STUDY OF A REOVIRUS PROTEIN INVOLVED IN VIRAL mRNA TRANSLATION

by

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SUMMARY

This thesis concerns the mechanisms responsible for preferential translation of reovirus mRNA in infected cells. The absence of a poly(A) tract at the 3′-end is one of the structural differences that distinguishes reovirus mRNA from cellular mRNA. Addition of free poly(A) inhibits *in vitro* translation at the level of initiation of protein synthesis, probably by competition between free poly(A) and mRNA. Reovirus mRNA is resistant to this inhibition and this property might confer a translational advantage to the viral mRNA. Another difference between reovirus mRNA and cellular mRNA is the absence of a 5′-cap structure on late viral mRNA. This mRNA is translated much more efficiently in lysates from infected than uninfected cells, even in the absence of inhibition of host cell protein synthesis. Addition to uninfected cell lysates of proteins from infected cells stimulates translation of late viral mRNA without effect on other mRNAs; the presence of the sigma 3 viral protein is shown to account for the stimulation of translation. Sigma 3 binds to ribosomes, probably during initiation of protein synthesis, but apparently does not interact directly with mRNA. The sigma 3 protein exists in multiple forms, differing in isoelectric point but possessing all the known properties of sigma 3. These forms possibly result from mutations in the gene encoding sigma 3. The cloned gene which encodes sigma 3 was expressed in L cells without apparent detrimental effect on the cells. Lysates prepared from these cells translate late viral mRNA with an increased efficiency. The sigma 3 protein is associated with the

ribosomes in these cells even if no viral mRNA is present. It is suggested that the sigma 3 protein acts as a viral-specific initiation factor of protein synthesis.

RÉSUMÉ

Cette thèse concerne les mécanismes responsables de la traduction préférentielle de l'ARNm de réovirus dans les cellules infectées. L'absence d'une extrémité 3'-poly(A) est une des différences de structure qui distingue l'ARNm de réovirus de l'ARNm cellulaire. L'addition de poly(A) libre inhibe la traduction in vitro au niveau de l'initiation de la synthèse protéique, probablement par compétition entre le poly(A) libre et l'ARNm. L'ARNm de réovirus est résistant à cette inhibition et cette propriété pourrait conférer un avantage traductionnel à l'ARNm viral. Une autre différence entre l'ARNm de réovirus et l'ARNm cellulaire est l'absence d'une structure 5'-chapeau sur l'ARNm viral tardif. Cet ARNm est traduit beaucoup plus efficacement dans des lysats de cellules infectées que non-infectées même en absence d'inhibition de la synthèse protéique cellulaire. L'addition, à un lysat de cellules non-infectées, de protéines de cellules infectées stimule la traduction de l'ARNm viral tardif sans effet sur d'autres ARNm; la présence de la protéine virale sigma 3 est responsable de la stimulation de traduction. Sigma 3 s'attache aux ribosomes, probablement durant l'initiation de la synthèse protéique, mais apparemment n'interagit pas directement avec l'ARNm. La protéine sigma 3 existe sous plusieurs formes, différant de point isoélectrique mais possédant toutes les propriétés connues de sigma 3. Ces formes découlent possiblement de mutations du gène codant pour sigma 3. Le gène cloné qui code pour la protéine sigma 3 a été exprimé dans des cellules L sans effet dommageable apparent sur les cellules. Des

lysats de ces cellules traduisent l'ARNm viral tardif avec une efficacité accrue. La protéine est associée avec les ribosomes dans ces cellules même si aucun ARNm viral n'est présent. Il est suggéré que la protéine agit comme facteur d'initiation de la synthèse protéique; facteur qui serait spécifiquement viral.

TO MY PARENTS,

À MES PARENTS,

afin qu'ils sachent que leur affection

reste la chose la plus importante à mes yeux.

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LIST OF ABBREVIATIONS

In addition to chemical nomenclature and abbreviations of the metric system, the following abbreviations have been used.

ATA: aurintricarboxylic acid

ATP: adenosine triphosphate

BSA: bovine serum albumin

CBP: cap-binding proteins

cDNA: complementary DNA

cpm: counts per minute

DNA: deoxyribonucleic acid

dT: deoxythymidine

DTT: dithiotreitol

EDTA: ethylenediamine tetraacetic acid

EGTA: ethyleneglycol-bis-(beta-aminoethyl ether)-N-N′-tetraacetic acid

EMC: encephalomyocarditis virus

FV3: Frog virus 3

GMP: guanosine monophosphate

GTP: guanosine triphosphate

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HTLV: human T-lymphotropic virus

IEF: isoelectric focusing

kbp: kilobase pair

kDa: kiloDalton

KIU: kallikrein inhibitory unit

MEM: minimum essential medium

m^7GTP: 7-methylguanosine triphosphate

mRNA: messenger RNA

MW: molecular weight

PBS: phosphate buffered saline

PFU: plaque forming unit

PMSF: phenylmethylsulfonylfluoride

poly(A): polyadenylic acid

poly(G): polyguanylic acid

poly(I)-poly(C): polyinosinic-polycytidylic acid (double-stranded

PPi: inorganic pyrophosphate

RNA: ribonucleic acid

rpm: revolutions per minute

RSW: ribosomal salt wash

S10: supernatant of 10,000 g. centrifugation (postnuclear supernatant)

S200: supernatant of 200,000 g. centrifugation (postribosomal supernatant)

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SDS: sodium dodecyl sulfate

SFV: Semliki Forest virus

TCA: trichloroacetic acid

TMV: tobacco mosaic virus

TRIS: Tris(hydroxymethyl)aminomethane

tRNA: transfer RNA

Vol: volume

PREFACE

This thesis concerns the mechanisms of translational control responsible for preferential translation of late viral mRNA in reovirus-infected cells.

