an important role of the promoter for regulated expression of TK, there is no doubt that additional controls exist post transcription. As discussed above, these could be at the level of mRNA stability but some evidence also points to involvement of translational and post-translational mechanisms (protein stability, possibly influenced by protein modification). This may become particularly relevant for the regulation of TK during the cell cycle or in terminally differentiating cells. Using cells synchronized by elutriation, Sherley and Kelly (1988) have, for instance, shown that the stability of human TK decreases dramatically after cell division. Little change in steady state levels of mRNA was observed; however, the efficiency of translation increased about 10-fold when cells entered the S phase.

A similar situation was observed by Merrill et al. (1984) in a study of TK regulation during muscle cell differentiation. Mouse muscle cells, stably transfected with several copies of the chicken TK gene, were induced to differentiate and the level of downregulation of TK was studied. It was found that during terminal differentiation TK activity and protein levels decreased to a much greater extent than TK mRNA, and evidence for translational control was presented (Gross et al. 1987; Gross and Merrill 1988, 1989). One drawback of these experiments is that the regulation of a chicken gene (present in multiple copies) was studied in

mouse cells. This may influence the results, because it can be expected that a regulatory system active at as many levels as that of TK expression may depend for its proper function on transcriptional activity, which can hardly be expected to function correctly when a chicken promoter, with little similarity to the mouse promoter, is to be activated by mouse transcription factors. The amounts of transcript produced may furthermore determine the efficiency of post-transcriptional control steps. These experiments should therefore be complemented by similar ones in a homologous system. Still, together with the data of Sherley and Kelly described above, the results suggest important controls at the level of TK mRNA translation. In a recent careful study Ito and Conrad (1990) examined the relative contribution of transcriptional and post-transcriptional processes during human TK expression using cDNA constructs under the control of the genuine promoter, the SV40 promoter or the herpes simplex virus type I (HSV) TK promoter. This study confirms the earlier observation (Hofbauer et al. 1987; Lieberman et al. 1988) that expression from a construct carrying TK cDNA under the promoter of HSV TK is growth-regulated much like expression from the endogenous gene, which must be due to sequences within the cDNA as the HSV TK promoter exhibits no response to growth conditions (Hofbauer et al. 1987). The different promoters in the constructs did, however, confer striking variations to the kinetics of mRNA production. Furthermore, the study underlines the role of translational regulation on TK expression. All these experiments were done by transfecting the constructs carrying human TK cDNA into TK-deficient rat cells followed by selection of TK^+ clones. Regulation was, therefore, studied against a heterologous background. Considering the multilevel control of TK expression and the differences in promoter sequences (the sequence of the rat TK promoter is not known so far) the caveat indicated above also applies for this study.

Two final points should be discussed concerning TK:

1. Despite the complex and tight growth regulation of TK levels in cells, TK negative mutants (TK^- cells) can be readily isolated from many mammalian cell types (notably from mouse fibroblasts), and such cells apparently grow and divide almost normally in culture. They do exhibit enhanced mutation rates though, which might be due to an imbalance in the precursor pool caused by the absence of TK. Such a situation could be expected to interfere with the normal development of an organism, explaining why TK is encoded (with very few exceptions, for example, yeast) in the genome of nearly all organisms. Cell lines growing in culture are abnormal in many respects and therefore do not show any obvious defects when lacking TK. Such TK^- cell lines have proved most valuable

in cell biology and in particular also for studies on TK regulation. They can be transfected with genes, minigenes or cDNA constructs carrying TK information, and TK^+ revertants can be isolated by selection in HAT (hypoxanthine-aminopterin-thymidine) medium (Littlefield 1964), which only allows TK^+ cells to grow. HAT-resistant cell clones can also be isolated after transfection of the TK gene from HSV TK. Such cells express TK in sufficient quantity (up to 5% of the activity present in rapidly growing fibroblasts) to allow growth in HAT medium. Contrary to the normal cellular enzyme and to enzyme expressed from various TK gene constructs, HSV TK is expressed constitutively (Schlosser et al. 1981; Hofbauer et al. 1987), and in this context it is remarkable that despite the isolation of a great number of cells transformed with HSV TK, we never obtained a cell line exhibiting more than a few percent of the normal TK activity (Wawra et al., unpublished). This could indicate that the unregulated expression of higher activities of TK is detrimental to cells and that downregulation of TK activity in G_2 or during quiescence is important.

2. Mouse cells carry one TK gene coding for the cytoplasmic enzyme consisting of 233 amino acids. In addition the mouse genome harbors two pseudogenes, one of which has been characterized as a processed pseudogene (Seiser et al. 1989), sequenced and found to carry a number of point mutations, small deletions and insertions if compared with the sequence of the cDNA (Seiser and Wintersberger 1989). In particular, a 2-bp deletion after the codon for amino acid 177 changes the reading frame, which causes the addition of 12 wrong amino acids and then termination. Within these 177 amino acids there are only three amino acid changes with respect to the wild type enzyme. Interestingly, the pseudogene, if transfected into $LMTK^-$ cells, gives rise to HAT-resistant clones expressing TK (Seiser et al. 1990). The number of TK^+ cells and the TK activity in the transformants can be increased dramatically by adding a strong promoter in place of the 5′ flanking region of the pseudogene. Cell lines can thus be obtained which express up to 95% of the activity normally present in wild type fibroblasts. Expression of this activity is growth dependent, and experiments have shown that the control is not at the level of mRNA synthesis because steady state amounts of mRNA remain the same in growing or resting cells (Seiser et al., unpublished). This is indicative of translational or post-translational regulation of TK levels and reminiscent of similar situations during the cell cycle or during differentiation as outlined above.

3.1.4 Proliferating Cell Nuclear Antigen and DNA Polymerase α

Proliferating cell nuclear antigen (PCNA), formerly also known as cyclin, is a protein which was detected by its appearance in the S phase of cells stimulated to proliferate (Bravo and Celis 1980). Originally a spot in two-dimensional gels, it is now known to be a protein cofactor of DNA polymerase δ, an enzyme involved in DNA replication (Bravo et al. 1987; Prelich et al. 1987a,b). PCNA increases the processivity of the polymerase, and both proteins together seem to carry out DNA synthesis at the leading strand of the replication fork (Prelich and Stillman 1988; Stillman 1989). PCNA was observed to change its location in nuclei during the S phase, possibly in conjunction with ongoing replication (Bravo and Macdonald-Bravo 1985; Celis and Celis 1985). More recently, cDNA clones for the 36-kDa protein were isolated and characterized (Almendral et al. 1987; Matsumoto et al. 1987). PCNA mRNA, just like the protein itself, was found to increase during G_1/S transition of cells stimulated to proliferate by growth factors (Jaskulski et al. 1988). Studies on the regulation of PCNA during the cell cycle using cells fractionated by centrifugal elutriation documented an approximately twofold increase of protein and mRNA in cells in the S phase. However, PCNA apparently is always in excess over the amount required for DNA replication so that the availability of the protein is not a limiting factor for DNA synthesis (Morris and Mathews 1989). The human gene for PCNA was isolated recently (Travali et al. 1989) and found to consist of six exons. Removal of intron 4 leads to abnormal regulation of the expression of the gene. In contrast to the normal case, lack of this intron causes the production of high levels of PCNA mRNA when cells are made quiescent by serum deprivation. Addition of serum results in an immediate decrease of PCNA mRNA which is followed by a steady increase with time, just as in the case of serum-induced expression of the normal gene (Ottavio et al. 1990). Hence, as in genes for other DNA synthesis enzymes (DHFR, TK) introns may play a role in gene regulation.

The 5′ flanking sequence was determined so that studies on the promoter activity and its regulation can now be carried out.

DNA polymerase α was the first eukaryotic DNA polymerase for which a role in DNA replication during the S phase was ascertained. It has been known for a long time that DNA polymerase α activity is lower in resting cells than in actively growing ones. The complex structure of the enzyme and the large size of its catalytic subunit have slowed down progress toward more detailed elucidation of the regulation of this DNA polymerase. Only recently, full-size cDNA clones for the catalytic subunit (165 kDa) were isolated and characterized (Wong et al. 1988). With these

at hand it could be shown that the production of mRNA is growth dependent. However, when similar experiments were carried out with populations of cells from different phases of the cell cycle (obtained by elutriation or by mitotic shake-off) only a very moderate increase in the amounts of polymerase mRNA could be detected in cells in the S phase compared to those in G_1 (Wong et al. 1988; Wahl et al. 1988). DNA polymerase δ, the second enzyme for which involvement in DNA replication has been proved (for reviews see Blow 1989b; Stillman 1989), has not yet been cloned, and no data on its regulation are available. On the other hand, DNA polymerase β, which most likely is involved in DNA repair only, seems to be neither growth nor cell cycle regulated (Zmudzka et al. 1988).

3.2 Common Elements in Regulatory Mechanisms Involving DNA Replication Enzymes

All of the enzymes described in this section are low in activity and/or amount in quiescent cells and increase when cells are stimulated to proliferate. The increase requires protein synthesis since even small amounts of cycloheximide interfere with enzyme induction. This has been attributed to the role of a labile protein or proteins in this reaction (see Pardee 1989). All the enzymes mentioned here, as well as others which are known to increase in activity in S phase [for example, ribonucleotide reductase, DNA ligase, topoisomerases (for which detailed studies are lacking so far)], are involved in DNA replication or precursor production. Active DNA synthesis is, however, not necessary for their induction no difference can be observed as to whether the cells are growth stimulated in the absence or presence of DNA synthesis inhibitors. This contrasts with the regulation of S phase-specific histone synthesis (see below). For many of these enzymes there was a distinction in regulation when proliferation stimulated cells were compared with cycling cells in various phases of the cell cycle. For all those enzymes for which growth regulation of expression is evident when at the same time no difference is observed during the cell cycle, a regulatory role in the initiation of DNA synthesis can very likely be excluded. A remaining possibility, so far unexplored, is that some enzymes are modified post-translationally and that this modification, although it may not greatly change the specific activity of the particular enzyme, influences its capability to interact with other proteins in a multienzyme complex such as that involved in DNA replication.

Comparisons of promoter sequences for different genes showing similar growth regulation have not yet yielded clues as to how the

coordinated regulation could be achieved at the transcriptional level. This holds in some cases (such as TK) even for the same gene in different organisms. Further studies will have to demonstrate whether unexpectedly small regions of the promoter sequences show similarity and bind common transcription factors, or whether quite different regulatory proteins (binding to specific sequences which differ in each case) give rise to the same results. Also worth considering is the possibility mentioned above, namely that growth regulation is performed by a protein which does not bind to DNA itself but interacts with different DNA-binding proteins, thereby regulating their activity.

4 The S Phase

4.1 Initiation of DNA Replication

Replication of the relatively small genomes of bacteria or viruses which constitute single replication units (replicons) normally starts at well-defined origins of replication. The much larger genomes of eukaryotes are split into a great number of replicons of different sizes which are switched on in clusters in a temporal order that may vary in a cell type-specific and developmentally regulated manner (Edenberg and Huberman 1975). Efforts to define origins of replication in eukaryotic cells have met with limited success. Sequences for which experimental evidence suggests their function as replication origins were defined only in the yeast *Saccharomyces cerevisiae*, where such sequences give plasmids the ability to replicate autonomously in yeast cells (Newlon 1988). Such sequences were therefore called ARS (autonomously replicating sequences). The search for ARS in other organisms was much less successful. Although many reports appeared in the literature describing sequences which provide plasmids with the capacity to replicate autonomously in mammalian cells, it is questionable whether they are true chromosomal origins of replication (see Umek et al. 1989 for a critical discussion on origins of replication). Many of these sequences carry elements of transcription factor binding sites, an observation which may be relevant for the function of such sequences as ARS but not necessarily as genuine origin. It has been suggested that the difficulty in defining ARS from mammalian cells may lie in the fact that episomal replication of plasmids in such cells requires more than just a site for replication initiation. Vectors on the basis of Epstein-Barr virus DNA (which characteristically replicates as episome in human cells) were therefore designed to provide a system for the isolation

of human origins of replication (Krysan et al. 1989). The usefulness of this vector system has yet to be shown on a broader basis.

One of the more convincing examples for a true mammalian origin of replication is provided by the studies on amplified genes coding for DHFR in Chinese hamster ovary cells. A cell line containing several hundred copies of the DHFR gene was used to look for the origin of replication from which the amplification could have started. Clear evidence for a region preferentially acting as a start site for replication was obtained (Leu and Hamlin 1989; Anachkova and Hamlin 1989; Handeli et al. 1989) and the sequence was determined recently (Caddle et al. 1990). This sequence exhibits many peculiarities which could play a role in its function as origin of replication. Characteristically (as in yeast ARS sequences) there are AT-rich regions prone to bending of the DNA, long stretches of purines capable of forming triple-stranded DNA and nuclease-sensitive sites.

Another example which (in contrast to many others) may survive future critical studies is the demonstration of the presence of a putative replication origin in the 5′ flanking sequence of the human *c-myc* gene. By analyzing the direction of replication through the *c-myc* gene, Leffak and James (1989) obtained evidence for the start site of replication some 4–5 kb 5′ to the gene. Very recently they could provide more conclusive evidence for an origin in that region by isolation of the respective DNA region, and showing that a 2.4-kb fragment of sequences 5′ to the *c-myc* gene can confer autonomous replication on a plasmid which in addition carries the selectable neomycin-resistance gene. The plasmid was stable episomally for many cell generations in copy numbers of five to ten per cell when the cells were under selection pressure. It was not integrated into the genome as long as extrachromosomal replication persisted (McWhinney and Leffak 1990). Ariga et al. (1989) found that the sequence most likely comprising a replication origin is an enhancer for the *c-myc* gene; both elements share protein binding sequences.

Drosophila chorion genes provide another interesting example of a system in which the question of specific origins of replication is open to experimentation. During the final part of oogenesis, genes coding for eggshell proteins are amplified more than 50-fold in a controlled way by the initiation of multiple rounds of replication. Recent experiments indicate that replication starts at few specific origins, the sequence of which shows similarities to those found in yeast ARS elements, in particular to the yeast ARS core structure (Heck and Spradling 1990).

Studies on the function of ARS elements in yeast clearly showed their involvement in the start of a replicon; however, these studies at the same time established that not all ARS sequences function as origins in every

cell cycle (Brewer and Fangman 1988; Linskens and Huberman 1988). This indicates that chromatin structure probably plays a critical role in defining origins of replication. Another factor definitely influencing the selection of sequences as origins is transcription and/or the presence of transcriptional promoters or enhancers, underlining the importance of the structure of chromatin. In fact, proteins isolated on the basis of their capacity to bind to ARS sequences in some cases were found to play a role in transcription (see, for example Diffley and Stillman 1989). A correlation between transcription control regions and initiation of DNA replication is also evident in many viruses. The origin regions of polyomavirus and of SV40, for instance, are associated with transcriptional promoters (active in divergent orientation) and with enhancers. The latter play an important, though to date not fully defined role in replication (for a review see Stillman 1989). Adenovirus DNA replication is also stimulated by transcription factors binding in the vicinity of the replication start site. The relation between the regulation of transcription and replication is therefore quite strong and was discussed recently (Wintersberger and Wintersberger 1987; dePamphilis 1988).

Related to the question of specific origins of replication and their activation during the S phase is the fact that the total genome must be replicated once and only once during one S phase. In other words, an origin which was activated once must not be activated for a second time during the same S phase. How this is achieved is one of the important questions for which an answer has yet to be found. Experiments on a model system, the replicon of bovine papilloma virus, have yielded interesting results. Viral DNA replicates in mouse cells up to a restricted copy number which then stays fairly constant during many cycles of the host cell. Experimental evidence suggests this is due to a negative regulatory element in the origin region at which a trans-acting factor, encoded by the virus, appears to act (Roberts and Weintraub 1986, 1988; Berg et al. 1986). This led to the proposal that there might exist in cell nuclei factors which block origins once they have fired. An alternative interpretation of the regulation of papilloma virus DNA replication in mouse cells has, however, been proposed (Gilbert and Cohen 1987), and the discrepancy is not yet resolved.

A quite distinct situation characterizes initiation of DNA replication in *Xenopus laevis* oocytes. DNA of various sources, including bacteriophages and plasmids, if injected into unfertilized eggs replicates under control of the cell cycle without requiring specific origins (Harland and Laskey 1980; see also Laskey et al. 1989). Hence, at least for this case, specific origins are not necessary to start replication. Subsequently it was demonstrated that cell-free extracts from *Xenopus* eggs can replicate

demembranated sperm nuclei or bacteriophage DNA (Blow and Laskey 1986; Hutchison et al. 1987; Newport 1987). This process was dependent on a prior assembly of chromosomes into nuclear structures. In addition to replicating enzymes and proteins required for formation of nuclei and of nuclear pores, the egg extract provides a vesicular fraction contributing to the formation of nuclear envelopes (Sheenan et al. 1988). Initiation of DNA replication affords an intact nucleus, but once DNA replication has started within an individual nucleus, it usually goes to completion (Blow and Watson 1987). Replication ceases once the DNA content has doubled, reflecting the situation of a single cell cycle. However, re-replication could be achieved when the nuclear membranes were deliberately permeabilized (Blow and Laskey 1988). This led to the proposal that a factor, called licensing factor, is required for initiation of DNA replication but is somehow used up or inactivated after its function. Newly synthesized licensing factor can enter the nucleus only when the nuclear envelope is permeabilized, as in the in vitro experiments, or broken down, as in mitosis. In vivo, therefore, nuclei would have to go through mitosis before reinitiation of DNA synthesis can occur. The model is in accord with the cell fusion data demonstrating that a G_2 nucleus cannot be triggered to start replication in heterokaryons containing S phase nuclei (Rao and Johnson 1970), but it does not explain why a G_1 nucleus by itself remains inactive in replication but initiates DNA synthesis if it is present together with an S phase nucleus in a heterokaryon. Cytoplasmic components present in S phase cells, or nuclear factors capable of moving from an S phase nucleus into that of a G_1 cell, are likely to be necessary in addition. There are other questions which have to be resolved in order to solidify the model of the licensing factor. For instance, many cells can remain quiescent for long periods of time but still can be stimulated to proliferate. Does licensing factor survive in nuclei of resting cells for a long time? The prevailing alternative model for the regulation of initiation by a positively acting factor assumes that such a factor is produced prior to the S phase in cycling or growth-stimulated cells. It must be able to act on DNA of early S phase chromatin. The site of action of the initiation protein (the origin of replication) might be dictated by the regional structure of the chromatin. Once a region of the chromatin is replicated it might change its structure such that it is not accessible to *trans*-acting initiation factor until the chromosome cycle has run through G_2, M and G_1.

It is worth remembering in this context that the number and thereby the sites of replication origins within the genome change dramatically during development of many multicellular organisms. In fertilized eggs replicons are very small, the number of origins is large and, as a consequence, the replication of the genome is fast. In cultured cells from the

same organism, replication takes much longer, there are far fewer origins and replicon sizes are much larger. This is one of the reasons why the question of the presence of specific origins of replication in multicellular eukaryotes is so heavily debated. Interestingly, there is no transcription in fertilized eggs as long as replication is fast. Hence, there is no interference of the replication apparatus with the transcription machinery. This is different in all other cell types where transcription and replication take place, and their temporal order in particular regions of the genome must somehow be controlled to avoid collisions between the two synthesis machines (for a discussion of this problem in bacteria see Brewer 1988). In fact, replication of ribosomal DNA in yeast is dictated by the heavy expression of these genes because the transcription apparatus creates a replication fork barrier in the direction opposite to transcription, so that replication is largely unidirectional in the direction of transcription (Linskens and Huberman 1988; Brewer and Fangman 1988).

The correlation of DNA replication with gene expression was also investigated in a study on the polarity of DNA replication through the α-globin locus in avian cells (James and Leffak 1986). In these experiments replication was continued in vitro from in vivo initiated replication sites (in vitro nuclear run on replication) and it was found that the α-globin locus is preferentially replicated in transcriptional direction, both in cells where the genes are active (and replicate early) and in those where they are inactive (and replicate late). However, in subsequent studies of the same question in a different locus, namely that of the histone H5 gene, Trempe et al. (1988) obtained a different result, because the direction of replication of that gene correlated with the direction of transcription only in cells expressing the gene; replication occurred in the opposite direction in cells where the gene was shut off. An analogous situation was noted in the case of the *c-myc* gene (Leffak and James 1989). The common feature of all results in this study is that transcribed genes always replicate in the direction of transcription.

4.2 Regulation of the S Phase

Replicons are switched on either in early or in mid-S phase in a regulated fashion. Much evidence indicates that the timing of the initiation of individual replicons in S phase is rather fixed from one cell cycle to the next in one particular cell type but may vary in different cell types depending on specialized transcriptional capacities of the cell.