There are two main structural differences between reovirus mRNA and eucaryotic cellular mRNA. 1) Cellular mRNA generally terminates with a poly(A) tail at its 3′-end; in contrast reovirus mRNA is never polyadenylated. 2) Cellular mRNA is blocked at the 5′-end by the so-called cap structure; in contrast it was demonstrated by previous investigators in our laboratory that the reovirus mRNA synthesized late in infection is uncapped.

In chapter 2 are reported and discussed some observations concerning the effect of free poly(A) during in vitro translation of mRNAs including reovirus mRNA.

The chapters 3 to 5 concern the main topic of this thesis, namely the study of a viral-encoded factor involved in the stimulation of translation of late (uncapped) reovirus mRNA.

At the beginning of each chapter is mentioned the part of the work that was published and which parts were done in collaboration. I also briefly mention the relationship between each chapter and the previous ones.

The references for all the chapters are presented in one list at the end of the thesis. The figures and tables are included at the end of each chapter but are numbered consecutively throughout the thesis.

CHAPTER 1

Introduction

THE REOVIRIDAE

The first members of the Reoviridae family were isolated in the 1950's and the term reovirus (respiratory enteric orphan virus) was suggested in 1959 (Sabin, 1959). The most important characteristics of the family are the presence of a double-layered capsid and of a genome made of segmented double-stranded RNA. Other features are the lack of complete uncoating of the viral genome, the possession of enzymes for the synthesis of messenger RNA, and the absence of a poly(A) tail at the 3'-end of messenger RNA. Members of the Reoviridae family have been isolated from a wide variety of organisms (reviewed in: Joklik, 1983a). At the molecular level the most widely studied examples of the family are the weakly pathogenic human reoviruses; in this thesis I will consider only the human reoviruses and especially reovirus serotype 3 (Dearing strain). Unless clearly mentioned in the text, all the information provided concerns this particular virus strain. The human reoviruses have been studied extensively, principally as model systems, even if they are relatively unimportant from the clinical point of view. Despite the fact that they cannot be considered typical with their double-stranded RNA genome they have been very useful as a tool to study transcription, translation, and virus-cell interactions.

REOVIRUS STRUCTURE

General structure

Human reoviruses have a double capsid made of protein subunits. The inner capsid or core has a hollow center and contains the viral genome. The virion has a diameter of 76 nm and the core a diameter of 52 nm with a central cavity about 38 nm when measured by electron microscopy (reviewed in: Joklik, 1983b). Digestion of virions with chymotrypsin *in vitro* removes the outer protein layer leaving intact the inner capsid containing the viral genome (Shatkin and Sipe, 1968; Smith, Zweerink and Joklik, 1969; Joklik, 1972). The same phenomenon called uncoating also occurs *in vivo* when, after phagocytosis of the virion, the outer capsid proteins are digested by lysosomal enzymes (Silverstein and Dales, 1968; Silverstein *et al.*, 1970, 1972; Chang and Zweerink, 1971). However, *in vivo*, the mu1C protein of the outer capsid is only partially removed and a 65 kDa fragment called the delta protein stays attached to the core (Silverstein *et al.*, 1972). The inner core is made of 6 different proteins and the outer capsid of 3 (reviewed in: Joklik, 1983b). The arrangement of the proteins in the core structure is still largely unknown. The general structure of the virion is schematized in Fig. 1 and the next two sections will describe in greater details the nucleic acid and proteins that composed this structure.

Nucleic acid composition

Removal of the outer capsid by proteolytic digestion releases small oligonucleotides from the virion (Bellamy and Hole, 1970). These oligonucleotides are possibly by-products of the transcription of the

viral genome (Bellamy et al., 1972; Zarbl, Hastings and Millward, 1980; Yamakawa, Furuichi and Shatkin, 1982) and their function is still unknown. It was suggested that the oligonucleotides may inhibit host cell protein synthesis (Zarbl and Millward, 1983). This last point will be discussed later. A first class of oligonucleotides comprised essentially the first 2 to 10 nucleotides normally present on mRNA, some of them are also capped and methylated. They are present at about 2000 per virion. Another class of oligonucleotides comprised oligo(A) of around 2 to 20 nucleotides long; these are present at about 1000 per virion (reviewed by: Zarbl and Millward, 1983).

The inner core contains the viral genome made of 10 segments of double-stranded RNA (reviewed in: Joklik, 1983b). Each segment gives rise after transcription to one species of mRNA that will be translated into one species of protein. The known exception, and most probably the only one (Kozak, 1982; Cenatiempo et al., 1984), being the S1 gene that encodes a second protein in another reading frame (Ernst and Shatkin, 1985; Jacobs and Samuel, 1985; Jacobs et al., 1985; Sarkar et al., 1985). The genomic segments can be subdivided in three size classes; there are three segments of L (large) class (about 3.8 kbp.), 3 segments of M (medium) class (about 2.2 kbp.), and 4 segments of S (small) class (about 1.3 kbp.). The sizes of these different segments were recently determined more precisely by molecular cloning of cDNA copies of each segment (Cashdollar et al., 1984).

Protein composition

The peculiar arrangement in three size classes observed with the

genomic segments is also evident at the protein level: 3 proteins of lambda class (about 145 kDa) plus the cleavage products of lambda 2 (lambda 2C about 125 kDa), 3 mu proteins (about 75 kDa) plus the cleavage product of mu1 and muNS (mu1C about 72 kDa and muNSC about 70 kDa), and 4 sigma proteins (about 35-40 kDa) plus the second reading frame of S1 (sigma 1bNS; about 14 kDa). In the nomenclature of reovirus proteins: NS stands for nonstructural proteins and C indicates a cleavage product. The apparent molecular weight, location and abundance of the different viral proteins are summarized in Table I (adapted from: Joklik, 1983b). A cDNA copy of four reovirus genes has been cloned and sequenced [S2: (Cashdollar et al., 1982); S3: (Richardson and Furuichi, 1983); S1: (Nagata et al., 1984; Bassel-Duby et al., 1985; Cashdollar et al., 1985); S4: (Giantini et al., 1984)] and the molecular weights deduced by compilation of the amino acid sequences are indicated in parentheses. In addition to the eleven primary translation products some reovirus proteins are cleaved. The best example is mu1C that is in fact more abundant than the primary translation product mu1 from which it is derived. The roles of the other cleavage products are unknown. Another level of diversity of the reovirus proteins was observed by two-dimensional gel electrophoresis. It was observed that some of the reovirus proteins are present in multiple forms differing in isoelectric point (Samuel, 1983; Ewing, Sargent and Borsa, 1985). Posttranslational modifications may explain the different isoelectric points in some cases. Carbohydrates are attached to reovirus proteins through O-glycosidic bonds but the amount of carbohydrate is quite low, the mu1C protein is the most heavily glycosylated and only 2% of the mu1C

molecules present in the virion have carbohydrates attached (Krystal, Perrault and Graham, 1976; Lee, 1983). It seems that most of the other reovirus protein species are also glycosylated but to an even lower extent, however this low overall level of glycosylation might be biologically important since an inhibitor of glycosylation inhibits viral growth (Lee, 1983). A low level of phosphorylation of mu proteins (Krystal et al., 1975) and polyadenylation of different structural proteins has also been reported (Carter, Lin and Metlay, 1980); it was suggested that some of the modifications might be important for virion morphogenesis (Mora and Carter, 1983) but the details are still unknown. In Fig. 1 the position of different proteins is indicated on the general scheme of the virion; further work is however needed to get a more precise view of the organization of the virion and especially for the inner capsid.

Some properties and functions of different reovirus proteins have been elucidated using structural, biochemical, or genetic analysis. It has been suggested that the lambda 3 protein has the transcriptase activity (Drayna and Fields, 1982); this activity is responsible for the synthesis of mRNA from the viral genome. This assignment was obtained using viral reassortants for genetic analysis, this method will be briefly described later; however physicochemical studies assign the same function to the lambda 1 protein (Powell, Harvey and Bellamy, 1984). The lambda 2 protein (see Fig. 1) makes up the spikes seen on the outer surface of the virus (White and Zweerink, 1976; Ralph et al., 1980) through which viral mRNA is probably extruded. Recently the guanylyltransferase activity (described in another section) has also been shown to be associated with this

protein (Shatkin et al., 1983; Cleveland, Zarbl and Millward, 1986). Sigma 1 is the receptor (Lee et al., 1981a) and hemagglutinin (Weiner et al., 1978) responsible for host range, cell attachment, and also has a role in determining pathogenicity in the animal (Weiner et al., 1977, Weiner and Fields, 1977; Weiner, Powers and Fields, 1980; Weiner, Ault and Fields, 1980; Weiner, Green and Fields, 1980). It might also have roles intracellularly in the observed inhibition of host DNA synthesis following reovirus infection (Sharpe and Fields, 1981) and association of the virus with microtubules (Babiss et al., 1977; Sharpe, Chen and Fields, 1982). However the recent discovery of another protein coded in a different reading frame by the same gene (as mentioned in the preceding section) suggests that these latter properties, previously associated with the S1 gene (encoding sigma 1) using genetic analysis, may be due to the smaller protein instead of sigma 1.