4.2.1 Experimental Observations

One of the first thorough studies on the timing of replication of some 25 genes for which probes were available was published by Goldman et al. (1984). They showed that DNA replication in mammals is temporally bimodal, consistent with the idea that the genome can be divided into two functionally distinct parts. Genes which are active in all cells, the "housekeeping" genes, replicate during the first half of the S phase. Tissue-specific genes also replicate early in those cells in which they are expressed but they replicate in the second half of S phase in those tissues in which they are not expressed. Methodology is important for an evaluation of the data obtained and must therefore be mentioned briefly. The principle of the method used in this and many subsequent studies was the following: cells were synchronized either by mitotic shake-off (Goldman et al. 1984) or by centrifugal elutriation (the studies by Schildkraut's group cited below). Bromodesoxyuridine was then incorporated into replicating DNA by adding the precursor analog to the growth medium for various periods of time. Thereafter, DNA was isolated and digested with a restriction endonuclease and bromodesoxyuridine-containing DNA was isolated by centrifugation in CsCl gradients. Heavy DNA fragments were then either separated by electrophoresis on agarose gels followed by hybridization with various labeled, gene-specific DNA probes or blotted directly onto filters using a slot blot device and then hybridized. Mitotic selection provides only rather limited numbers of cells compared to the more elaborate centrifugal elutriation. Still, the main conclusions drawn from results using the two methods were similar and generally are corroborated by experiments using a quite different method (see below, p. 73).

Schildkraut and coworkers initially studied the replication time of α-globin genes in mouse cells. Extending their studies to the immunoglobulin heavy chain constant region of the mouse, they made the important observation that changes in gene position have an effect on the time of replication (Calza et al. 1984). In other words, the replication time of a gene does not depend on its sequence but rather on its surroundings within the genome. Another multigene family studied was that of the human histone genes which were also found to replicate during the first half of the S phase (Iqbal et al. 1984), leading to the suggestion that replication of genes may play a role in their expression, perhaps by causing a change in chromatin structure. This point is discussed more fully below. In an extensive study the replication of multigene families (α-globin, β-globin, immunoglobulin) and of expressed or non-expressed genes in a variety of cell lines was examined (Hatton et al. 1988a,b). The general

conclusions were: actively transcribed genes are replicated during the first quarter of the S phase; some of these genes replicate later in cell lines in which they are not expressed. Other genes replicate in the first half of the S phase, although they are not expressed; they replicate even earlier in cell lines in which they are transcribed. Some genes replicate early whether they are transcribed or not, and genes which are not transcribed can replicate during any interval of the S-phase. There is coordinated timing of replication of sequences within a multigene family. The coordinated replication of the human β-globin gene locus also reflects its transcriptional activity, and in addition was found to correlate with nuclease sensitivity of the chromatin region (Dhar et al. 1988; Epner et al. 1988).

All these results make it quite clear that replication of a gene can start from alternative origins, and different cell types use different sets of origins of replication. Since the selection of origins depends on nuclear location, the three-dimensional organization of the genome must play an important role.

Another group of genes whose replication time was particularly interesting is that of the proto-oncogenes. Their replication was found to be mostly in early S phase in human, hamster and mouse cells (Iqbal et al. 1987; Doggett et al. 1988).

Finally, an interesting approach was taken by Dhar et al. (1989), namely that activation or repression of genes was not the result of rearrangements within the genome as was the case in earlier work on immunoglobulin genes but was achieved by cell hybridization (leaving the gene in question in the same position as in the parent cell). The replication time of β-globin genes from mouse cells was studied in mouse hepatoma–mouse erythroleukemia hybrid cells in which the gene, which is active in the erythroleukemia cells, is inactivated. The inactivated gene in the hybrid was found to replicate later than the active one in the parent cell. On the contrary, in a hybrid between a human fibroblast line (not expressing the β-globin gene) and mouse erythroleukemia cells the β-globin gene is transcriptionally activated and replicates earlier in the hybrid than in the fibroblast. In addition, the β-globin gene in the hybrid is more sensitive to nucleases than in the parent fibroblast. These results show that the timing of replication can be altered even in differentiated cells when the expression of the gene in question is changed. Early replication thus definitely coincides with transcriptional activity.

The temporal order of replication of a large group of genes, 29 in all, in Chinese hamster ovary cells was studied by Taljanidisz et al. (1989) using a different method. These authors separated exponentially growing cells by centrifugal elutriation into populations at different stages of the cell cycle and analyzed the replication of specific genes by labeling the

DNA with 5-mercuri-dCTP (deoxytidine triphosphate) and ^{3}H-dTTP (deoxythymidine triphosphate) in permeabilized cells. Newly synthesized mercurated DNA was purified by affinity chromatography on thiol-agarose, then labeled with ^{32}P using the random primer method, and hybridized to plasmid DNA containing cloned genes fixed to nitrocellulose filters. The extent of hybridization is a measure of the replication activity of the particular gene in a cell population at a defined stage in the cell cycle. This study supports results of earlier ones obtained with a different method and includes several genes not dealt with in previous experiments. Furthermore, data are summarized in a more objective way by not simply stating early versus middle or late S phase, but by relating the extent of replication of a gene with the C-value of cells as determined by flow cytometry. A C-value of 1 corresponds to the haploid DNA content per cell; hence, within one S phase of a diploid cell the C-value increases from 2 to 4. The genes chosen span almost the entire S phase of hamster cells. Whereas many genes were found to replicate early (C-values 2–2.5), some others replicate late (C-values 3.5–4). Only three genes were observed to replicate at C-values of 2.5–3 (among these is the transcriptionally active gene of TK) and none were found to replicate at C-values of 3–3.5, providing support for the "3 C pause" (a significant decrease in the DNA synthesis capacity) in the middle of S phase described by Goldman et al. in 1984 (see also the discussion below, p. 74). Otherwise the following conclusions were drawn by the authors:

1. Housekeeping genes replicate during the first half of the S phase.
2. Proto-oncogenes fall into two classes: six transcriptionally active and three transcriptionally inactive genes replicate in the first quarter of S phase, three others replicate late.
3. Although there was a correlation between transcriptional activity and replication in the first half of the S phase, there are a number of exceptions to this rule. (About a quarter of the total mRNA was transcribed from late-replicating DNA.) In other words, although there is a strong preference of expressed genes to replicate early and of unexpressed genes to replicate late, there are cases where unexpressed genes replicate early and expressed genes replicate late.

4.2.2 Interpretations of Experimental Results

Reviewing the earlier work on replication timing, Holmquist (1987) concluded that there may be two classes of genes, one class present in the early replicating part of the genome which cytologically is characterized by R bands (Giemsa light bands) comprising transcriptionally active

GC-rich sequences, the other in the late replicating part of the genome characterized by G bands (Giemsa dark bands) comprising AT-rich sequences which are transcriptionally repressed (Taylor 1984). R bands finish replication before G bands start, giving rise to the 3 C pause.

The connection between early replication and transcriptional activity can be interpreted in two ways: early replication is a prerequisite for the expression of genes (see, for example, Goldman 1988) or, vice versa, transcriptional activity causes a change in chromatin structure making early replication possible. There is support for both interpretations. For the former one (replication as a prerequisite for transcription) recourse is often taken to the regulation of the expression of somatic and oocyte-type 5S ribosomal RNA genes of *Xenopus laevis*. In somatic cells the first type is expressed but not the latter one. When transcription factors involved in the regulation of 5S RNA transcription, in particular TFIIIA, became known, it was suggested that this factor might be present in limiting amounts and might bind first to replicated somatic genes, leaving oocyte-specific genes silent (Gottesfeld and Bloomer 1982). This, of course, would mean that somatic genes should be replicated early and oocyte genes late, which indeed was found to be the case (Gilbert 1986; Guinta and Korn 1986). Furthermore, in vitro DNA replication was found to erase the 5S RNA transcription complex (Wolffe and Brown 1986). This, however, means no less than that at least some transcription complexes, once established, can be disrupted (whether active or not) by a passing replication fork. Hence, cell cycle-regulated provision of transcription factors and their exhaustion by binding to early replicated DNA sequences, such as those of somatic 5S RNA genes in *Xenopus*, would not be necessary, since the late replication of the oocyte-specific genes would inevitably lead to destruction of transcription complexes which might have formed at those genes (Wolffe and Brown 1988) and which probably cannot be reestablished before chromosome condensation.

Another particularly interesting case, where a difference in replication time dramatically coincides with transcriptional activity, is that of the X chromosomes of female mammals, one copy of which is expressed and replicates early, while the other one is inactive and replicates late.

More distantly related investigations relevant to the problem of S phase regulation involve the replication-dependence of the expression of genes from viral promoters active late in the viral replication cycle. During virus replication such genes are active only after replication of viral DNA has started. This dependence on replication can be mimicked with transfected plasmids carrying viral late genes or reporter genes under the control of a viral late promoter and a viral origin of replication, located distant from the promoter so as to avoid interference between signals on

the promoter and those on the origin. (Remember that the SV40 origin, for instance, is closely associated with both promoter and enhancer sequences.) In several studies, differing in detail, the conclusion was that replication of the plasmid is required for high expression from the late promoter (Matsui et al. 1986; Venkatesh and Chinnadurai 1987; Grass et al. 1987; Enver et al. 1988). The molecular basis of this control is so far unknown but may lie in the requirement of replication for proper assembly of the late transcription complex. Interestingly, replication dependence of transcription was not observed in some control experiments in which viral early promoters (from which transcription is started prior to initiation of DNA replication) were used. A dependence on replication of transcription from late promoters is already seen in bacteriophages. In the case of phage T4 this was recently shown to be due to stimulation of transcription by components of the replication apparatus (Herendeen et al. 1989).

While these data together with those on 5S RNA genes of *Xenopus* and on the mammalian X chromosome support the idea that early versus late replication may control gene expression during the cell cycle and in development, there are examples favoring the reverse interpretation – that transcription enables early replication. First, this is in accord with the striking correlation mentioned above between replication origins (or whatever is described as such in animal cells) and transcription signals, promoters and enhancers. This correlation is reminiscent of the situation in some phages (notably phage λ) and bacteria (*Escherichia coli*) where transcription around the origin of replication seems to play a role in replication, a correlation which is referred to as "transcriptional activation" of DNA replication (see DePamphilis 1988). Most likely transcriptional activation facilitates the establishment of the replication apparatus at the replication initiation site. Furthermore, contrary to the belief that replication is a prerequisite for gene expression, many housekeeping genes which are transcriptionally active and replicate early are expressed in the absence of ongoing DNA replication. For instance, induction of enzymes like DHFR, TS or TK in growth-stimulated cells does occur even if cells are prevented from starting the S phase due to the intentional addition of DNA replication inhibitors such as fluorodesoxyuridine, hydroxyurea, methotrexate or aphidicolin. In addition, while the expression of both DHFR and TK is activated during G_1/S transition and in early S phase in growth-stimulated cells (see above), only the DHFR gene replicates early; the TK gene replicates towards the middle of S phase (Taljanidisz et al. 1989).

In conclusion, despite many cases where transcriptionally active genes are replicated early in the S phase and non-expressed genes are replicated

late, there is a significant number of exceptions to this rule. And similarly, while there are cases where replication may be a prerequisite for transcription, others can be interpreted in the opposite direction.

In the case of viruses, there appear to exist two classes of promoters, those active in the absence of DNA replication (their firing may or may not activate DNA synthesis by a mechanism separate from and in addition to the need for an early viral gene product for replication) and those which are strongly stimulated by prior replication. An analogous situation can be envisaged for cellular promoters, which may also fall into two classes. The difference between these could lie in a different structure of the chromatin and/or in variable binding constants of the transcription factors involved. This might or might not allow transcription factors to bind in the absence of replication. Cases in question are some viral origins of replication (e.g., those of adenovirus, SV40 or polyomavirus) which are stimulated by the binding of transcription factors. Other cellular transcription start sites might require the previous passing of the replication machinery for their activation.

According to this view, the local three-dimensional structure of chromatin appears to be decisive in determining where and when replication starts. Important in the selection of replication sites could be an association of replicating chromatin with the nuclear matrix or scaffold structure, both during initiation (see Amati and Gasser 1988 and references therein) and during elongation (see Paff and Fernandes 1990 and references therein). The view that changes in chromatin structure have a strong effect on DNA replication is supported by the observation that a so-called linker histone, H5, which is involved in condensation of chromatin, plays an active role in the control of DNA replication (Sun et al. 1989). The question of whether fixed origins of replication exist in the genome of higher eukaryotes also has to be seen against the background of the above discussion on the timing of replication. In complete contrast to the simple replicons of viruses and bacteria, comprising the whole genome, the replication of which is regulated by the use of specific origins of replication, other factors may be more important in the ordered replication of the eukaryotic genome. One of the most important regulatory devices must be the "once per cell cycle rule". In other words, it may not be so important where and when precisely replication starts; much more important is that once a stretch of the genome is replicated it is immediately protected against re-replication until cells have gone through the cycle and arrived at the G_1/S border again. The molecular basis of this control is unknown but it is likely to reside in the structure of chromatin which probably changes continuously throughout the cell cycle. According to this view, the defined ARS sequences in the yeast

Saccharomyces cerevisiae, which in many cases are used as origins in vivo, are the exception awaiting an explanation, rather than a model for higher eukaryotes. One reason for the requirement of specific initiation sites for DNA replication in yeast could be the extensive transcription of most of the genome throughout the cell cycle. In this respect *Saccharomyces cerevisiae* is an extreme case (accompanied only by prokaryotes and viruses). A quite opposite position is that of the fertilized egg in which no transcription takes place and replication is fast. Whereas replication starts at a very large number of sites in the genome of fertilized eggs (frog eggs even support replication of phage and plasmid DNA) because there is no interference with transcription, the situation is at the other extreme in *Saccharomyces cerevisiae* where the genome is heavily transcribed and as a consequence replication has to start at rather defined origins. Mammalian somatic cells might be somewhere in between these two extreme cases. This is not to say that replication could start anywhere in a mammalian genome; on the contrary, it is likely that there are preferred sites which function as origins of replication, but there may not be precisely defined sequences as there are in viral or "modern" bacterial genomes. In this connection it is also worth remembering that under stress conditions even *Escherichia coli* can start replication at sites different from the main origin of replication, *ori-C*. In fact, *ori-C* may have become the preferential replication origin of *E. coli* only because this bacterium inhabits the mammalian gut, where it finds conditions for relatively rapid growth for which the ill-controlled replication starts at the other origins (*ori-K*) are not well suited (von Meyenburg et al. 1987), probably because replication from such origins interferes with high rates of transcription (Brewer 1988).

The division of the genome into early and late replicating parts raises another interesting question: is this division accomplished by the existence of early and late firing origins of replication? Alternatively, it is possible that late replicating sequences are simply far away from an active origin and a long time is required for the fork to move into them. The time required could be further lengthened by a more condensed structure of the chromatin in late replicating parts of the genome. One barrier to movement of replication forks could certainly be active transcription in the opposite direction as discussed above for yeast ribosomal RNA genes. There are as yet no data available which would prove or exclude the existence of origins initiated late in the S phase. In *Saccharomyces cerevisiae* initiation of origins was found to occur for at least the first half of the S phase (Newlon 1988 for review). Whether there are truly late initiating ARS sequences is not known, although Reynolds et al. (1989) have started to analyze this question by examining the temporal pattern

of replication of 200 kb of chromosome III of *Saccharomyces cerevisiae* in which several ARS elements are known to be present. They found that some ARS elements near the telomers replicate late while internal ones replicate early. But it is not known, so far, whether initiation takes place at late replicating ARS elements, because, as in earlier work, some ARS elements were found to be inactive as replication origins at least in some cells. Delayed readthrough from an early started replicon could equally well explain the results obtained.

The two models have, however, testable consequences: If late replication were due to late initiation this would mean that sequences must exist acting as boundaries of early replicating replicons. In other words, it would be expected that termination sites for replication exist at the ends of replicon clusters. If, on the other hand, some replication forks were stalled until late in S phase at certain regions of the genome (due to a more condensed chromatin structure or an association of sequences with the nuclear matrix or a chromosome scaffold) these late replicating replicons would be expected to be larger than early replicating ones. The size of replicons is indeed quite variable in yeast as well as in animal cells, but as yet there is no indication as to whether there is a correlation of the size with the time of replication. An interesting system, which might provide answers to these questions, is that of the amplification unit of the DHFR locus in methotrexate-resistant Chinese hamster cell lines. Ma et al. (1990) recently obtained evidence for three early firing origins within the 240 kb DHFR amplicon as well as for the presence of a replication terminus.

4.3 Mitosis Depends on Completion of the S Phase

To assure that mitosis does not begin before DNA is completely replicated (otherwise daughter cells would not receive a full genome) there must exist a checkpoint between the S and the M phase of the cell cycle. Cell fusion experiments clearly show that in heterokaryons harboring a G_2 and a G_1 or an S phase nucleus, the G_2 nucleus is prevented from starting mitosis until the other nucleus has completed DNA synthesis (Zelenin and Prudovsky 1989). Also, cells having suffered damage to their DNA are delayed in entering mitosis (Adlakha et al. 1984). Recently a start towards elucidation of the mechanism involved was made (Weinert and Hartwell 1988; Hartwell and Weinert 1989). The product of a gene, *RAD 9*, of *Saccharomyces cerevisiae* that causes radiation sensitivity if mutated, was found to assure G_2 arrest after DNA damage by mutagenic agents, suggesting that a negative control system may delay passage of cells from S to M as long as DNA is not fully replicated and DNA damage is not

repaired. Also in the fission yeast, *Schizosaccharomyces pombe*, a mutation was observed which abolishes the dependence of mitosis on DNA replication (Enoch and Nurse 1990). These studies will certainly throw light on how the cell couples mitosis with completion of DNA replication. In vitro experiments using cell-free extracts from *Xenopus* eggs revealed a feedback pathway which suppresses mitosis until replication is completed. This appears to be governed by threshold amounts of unreplicated DNA which somehow block the activation of maturation promoting factor (Dasso and Newport 1990). According to recent evidence, it is likely that the well-known effect of caffeine in inducing premature chromosome condensation is due to a relaxation of this blockade (Downes et al. 1990). Both effects, the blockade by unreplicated DNA and its relaxation by caffeine, may involve post-translational modifications critical for the activation of maturation promoting factor.

4.4 Regulation of Histone Synthesis

In eukaryotes, replication of chromatin necessitates not just DNA replication but also the synthesis of histones. This takes place in the S phase and is tightly linked to and dependent upon DNA synthesis. The replication-dependent histone genes are coded by a multigene family consisting of up to 20 members for each histone type, namely H1, H2A, H2B, H3 and H4. Dimers of H2A, H2B, H3 and H4 make up the nucleosome and therefore must be produced in equal molar amounts. Replication-dependent histone genes lack introns and the mRNAs are devoid of poly(A) stretches at the 3′ end. These features distinguish this group of histone genes from another one which is constitutively expressed, contains polyadenylation signals and sometimes introns and codes for so-called replacement histones. The tight regulation of replication-dependent histone gene expression has been known for some time, and the mechanisms underlying this regulation are under intensive study. A short review by Schümperli (1988) summarizes the recent state of the art.

Histone synthesis is regulated at several levels starting with transcription. The rates of histone gene transcription increase three- to fivefold during the S phase, a range which does not nearly come up to the 20- to 100-fold increase in steady state levels of histone mRNA, indicating that post-transcriptional regulation must play an important role. Studies on the mechanism of transcriptional regulation have been carried out both in vivo and in vitro. The possibility of reintroducing cloned histone genes with wild type or mutated promoter sequences has facilitated analysis of transcriptional regulation. Studies of protein binding onto isolated

promoter sequences led to the characterization of protein binding elements in the upstream region of the gene for histone H4 (Capasso and Heintz 1985; Dailey et al. 1986; Pauli et al. 1987; Kroeger et al. 1987; Helms et al. 1987; Seiler-Tuyns and Paterson 1987), histone H3 (Pauli et al. 1989), histone H2B (Sive et al. 1986; La Bella et al. 1988) and histone H1 (Dalton and Wells 1988a). There are binding sites for general transcription factors (CCAAT and TATA) in each one of the promoters of histone genes, but in addition there are binding sites for transcription factors which are specific for one particular histone subtype. Some of these subtype-specific transcription factors have been purified and characterized in recent years (Fletcher et al. 1987; van Wijnen et al. 1987, 1989; Dailey et al. 1988; Dalton and Wells 1988b; Gallinari et al. 1989; La Bella et al. 1989), and although for some of them no change in DNA binding activity could be observed during the cell cycle, it is anticipated that they do play an important role in cell cycle regulated transcription. This could be the result of covalent modification of the factors during G_1/S transition, or of their interaction with specific non-DNA-binding proteins. Changes in histone type-specific transcription factor levels or activity during G_1/S transition are accompanied by alterations in the nuclease sensitivity of chromatin in the vicinity of histone genes (Chrysogelos et al. 1985, 1989; Pauli et al. 1989).

While regulation at the level of transcription is responsible for only part of the cell cycle regulation of histone gene expression, it does seem to play the dominant role in growth regulation of these genes. During growth arrest of differentiating human HL-60 cells, for instance, histone gene transcription is downregulated apparently at the level of the amount of type-specific transcription factors (Stein et al. 1989). Hence, as in the case of the regulation of expression of DNA synthesis enzymes described above, control of histone gene expression differs in cycling versus growth-stimulated cells.

As stated above, replication-dependent histone mRNAs lack a poly(A) sequence at the 3′ end; hence, the mechanism of formation of the 3′ end must differ from that making use of a polyadenylation signal. Histone mRNAs are characterized by the presence of a stem-loop structure at the 3′ end, and it was found that this structure plays a critical role in the formation of the 3′ end of the mature mRNA (see, for example, Lüscher et al. 1985 and references therein). This structure is a recognition site for an enzyme system cleaving the precursor RNA close by. Also required for this cleavage is a small nuclear RNA, known as U7 RNA, in the form of a ribonucleotide complex (snRNP). This RNA has a sequence which can base pair with a sequence immediately 3′ to the stem-loop in the mRNA precursor (Soldati and Schümperli 1988). Apart from the U7

snRNP, a heat-labile protein factor is necessary. This factor, unlike U7 snRNP, is present only in proliferating cells, thus providing a link between the proliferation state of a cell and the production of mature histone mRNA (Gick et al. 1986, 1987; Lüscher and Schümperli 1987). More recently a protein factor was detected which specifically binds to the conserved hairpin structure of histone mRNA precursors and differs from the heat-labile factor mentioned above (Vasserot et al. 1989).

The same region at the 3′ end of histone mRNA is the site of action of an enzyme or enzymes determining the stability of the mature mRNA (Stauber et al. 1986; Levine et al. 1987; Pandey and Marzluff 1987; Capasso et al. 1987). The half-life of histone mRNA varies dramatically during the cell cycle, being 30–60 min during the S phase but decreasing to about 10 min at other times or in cells in which DNA synthesis has been inhibited. Degradation of the mRNA starts at the 3′ terminus and continues towards the 5′ end (Ross et al. 1986). mRNA turnover was shown to be coupled to translation (Graves et al. 1987); inhibition of protein synthesis by cycloheximide stabilizes histone mRNA. In order to unravel the mechanism of regulation of histone mRNA turnover, an in vitro system was developed (Ross and Kobs 1986) which mimics the events occurring in vivo. With this system the suggestion that the free histone proteins themselves trigger histone mRNA degradation (Butler and Mueller 1973; Stein and Stein 1984) was tested, and it was found that addition of core histones, in the presence of a cytoplasmic high speed supernatant fraction, did increase the degradation of histone mRNA 4–6-fold (Peltz and Ross 1987). The effect was specific, in that the rate of degradation of a number of other poly(A^-) or poly(A^+) mRNAs was not changed by addition of histones. The exonuclease activity possibly involved in the degradation of histone mRNA was found to be ribosome-bound (Graves et al. 1987; Ross et al. 1987). Attempts at the characterization and isolation of the nuclease have started (Peltz et al. 1987).

4.5 Assembly of Chromatin

The S phase is the time point when newly replicated DNA assembles with histones and non-histone proteins to form chromatin. This allows for gross changes of chromatin structure as, for instance, in development and differentiation (an early summary of the role of the cell cycle and of cell proliferation in differentiation can be found in several articles in Reinert and Holtzer 1975; for a more recent review see Weintraub 1985). It is not surprising, therefore, that much attention has been paid to the events following DNA replication. Due to the great abundance of histones, most

of the studies on chromatin assembly are limited to the binding of these proteins to DNA during the formation of nucleosomes (see, for example, Worcel et al. 1978). Nucleoplasmin, a protein isolated from *Xenopus laevis* oocytes, may play a role in the assembly of nucleosomes (Dilworth and Dingwall 1988). Other proteins have also been found to promote nucleosome formation, and include two proteins isolated from *Xenopus laevis* somatic cells called N_1/N_2 (Cotten and Chalkley 1987). Nucleoplasmin and N_1/N_2 seem to act separately in nucleosome assembly in *Xenopus laevis*. In vitro experiments with extracts from oocyte nuclei have shown that N_1/N_2 forms complexes with histones H3 and H4 while nucleoplasmin is present in complexes of histones H2A and H2B. Both complexes transfer their histones to DNA (Kleinschmidt et al. 1990). In human cells, a protein (CAF-1) was detected which catalyzes nucleosome formation in an in vitro replications system employing SV40 DNA (Smith and Stillman 1989). Using the same cell-free DNA replication system, it was shown that replication-dependent nucleosome formation (Stillman 1986) occurs in steps, only the first one requiring DNA synthesis (Fotedar and Roberts 1989). However, specificity of this process as regards phasing of nucleosomes must come through binding of non-histone proteins, such as transcription factors (plus SV40 T-antigen in the in vitro system mentioned above). These provide "nucleation regions" from where nucleosome formation could start, guaranteeing that parts of the DNA sequence remain free of nucleosomes, as most clearly exemplified in case of the genomes of the small DNA tumor viruses, SV40 and polyoma (reviewed by Fried and Prives 1986). Such regions characteristically are sensitive to digestion by nucleases. In order to influence the phasing of nucleosome formation, transcription factors or other regulatory binding proteins, for instance those fixing particular parts of the DNA to the nuclear matrix (Amati and Gasser 1988; Hofmann et al. 1989) must bind to newly replicated DNA before nucleosome formation. In fact, a competition between the assembly of transcription complexes and the formation of chromatin was found to take place when *Xenopus* 5S RNA genes were incubated with egg extracts (Almouzni et al. 1990). Also important in this context is what happens with the old nucleosomes present in the chromatin, how they are distributed, and in which way their distribution influences the formation of new nucleosomes. This point has been heavily debated, due to the technical difficulty of the experiments necessary to answer the question unequivocally, and the debate is still going on (for a recent opinion on this problem see Svaren and Chalkley 1990). The recent observations with nuclear extracts from *Xenopus* oocytes mentioned above (and considered by Svaren and Chalkley) greatly advance our knowledge in this area.

Important as the change of chromatin structure in particular regions of the genome is during differentiation, an equally remarkable property of chromatin is the constancy of its structure in other regions, that is, the inheritance of areas of chromatin structure through many cell cycles and cell divisions. Practically nothing is known about how this is achieved. Another open but important question concerns the molecular mechanism by which replicated chromatin is labeled such that re-replication within the same S phase is prevented.

5 Epilogue

Despite a vast literature on cell cycle and growth regulation, progress at the molecular level is presently limited to few areas of research. These concern primarily those events involving the protein kinase p34/cyclin, which controls the initiation of mitosis and, in a different form, probably reactions at the G_1/S border. Recent progress in this area has come through the fusion of studies on different organisms. Of great help was the availability of mutants in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* which, together with originally completely independent experiments on *Xenopus* and later on HeLa cells, led to the present concept of the role of cyclins and maturation promoting factor (Norbury and Nurse 1989). Another area coming of age is that of the regulation of enzymes and proteins involved in DNA replication during transition from G_1 into S phase, discussed in this review. It is remarkable in the present situation that there are as yet no common elements in the results of studies on different enzymes that would allow conclusions to be drawn as to the mechanism of this seemingly coordinated control of the various enzymes. Most of these studies were carried out with different lines of mammalian cells, a fact which may contribute to the divergence seen. This divergence, however, extends to the structure of promoters, which is a property of the organism and cannot be expected to depend on the use of particular cell lines. Hence, either the regulation of different enzymes is indeed quite variable as far as the mechanisms involved are concerned, or the common elements have not yet been found. The active research in this area will probably resolve this question in the next few years.

While these problems are amenable to fruitful experimentation, others are much farther away from any solution, in particular those dealing with the regulation of the initiation of replicons during the S phase and with the mechanism which restricts replication to once per cell cycle.

As any deviation from this rule can lead to defects in gene dosage or even to aneuploidy, the problem is of central importance. Although for one aspect, namely early versus late replication, data exist which can be reconciled, despite the fact that they were obtained with different cell lines and cell types, in other cases the fact that such studies are mostly carried out with established cell lines might influence the results. One central property of such cell lines is their at least partial escape from normal control mechanisms, resulting in immortalization which is nearly always accompanied by aneuploidy and chromosomal abnormalities. This might indeed have some effect on regulatory mechanisms and could explain why extraordinary differences are often found with various cell systems. One interesting example underlining this aspect is the recent report that, contrary to what is observed with established cell lines and with tumor cells, DNA amplification is very rare in *normal* human cells (Wright et al. 1990; see also Wintersberger 1984). Considering that many of the results on growth regulation and cell cycle regulation of DNA replication enzymes were obtained with cell lines in which the gene under study was amplified often more than 100-fold, it can be asked to what extent the results obtained in these cases may reflect processes occurring in normal cells. As helpful as research on such cell systems may be, it should be considered as a starting point for the isolation of genomic sequences and cDNAs and as a beginning towards an understanding of the regulation; eventually, however, it should be complemented by control experiments with cells having a normal gene dosage. This may be all the more necessary since regulation seems to take place at several levels, in particular also at the post-transcriptional and the translational level, the relative contributions of which may well depend on the amount of transcript present to start with.

Established cell lines have gone through a phase called "crisis" that obviously is characterized by strong selection pressure. This is survived by very few cells, all of which have acquired a destabilization of the genome, a fact which should be of concern also for another reason: The mechanism by which gene amplification is initiated probably involves reinitiation of DNA replication at a site of the genome already replicated during the ongoing S phase (see reviews by Schimke 1988; Stark et al. 1989). It is precisely this reaction which must not take place and in normal cells does not take place. The strict control of the once per cell cycle rule may be the reason why normal cells cannot amplify DNA. The relaxation of this rule in continuously growing cell lines gives rise to doubt as to whether such cells are suitable for studies on the mechanism underlying this control.

Insight into chromatin structure and changes taking place at this level in the cell cycle is being gained only slowly. This is mainly due to technical

problems. Only gross structures can be examined so far; however, the solution probably lies within the arrangement in particular, sometimes small, areas of chromatin the study of which has to await the development of new methods. An interesting start in this direction was recently reported by Tazi and Bird (1990). These authors have taken advantage of the fact that many active genes are characterized by so-called GpC-rich islands which are unmethylated and susceptible to cleavage by specific restriction endonucleases, thus allowing isolation and characterization of chromatin from transcriptionally active parts of the genome.

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References

Adlakha RC, Wang YC, Wright DA, Sahasrabuddhe CG, Bigo H, Rao PN (1984) Inactivation of mitotic factors by ultraviolet irradiation of HeLa cells in mitosis. J Cell Sci 65:279–295

Almendral JM, Huebsch D, Blundell PA, Macdonald-Bravo H, Bravo R (1987) Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. Proc Natl Acad Sci USA 84:1575–1579

Almouzni G, Mechali M, Wolffe AP (1990) Competition between transcription complex assembly and chromatin assembly on replicating DNA. EMBO J 9:573–582

Amati BB, Gasser SM (1988) Chromosomal ARS and CEN elements bind specifically to the yeast nuclear scaffold. Cell 54:967–978

Anachkova B, Hamlin JL (1989) Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element. Mol Cell Biol 9:532–540

Arcot SS, Flemington EK, Deininger PL (1989) The human thymidine kinase gene promoter. J Biol Chem 264:2343–2349

Ariga H, Imamura Y, Iguchi-Ariga SMM (1989) DNA replication origin and transcriptional enhancer in c-*myc* gene share the c-*myc* protein binding sequences. EMBO J 8:4273–4279

Ayusawa D, Shimizu K, Koyama H, Kaneda S, Takeishi K, Seno T (1986) Cell-cycle directed regulation of thymidylate synthase messenger RNA in human diploid fibroblasts stimulated to proliferate. J Mol Biol 190:559–567

Baserga R (1985) The biology of cell reproduction. Harvard University Press, Cambridge

Bello LJ (1974) Regulation of thymidine kinase synthesis in human cells. Exp Cell Res 89:263–274

Berg L, Lusky M, Stenlund A, Botchan MR (1986) Repression of bovine papilloma virus replication is mediated by a virally encoded trans-acting factor. Cell 46:753–762

Berger H, Wintersberger E (1986) Polyomavirus small T antigen enhances replication of viral genomes in 3T6 mouse fibroblasts. J Virol 60:768–770

Blow J (1989a) Mitosis comes apart. Trends in Genetics 5:166–167

Blow J (1989b) Eukaryotic chromosome replication requires both α and δ DNA polymerases. Trends in Genetics 5:134–136
Blow JJ, Laskey RA (1986) Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. Cell 47:577–587
Blow JJ, Laskey RA (1988) A role for the nuclear envelope in controlling DNA replication within the cell cycle. Nature 332:546–548
Blow JJ, Watson JV (1987) Nuclei act as independent and integrated units of replication in a *Xenopus* cell-free DNA replication system. EMBO J 6:1997–2002
Bradshaw HD Jr (1983) Molecular cloning and cell cycle-specific regulation of a functional human thymidine kinase gene. Proc Natl Acad Sci USA 80:5588–5591
Bradshaw HD Jr, Deininger P (1984) Human thymidine kinase gene: molecular cloning and nucleotide sequence of a cDNA expressible in mammalian cells. Mol Cell Biol 4:2316–2320
Bravo R, Celis JE (1980) A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. J Cell Biol 84:795–802
Bravo R, Macdonald-Bravo H (1985) Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. EMBO J 4:655–661
Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. Nature 326:515–517
Brewer BJ (1988) When polymerases collide: replication and the transcriptional organization of the *E. coli* chromosome. Cell 53:679–686
Brewer BJ, Fangman WL (1988) A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. Cell 55:637–643
Brooks RF, Riddle PN, Richmond FN, Marsden J (1983) The G_1 distribution of 'G_1-less' V79 Chinese hamster cells. Exp Cell Res 148:127–142
Butler WB, Mueller GC (1973) Control of histone synthesis in HeLa cells. Biochim Biophys Acta 294:481–496
Caddle MS, Lussier RH, Heintz NH (1990) Intramolecular DNA triplexes, bent DNA and DNA unwinding elements in the initiation region of an amplified dihydrofolate reductase replicon. J Mol Biol 211:19–33
Cadman E, Heimer R (1986) Levels of thymidylate synthetase during normal culture growth of L1210 cells. Cancer Res 46:1195–1198
Calza RE, Eckhardt LA, DelGuidice T, Schildkraut CL (1984) Changes in gene position are accompanied by a change in time of replication. Cell 36:689–696
Capasso O, Heintz N (1985) Regulated expression of mammalian histone H4 genes in vivo requires a *trans*-acting transcription factor. Proc Natl Acad Sci USA 82:5622–5626
Capasso O, Bleecker GC, Heintz N (1987) Sequences controlling histone H4 mRNA abundance. EMBO J 6:1825–1831
Celis JE, Celis A (1985) Cell cycle-dependent variations in the distribution of the nuclear protein cyclin (proliferating cell nuclear antigen) in cultured cells: subdivision of S phase. Proc Natl Acad Sci USA 82:3262–3266
Chrysogelos S, Riley DE, Stein G, Stein J (1985) A human histone H4 gene exhibits cell cycle-dependent changes in chromatin structure that correlate with its expression. Proc Natl Acad Sci USA 82:7535–7539
Chrysogelos S, Pauli U, Stein G, Stein J (1989) Fine mapping of the chromatin structure of a cell cycle-regulated human H4 histone gene. J Biol Chem 264:1232–1237
Coppock DL, Pardee AB (1985) Regulation of thymidine kinase activity in the cell cycle by labile protein. J Cell Physiol 124:269–274
Coppock DL, Pardee AB (1987) Control of thymidine kinase mRNA during the cell cycle. Mol Cell Biol 7:2925–2932
Cotten M, Chalkley R (1987) Purification of a novel, nucleoplasmin-like protein from somatic nuclei. EMBO J 6:3945–3954
Cross F, Roberts J, Weintraub H (1989) Simple and complex cell cycles. Annu Rev Cell Biol 5:341–396

Crouse GF, Leys EJ, McEwan RN, Frayne EG, Kellems RE (1985) Analysis of the mouse *dhfr* promoter region: existence of a divergently transcribed gene. Mol Cell Biol 5:1847–1858

Dailey L, Hanly SM, Roeder RG, Heintz N (1986) Distinct transcription factors bind specifically to two regions of the human histone H4 promoter. Proc Natl Acad Sci USA 83:7241–7245

Dailey L, Boseman Roberts S, Heintz N (1988) Purification of the human histone H4 gene-specific transcription factors H4TF-1 and H4TF-2. Genes Dev 2:1700–1712

Dalton S, Wells JRE (1988a) A gene-specific promoter element is required for optimal expression of the histone H1 gene in S phase. EMBO J 7:49–56

Dalton S, Wells JRE (1988b) Maximal binding levels of an H1 histone gene-specific factor in S-phase correlate with maximal H1 gene transcription. Mol Cell Biol 8:4576–4578

Dasso M, Newport JW (1990) Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in *Xenopus*. Cell 61:811–823

Deng T, Li D, Jenh CH, Johnson LF (1986) Structure of the gene for mouse thymidylate synthase. J Biol Chem 261:16000–16005

Deng T, Li Y, Jollif K, Johnson LF (1989) The mouse thymidylate synthase promoter: essential elements are in close proximity to the transcriptional initiation sites. Mol Cell Biol 9:4079–4082

Denhardt DT, Edwards DR, Parfett CLJ (1986) Gene expression during the mammalian cell cycle. Biochim Biophys Acta 865:83–125

DePamphilis ML (1988) Transcriptional elements as components of eukaryotic origins of DNA replication. Cell 52:635–638

Dhar V, Mager D, Igbal A, Schildkraut CL (1988) The coordinate replication of the human β-globin gene domain reflects its transcriptional activity and nuclease hypersensitivity. Mol Cell Biol 8:4958–4965

Dhar V, Skoultchi AI, Schildkraut CL (1989) Activation and repression of a β-globin gene in cell hybrids is accompanied by a shift in its temporal replication. Mol Cell Biol 9:3524–3532

Diffley JFX, Stillman B (1989) Similarity between the transcriptional silencer binding proteins ABF1 and RAP1. Science 246:1034–1038

Dilworth SM, Dingwall C (1988) Chromatin assembly in vitro and in vivo. Bio Essays 9:44–49

Doggett NA, Cordeiro-Stone M, Chae CB, Kaufman DG (1988) Timing of proto-oncogene replication: a possible determination of early S phase sensitivity of C3H 10T1/2 cells to transformation by chemical carcinogens. Mol Carcinogenesis 1:41–49

Downes CS, Musk SRR, Watson JV, Johnson RT (1990) Caffeine overcomes a restriction point associated with DNA replication, but does not accelerate mitosis. J Cell Biol 110:1855–1859

Edenberg HI, Huberman JA (1975) Eukaryotic chromosome replication. Annu Rev Genet 9:245–284

Enoch T, Nurse P (1990) Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell 60:665–673

Enver T, Brewer AC, Patient RK (1988) Role for DNA replication in β-globin gene activation. Mol Cell Biol 8:1301–1308

Epner E, Forrester WC, Groudine M (1988) Asynchronous DNA replication within the human β-globin gene locus. Proc Natl Acad Sci USA 85:8081–8085

Farnham PJ, Means AL (1990) Sequences downstream of the transcription initiation site modulate the activity of the murine dihydrofolate reductase promoter. Mol Cell Biol 10:1390–1398

Farnham PJ, Schimke RT (1985) Transcriptional regulation of mouse dihydrofolate reductase in the cell cycle. J Biol Chem 260:7675–7680