The sigma 3 protein is the main topic of this thesis and some of its properties will be described in greater details later. Already known was the fact that this protein has an affinity, of unknown significance, for double-stranded RNA (Huismans and Joklik, 1976). Genetic analysis also indicates a possible involvement of this protein in the inhibition of protein synthesis in infected cells (Sharpe and Fields, 1982). A complex made of sigma 3 and mulC in a ratio of 2 to 1 forms the major part of the outer capsid. In the infected cell the bulk of these two proteins is complexed (Huismans and Joklik, 1976; Lee, Hayes and Joklik, 1981b).

Some of the proteins encoded by viral genes and mentioned in Table I are not part of the mature virus particles and are called

nonstructural proteins (NS proteins). They may be responsible for some of the effects of virus infection on the host-cell or alternatively may be involved during virus morphogenesis. The muNS protein was shown to be transiently associated with intermediate subviral particles suggesting a role during morphogenesis (Morgan and Zweering, 1974, 1975). The sigma NS protein has an affinity for single-stranded RNA (Huismans and Joklik, 1976) and possibly also for DNA (Shelton et al., 1981). Its exact role is unknown. It was suggested that this protein by binding to different segments of reovirus mRNA may form a particle playing a role in the grouping of the different mRNA segments prior to the synthesis of the second strand of genomic RNA (Gomatos, Prakash and Stamatos, 1981; Stamatos and Gomatos, 1982) as will be described later.

Genetic analysis

There are three serotypes of human reoviruses exhibiting small but significant differences in properties. The genomic segments and proteins of these serotypes can be differentiated by small differences in their sizes (Ramig, Gross and Fields, 1977; Sharpe et al., 1978). Upon mixed infection with two different serotypes it is possible to generate viral reassortants containing segments of both viruses. As mentioned previously, this powerful technique of genetic analysis (reviewed in: Ramig and Fields, 1983) allowed the assignment of different viral functions to different genomic segments and thus to proteins encoded by them. Except for the sigma 1 protein, which is the serotype-specific protein, there is homology between the corresponding genes and gene products of the different serotypes. This similarity can be demonstrated by the conservation of epitopes

(Gaillard and Joklik, 1980) and peptides obtained by tryptic digestion (Gentsch and Fields, 1981) between the serotypes and by the homology between their genes (Gaillard and Joklik, 1982). Except for the S1 segment, the homology between serotype 1 and 3 varies between 90% for the L segments and 35% for segment M2. By contrast the homology between serotype 2 and 3 genes is much lower, varying between about 20% for L1 to only 5% for the M segments.

VIRAL mRNA

Synthesis

The occurrence of DNA viruses (like vaccinia virus) and double-stranded RNA viruses (like reovirus) that replicate in the cytoplasm involves the problem of synthesizing mRNA and lead to the discovery of viral RNA polymerase; first in vaccinia (Kates and McAuslan, 1967; Munyon, Paoletti and Grace, 1967), and shortly thereafter in reovirus (Borsa and Graham, 1968; Shatkin and Sipe, 1968). During reovirus infection the genomic RNA stays in the core and the transcriptase enzyme is activated by removal of the outer capsid (reviewed in: Zarbl and Millward, 1983; Shatkin and Kozak, 1983). *In vivo* this is the result of digestion by lysosomal enzymes (Silverstein *et al.*, 1970, 1972; Chang and Zweerink, 1971); *in vitro* this is generally accomplished by chymotrypsin digestion (Shatkin and Sipe, 1968) but heat shock was also proved efficient (Borsa and Graham, 1968) probably by relaxing the outer capsid structure. In fact, recent findings indicate that what is "activated" is not the enzyme itself but rather the movement of genome segments relative to the transcriptase catalytic site (Yamakawa, Furuichi and Shatkin, 1982; Powell, Harvey and Bellamy, 1984; reviewed in: Joklik, 1985). Until now it has been impossible to isolate the transcriptase, an intact core structure is apparently necessary for its activity (White and Zweerink, 1976). Only the so-called plus-strand of genomic RNA is transcribed both *in vitro* and *in vivo* (Hay and Joklik, 1971). *In vitro* the frequency of transcription of the different segments is inversely proportional to their length (Skehel and Joklik, 1969), a conclusion consistent with an uniform speed on each segment without preferential utilisation of

particular segments. In vivo however there is a control over the frequency of transcription of different segments. The relative frequencies of transcription and translation (discussed later) are summarized in Table II (Zweerink and Joklik, 1970; reviewed in: Joklik, 1981). Furthermore it was observed that early in infection there is a preferential transcription of L1, M3, S3, S4 [called pre-early mRNAs (Watanabe, Millward and Graham, 1968; Nonoyama, Millward and Graham, 1974)]. It was also shown that these segments are transcribed in the presence of inhibitors of protein synthesis; protein synthesis is however required for transcription of the other genes (Lau et al., 1975). Furthermore when nonpermissive (avian) cells are infected with the virus there is transcription of the four genes only (Spandidos and Graham, 1976). Presumably a viral or viral-induced factor has to be synthesized in order to release the transient inhibition of transcription of the other genes but the nature of this hypothetical factor is still unknown.