Farnham PJ, Schimke RT (1986a) Murine dihydrofolate reductase transcripts through the cell cycle. Mol Cell Biol 6:365–371
Farnham PJ, Schimke RT (1986b) In vitro transcription and delimitation of promoter elements of the murine dihydrofolate reductase gene. Mol Cell Biol 6:2392–2401
Farnham PJ, Abrams JM, Schimke RT (1985) Opposite-strand RNAs from the 5′ flanking region of the mouse dihydrofolate reductase gene. Proc Natl Acad Sci USA 82:3978–3982
Feder JN, Assaraf YG, Seamer LC, Schimke RT (1989) The pattern of dihydrofolate reductase expression through the cell cycle in rodent and human cultured cells. J Biol Chem 264:20583–20590
Fernandez-Sarabia MJ, Fantes PA (1990) Ribonucleotide reductase and its regulation during the cell cycle. Trends in Genetics 6:275–276
Flemington E, Bradshaw H, Traina-Dorge V, Slagel V, Deininger P (1987) Sequence, structure and promoter characterization of the human thymidine kinase gene. Gene 52:267–277
Fletcher C, Heintz N, Roeder RG (1987) Purification and characterization of OTF1, a transcription factor regulating cell cycle expression of a histone H2b gene. Cell 51:773–781
Fotedar R, Roberts JM (1989) Multistep pathway for replication-dependent nucleosome assembly. Proc Natl Acad Sci USA 86:6459–6463
Fried M, Prives C (1986) The biology of simian virus 40 and polyomavirus. Cancer Cells 4:1–16
Fujii H, Shimada T (1989) Isolation and characterization of cDNA clones derived from the divergently transcribed gene in the region upstream from the human dihydrofolate reductase gene. J Biol Chem 264:10057–10064
Gallinari P, La Bella F, Heintz N (1989) Characterization and purification of H1TF2, a novel CCAAT-binding protein that interacts with a histone H1 subtype specific consensus element. Mol Cell Biol 9:1566–1575
Gick O, Krämer A, Keller W, Birnstiel ML (1986) Generation of histone mRNA 3′ ends by endonucleolytic cleavage of the pre-mRNA in a snRNP-dependent in vitro reaction. EMBO J 5:1319–1326
Gick O, Krämer A, Vasserot A, Birnstiel ML (1987) Heat-labile regulatory factor is required for 3′ processing of histone precursor mRNAs. Proc Natl Acad Sci USA 84:8937–8940
Gilbert DM (1986) Temporal order of replication of Xenopus laevis 5S ribosomal RNA genes in somatic cells. Proc Natl Acad Sci USA 83:2924–2928
Gilbert DM, Cohen SN (1987) Bovine papilloma virus plasmids replicate randomly in mouse fibroblasts throughout S phase of the cell cycle. Cell 50:59–68
Goldman MA (1988) The chromatin domain as a unit of gene regulation. Bioessays 9:50–55
Goldman MA, Holmquist GP, Gray MC, Caston LA, Nag A (1984) Replication timing of genes and middle repetitive sequences. Science 224:686–692
Gottesfeld I, Bloomer LS (1982) Assembly of transcriptionally active 5S RNA gene chromatin in vitro. Cell 28:781–791
Grass DS, Read D, Lewis ED, Manley JL (1987) Cell- and promoter-specific activation of transcription by DNA replication. Genes Dev 1:1065–1074
Graves RA, Pandey NB, Chodchoy N, Marzluff WF (1987) Translation is required for regulation of histone mRNA degradation. Cell 48:615–626
Gross MK, Merrill GF (1988) Regulation of thymidine kinase protein levels during myogenic withdrawal from the cell cycle is independent of mRNA regulation. Nucleic Acids Res 16:11625–11643
Gross MK, Merrill GF (1989) Thymidine kinase synthesis is repressed in nonreplicating muscle cells by a translational mechanism that does not affect the polysomal distribution of thymidine kinase mRNA. Proc Natl Acad Sci USA 86:4987–4991

Gross MK, Kainz MS, Merrill GF (1987) The chicken thymidine kinase gene is transcriptionally repressed during terminal differentiation: the associated decline in TK mRNA cannot account fully for the disappearance of TK enzyme activity. Dev Biol 122:439–451

Groudine M, Casimir C (1984) Post-transcriptional regulation of the chicken thymidine kinase gene. Nucleic Acids Res 12:1427–1446

Gudas JM, Knight GB, Pardee AB (1988) Nuclear posttranscriptional processing of thymidine kinase mRNA at the onset of DNA synthesis. Proc Natl Acad Sci USA 85:4705–4709

Guinta DR, Korn LJ (1986) Differential order of replication of *Xenopus laevis* 5S RNA genes. Mol Cell Biol 6:2536–2542

Handeli S, Klar A, Meuth M, Cedar H (1989) Mapping replication units in animal cells. Cell 57:909–920

Harland RM, Laskey RA (1980) Regulated replication of DNA microinjected into eggs of *Xenopus laevis*. Cell 21:761–771

Hartwell LH, Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. Science 246:629–634

Hatton KS, Dhar V, Brown EH, Iqbal MA, Stuart S, Didamo VT, Schildkraut CL (1988a) Replication program of active and inactive multigene families in mammalian cells. Mol Cell Biol 8:2149–2158

Hatton KS, Dhar V, Gahn TA, Brown EH, Mager D (1988b) Temporal order of replication of multigene families reflects chromosomal location and transcriptional activity. Cancer Cells 6:335–340

Heck MMS, Spradling AC (1990) Multiple replication origins are used during *Drosophila* chorion gene amplification. J Cell Biol 110:903–914

Helms SR, van Wijnen AJ, Kroeger P, Shiels A, Stewart C, Hirshman J, Stein JL, Stein GS (1987) Identification of an enhancer-like element upstream from a cell cycle dependent human H4 histone gene. J Cell Physiol 132:552–558

Herendeen DR, Kassavetis GA, Barry J, Alberts BM, Geiduschek EP (1989) Enhancement of bacteriophage T4 late transcription by components of the T4 DNA replication apparatus. Science 245:952–958

Hofbauer R, Müllner E, Seiser C, Wintersberger E (1987) Cell cycle regulated synthesis of stable mouse thymidine kinase mRNA is mediated by a sequence within the cDNA. Nucleic Acids Res 15:741–752

Hofmann JFX, Laroche T, Brand AH, Gasser SM (1989) RAP-1 factor is necessary for DNA loop formation in vitro at the silent mating type locus HML. Cell 57:725–737

Holmquist GP (1987) Role of replication time in the control of tissue-specific gene expression. Am J Hum Genet 40:151–173

Howard A, Pelc SR (1953) Synthesis of desoxyribonucleic acid in normal and irradiated cells and its relation to chromosomal breakage. Heredity [Suppl] (Lond) 6:261–273

Hutchison CJ, Cox R, Drepaul R, Gomperts M, Ford CC (1987) Periodic DNA synthesis in cell-free extracts of *Xenopus* eggs. EMBO J 6:2003–2010

Imam AAM, Crossley PH, Jackman AL, Little PFR (1987) Analysis of thymidylate synthase gene amplification and of mRNA levels in the cell cycle. J Biol Chem 262:7368–7373

Iqbal MA, Plumb M, Stein J, Stein G, Schildkraut CL (1984) Coordinate replication of members of the multigene family of core and H1 human histone genes. Proc Natl Acad Sci USA 81:7723–7727

Iqbal MA, Chinsky J, Didamo V, Schildkraut CL (1987) Replication of proto-oncogenes early during the S phase in mammalian cell lines. Nucleic Acids Res 15:87–103

Ito M, Conrad SE (1990) Independent regulation of thymidine kinase mRNA and enzyme levels in serum-stimulated cells. J Biol Chem 265:6954–6960

James CD, Leffak M (1986) Polarity of DNA replication through the avian alpha-globin locus. Mol Cell Biol 6:976–984

Jaskulski D, Gatti C, Travali S, Calabretta B, Baserga R (1988) Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. J Biol Chem 263:10175–10179

Jenh CH, Geyer PK, Johnson LF (1985) Control of thymidylate synthase mRNA content and gene transcription in an overproducing mouse cell line. Mol Cell Biol 5:2527–2532

Johnson LF, Rao LG, Muench AJ (1982) Regulation of thymidine kinase enzyme level in serum-stimulated mouse 3T6 fibroblasts. Exp Cell Res 138:79–85

Johnson PF, McKnight SL (1989) Eukaryotic transcriptional regulatory proteins. Annu Rev Biochem 58:799–839

Johnston GC, Singer RA (1983) Growth and the cell cycle of the yeast *Saccharomyces cerevisiae*. I. Slowing S phase or nuclear division decreases the G_1 cell cycle period. Exp Cell Res 149:1–13

Johnston RN, Feder J, Hill AB, Sherwood SW, Schimke RT (1986) Transient inhibition of DNA synthesis results in increased dihydrofolate reductase synthesis and subsequent increased DNA content per cell. Mol Cell Biol 6:3373–3381

Kasid A, Davidson NE, Gelmann EP, Lippman ME (1986) Transcriptional control of thymidine kinase gene expression by estrogen and antiestrogens in MCF-7 human breast cancer cells. J Biol Chem 261:5562–5567

Kaufmann RJ, Sharp PA (1983) Growth dependent expression of dihydrofolate reductase mRNA from modular cDNA genes. Mol Cell Biol 3:1598–1608

Kim YK, Wells S, Lau YFC, Lee AS (1988) Sequences contained within the promoter of the human thymidine kinase gene can direct cell-cycle regulation of heterologous fusion genes. Proc Natl Acad Sci USA 85:5894–5898

Kleinschmidt JA, Seiter S, Zentgraf H (1990) Nucleosome assembly in vitro: separate histone transfer and synergistic interaction of native histone complexes purified from nuclei of *Xenopus laevis* oocytes. EMBO J 9:1309–1318

Knight GB, Gudas JM, Pardee AB (1987) Cell-cycle-specific interaction of nuclear DNA-binding proteins with a CCAAT sequence from the human thymidine kinase gene. Proc Natl Acad Sci USA 84:8350–8354

Kreidberg JA, Kelly TJ (1986) Genetic analysis of the human thymidine kinase gene promoter. Mol Cell Biol 6:2903–2909

Kroeger P, Stewart C, Schaap T, Van Wijnen A, Hirshman J, Helms S, Stein G, Stein J (1987) Proximal and distal regulatory elements that influence in vivo expression of a cell cycle-dependent human H4 histone gene. Proc Natl Acad Sci USA 84:3982–3986

Krysan PJ, Haase SB, Calos MP (1989) Isolation of human sequences that replicate autonomously in human cells. Mol Cell Biol 9:1026–1033

La Bella F, Sive HL, Roeder RG, Heintz N (1988) Cell-cycle regulation of a human histone H2b gene is mediated by the H2b subtype-specific DNA consensus element. Genes Dev 2:32–39

La Bella F, Gallinari P, McKinney J, Heintz N (1989) Histone H1 subtype-specific consensus elements mediate cell cycle-regulated transcription in vitro. Genes Dev 3:1982–1990

Laskey RA, Fairman MP, Blow JJ (1989) S phase of the cell cycle. Science 246:609–614

Lau YF, Kan YW (1984) Direct isolation of the functional human thymidine kinase gene with a cosmid shuttle vector. Proc Natl Acad Sci USA 81:414–418

Lawson R, Cohen P, Lane DP (1990) Simian virus 40 large T-antigen-dependent DNA replication is activated by protein phosphatase 2A in vitro. J Virol 64:2380–2383

Lee M, Nurse P (1988) Cell cycle control genes in fission yeast and mammalian cells. Trends in Genetics 4:287–290

Leffak M, James CD (1989) Opposite replication polarity of the germ line *c-myc* gene in HeLa cells compared with that of two Burkitt lymphoma cell lines. Mol Cell Biol 9:586–593

Leu TH, Hamlin JL (1989) High-resolution mapping of replication fork movement

through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation analysis. Mol Cell Biol 9:523–531

Levine BJ, Chodchoy N, Marzluff WF, Skoultchi A (1987) Coupling of replication type histone mRNA levels to DNA synthesis requires the stem-loop sequence at the 3′ end of the mRNA. Proc Natl Acad Sci USA 84:6189–6193

Lewis JA (1986) Structure and expression of the Chinese hamster thymidine kinase gene. Mol Cell Biol 6:1998–2010

Lewis JA, Matkovich D (1986) Genetic determinants of growth phase-dependent and adenovirus 5-responsive expression of the Chinese hamster thymidine kinase gene are contained within thymidine kinase mRNA sequences. Mol Cell Biol 6:2262–2266

Lewis JA, Shimizu K, Zipser D (1983) Isolation and preliminary characterization of the Chinese hamster thymidine kinase gene. Mol Cell Biol 3:1815–1823

Lieberman HB, Lin PF, Yeh DB, Ruddle FH (1988) Transcriptional and posttranscriptional mechanisms regulate murine thymidine kinase gene expression in serum-stimulated cells. Mol Cell Biol 8:5280–5291

Lin PF, Zhao SY, Ruddle FH (1983) Genomic cloning and preliminary characterization of the human thymidine kinase gene. Proc Natl Acad Sci USA 80:6528–6532

Lin PF, Lieberman HB, Yeh DB, Xu T, Zhao SY, Ruddle FH (1985) Molecular cloning and structural analysis of murine thymidine kinase genomic and cDNA sequences. Mol Cell Biol 5:3149–3156

Linskens MHK, Huberman JA (1988) Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. Mol Cell Biol 8:4927–4935

Linton JP, Yen J-YJ, Selby E, Chen Z, Chinsky JM, Liu K, Kellems RE, Crouse GF (1989) Dual bidirectional promoters at the mouse dhfr locus: cloning and characterization of two mRNA classes of the divergently transcribed *rep-1* gene. Mol Cell Biol 9:3058–3072

Lipson KE, Baserga R (1989) Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific probe. Proc Natl Acad Sci USA 86:9774–9777

Liskay RM, Prescott DM (1978) Genetic analysis of the G_1 period: isolation of mutants (or variations) with a G_1 period from a Chinese hamster cell line lacking G_1. Proc Natl Acad Sci USA 75:2873–2877

Liskay RM, Kornfeld B, Fullerton P, Evans R (1980) Protein synthesis and the presence or absence of a measurable G_1 in cultured Chinese hamster cells. J Cell Physiol 104:461–467

Littlefield IW (1964) Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145:709–712

Lüscher B, Schümperli D (1987) RNA 3′ processing regulates histone mRNA levels in a mammalian cell cycle mutant. A processing factor becomes limiting in G_1-arrested cells. EMBO J 6:1721–1726

Lüscher B, Stauber C, Schindler R, Schümperli D (1985) Faithful cell-cycle regulation of a recombinant mouse histone H4 gene is controlled by sequences in the 3′-terminal part of the gene. Proc Natl Acad Sci USA 82:4389–4393

Ma C, Leu T-H, Hamlin JL (1990) Multiple origins of replication in the dihydrofolate reductase amplicons of a methotrexate-resistant Chinese hamster cell line. Mol Cell Biol 10:1338–1346

Matsui T, Murayama M, Mitra T (1986) Adenovirus 2 peptide IX gene is expressed only on replicated DNA molecules. Mol Cell Biol 6:4149–4154

Matsumoto K, Moriuchi T, Koji T, Nakane PK (1987) Molecular cloning of cDNA coding for rat proliferating cell nuclear antigen (PCNA)/cyclin. EMBO J 6:637–642

McVey D, Brizuela L, Mohr I, Marshak DR, Gluzman Y, Beach D (1989) Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. Nature 341:503–507

McWhinney C, Leffak M (1990) Autonomous replication of a DNA fragment containing the chromosomal replication origin of the human *c-myc* gene. Nucleic Acids Res 18:1233–1242

Means AL, Farnham PJ (1990) Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site. Mol Cell Biol 10:653–661

Merrill GF, Harland RM, Groudine M, McKnight SL (1984) Genetic and physical analysis of the chicken *tk* gene. Mol Cell Biol 4:1769–1776

Mitchell PJ, Carothers AM, Han JH, Harding JD, Kas E, Venolia L, Chasin LA (1986) Multiple transcription start sites, DNAse I-hypersensitive sites, and an opposite-strand exon in the 5′ region of the CHO dhfr gene. Mol Cell Biol 6:425–440

Morris GF, Mathews MB (1989) Regulation of proliferating cell nuclear antigen during the cell cycle. J Biol Chem 264:13856–13864

Murphy AJM, Efstratiadis A (1987) Cloning vectors for expression of cDNA libraries in mammalian cells. Proc Natl Acad Sci USA 84:8277–8281

Murray AW, Kirschner MW (1989) Dominoes and clocks: the union of two views of the cell cycle. Science 246:614–621

Nagarajan M, Johnson LF (1989) Regulation of thymidylate synthase gene expression in mouse fibroblasts synchronized by mitotic selection. Exp Cell Res 181:289–297

Newlon CS (1988) Yeast chromosome replication and segregation. Microbiol Rev 52:568–601

Newport J (1987) Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. Cell 48:205–217

Norbury CJ, Nurse P (1989) Control of the higher eukaryote cell cycle by $p34^{cdc2}$ homologues. Biochim Biophys Acta 989:85–95

Nurse P (1990) Universal control mechanism regulating onset of M-phase. Nature 344:503–508

Okuda A, Cooper S (1989) The continuum model: an experimental and theoretical challenge to the G_1 model of cell cycle regulation. Exp Cell Res 185:1–7

Ottavio L, Chang C-D, Rizzo M-G, Travali S, Casadevall C, Baserga R (1990) Importance of introns in the growth regulation of mRNA levels of the proliferating cell nuclear antigen gene. Mol Cell Biol 10:303–309

Paff MT, Fernandes DJ (1990) Synthesis and distribution of primer RNA in nuclei of CCRF-CEM leukemia cells. Biochemistry 29:3442–3450

Pallas DC, Shahrik LK, Martin BL, Jaspers S, Miller TB, Brautigan DL, Roberts TM (1990) Polyoma small and middle T antigens and SV40 small T antigen form stable complexes with protein phosphatase 2A. Cell 60:167–176

Pandey NB, Marzluff WF (1987) The stem-loop structure at the 3′ end of histone mRNA is necessary and sufficient for regulation of histone mRNA stability. Mol Cell Biol 7:4557–4559

Pardee AB (1989) G_1 events and regulation of cell proliferation. Science 246:603–608

Pauli U, Chrysogelos S, Stein G, Stein J, Nick H (1987) Protein-DNA interactions in vivo upstream of a cell cycle-regulated human H4 histone gene. Science 236:1308–1311

Pauli U, Chrysogelos S, Nick H, Stein G, Stein J (1989) In vivo protein binding sites and nuclease hypersensitivity in the promoter region of a cell cycle regulated human H3 histone gene. Nucleic Acids Res 17:2333–2350

Peltz SW, Ross J (1987) Autogenous regulation of histone mRNA decay by histone proteins in a cell-free system. Mol Cell Biol 7:4345–4356

Peltz SW, Brewer G, Kobs G, Ross J (1987) Substrate specificity of the exonuclease activity that degrades H4 histone mRNA. J Biol Chem 262:9382–9388

Perucho M, Hanahan D, Lipsich L, Wigler M (1980) Isolation of the chicken thymidine kinase gene by plasmid rescue. Nature 285:207–210

Prelich G, Stillman B (1988) Coordinated leading and lagging strand synthesis during SV40 DNA replication in vitro requires PCNA. Cell 53:117–126

Prelich G, Kostura M, Marshak DR, Mathews MB, Stillman B (1987a) The cell-cycle

regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. Nature 326:471–475
Prelich G, Tan CK, Kostura M, Mathews MB, So AG, Downey KM, Stillman B (1987b) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. Nature 326:517–520
Rao PN, Johnson RN (1970) Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature 225:159–164
Reinert I, Holtzer H (eds) (1975) Cell cycle and cell differentiation. Springer, Berlin Heidelberg New York (Results and problems in cell differentiation, vol 7)
Reynolds AE, McCarroll RM, Newlon CS, Fangman WL (1989) Time of replication of ARS elements along yeast chromosomes. Mol Cell Biol 9:4488–4494
Richardson HE, Wittenberg C, Cross F, Reed SI (1989) An essential G_1 function for cyclin-like proteins in yeast. Cell 59:1127–1133
Rizzo MG, Ottavio L, Travali S, Chang C-D, Kaminska B, Baserga R (1990) The promoter of the human proliferating cell nuclear antigen (PCNA) gene is bidirectional. Exp Cell Res 188:286–293
Roberts JM, d'Urso G (1988) An origin unwinding activity regulates initiation of DNA replication during mammalian cell cycle. Science 241:1486–1489
Roberts JM, Weintraub H (1986) Negative control of DNA replication in composite SV40-bovine papilloma virus plasmids. Cell 46:741–752
Roberts JM, Weintraub H (1988) *Cis*-acting negative control of DNA replication in eukaryotic cells. Cell 52:397–404
Ross J, Kobs G (1986) H4 histone messenger RNA decay in cell-free extracts initiates at or near the 3′ terminus and proceeds 3′ to 5′. J Mol Biol 188:579–593
Ross J, Peltz SW, Kobs G, Brewer G (1986) Histone mRNA degradation in vivo: the first detectable step occurs at or near the 3′ terminus. Mol Cell Biol 6:4362–4371
Ross J, Kobs G, Brewer G, Peltz SW (1987) Properties of the exonuclease activity that degrades H4 histone mRNA. J Biol Chem 262:9374–9381
Sazer S, Schimke RT (1986) A re-examination of the 5′ termini of mouse dihydrofolate reductase RNA. J Biol Chem 261:4685–4690
Schilling LJ, Farnham PJ (1989) Identification of a new promoter upstream of the murine dihydrofolate reductase gene. Mol Cell Biol 9:4568–4570
Schimke RT (1988) Gene amplification in cultured cells. J Biol Chem 263:5989–5992
Schlosser CA, Steglich C, de Wet JR, Scheffler I (1981) Cell cycle-dependent regulation of thymidine kinase activity introduced into mouse $LMTK^-$ cells by DNA and chromatin-mediated gene transfer. Proc Natl Acad Sci USA 78:1119–1123
Schmidt EE, Merrill GF (1989) Transcriptional repression of the mouse dihydrofolate reductase gene during muscle cell commitment. J Biol Chem 264:21247–21256
Schümperli D (1988) Multilevel regulation of replication-dependent histone genes. Trends in Genetics 4:187–191
Seiler-Tuyns A, Paterson BM (1987) Cell cycle regulation of a mouse histone H4 gene requires the H4 promoter. Mol Cell Biol 7:1048–1054
Seiser C, Wintersberger E (1989) Mouse thymidine kinase: sequence of a processed pseudogene. Nucleic Acids Res 17:2128
Seiser C, Knöfler M, Rudelstorfer I, Haas R, Wintersberger E (1989) Mouse thymidine kinase: the promoter sequence and the gene and pseudogene structures in normal cells and in thymidine kinase deficient mutants. Nucleic Acids Res 17:185–195
Seiser C, Beck G, Wintersberger E (1990) The processed pseudogene of mouse thymidine kinase is acive after transfection. FEBS Lett 270:123–126
Sheenan MA, Mills AD, Sleeman AM, Laskey RA, Blow JJ (1988) Steps in the assembly of replication-competent nuclei in a cell-free system from *Xenopus* eggs. J Cell Biol 106:1–12
Sherley JL, Kelly TJ (1988) Regulation of human thymidine kinase during the cell cycle. J Biol Chem 263:8350–8358