Structure

Reovirus mRNA presents some characteristic features. Unlike the vast majority of cellular and viral mRNAs there is no splicing and no 3'-terminal polyadenylation (Stoltfus, Shatkin and Banerjee, 1973). The 5' non-coding region is relatively short varying between 12 nucleotides (segment S1) and 32 nucleotides (segment S4) (Antczak et al., 1982). The first 4 nucleotides at the 5'-end are GCUA and the last 5 at the 3'-end are UCAUC (Antczak et al., 1982; Gaillard et al., 1982). These conserved sequences may be more important for the packaging of genomic RNA or the replication of the genome and may be present on the messenger RNAs only because they are complete copies of

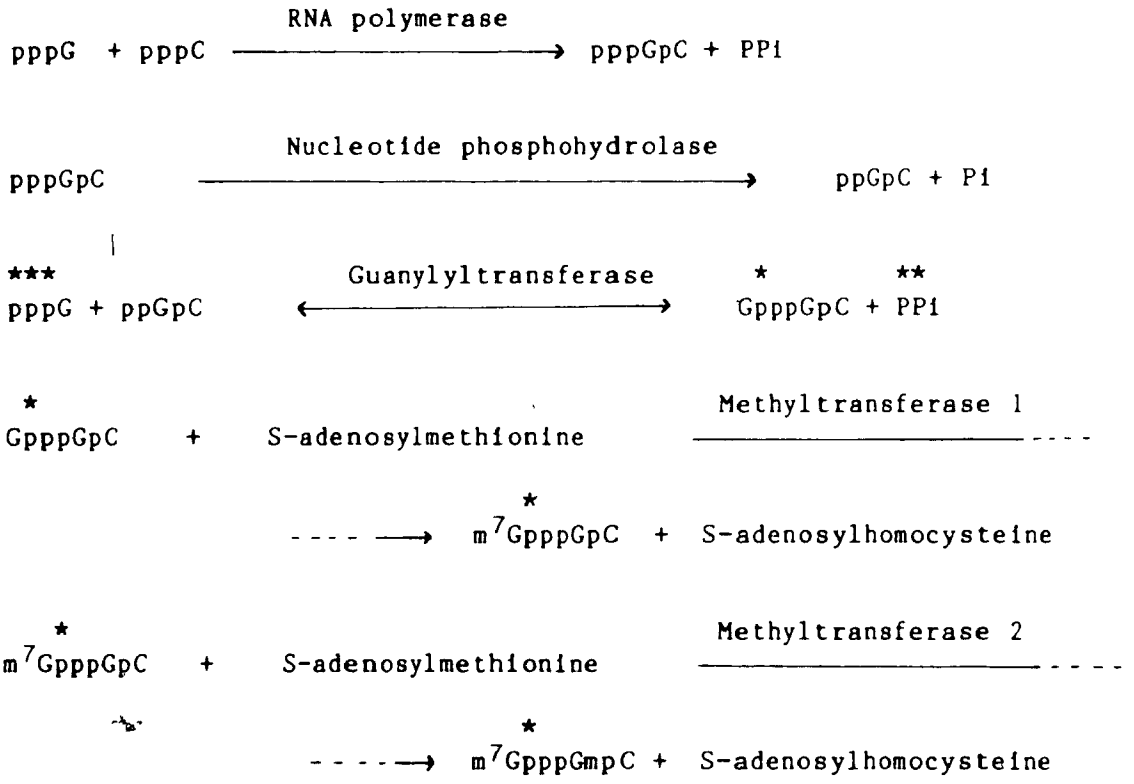
genomic segments (Li et al., 1980; Hastings and Millward, 1981). It is also possible that these sequences play a role in translation as will be discussed later.

THE CAP STRUCTURE

Structure and synthesis

Reovirus cores can modify the 5′-end of the newly synthesized mRNA by addition of the so-called cap structure, similar to the cap structure present on cellular mRNAs. The cap structure of the 5′-end of reovirus mRNA was in fact discovered (Furuichi, Muthukrishnan and Shatkin, 1975; Furuichi et al., 1975; Faust, Hastings and Millward, 1975) shortly after the capping of vaccinia virus messages (Martin and Moss, 1975) and even before cellular mRNA capping (Furuichi et al., 1975; reviewed in: Banerjee, 1980). The cap structure consists of an unusual 5′ to 5′ triphosphate linkage between GTP and the first nucleotide of the mRNA. This GTP is methylated at the N-7 position of the purine ring generating a positive charge on this group. In reovirus mRNA, as in most cases, only one phosphate is derived from the incoming GTP while the other two phosphates are derived from the 5′-end of the mRNA (Furuichi et al., 1976; reviewed in: Banerjee, 1980). The penultimate nucleotide is very often a purine and in the case of reovirus, as already mentioned, it is always a G. When this nucleotide is not methylated the structure is named type 0 cap, when it is methylated (in position 2′-O of the sugar ring) the structure is type 1 cap, and finally when the next nucleotide is also 2′-O-methylated it is a type 2 cap (Shatkin, 1976; reviewed in: Banerjee, 1980). Synthesis of mRNA in vitro with reovirus cores generates type 0 and 1 cap in the presence of a methyl group donor (S-adenosylmethionine) (Faust, Hastings and Millward, 1975; Furuichi et al., 1976). In vivo it is also possible to find type 2 structure suggesting that an host methyltransferase is involved in the last

step. Mammalian cell mRNAs have mostly, but not exclusively, type 2 cap but more primitive organisms may have only type 1 or even type 0 (reviewed in: Shatkin, 1976; reviewed in: Banerjee, 1980). The reactions involved in the synthesis of reoviral cap (Furuichi et al., 1976; Furuichi and Shatkin, 1976) are schematized below.