Singer RA, Johnston GC (1983) Growth and the cell cycle of the yeast *Saccharomyces cerevisiae*. II. Relief of cell-cycle constraints allows accelerated cell divisions. Exp Cell Res 149:15–26

Sive HL, Heintz N, Roeder RG (1986) Multiple sequence elements are required for maximal in vitro transcription of a human histone H2B gene. Mol Cell Biol 6:3329–3340

Smith S, Stillman B (1989) Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. Cell 58:15–25

Soldati D, Schümperli D (1988) Structural and functional characterization of mouse U7 small nuclear RNA active in 3′ processing of histone pre-mRNA. Mol Cell Biol 8:1518–1524

Stark GR, Debatisse M, Giulotto E, Wahl GM (1989) Recent progress in understanding mechanisms of mammalian DNA amplification. Cell 57:901–908

Stauber C, Lüscher B, Eckner R, Lötscher E, Schümperli D (1986) A signal regulating mouse histone H4 mRNA levels in a mammalian cell cycle mutant and sequences controlling RNA 3′ processing are both contained within the same 80-bp fragment. EMBO J 5:3297–3303

Stein G, Lian J, Stein J, Briggs R, Shalhoub V, Wright K, Pauli U, van Wijnen A (1989) Altered binding of human histone gene transcription factors during the shutdown of proliferation and onset of differentiation in HL-60 cells. Proc Natl Acad Sci USA 86:1865–1869

Stein GS, Stein JL (1984) Is histone gene expression autogenously regulated? Mol Cell Biochem 64:105–110

Stewart CJ, Ito M, Conrad SE (1987) Evidence for transcriptional and post-transcriptional control of the cellular thymidine kinase gene. Mol Cell Biol 7:1156–1163

Stillman B (1986) Chromatin assembly during SV40 DNA replication in vitro. Cell 45:555–565

Stillman B (1989) Initiation of eukaryotic DNA replication in vitro. Annu Rev Cell Biol 5:197–245

Stuart P, Ito M, Stewart C, Conrad SE (1985) Induction of cellular thymidine kinase occurs at the mRNA level. Mol Cell Biol 5:1490–1497

Sun JM, Wiaderkiewicz R, Ruiz-Carrillo A (1989) Histone H5 in the control of DNA synthesis and cell proliferation. Science 245:68–71

Svaren J, Chalkley R (1990) The structure and assembly of active chromatin. Trends in Genetics 6:52–56

Taljanidisz J, Popowski J, Sarkar N (1989) Temporal order of gene replication in Chinese hamster ovary cells. Mol Cell Biol 9:2881–2889

Taylor JH (1984) Origins of replication and gene replication. Mol Cell Biochem 61:99–109

Tazi J, Bird A (1990) Alternative chromatin structure of CpG islands. Cell 60:909–920

Travali S, Lipson KE, Jaskulski D, Lauret E, Baserga R (1988) Role of the promoter in the regulation of the thymidine kinase gene. Mol Cell Biol 8:1551–1557

Travali S, Ku DH, Rizzo MG, Ottavio L, Baserga R, Calabretta B (1989) Structure of the human gene for the proliferating cell nuclear antigen. J Biol Chem 264:7466–7472

Trempe JP, Lindstrom YI, Leffak M (1988) Opposite replication polarities of transcribed and nontranscribed histone H5 genes. Mol Cell Biol 8:1657–1663

Umek RM, Linskens MHK, Kowalski D, Huberman JA (1989) New beginnings in studies of eukaryotic DNA replication origins. Biochim Biophys Acta 1007:1–14

Urlaub G, Mitchell PJ, Ciudad CJ, Chasin LA (1989) Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. Mol Cell Biol 9:2868–2880

Van Wijnen A, Stein JL, Stein GS (1987) A nuclear protein with affinity for the 5′ flanking region of a cell cycle dependent human H4 histone gene in vitro. Nucleic Acids Res 15:1679–1698

Van Wijnen AJ, Wright KL, Lian JB, Stein JL, Stein GS (1989) Human H4 histone gene transcription requires the proliferation-specific nuclear factor HiNF-D. J Biol Chem 264:15034–15042

Vasserot AP, Schaufele FJ, Birnstiel M (1989) Conserved terminal hairpin sequences of histone mRNA precursors are not involved in duplex formation with the U7 RNA but act as a target site for a distinct processing factor. Proc Natl Acad Sci USA 86:4345–4349
Venkatesh LK, Chinnadurai G (1987) Activation of the adenovirus 2 protein IX promoter by DNA replication in a transient expression assay. Nucleic Acids Res 15:2235–2250
Virshup DM, Kelly TJ (1989) Purification of replication protein C, a cellular protein involved in the initial stages of simian virus 40 DNA replication in vitro. Proc Natl Acad Sci USA 86:3584–3588
Virshup DM, Kauffman MG, Kelly TJ (1989) Activation of SV40 DNA replication in vitro by cellular protein phosphatase 2A. EMBO J 8:3891–3898
Von Meyenburg K, Boye E, Skarstad K, Koppes L, Kogoma T (1987) Mode of initiation of constitutive stable DNA replication in RNase H-defective mutants of *Escherichia coli* K-12. J Bacteriol 169:2650–2658
Wahl AF, Geis AM, Spain BH, Wong SW, Korn D, Wang TSF (1988) Gene expression of human DNA polymerase α during cell proliferation and the cell cycle. Mol Cell Biol 8:5016–5025
Wawra E. Pöckl E, Müllner E, Wintersberger E (1981) Effect of sodium butyrate on induction of cellular and viral DNA synthesis in polyoma virus-infected mouse kidney cells. J Virol 38:973–981
Weinert TA, Hartwell LH (1988) The *RAD 9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science 241:317–322
Weintraub H (1985) Assembly and propagation of repressed and derepressed chromosomal states. Cell 42:705–711
Weichselbraun I, Ogris E, Wintersberger E (1990) Bidirectional promoter activity of the 5′ flanking region of the mouse Thymidine kinase gene. FEBS Lett 275:48–52
Wintersberger E (1990) Control of gene expression during cell cycle and growth of mammalian cells. In: Paukovits WR (ed) Growth regulation and carcinogenesis. Vol I. CRC Uniscience Boca Raton, pp 13–30
Wintersberger U (1984) The selective advantage of cancer cells: a consequence of genome mobilization in the course of the induction of DNA repair processes? (Model studies on yeast). Adv Enzyme Regul 22:311–323
Wintersberger U, Wintersberger E (1987) RNA makes DNA: a speculative view of the evolution of DNA replication mechanisms. Trends in Genetics 3:198–202
Wolffe AP, Brown DD (1986) DNA replication in vitro erases a *Xenopus* 5S RNA gene transcription complex. Cell 47:217–227
Wolffe AP, Brown DD (1988) Developmental regulation of two 5S ribosomal RNA genes. Science 241:1626–1632
Wong SW, Wahl AF, Yuan PM, Arai N, Pearson BE, Arai KI, Korn D, Hunkapiller MW, Wang TSF (1988) Human DNA polymerase α gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. EMBO J 7:37–47
Worcel A, Han S, Wong ML (1978) Assembly of newly replicated chromatin. Cell 15:969–977
Wright JA, Smith HS, Watt FM, Hancock MC, Hudson DL, Stark GR (1990) DNA amplification is rare in normal human cells. Proc Natl Acad Sci USA 87:1791–1795
Yen JY, Kellems RE (1987) Independent 5′- and 3′-end determination of multiple dihydrofolate reductase transcripts. Mol Cell Biol 7:3732–3739
Zelenin AV, Prudovsky IA (1989) Regulation of DNA synthesis investigated in heterokaryons of dividing and nondividing cells. Int Rev Cytol 117:179–214
Zmudzka BZ, Fornace A, Collins J, Wilson SH (1988) Characterization of DNA polymerase β mRNA: cell-cycle and growth response in cultured human cells. Nucleic Acids Res 16:9587–9596

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Synergism in the Control of Force and Movement of the Forearm

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Contents

1 Introduction

Until recently there seemed to be general agreement in the literature that the motor nucleus together with the muscle it innervates behaved as a functional unit: one single activation parameter (i.e., total synaptic drive) would account for the activity of the motoneuron pool as a whole. The activity of an individual motoneuron was believed to be determined by this activation parameter and by size-related properties of that motoneuron. Motoneurons were recruited in an orderly way according to the size principle (Henneman 1957, 1980; Henneman and Mendell 1981), and at recruitment each motoneuron started firing with an initial frequency typical for that neuron. A further increase in the activation parameter would increase the motoneuron firing frequency in a reproducible way until saturation occurred.

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Recent investigations indicate that the notion as outlined above needs to be readjusted. Increasing evidence has become available contradicting the view that individual muscles should be regarded as functional units in the control of the motor apparatus. Ter Haar Romeny et al. (1982) found that the relative activity of motoneurons in the m. biceps brachii was task-dependent. This clearly indicated the presence of more than one control or activation parameter for the motoneuron pool of the m. biceps brachii. This finding was deepened and elaborated in a number of follow-up studies which revealed among other things that the firing frequency at recruitment and at decruitment is often quite different. Also, the firing frequency at recruitment does not seem to be fixed, but seems to depend on the task. These studies are summarized in this review.

The results agree with some other recent findings and concepts presented in the literature. For instance, Loeb (1985) has proposed the concept of task groups as a functional compartmentalization of the motor apparatus during locomotion. This view is supported by results found in the cat. The activity of the biarticular anterior sartorius muscle during the stance phase (active lengthening) and the swing phase (active shortening) occurs in completely different groups of motor units (Hoffer et al. 1980, 1987). In addition, Kandou and Kernell (1989) have reported that the activity of the peroneus longus muscle is distributed differently over its anterior and posterior part after stimulation at different locations (motor cortex versus superficial peroneal nerve). Windhorst et al. (1989) have even proposed a combined sensory and neuromuscular partitioning, such that segmental control mechanisms are based on subdivisions of motoneuron pool – muscle complexes. Size-related recruitment would occur within these subdivisions rather than in the motoneuron pool as a whole.

Because of the inhomogeneous activation of motoneuron pools, the organization of muscle coordination cannot be based on the principle that whole muscles are the functional units of control. The classification of muscles is merely an anatomical concept. In this paper we will pursue this matter further by examining the coordination of muscles acting around the elbow joint. This will be done by studying single-unit electromyograms (EMGs) rather than surface EMGs. The latter method is too gross to assess the recruitment and firing frequency behavior of individual motor units and to reveal possible inhomogeneities in muscle activation. The elbow-joint configuration together with the most important forearm and upper-arm muscles are schematically drawn in Fig. 1.

In order to gain more insight into concepts that underlie the coordination of the elbow muscles, we will describe and compare the recruitment and firing frequency behavior of motor units under different conditions: (a) isometric tasks in which forces have to be exerted in

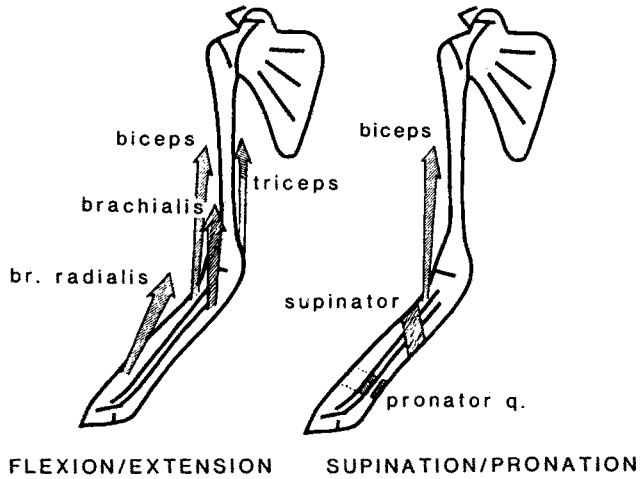


Fig. 1. Schematic anatomical representation of six major muscles acting in flexion/extension and pronation/supination direction. (From Jongen et al. 1989a)

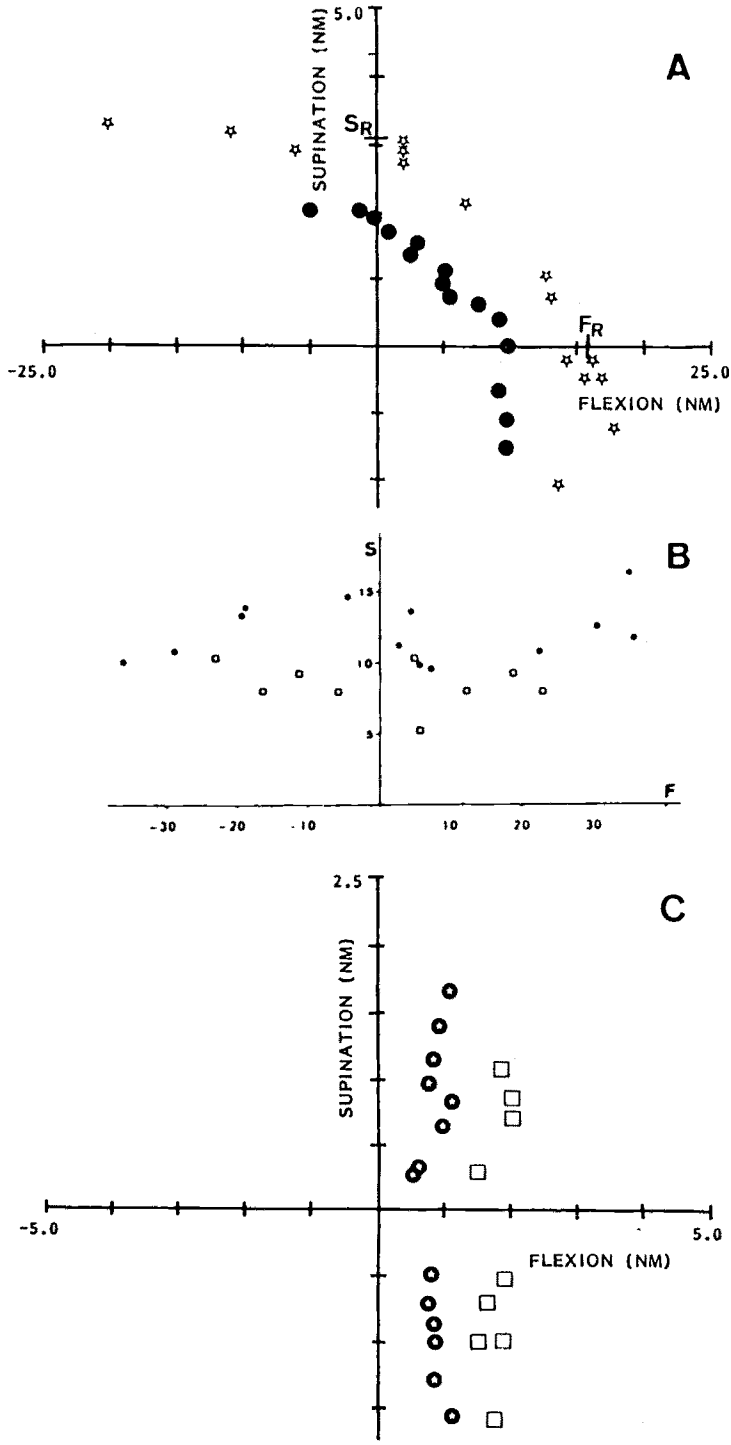
different directions, (b) isometric contraction versus isometric relaxation, (c) reflexively induced recruitment, and (d) force tasks versus movement tasks. On the basis of the experimental evidence some coordination concepts as well as some theoretical models will be discussed.

2 Inhomogeneous Activation of Human Arm Muscles

In this chapter we will discuss the activation of motor units in a number of muscles in the human arm. The recruitment behavior of motor units was studied as a function of isometric torque in the directions of flexion/extension and supination/pronation. Subjects were instructed to increase isometric torque in one direction up to a particular level and then to increase isometric torque in another direction while keeping the torque in the first direction constant.

2.1 Activation and Recruitment Order of Motor Units in the Musculus Biceps

Figure 2 shows examples of motor-unit behavior in m. biceps brachii (caput longum). Motor units with a recruitment behavior such as is shown in Fig. 2A are recruited when isometric torque in the flexion direction


A
5.0
SUPINATION (NM)
SR
FR
-25.0
25.0
FLEXION (NM)
B
S
15
10
5
F
-30
-20
-10
10
20
30
C
2.5
SUPINATION (NM)
-5.0
5.0
FLEXION (NM)

exceeds a particular threshold. For these motor units the activation is not changed if a torque in the supination or pronation direction is superimposed on the flexion torque. Motor units, the recruitment of which is shown in Fig. 2B, are recruited only when a particular torque in the supination direction is exceeded. The activation of these motor units is not affected by the amount of torque in the flexion or extension direction.

By far the majority of motor units in m. biceps brachii (caput longum) demonstrated the recruitment behavior shown in Fig. 2C. For torques in the flexion and supination directions, the motor units are recruited when a linear combination of torques in the flexion and supination directions exceeds a particular threshold. Extension torques do not affect the recruitment threshold of these motor units significantly, nor do pronation torques.

The three motor units that show the recruitment behavior illustrated in Fig. 2 can be considered to belong to three different subpopulations. The motor units in a subpopulation have different recruitment thresholds, but in all other respects the recruitment behavior is the same. This is illustrated in Fig. 2 where the recruitment thresholds of motor units of the same type fall along parallel straight lines with junctions at or near the horizontal or vertical axes. This is more clearly illustrated in Fig. 3, where the recruitment thresholds for flexion and supination are plotted along the horizontal and vertical axes, respectively. Data from motor units (all of the type shown in Fig. 2C) of different subjects are pooled. All data seem to fall along a straight line, indicating that the ratio of recruitment thresholds in the flexion and supination directions is constant for all motor units.