Except for the enzymes involved, the cap structure of cellular mRNAs are synthesized by the same general mechanism (reviewed in: Banerjee, 1980). All cellular eucaryotic mRNAs examined to date have a cap structure except for the mitochondrial, procaryotic like, mRNAs (reviewed in: Banerjee, 1980). Eucaryotic viral mRNAs are also capped except for the picornaviral mRNAs (reviewed in: Banerjee, 1980; Ehrenfeld, 1984) and, as will be discussed later, reovirus late mRNA (Zarbl, Skup and Millward, 1980).

Cap-binding proteins

The general occurrence of the cap structure reflects its importance. The cap structure is implicated in stability of mRNA. It was shown that the 5′ to 5′ linkage is essential to this effect but not the methylation. The m^7G methylation is important however for efficient initiation of protein synthesis in eucaryotic cells (reviewed in: Filipowitz, 1978; Banerjee, 1980). It was shown that for reovirus mRNA the cap structure is essential for the binding of mRNA to initiation complexes (Both, Banerjee and Shatkin, 1975; Both et al., 1975; Muthukrishnan et al., 1976). Proteins that specifically interacts with the cap structure (cap binding proteins) were discovered and isolated using crosslinking to labeled cap groups (Sonenberg and Shatkin, 1977; Sonenberg et al., 1978; Sonenberg et al., 1979; Pelletier and Sonenberg, 1985) or affinity chromatography using cap analog (Sonenberg et al., 1979; Tahara, Morgan and Shatkin, 1981). These proteins are in fact a polyprotein complex (Tahara, Morgan and Shatkin, 1981; Grifo et al., 1982; Edery et al., 1983; Sonenberg et al., 1983) playing the role of an initiation factor by stimulating translation of capped mRNA (Sonenberg et al., 1980; Tahara, Morgan and Shatkin, 1981; Edery, Lee and Sonenberg, 1984; reviewed by: Shatkin, 1985). The exact activities of these proteins are not completely known, but several lines of evidence suggest that the cap binding protein complex, after recognition of the capped 5′-end, is involved in relaxation (or melting) of the secondary structure of mRNA (Sonenberg, 1981; Sonenberg et al., 1981b; Grifo et al., 1982; Ray et al., 1985; reviewed by: Shatkin, 1985). This melting could be important for the binding and scanning of 40S ribosomal subunit

(Kozak, 1980) until it encounters an initiation codon on the mRNA. In accordance with this model the dependence on cap structure and cap binding proteins for efficient translation correlates with the degree of secondary structure at the 5'-end of the mRNA, considering the nature of the mRNA as well as ionic and temperature conditions (Weber, Hickey and Baglioni, 1977; Weber et al., 1977; Bergmann and Lodish, 1979; Morgan and Shatkin, 1980; Sonenberg et al., 1981; Sonenberg, Guertin and Lee, 1982; Gehrke et al., 1983; Lee, Guertin and Sonenberg, 1983).

The cap structure and nuclear cap binding proteins (Patzelt, Blaas and Kuechler, 1983) have also been recently shown to be implicated in the splicing of mRNA (Konarska, Padgett and Sharp, 1984; Edery and Sonenberg, 1985). They may also play a role in the attachment of polysomes to cytoskeletal elements, this attachment is believed to have a role in the translation process (Zumbe, Stahli and Trachsel, 1982; Cervera, Dreyfus and Penman, 1981; Howe and Hershey, 1984; Bonneau, Darveau and Sonenberg, 1985).

EVENTS IN THE REOVIRUS-INFECTED CELL

Growth cycle of reovirus

When a virus infects a cell it needs to replicate the viral genome, transcribe it, and translate the mRNA into viral proteins. Concomitantly it may have an effect on host cell DNA, RNA, and protein synthesis (See: Luria et al., 1978). My work deals with the control of protein synthesis in reovirus infected L cells and this last point will be treated in greater detail. Recent studies indicate that there is a shift from cap dependence to cap independence in late reovirus serotype 3 infected L cells (Skup and Millward, 1980a; Skup Zarbl and Millward, 1981; Sonenberg et al., 1981a). From these and other studies a general scheme of the events occurring in the reovirus-infected cell was deduced (Skup, Zarbl and Millward, 1981; reviewed in Zarbl and Millward). This whole series of events is schematized in Fig. 2; in Fig. 3 the time course at 37 °C of the different events is illustrated. After adsorption of viral receptors onto cell receptors there is phagocytosis of the virion and digestion by lysosomal enzymes of the outer capsid proteins. The viral transcriptase and capping enzymes are thus activated and synthesize early viral mRNA (capped) as described earlier. The maximum rate of early mRNA synthesis occurs around 7 h post-infection at 37 °C and decreases thereafter. This mRNA is translated by the normal host-cell protein synthesis machinery and generates viral proteins with varying translational efficiency as indicated in Table II (Zweerink and Joklik, 1970; Gaillard and Joklik, 1985; reviewed in: Joklik, 1981, 1985). These relative efficiencies are probably related to some aspects of the secondary structure of the different species of mRNA but the details are not known yet. The

early mRNA has a dual function; it is used as mRNA as well as the template strand (called plus strand) for double-strand (genomic) RNA synthesis (Schonberg et al., 1971). Some of these molecules associates with viral proteins generating intermediate replicative structures (Zweerink, 1974; Morgan and Zweerink, 1974; 1975) where the second strand (minus strand) is synthesized by a viral double-strand RNA polymerase single-strand RNA dependent (viral replicase). The replication of the viral genomic RNA is thus conservative (Silverstein et al., 1970; Schonberg et al., 1971) but the details of the viral enzymes implicated as well as exact mechanisms are not known. The plus strand of the genomic RNA is thus capped (Furuichi et al., 1975) and it was shown that after denaturation it can indeed serve as mRNA (McCrae and Joklik, 1978; Mustoe et al., 1978). After completion of replication the particles generated are called progeny subviral particles (Morgan and Zweerink, 1974). These particles are transcriptionally active (Morgan and Zweerink, 1975; Zweerink, Morgan and Skyler, 1976). However, their capping activities are either masked or absent (Skup and Millward, 1980b) and the mRNA synthesized is thus uncapped (Zarbl, Skup and Millward, 1980). This is consistent with the apparent absence of lambda 2 spikes on the progeny subviral particles (Morgan and Zweerink, 1974; 1975); the lambda 2 protein is responsible for the first reaction of cap formation (Shatkin et al., 1983; Cleveland, Zarbl and Millward, 1986). The synthesis of late viral mRNA peaks around 13 hours post-infection at 37°C. Different lines of evidence indicate that 85% of viral mRNA produced during infection is synthesized from the progeny subviral particles (reviewed in: Zarbl and Millward, 1983). The viral mRNA present late in infection is

thus mostly uncapped and the bulk of the viral mRNA produced is also uncapped. After translation to produce more viral proteins the outer capsid is added to both the parental and progeny subviral particles (Chang and Zweerink, 1971). The completion of the infection results in lysis of the cell accompanied by release of mature virus.

Effects on host cell protein synthesis

The events already described are accompanied by different effects on cellular metabolism. Early studies on reovirus have shown that there is an inhibition of DNA synthesis (Ensminger and Tamm, 1966) and protein synthesis (Kudo and Graham, 1965) following reovirus infection. The effect on DNA occurs at the level of initiation of replication (Hand and Tamm, 1974). The nature of the viral function responsible for this effect is still controversial (Shelton et al., 1981; Sharpe and Fields, 1981; reviewed in: Fields, 1983).

Some authors suggested that the inhibition of protein synthesis results from cell deterioration when cells are grown in suspension culture (Ensminger and Tamm, 1969). These studies were conducted at 37 °C and it is true that late in infection, when cells are damaged, there is a general and non-specific decrease of protein synthesis (see Fig. 3). Later it was observed that by looking at host-specific instead of total protein synthesis there is indeed an inhibition of host cell protein synthesis that precedes total protein synthesis inhibition. A transition gradually occurs between synthesis of host proteins and synthesis of viral proteins resulting in preferential synthesis of viral proteins (Zweerink and Joklik, 1970). More recent studies also indicate that a similar transition occurs during infection of L cells grown in monolayer (Munemitsu and Samuel, 1984).

Using lower incubation temperature lengthens the different phases of the infection course and facilitates the study of this transition in protein synthesis; at 30 °C the length of each phase is doubled compared to 37 °C. Using this lower temperature and mRNA-dependent *in vitro* translation systems prepared from uninfected or reovirus late-infected cells (Skup and Millward, 1977), it was confirmed that a transition in protein synthesis occurs in infected cells (Skup and Millward, 1980a). Late in the infection, when most of the protein synthesis is viral specific, the bulk of polysomal viral mRNA was shown to be uncapped (Skup and Millward, 1981). *In vitro* translation system showed that late-infected cells are less efficient at translating capped mRNA, either cellular or viral. This phenomenon is probably at least partially responsible for the inhibition of host cell protein synthesis. It was also shown that the inhibition is even stronger for capped (early) viral mRNA (Lemieux, Zarbl and Millward, 1984) possibly helping to free the capped mRNA to serve as genomic plus-strand RNA (Zarbl and Millward, 1983), while the uncapped (late) viral mRNA is used for protein synthesis.