Within a subpopulation the recruitment order for isometric contractions is fixed. Moreover, no significant differences in firing rate and recruitment order were observed for various torque directions. The results

Fig. 2A-C. Examples of motor unit behavior recorded in the m. biceps. Each point indicates the combination of torques at which the motor unit was recorded. Different symbols indicate different motor units. **A** Motor units which are recruited when torques in both flexion and supination direction are exerted. Such motor units are called "summing units". *Closed symbols* represent results from a unit recorded in the long head of the m. biceps, *open symbols* represent results from a motor unit recorded in the short head. For the latter motor unit the recruitment thresholds for flexion (F_R) and supination (S_R) are indicated. These results were obtained at an elbow angle of 110°. (From van Zuylen et al. 1988a). **B** Motor units which are recruited only when a particular torque in supination direction is exerted. Such motor units are called "supination units". These results were obtained at an elbow angle of 110°. (From ter Haar Romeny et al. 1984). **C** Motor units from the long head of the m. biceps which are recruited only when a particular torque in flexion direction is exerted. Such motor units are called "flexion units". These results were obtained at an elbow angle of 100° (180° is full extension). (From van Zuylen et al. 1988a)

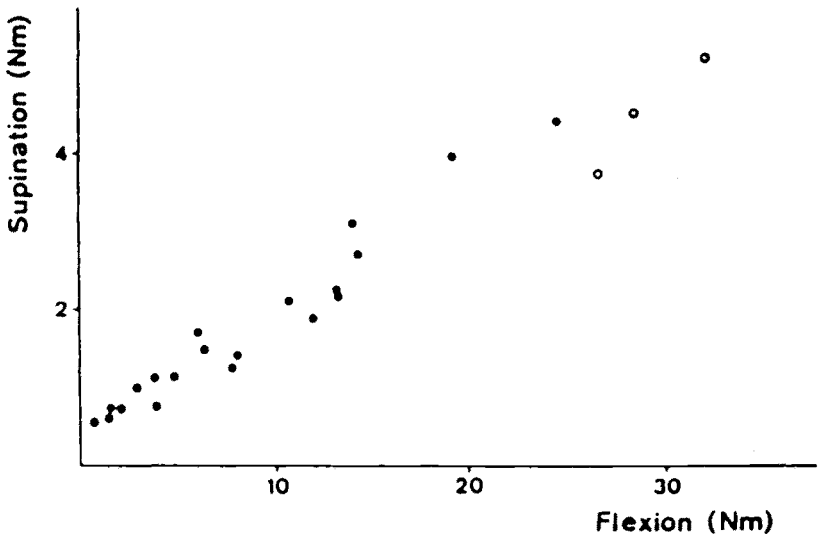


Fig. 3. Recruitment thresholds for supination as a function of recruitment thresholds for flexion of "summing units". Motor units were recorded in the long head (*filled circles*) and in the short head (*open circles*) of the m. biceps. Results of different experiments and different subjects are pooled. (From ter Haar Romeny et al. 1984)

suggest homogeneous activation of motor units in a given subpopulation. These data are also in agreement with the notion of the "size principle" as originally proposed by Henneman (1957, 1981). The size principle assumes that the order of recruitment is related to the size of the soma of each motoneuron. Although some evidence has been presented that factors other than the size of the soma of the motoneuron may be relevant (see Gustafsson and Pinter 1985) the "size principle" is still one of the main hypotheses on which current research on muscle activation is based.

The data in Fig. 2 demonstrate that the recruitment order within a muscle is not necessarily fixed. For example, the motor units of the type shown in Fig. 2A are recruited for flexion torques, but not for supination torques, whereas the motor units of the type shown in Fig. 2B are recruited if one starts by exerting a supination torque.

Motor units of a particular subpopulation are not scattered randomly in the muscle but seem to be grouped together. Motor units from which activity can be recorded at the same site generally show similar behavior, whereas recordings from different sites may reveal clearly different behavior. An example of this finding is shown in Fig. 4. Intramuscular wire electrodes were inserted in the lateral and medial side of the long head of m. biceps branchii. With the increasing isometric torque in the

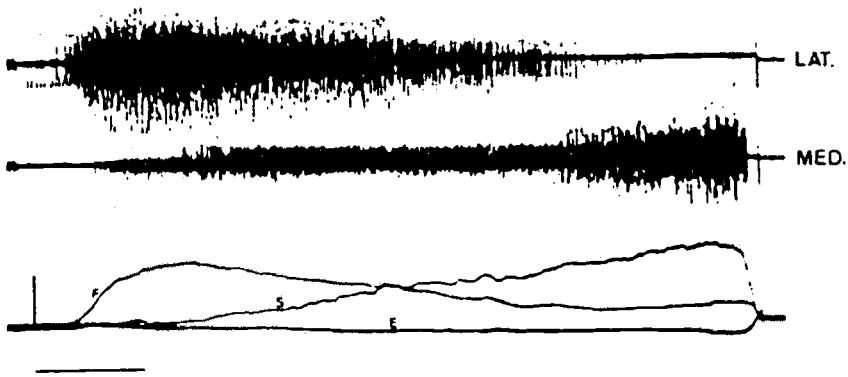


Fig. 4. Firing behavior of motor units located in the lateral (*LAT*) and the medial (*MED*) sites of the long head of the m. biceps. In this experiment the force exerted in the flexion direction (*F*) was interchanged with a force in the supination direction (*S*) while the force in the exorotation direction (*E*; i.e., the rotation of the humerus) was kept as low as possible. Time calibration: 2 s; torque calibration: 2.7 Nm flexion, 0.6 Nm supination, 0.6 Nm exorotation. (From ter Haar Romeny et al. 1984)

flexion direction, the activity in the electrode at the lateral side increased, whereas the activity in the medial electrode was relatively small. When the isometric flexion torque was decreased with simultaneous increase of isometric supination, the activity gradually decreased in the lateral recording, but increased in the medial recording.

Since motor units with different task-dependent activation were located in different regions of the muscle, the question that arose was whether the inhomogeneous activation might be related to differences in the mechanical advantage of motor units in different parts of the muscle. The twitch torque in the flexion and supination directions was obtained by spike-triggered averaging (for a detailed description of this techique, see ter Haar Romeny et al. 1984). Although motor units revealed twitches of different amplitude, the ratio of amplitudes for twitches in the flexion and supination directions was approximately constant for all motor units and did not depend on the recording site or on the subpopulation to which the motoneuron belonged. The electrodes were also used for electrical stimulation of small parts of the muscle. Independent of the location of the stimulation wires (lateral or medial), the ratio of amplitudes of the electrically induced twitches in the flexion and supination directions was the same. This indicates that all motor units in m. biceps have the same mechanical advantage relative to the elbow. Therefore, the existence of motor-unit subpopulations in the long head of m. biceps brachii reflects a neural organization rather than differences in the mechanical effect of motor units.

Summarizing, the results presented in this section have shown an inhomogeneous activation of the population of motor units in m. biceps brachii (caput longum.) Depending on the task (isometric flexion or isometric supination) different groups of motor units may be active. Due to this inhomogeneous activation, the recruitment order within a muscle is not fixed, but is task-dependent. Within a subpopulation there is a fixed recruitment order. The long head of m. biceps cannot be considered as one functional neuromuscular unit because of the task-dependent activation of different subpopulations of motor units. This muscle, therefore, probably consists of at least three functional subunits. It appears that a muscle is only an anatomical concept and that an understanding of the coordination of muscle-activation patterns requires a more detailed analysis of the activation patterns of motor-unit subpopulations.

2.2 Coordination of the Activation of Subpopulations in Various Muscles in Isometric Contractions

Recently, van Zuylen et al. (1988a) showed that subpopulations of motor units, each with a different recruitment behavior, exist in almost all arm muscles. Therefore, subpopulations exist not only in muscles where the mechanical advantage is such that the muscle contributes to different tasks (e.g., flexion and supination), but also in muscles that contribute to flexion or extension only. For a detailed survey of the various subpopulations in other human arm muscles and their recruitment behavior, see van Zuylen et al. (1988a).

Figure 5 shows the recruitment behavior for two motor units from a particular subpopulation in m. triceps. The recruitment threshold of these motor units depends on the torque in the extension direction as well as on the torques in the supination and pronation directions. At first sight, it may seem strange that a muscle which has a mechanical advantage in the extension direction only is also activated for torques in the supination direction. However, this can easily be understood if one recalls that m. biceps, which is activated for torques in the supination direction, contributes to torque in the flexion direction. In order to obtain a torque in the supination direction only, the contribution to the flexion torque of m. biceps has to be compensated by m. triceps. This example illustrates that the activation of a muscle cannot be understood from the mechanical advantage of that single muscle, but that the contribution of all muscles acting across the joint has to be taken into account.

Another example which illustrates that the activatioin of a muscle cannot be understood simply from the mechanical advantage of that

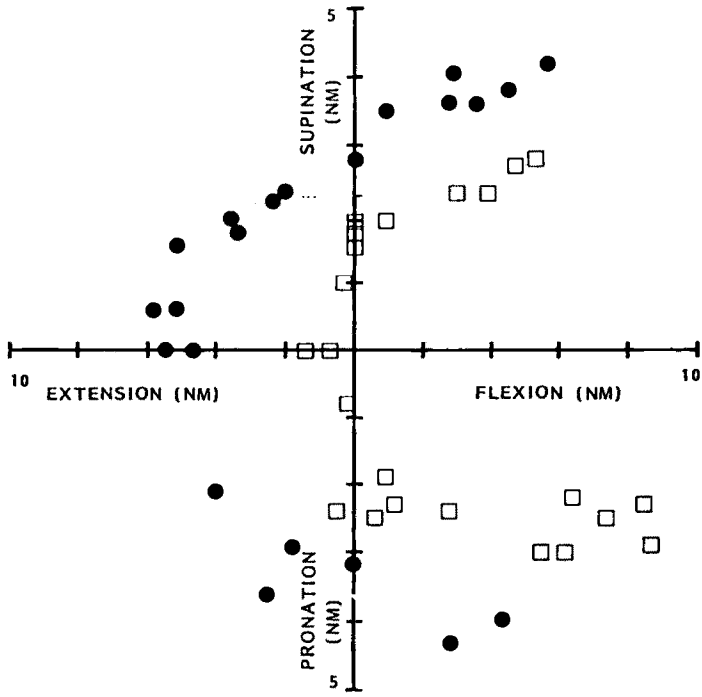
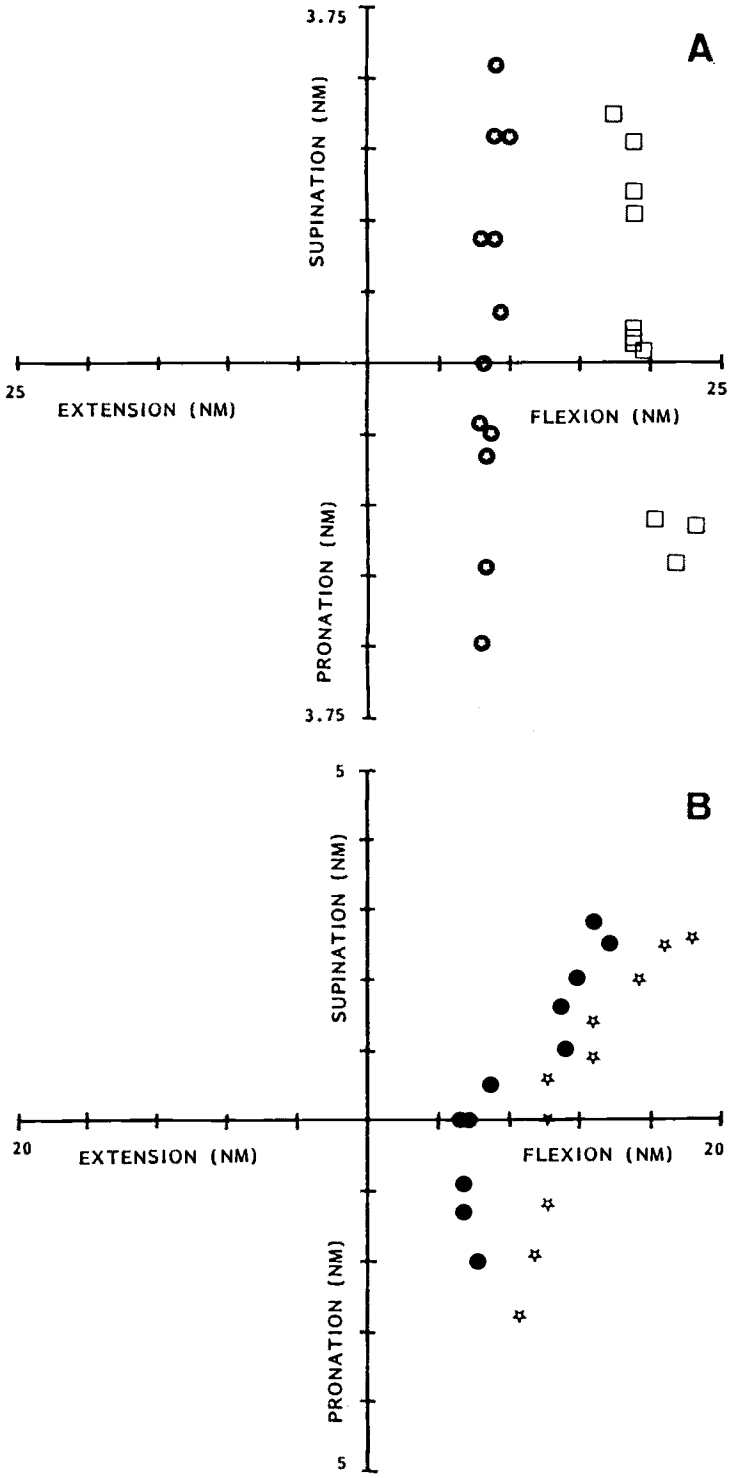


Fig. 5. Recruitment behavior of motor units in the m. triceps. Motor units were activated during extension, supination and pronation torques. Flexion increased the recruitment threshold for supination, but hardly affected the recruitment threshold for pronation. Motor units were recorded in the long head (*filled circles*) and in the lateral head (*open squares*) of the m. triceps. Recordings were obtained at an elbow angle of 100°. (From van Zuylen et al. 1988a)

single muscle is shown in Fig. 6. For one subpopulation of motor units in m. brachialis the recruitment data fall along a vertical line (Fig. 6A): the recruitment threshold depends on the torque in the flexion direction only. For the other subpopulation the recruitment data fall along oblique lines (Fig. 6B): the recruitment threshold in the flexion direction increases when torque in the supination direction is increased. This can be understood in the same way as the activation of m. triceps for torques in the supination direction. When supination torque increases at a constant flexion torque, the activation of m. biceps increases. This contributes an additional torque component in the flexion direction which can be cancelled either by activating m. triceps, or by decreasing the activation of m. brachialis. The latter may explain the oblique recruitment lines for flexion and supination torques for m. brachialis.

An important question with regard to the subpopulations of motor units is whether motor units can indeed be grouped in a few sub-


A
3.75
SUPINATION (NM)
25
EXTENSION (NM)
FLEXION (NM)
25
PRONATION (NM)
3.75
B
5
SUPINATION (NM)
20
EXTENSION (NM)
FLEXION (NM)
20
PRONATION (NM)
5

populations, each with a distinct homogeneous activation, or whether the recruitment behavior of motor units in a muscle shows a broad distribution; if the latter explanation is the correct one, this would argue against discrete subpopulations and favor a continuous spectrum of motor unit behavior. This was investigated by calculating the slope of the line that fits the recruitment data for torques in the flexion, supination, pronation, and extension directions.

Discrete sets of motor units are clearly found in m. biceps. Either the data fall along vertical lines (see Fig. 2A; angle of 90°), or along horizontal lines (see Fig. 2B; angle of 0°), or along oblique lines (see Fig. 2C; angle of 170°). The latter results are summarized in Fig. 3 by plotting the intercepts of the recruitment lines for these motor units.

For torques in the flexion and supination directions the motor-unit behavior in m. brachialis clearly shows a bimodal, rather than a unimodal distribution (Fig. 7). However, if one accepts the suggestion of two subpopulations of motor units, the motor-unit properties do not have a narrow-peaked distribution. Rather, the data suggest some variation in motor-unit properties within each subpopulation.

A rather wide distribution of motor-unit properties is certainly borne out by the data for m. triceps. Some motor units could not be recruited by torques in the pronation direction. However, for the motor units that could be recruited by torques in the pronation direction the slopes could be quite different.

In order to investigate the effect of joint angle on the recruitment behavior of motor units, the recruitment thresholds were determined at various elbow-joint angles. For all motor units in a particular subpopulation, the recruitment behavior changed as a function of elbow-joint angle in the same way. However, we never observed that a motor unit classified in a particular subpopulation at a given elbow angle had to be classified in another subpopulation for another elbow angle.

An example of the effect of elbow-joint angle on the recruitment behavior of motor units in m. biceps and m. brachialis is shown in Fig. 8. For motor units in m. biceps the recruitment threshold for supination is about the same for all elbow-joint angles. However, the recruitment

Fig. 6A,B. Recruitment behavior of two types of motor units in the m. brachialis. Different symbols represent data from different motor units. **A** Recruitment threshold for flexion is independent of exerted supination or pronation torques. Recordings were obtained at an elbow angle of 90°. **B** Recruitment threshold for flexion is independent of exerted supination, but depends on exerted pronation torques. Recordings were obtained at an elbow angle of 100°. (From van Zuylen et al. 1988a)

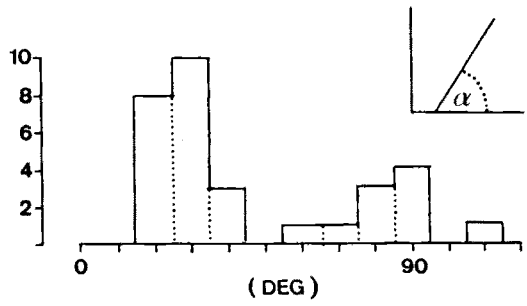


Fig. 7. Histogram for the number of motor units found with a particular slope for the line fitted to the recruitment thresholds for m. brachialis. The angle α refers to the angle of the line with the flexion/supination quadrant. (From van Zuylen et al. 1988a)

threshold for flexion decreases for more extended elbow-joint angles. A decrease in recruitment threshold is in agreement with the fact that the mechanical advantage and exerted force of all flexor muscles decrease for larger elbow angles (van Zuylen et al. 1988b). Therefore, the same activation will result in a smaller torque at the elbow joint and motor units will be recruited at lower thresholds. A decrease in recruitment threshold for flexion is observed for motor units in m. brachialis and m. brachioradialis, just as it was in m. biceps.

3 Examples of Task-Dependent Coordination of Arm Muscles

In Sect. 2 we showed that subpopulations of motor units, each with a different task-dependent homogeneous activation, exist in several arm muscles. Within such a subpopulation motor units are recruited in the same order, irrespective of the direction of contraction. The motor units are characterized by the force level at which they are recruited (the recruitment threshold) during a voluntary isometric contraction task and by the frequency of firing at recruitment (the initial firing frequency). In this section we will show that the recruitment threshold and the initial firing frequencies are different for different types of tasks. For instance, the characteristics of motor units change when we ask subjects to relax isometrically or to move their arm. As a result of these changes contributions of different muscles to a particular force must be task-dependent. We will give various examples of task-dependent coordination of arm muscles and discuss possible implications for central control of muscle force.

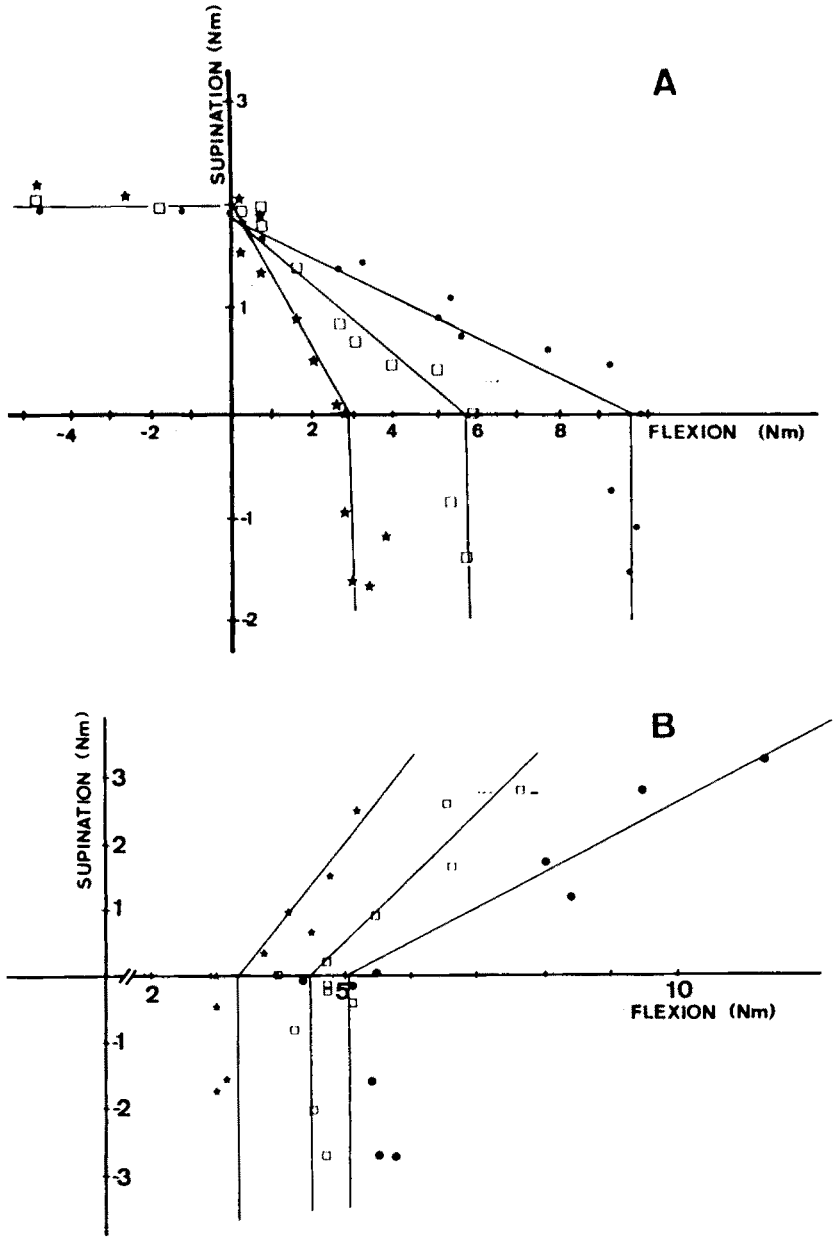


Fig. 8A,B. Recruitment thresholds as a function of elbow angle. **A** Motor unit located in the long head of the m. biceps. Each data point indicates the threshold for a combination of forces in flexion/extension and supination/pronation. Different symbols refer to different elbow angles: 100°, *dots*; 145°, *squares*; 175°, *asterisks*. **B** Motor unit located in the m. brachialis. Different symbol refer to different elbow angles: 100°, *dots*; 135°, *squares*; 170°, *asterisks*. (From Gielen and van Zuylen 1986)

3.1 Isometric Relaxation

Denier van der Gon et al. (1985) investigated the behavior of motor units during slow isometric contraction and relaxation of arm muscles. Recruitment thresholds and initial firing frequencies were studied for motor units in m. biceps brachii (caput longum), in m. brachialis and in m. supinator while subjects were performing flexion of the elbow or supination of the forearm.

Figure 9 shows the results for motor units located at medial, central, and lateral sites in the long head of the m. biceps, in the m. brachialis and in the m. supinator. The end points of each line in this figure represent the average behavior of motor units at recruitment or decruitment obtained in two to five experiments. The upper points indicate recruitment thresholds together with their accompanying initial firing frequencies. The lower points indicate decruitment thresholds together with the accompanying final firing frequencies. The figure shows that firing frequencies at recruitment were always higher than firing frequencies at decruitment. However, differences between recruitment and decruitment thresholds depended on the location of the motor units. The recruitment thresholds were higher than the decruitment thresholds for units at the medial and central sites of the long head of the m. biceps during flexion as well as during supination, but they were lower for motor units located in the m. brachialis and in the m. supinator. The lateral site of the m. biceps showed no significant difference in the two force thresholds.

The different behavior of motor units with respect to their threshold and the similar behavior with respect to their firing frequency suggest that the relative contribution of synergists to a task differs for isometric contraction and isometric relaxation. During contraction, m. brachialis and m. supinator contribute more to flexion and supination, respectively, than during relaxation. An interesting question is whether this behavior reflects different activation of motoneurons of synergists involved in the same task, or whether properties intrinsic to motor units can provide an explanation for the experimental results. We will address this question in Sect. 3.2.

3.2 Slow Movement

A clear example of task-dependent coordination of arm muscles was found in slow-movement tasks. The behavior of synergistic elbow flexor muscles in such tasks was investigated in a series of experiments (Tax et al. 1989, 1990a, b). Recruitment thresholds and initial firing frequencies of motor units in m. biceps, m. brachialis, and m. branchioradialis were

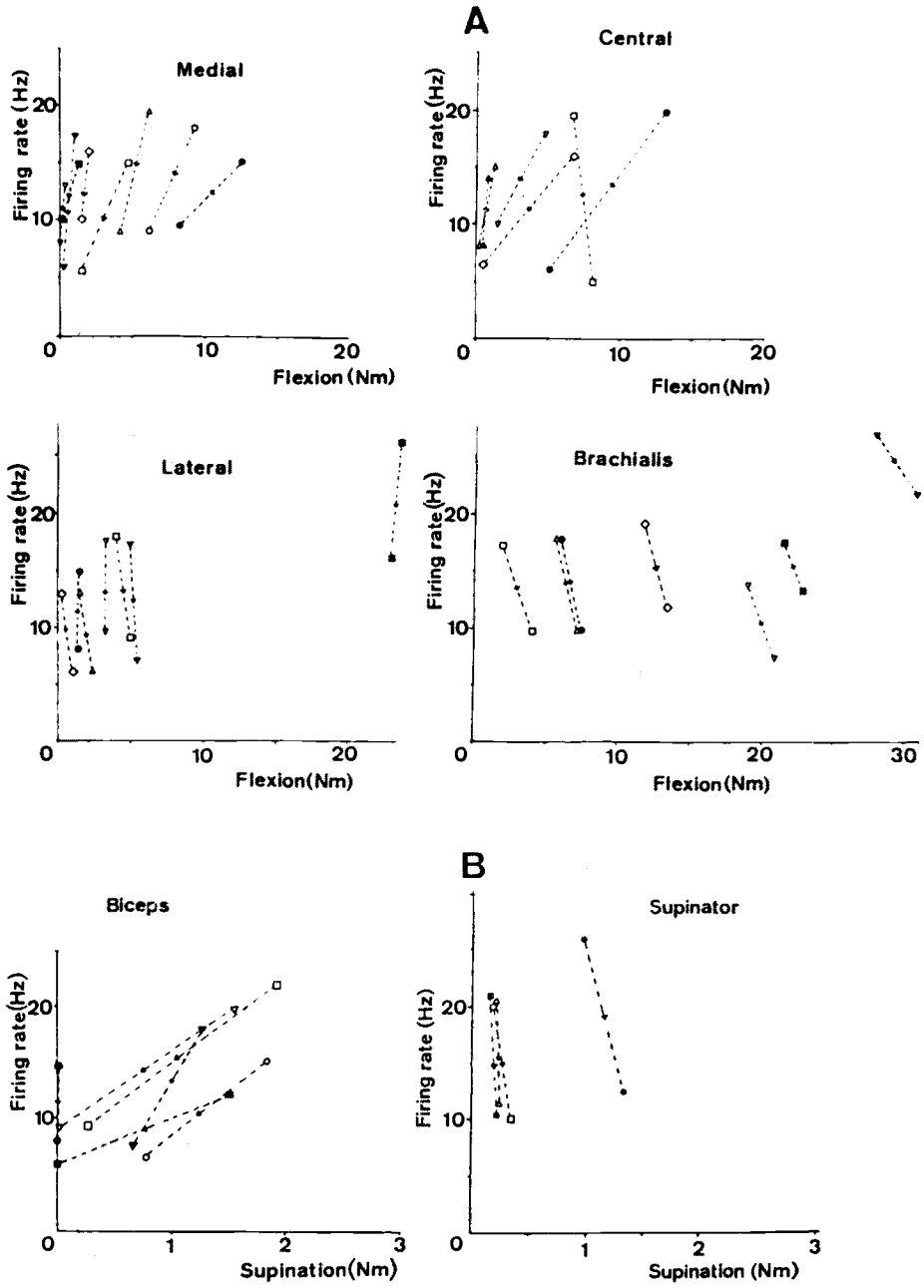


Fig. 9A-B. Recruitment and decruitment thresholds with accompanying first and last regular firing frequencies. The two thresholds of each neuron are connected by a *dashed line*; the higher frequencies correspond to recruitment. **A** Motor units recorded at medial, central and lateral locations in the long head of the m. biceps and in the m. brachialis active during flexion. **B** Motor units recorded at medial (*filled symbols*) and central (*open symbols*) locations in the long head of the m. biceps and in the m. supinator active during supination. (From Denier van der Gon et al. 1985)

examined under three experimental conditions: slow (3°/s) voluntary movement in the flexion direction during which an increasing external torque in the extension direction had to be overcome (movement task), slow (3°/s) imposed movement during which an increasing flexion torque had to be exerted (force task), and isometric flexion contraction (force task).

Figure 10 shows a result which is typical for motor units in the m. brachialis and m. brachioradialis on the one hand and for motor units in the m. biceps on the other hand. Recruitment thresholds of motor units were higher in the m. brachialis/brachioradialis and lower in the m. biceps during voluntary movement in the flexion direction than during isometric contraction. The results for the initial firing frequency were similar in all muscles tested. Initial firing frequencies were higher during flexion movements and lower during extension movements than during isometric contraction. During imposed movements both initial firing frequencies and recruitment thresholds were very similar to those found during isometric contraction. Therefore, a particular type of coordination does not seem to be related to movement or isometric contraction per se, but is probably related to the instruction to perform a specific task. This could mean that α- and/or γ-motoneuron pools are activated differently in force or movement tasks. On the other hand, it is difficult to see how initial firing frequencies at recruitment can be changed by α- and/or γ-signals alone. Kuypers and Huisman (1982) suggested that monoaminergic systems could serve as a "gain"-setting component of the motor system. Recently, it has been shown that serotonergic and noradrenergic projections are able to change both the excitability and the firing frequency of motoneurons (Conway et al. 1988). In our case this could

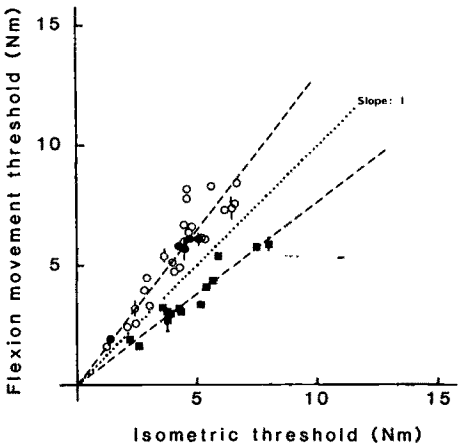


Fig. 10. Recruitment data of m. biceps (*filled squares*), m. brachialis (*open circles*) and m. brachioradialis (*filled circles*) gathered from different experiments and different subjects. For each motor unit the estimated recruitment threshold during slow voluntary flexion movements is plotted against the average isometric recruitment threshold. Linear regression analysis is used to fit the brachialis/brachioradialis data and the biceps data. (From Tax et al. 1990b)

mean that motoneuron pools involved in the flexion of the arm receive different monoaminergic input in force tasks and in slow-movement tasks.

There is a marked resemblance between the behavior of motor units during isometric relaxation and voluntary movement. In both tasks recruitment thresholds were higher in m. brachialis/brachioradialis and lower in m. biceps than during isometric contractions. This implies that the ratio between contributions of these muscles was shifted towards the m. biceps during relaxation or movement. This observation would mean that relaxation is to be seen as a task that is clearly different from contraction.

3.3 Cocontraction of Antagonistic Arm Muscles

We have presented evidence that motoneuron pools in several arm muscles are activated inhomogeneously and differently during various tasks. Current theories on muscle coordination do not provide a reasonable explanation for this inhomogeneous activation (see Sect. 5). Many authors have suggested that simultaneous activation of antagonistic muscles may be inhibited by Iα-reciprocal pathways (e.g., Tanaka 1974; Pierrot-Desseilligny et al. 1981; Day et al. 1984; Baldissera et al. 1987). Jongen et al. (1989b) showed, from experiments in which subjects performed a cocontraction task, that inhibitory mechanisms may also be at the basis of inhomogeneous activation. In these experiments subjects started by exerting isometric torques in the flexion or extension direction. They had to increase the level of contraction gradually from zero to about 40% of the maximum voluntary torque, keeping torques in other directions as small as possible. Thereafter, subjects had to perform the cocontraction task, in which they had to keep the external torques in all directions as close as possible to zero, while producing a prescribed EMG level which had to kept constant for about 5 s.

Figure 11A shows typical results for the EMG activity (wire electrodes, multi-unit recordings) from m. biceps (Medial Part MP, of caput longum), m. brachialis, m. brachioradialis, and m. supinator for increasing isometric torques in the flexion direction. In Fig. 11B the same EMG data are plotted, but now as a function of the EMG activity obtained from the m. biceps MP. Plotting EMG activity in this way allows us to compare muscles activity during isometric flexion and during cocontraction. Figure 11C shows EMG activities recorded during cocontraction of agonistic and antagonistic muscles. EMG activity in m. brachialis and m. brachioradialis is very small relative to that from m. biceps in clear contrast to the data shown in Fig. 11b. This means that parts of these

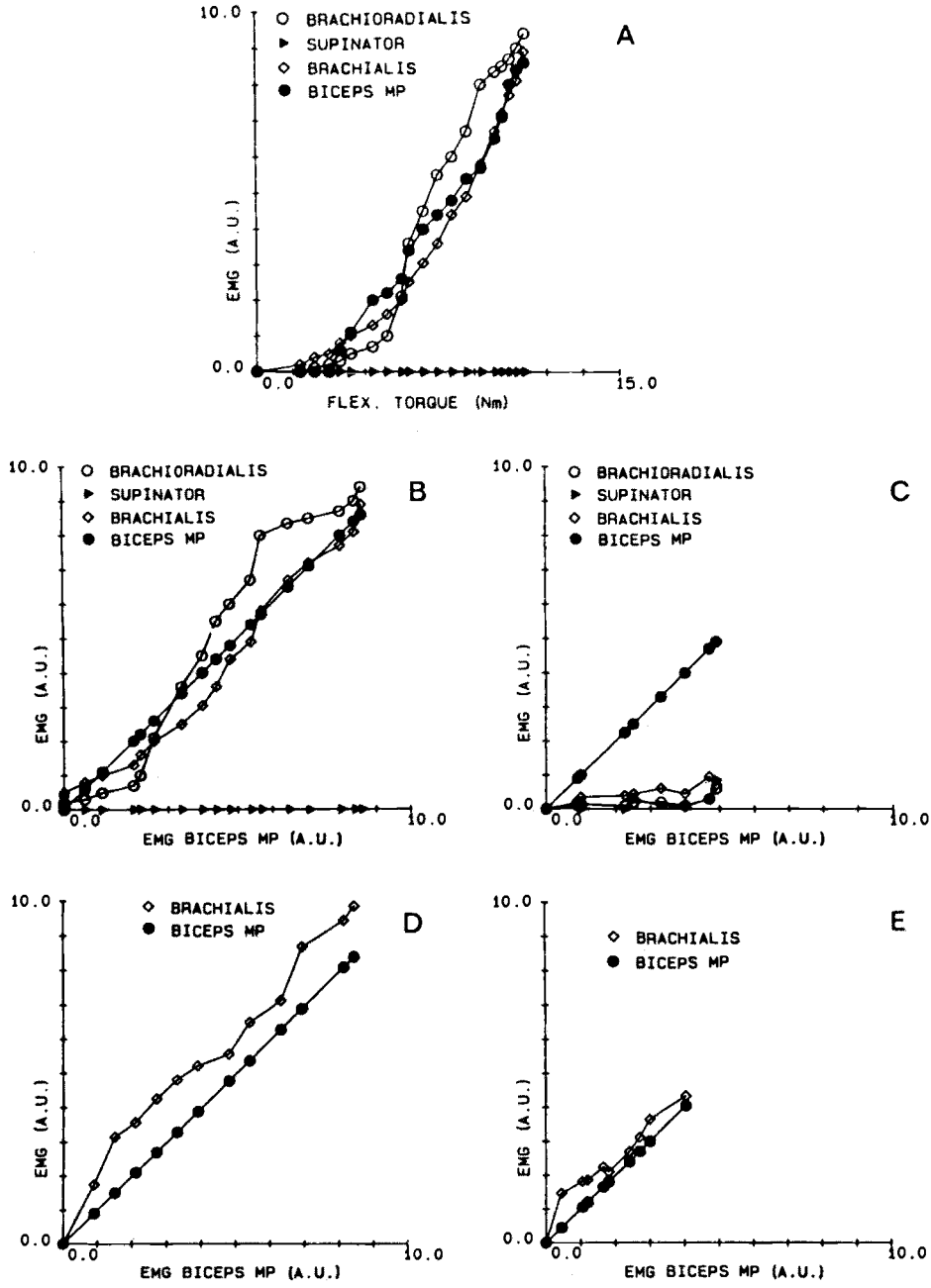


Fig. 11. **A** EMG activity (multi-unit recording) expressed in arbitrary units (A.U.) in several arm muscles of one subject as a function of the total flexion torque. **B** The same data as in **A**, but not plotted as a function of the EMG activity of the medial part of the long head of the m. biceps. **C** The same relationship as in **B**, but now during cocontraction of the arm muscles. **D** Similar data as in **B**, obtained from the same subject but in a different experiment (with different recording sites). **E** Similar data as in **C**, but now obtained from the same experiment as in **D**. (From Jongen et al. 1989b)

muscles are hardly activated during cocontraction of elbow flexion and extension muscles. Figure 11D, E show recordings from another site in m. brachialis. It appears that the low level of activation during cocontraction is not representative for all motor units in the m. brachialis. The results illustrate that the relative activation of motoneuron pools during cocontraction of agonistic and antagonistic muscle groups may be different from the relative activation observed when forces are exerted without cocontraction. This shows again that differences in relative activation do not occur only between muscles, but also within muscles.

3.4 Stretch Reflexes

Until now we have emphasized the inhomogeneous, task-dependent activation of arm muscles in voluntary motor tasks. Arm muscles, however, are also activated in response to perturbations of the position of the arm. Fast changes in arm position lead to segmented reflexes. Most authors agree that the short-latency reflex activity observed in a muscle is due directly to the stretching of that muscle. In many cases this reflex will be an inadequate response. For instance, when a pronation torque is applied to the forearm of a subject, the muscles that make a torque contribution in the supination direction (i.e., supinator and m. biceps) will be stretched. This means that a stretch reflex can be expected in both muscles and a counteracting supination torque will be generated. However, m. biceps will aso exert a torque in the flexion direction. The reflex would, therefore, lead to an unintentional movement component in the flexion direction. Gielen et al. (1988a) and Jongen (1989) investigated whether long-latency reflexes induce more adequate responses to perturbations. They proposed that the muscles acting around a joint which are activated in long-latency reflexes are the same as those activated during a voluntary contraction that would counteract the perturbation. We will illustrate this hypothesis for the aforementioned example. More circumstantial evidence can be found in Gielen et al. (1988a) and Jongen (1989). If a perturbation torque is applied in the pronation direction, a counteracting supination torque should be generated by the reflex-induced activation of not only the m. supinator and the m. biceps but also of the m. triceps. The activation of the latter muscle counteracts the flexion torque exerted by the m. biceps in a voluntary contraction in the supination direction (see Sect. 2.2). Thus, m. triceps, which is not stretched by pronation perturbations, should demonstrate increased activity at long latency if the hypothesis is correct.

Figure 12A shows that a relatively small short-latency response is followed by a much larger long-latency response when m. triceps is

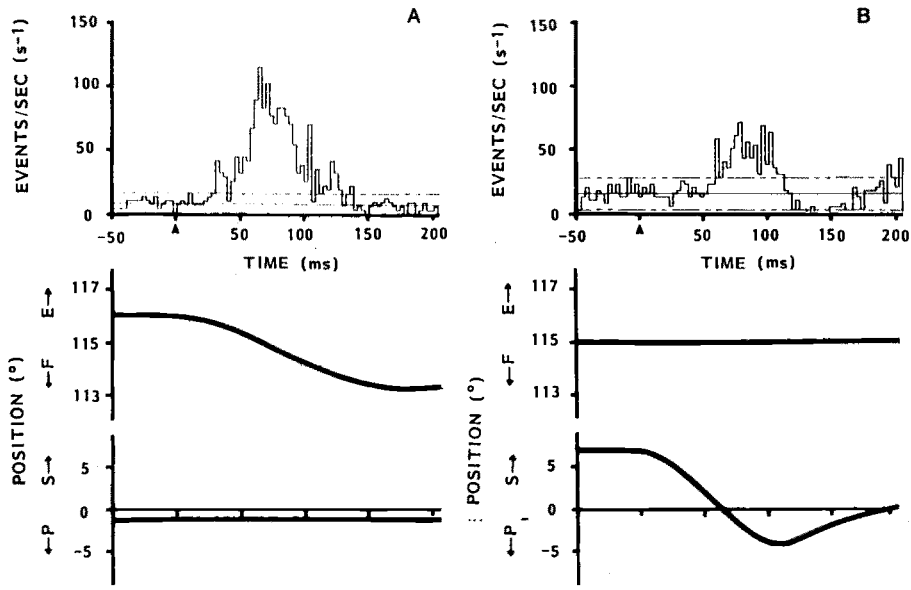


Fig. 12A,B. Poststimulus time histograms (PSTHs) which reveal the response of motor units of the m. triceps to perturbations with a duration of 200 ms in different directions, together with the corresponding positions of the wrist in the flexion/extension (*F*/*E*) and in the supination/pronation directions (*S*/*P*). Average motor unit activity is shown in the time interval from 50 ms before to 206 ms after the perturbation. In these experiments the m. triceps counteracts a flexion preload of 5.9 Nm. The *arrows* indicate the start of the perturbation ($t = 0$). In each PSTH three *horizontal lines* have been indicated. The *continuous line* gives the average number of events per second before the perturbation. The *broken lines* give the confidential levels for $p = 0.05$ based on the activity of the motor unit before the perturbation. **A** Average response to 104 perturbations of 5.2 Nm in flexion direction. The elbow angle changes by about 3° and the m. triceps is stretched. A relatively small short-latency response at about 30 ms is followed by a much larger long-latency response which starts at about 47 ms. **B** Average response to 130 perturbations of 1.3 Nm in pronation direction. The m. triceps is not stretched by this perturbation; this can be concluded from the flexion/extension position trace. There is no significant short-latency response. The activity is significantly increased starting at about 60 ms. (From Gielen et al. 1988a)

stretched by perturbations in the flexion direction. This result is to be expected when a muscle is stretched. Figure 12B shows that when perturbation torques in the pronation direction are applied which do not stretch m. triceps (see flexion/extension trace) a short-latency response is absent, but long-latency responses are present. This and other examples showed that there is a great similarity between the distribution of activation in long-latency reflexes and the distribution of activity during voluntary contractions.

4 Relative Contributions of Muscles to Motor Tasks

For studies on muscle coordination in motor tasks it is important to know the contribution made by each single muscle, especially in those cases where several muscles act across a single joint. In humans this contribution and its dependence on joint angle and muscle length are hard to determine. Van Zuylen et al. (1988b) developed a biomechanical model based on the anatomy of the human arm as well as on known muscle properties. The model computes relative twitch torques of motor units from the mechanical advantages of muscles, the lengths of the contractile part of muscles, and the length–tension relationships of actin-myosin filaments. Figure 13 shows the twitch torque as a function of the elbow angle for m. biceps and m. brachialis.

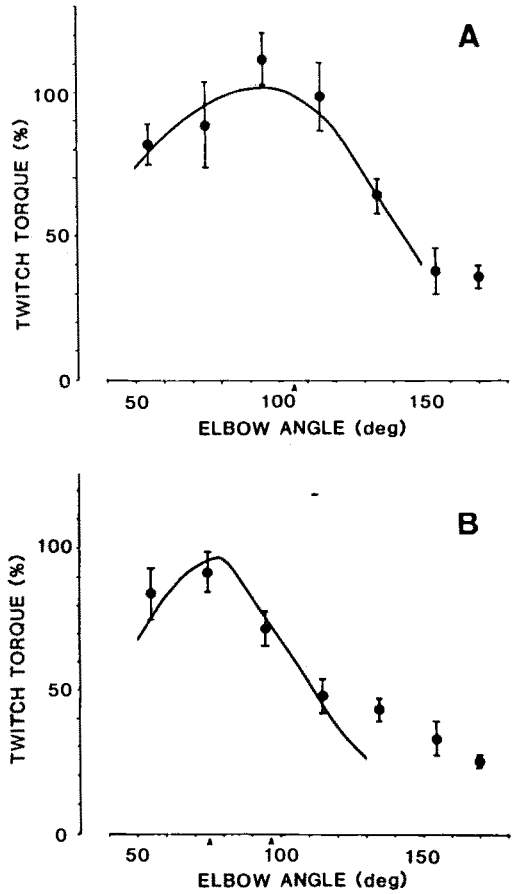


Fig. 13. Mean twitch amplitude and standard error as a function of elbow angle for motor units in m. biceps and m. brachialis. The data of different morot units were distributed in 20° intervals and averaged after normalization of the data for each motor unit. The curves give the model prediction for each muscle. **A** Twitches of motor units in the long head of the m. biceps ($n = 6$). For the fitting procedure the data obtained at elbow angles greater than 150° were excluded. The *arrow* indicates the angle at which the twitch force optimum is reached. **B** Twitches of motor units in the m. brachialis ($n = 6$). For the analysis the data obtained at elbow angles greater than 130° were excluded. The *arrow* indicates the angle at which the twitch force is optimal for the two fibers used in the model. (From van Zuylen et al. 1988b)

The theoretical results were tested and verified using a special spike-triggered averaging technique. The averaged data for all motor units tested are also shown in Fig. 13. Predictions for m. biceps are somewhat better than for m. brachialis. This is presumably due to the fact that recorded motor units were located at different positions in the muscles. This procedure influenced the result for m. brachialis negatively because in this muscle mechanical advantage and fiber length depend on the position of motor units. Besides, it may be argued that the model assumptions are less well fulfilled at large elbow angles.

Relative contributions of muscles to a motor task can be estimated from diagrams of recruitment thresholds of the different muscles such as were shown in Figs. 2, 5, and 6. A detailed explanation of the analysis technique is given by van Zuylen et al. (1988a). A necessary assumption for this analysis is that the torque exerted by all activated motor units of a particular subpopulation remains the same along the recruitment line of a motor unit of that subpopulation. Or, in other words, the recruitment order and initial firing frequency of motor units within a subpopulation have to remain the same in different tasks. The method can be used to obtain quantitative information about the contributions of muscles in different tasks. For instance, it was used to estimate and compare the contributions of different flexion muscles to isometric contraction with the contributions of the same muscles to slow voluntary movement in the flexion direction. Van Zuylen et al. (1988a) and Jongen et al. (1989a) estimated the relative contribution of m. biceps, m. brachialis/brachioradialis, and m. pronator teres to the total isometric torque at the wrist to be 36%, 57%, and 7%, respectively. Tax et al. (1990b) estimated that the contributions of m. biceps and m. brachialis/brachioradialis were 57% and 45%, respectively, during slow flexion movements against a preload. This means that the relative activation of synergists changes considerably in different motor tasks. Such changes affect not only elbow muscles but also shoulder muscles because m. biceps is biarticular muscle. Tax et al. (1989) showed that these changes were not accompanied by activity of m. triceps. This means that other muscles acting around the shoulder joint must account for stabilization of the shoulder joint.

5 Discussion

As illustrated in the foregoing sections, motoneuron pools of arm muscles are in general not activated homogeneously, but the available data show

that subpopulations of motor units are. Furthermore, in isometric-contraction tasks the initial firing frequency of the motor units was found to be independent of the direction of the exerted force. These findings may be indicative of different sources projecting to the motoneuron pools rather than of a dependence of intrinsic motoneuron properties on force direction. These sources may be related to the main anatomical directions of a joint or its stiffness (cocontraction) and would supply a common drive to one or more subpopulations (see e.g., De Luca 1988). The ratio of the weights with which these sources project onto the motoneurons may be different, but groups or subpopulations of the motoneurons within a pool are activated with more or less the same ratios. Ter Haar Romeny et al. (1984) presented a model of the innervation of the motoneuron pool of m. biceps. In that model the flexion input is assumed to be spread out over a greater part of the motoneuron pool than the supination input. Those units that would receive input from both sources would be of the summing-unit type. Flexion units would not receive a supination input and vice versa. Such a differential activation is not an unusual finding in muscle control (Kandou and Kernell 1989, Windhorst 1989).

Notwithstanding the fact that there is apparently a redundant number of muscles that act across the elbow joint for simple tasks such as flexion and supination, there is a clear resemblance of the innervation ratios of subpopulations between subjects. When comparing different types of tasks, e.g., isometric contraction, isometric relaxation, and movement tasks, it was found that the firing frequency behavior of the motor units is not fixed, but can be adjusted to the task to be performed or to the instruction given to the subject. For instance, the initial firing frequency is higher during voluntary flexion and lower during voluntary extension movements than it is during isometric contractions. Furthermore, firing frequencies may become much lower in relaxation tasks and in movement tasks than in isometric contraction tasks (force tasks). However, within a subpopulation the recruitment order never changed.

These results raise questions about the functional significance of differences in recruitment behavior of subpopulations of motor units, about principles of muscle coordination, and about the controllability of muscle forces. One single activation parameter (total synaptic drive) cannot account for the motor-unit activity in different tasks. The similar recruitment order for both force tasks and movement tasks and the inverse order for relaxation tasks do indicate a homogeneous activation of the subpopulations in these conditions. However, the firing frequency of the motoneurons can be adjusted rather independently: the motoneurons seem to posses several different "modi operandi" related to the required task. This means that the relative contribution of the two force-grading

mechanisms, i.e., the recruitment of motor units and the modulation of their firing frequency, is task-dependent. This finding and the fact, as outlined in Sect. 4, that the coordination of synergistic forearm muscles is task-dependent point to different modes of central control in posture versus movement or contraction versus relaxation. It is known that serotonergic raphe–spinal and noradrenergic coeruleus–spinal projections are able to change both the excitability and the firing frequency of the motoneurons (Conway et al. 1988). These projections, which might be different for different motoneuron pools, might be used to adjust the "modi operandi" of the motoneurons for different tasks. This solution would allow adequate reflexive control for different tasks without any adaptation of the reflex organization itself. In other words, the effects found are part of a more (central) general reprograming of the motor system in which reflex gains are also adjusted. However, it is not at all clear what the functional significance might be of different modes of control in posture versus movement or contraction versus relaxation. As far as we know, no adequate models exist that incorporate this task-dependent sensorimotor control.

In the case of the m. biceps it is hard to find a functional explanation for the differences of recruitment behavior of the motor-unit populations, since no corresponding differences in mechanical advantage could be found. Because there are clear indications that by far most of the motor units of the m. biceps are certainly of the summing type, one could argue that the motor units of the flexion and supination type may only represent a kind of physiological imperfection, e.g., incomplete outgrowth of the innervation of the motoneuron pool or an aberrant location of some motoneurons. But this line of reasoning does not hold for the rather large subpopulations found in the m. triceps. Some other muscles, e.g., the m. brachialis, show a much broader site of attachment and the different behavior of groups of motor units may have functional significance here. Summarizing, partitioned activation was found in almost all forearm muscles, but the occurrence and the significance of the so-called subpopulations are not yet fully understood.

From Sect. 3.4 it was concluded that there is a great similarity in the distribution of activation at long latency and the distribution of activation during voluntary isometric contractions. The afferent input originating from stretched muscles must be transformed to cause an adequate response that effectively counteracts a perturbation. Transmission through a transcortical loop takes almost the entire time between the short-latency and long-latency response and, therefore, a great number of synaptic transmissions are excluded (Houk and Rymer 1981). This means that the network mediating the long-latency activity is likely to be located quite

close to the motoneurons. Therefore, we suggest that there is an interneuronal network that acts as a transformer station for both descending pathways (voluntary contractions) and afferent pathways, e.g., type II and cutaneous afferents (Darton et al. 1985; Becker et al. 1987; Lundberg et al. 1987). Such a system would imply the observed similarity in activation during voluntary and reflexive (long-latency) contractions.

Theories on muscle coordination and solutions to the problem of redundancy are often based upon minimization of a cost function, e.g., fatigue (Dul et al. 1984a,b) or energy consumption (Crowninshield 1978). Another group of theories is based upon a matching of efferent signals to the muscles and afferent signals from the muscle sense organs (Gielen and van Zuylen 1986; Jongen et al. 1989a). Such matching permits a simple form of control at different levels in the central nervous system. Also, a simple learning algorithm based on this matching principle generates the recruitment characteristics of the main subpopulations (Denier van der Gon et al. 1990). In the case of a combined sensory and neuromuscular partitioning (Windhorst et al. 1989), parts of muscles may be seen as separate units, presumably with size-related recruitment within those subdivisions, and different behavior may come to the fore if these parts have on average different mechanical advantages. However, it remains unclear how the models mentioned above should account for the different subpopulations found in, for instance, m. biceps and m. triceps.

Finally, it should be stressed that a number of inhomogeneities in motoneuron-pool innervation as well as a number of nonlinearities in muscle transfer properties do not necessarily imply more complex control mechanisms. In a model study it was shown that known physiological properties, such as the size principle (Henneman and Mendell 1981), the potentiation (Burke et al. 1976), and the recruitment density (Milner-Brown et al. 1973) of motor units, appear to support the linearity of neuromuscular systems. In the case of different relative contributions by the two force-grading mechanisms within one muscle for different tasks, the input-output relation in each task may still be linear. In other words, the generated muscle force resulting from a homogeneously activated subpopulation of motoneurons can be shown to be proportional to a weighted sum of activities (firing frequencies) of the nerve fibers that project onto that part of the motoneuron pool (Denier van der Gon 1988; Tax and Denier van der Gon 1990). This linearity of the neuromuscular system is advantageous as far as control is concerned. Superposition of different motor tasks will then simply require superposition of corresponding input programs (Ruegg and Bongoianni 1989). Furthermore, the neuromuscular system will be stable under a variety of feedback conditions.

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References

Baldissera F, Cavallari P, Fournier E, Pierrot-Desseilligny E, Shindo M (1987) Evidence for mutual inhibition of opposite I_α interneurones in the human upper limb. Exp Brain Res 66:106–114

Becker WJ, Hayashi R, Lee RG, White D (1987) Effect of cutaneous nerve stimulation on voluntary and stretch reflex electromyographic activity in wrist flexors in humans. J Physiol (Lond) 382:509–522

Burke RE, Rudomin P, Zajac FE III (1976) The effect of activation history on tension production by individual muscle units. Brain Res 109:515–529

Conway BA, Hultborn H, Kiehn O, Mintz I (1988) Plateau potentials in α-motoneurones induced by intravenous injection of L-dopa and clonidine in the spinal cat. J Physiol 405:369–384

Crowninshield RD (1978) Use of optimization techniques to predict muscle forces. J Biomech Eng 100:88–92

Darton K, Lippold OCJ, Shahani M, Shahani U (1985) Long-latency spinal reflexes in humans. J Neurophysiol 53:1604–1617

Day BL, Marsden CD, Obeso JA, Rothwell JC (1984) Reciprocal inhibition between the muscles of the human forearm. J Physiol 349:519–534

De Luca CJ (1988) The common drive of motor units. In: Wallinga W, Boom HBK, de Vries J (eds) Electrophysiological kinesiology. Elsevier, Amsterdam, pp 35–38

Denier van der Gon JJ (1988) Motor control: aspects of its organisation, control signals and properties. In: Wallinga W, Boom HBK, de Vries J (eds) Electrophysiological kinesiology. Elsevier, Amsterdam, pp 9–17

Denier van der Gon JJ, ter Haar Romeny BM, van Zuylen EJ (1985) Behaviour of motor units of human arm muscles: differences between slow isometric contraction and relaxation. J Physiol (Lond) 359:107–118

Denier van der Gon JJ, Coolen ACC, Erkelens CJ, Jonker HJJ (1990) Self-organizing neural mechanisms possibly responsible for muscle coordination. In: Winters JM, Woo SL-Y (eds) Multiple muscle systems: biomechanics and movement organization. Springer, Berlin Heidelberg New York, pp 335–342

Dul J, Townsend MA, Shiavi R, Johnson GE (1984a) Muscular synergism – I. On criteria for load sharing between synergistic muscles. J Biomech 17:663–673

Dul J, Johnson GE, Shiavi R, Townsend MA (1984b) Muscular synergism – II. A minimum-fatigue criterion for load sharing between synergistic muscles. J Biomech 17:675–684

Gielen CCAM, van Zuylen EJ (1986) Coordination of forearm muscles during flexion and supination: application of the tensor analysis approach. Neuroscience 17:527–539

Gielen CCAM, Ramaekers L, van Zuylen EJ (1988a) Long-latency stretch reflexes as co-ordinated functional responses in man. J Physiol (Lond) 407:275–292

Gielen CCAM, van Zuylen EJ, Denier van der Gon JJ (1988b) Coordination of arm muscles in simple motor tasks. In: de Groot G, Hollander AP, Huying PA, van Ingen Schenau GJ (eds) International series on biomechanics, vol 7A. Free University Press, Amsterdam, pp 155–166

Gustafsson B, Pinter MJ (1985) On factors determining orderly recruitment of motor units: a role for intrinsic membrane properties. Trends Neurosci 431–433

Henneman E (1957) Relation between size of neurons and their susceptibility to discharge. Science 126:1345–1347

Henneman E (1980) Organisation of the motoneuronpool: the size principle. In: Mountcastle VB (ed) Medical physiology. Mosby, St Louis, pp 718–741

Henneman E (1981) Recruitment of motoneurons: the size principle. In: Desmedt JE (ed) Motor unit types, recruitment, and plasticity in health and disease progress in clinical neurophysiology, vol 9. Karger, Basel, pp 26–60

Henneman E, Mendell LM (1981) Functional organisation of motoneuron pool and its inputs. In: Brookhart JM, Mountcastle VB (eds) Handbook of physiology. Sect.1, the nervous system. Vol II, part 1, motor control. American Physiological Society, Bethesda MD, pp 423–507

Hoffer JA, Loeb GE, O'Donovan MJ, Pratt CA (1980) Unitary activity patterns during walking confirm the existence of two functionally distinct classes of motoneurones in cats. J Physiol (Lond) 308:20P

Hoffer JA, Loeb GE, Sugano N, Marks WB, O'Donovan MJ, Pratt CA (1987) Cat hindlimb motoneurons during locomotion. III Functional segregation in sartorius. J Neurophysiol 37:554–562

Houk JC, Rymer WZ (1981) Neural control of muscle length and tension. In: Brookhart JM, Mountcastle VB (eds) Handbook of Physiology. Sect. 1, the nervous system. Vol II, part 1, motor control. American Physiological Society Bethesda, MD, pp 257–323

Jongen HAH (1989) Theories and experiments on muscle coordination during isometric contractions. Thesis, University of Utrecht

Jongen HAH, Gielen CCAM, Denier van der Gon JJ (1989a) Activation of human arm muscles during flexion/extension and supination/pronation tasks: a theory on muscle coordination. Biol Cybern 61:1–9

Jongen HAH, Denier van der Gon JJ, Gielen CCAM (1989b) Inhomogeneous activation of the motoneurone pool as revealed by co-contraction of antagonistic arm muscles. Exp Brain Res 75:555–562

Kandou TWA, Kernell D (1989) Distribution of activity within the cat's peroneus longus muscle when activated in different ways via the central nervous system. Brain Res 486:340–350

Kuypers HJGM, Huisman AM (1982) The new anatomy of the descending brain pathway. In: Sjölund B, Björklund A (eds) Brain stem control of spinal mechanisms, vol 2. Elsevier, Amsterdam, pp 29–54

Loeb GE (1985) Motorneurone task groups: coping with kinematic heterogeneity. J Exp Biol 115:137–146

Lundberg A, Malmgren K, Schomburg ED (1987) Reflex pathways from group II muscle afferents. 3. Secondary spindle afferents and FRA: a new hypothesis. Exp Brain Res 65:294–306

Milner-Brown HS, Stein RB, Yemm R (1973) The orderly recruitment of human motor units during voluntary isometric contractions. J Physiol 230:359–370

Pierrot-Desseilligny E, Morin C, Bergego C, Tankov N (1981) Pattern of group I fibre projections from ankle flexor and extensor muscles in man. Exp Brain Res 42:337–350

Rüegg DG, Bongoianni F (1989) Superposition of ballistic on steady contractions in man. Exp Brain Res 77:412–420

Tanaka R (1974) Reciprocal I_a inhibition during voluntary movements in man. Exp Brain Res 21:529–540

Tax AAM, Denier van der Gon JJ (1990) A model for neural control of gradation of muscle force. Biol Cybern (to be published)

Tax AAM, Denier van der Gon JJ, Gielen CCAM, van den Tempel CMM (1989) Differences in the activation of m. biceps brachii in the control of slow isotonic movements and isometric contractions. Exp Brain Res 76:55–63

Tax AAM, Denier van der Gon JJ, Gielen CCAM, Kleyne M (1990a) Differences in the central control of m. biceps brachii in movement tasks and force tasks. Exp Brain Res 79:138–142

Tax AAM, Denier van der Gon JJ, Erkelens CJ (1990b) Differences in coordination of elbow flexor muscles in force tasks and in movement tasks. Exp Brain Res 81:567–572

ter Haar Romeny BM, Denier van der Gon JJ, Gielen CCAM (1982) Changes in recruitment order of motor units in the human biceps muscle. Exp Neurol 78:360–368

ter Haar Romeny BM, Denier van der Gon JJ, Gielen CCAM (1984) Relation of the location of a motor unit in human biceps muscle and its critical firing levels for different tasks. Exp Neurol 85:631–650

van Zuylen EJ, Gielen CCAM, Denier van der Gon JJ (1988a) Coordination and inhomogeneous activation of human arm muscles during isometric torques. J Neurophysiol 60:1523–1548

van Zuylen EJ, van Velzen A, Denier van der Gon JJ (1988b) A biomechanical model for flexion torques of human arm muscles as a function of elbow angle. J Biomech 21:183–190

Windhorst U, Hamm TM, Stuart DG (1989) On the function of muscle and reflex partitioning. Behav Brain Sci 12:629–681

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