Initiation factors in reovirus-infected cells

Studies aiming to understand the molecular basis of these modifications of protein synthesis revealed that addition of crude initiation factors prepared from uninfected cells, but not from infected cells, can restore translation of capped mRNA in reovirus-infected cells to a nearly normal level. It seems that the cap binding protein activity is somewhat altered in reovirus-infected cells and this is probably one of the causes of the decrease in translation of capped mRNA *in vitro* and host cell protein synthesis inhibition *in*

vivo (Sonenberg et al., 1981; Zarbl, Lemieux and Millward, unpublished data). Using the technique of crosslinking to labeled cap groups it was shown that the ability of two proteins to recognize cap groups is decreased in infected cells while the crosslinking of a third protein species is essentially abolished (Zarbl, Lemieux and Millward, unpublished data). However, in contrast with poliovirus infection (discussed later), where cap binding proteins activity is altered by cleavage of a 220 kDa protein component of the cap binding protein complex, there is apparently no such cleavage in reovirus - infected cells (Etchison and Fout, 1985). Concomitant with the inhibition of cap-dependent translation, there is an increase in the capability of the reovirus-infected cell to translate reovirus uncapped mRNA (Skup and Millward, 1980a, Skup, Zarbl and Millward, 1981; Lemieux, Zarbl and Millward, 1984). This phenomenon was shown to be specific for reovirus uncapped mRNA since other uncapped mRNAs are not more efficiently translated in infected compared to uninfected cell lysates (Lemieux, Zarbl and Millward, 1984). In vitro competition experiments between L cell mRNA and late viral mRNA revealed that these two populations are not in competition in late-infected cells (Lemieux, Zarbl and Millward, 1984). In lysates prepared from late infected cells and saturated with L cell mRNA it is still possible to increase total incorporation of amino acids by adding late viral mRNA but no other type of mRNA is efficient. The existence of a different set of factors (most probably initiation factors) responsible for the efficient translation of late viral mRNA was thus suggested (Zarbl and Millward, 1983; Lemieux, Zarbl and Millward, 1984).

Preliminary results (obtained by Réal Lemieux in our laboratory)

indicated that proteins from infected cells can specifically stimulate translation of late viral mRNA when added to lysates from uninfected cells. Most of the experiments described in this thesis concern the identification and characterization of the specific factor involved in late viral mRNA translation.

Effects of other reovirus serotypes on protein synthesis

It was observed that the serotype 2 of human reovirus produces a much more rapid protein synthesis inhibition than serotype 3. Using genetic analysis it was shown that viruses having all the genes of reovirus serotype 3 except for a serotype 2 gene encoding sigma 3 (S4 gene), have the rapid inhibition phenotype of serotype 2 and vice-versa. It was thus suspected that the sigma 3 protein plays a role in the control (or inhibition) of protein synthesis in infected cells (Sharpe and Fields, 1982). The protein synthesis in L cells infected with reovirus serotype 1 or 3 has also been studied (Munemitsu and Samuel, 1984). The serotype 1 virus has apparently no specific inhibitory effect even at times when viral protein synthesis is maximal. These authors suggest a correlation between the slower multiplication rate of serotype 1 and this absence of inhibition.

Other models of translation in reovirus-infected cells

It must be pointed out that some reports present apparently conflicting results concerning the control of protein synthesis in reovirus-infected cells. These divergences can be mostly explained by failure to differentiate between host and viral protein synthesis or failure to take into account the relative length of viral cycle in different strains of cells. Particularly significant in this last

regard are the studies in SC1 cells (Walden, Godefroy-Colburn and Thach, 1981; Detjen, Walden and Thach, 1982) where the authors deduce that there is no transition from cap dependence to cap independence but instead a competition between viral and cellular capped mRNA for the same pool of initiation factors. Their results showed that reovirus mRNA is a poor competitor, for the binding of an initiation factor, compared to cellular mRNA (Ray et al., 1983). The different translational efficiencies of the different species of reovirus mRNA are apparently due to a similar type of competition. However, the experiments were done at a time point that probably corresponds to early viral mRNA synthesis in SC1 cells, as the same authors admitted themselves in a more recent publication (Ray et al., 1985). The kind of competition that they described may well exist in L cells early in infection (Zarbl and Millward, 1983) between different capped viral mRNA and cellular mRNA, and late in infection between different viral uncapped mRNA, explaining the relative efficiencies of translation of the different mRNA species. However competition between cellular mRNA and late viral mRNA is not observed in late infected L cells (Lemieux, Zarbl and Millward, 1984).

Another group recently presented data apparently conflicting with both the competition and cap-discrimination models (Munoz, Alonso and Carrasco, 1985). However these experiments were performed mostly in HeLa cells where the growth of reovirus is not as well characterized as in L cells. Furthermore, they use superinfection with other viruses that also interfere with host cell protein synthesis rendering the situation very complex. The few experiments in L cells indicate that both vesicular stomatitis virus (capped RNA) and

encephalomyocarditis virus (uncapped RNA) mRNAs are translated early but not late in reovirus-infected cells. This last result is in accordance with our observation that only translation of uncapped reovirus mRNA is stimulated in late infected cells. The same authors also made a proposal, based mostly on experiments performed with HeLa cells, that an effect at the level of cellular membrane permeability is responsible for the translational effects of reovirus infection. The same authors have previously suggested a similar model for poliovirus infection that is still subject to controversy (described later).

Some of these experiments and models concerning translational control in reovirus infection will be mentioned again in this thesis, especially under General Discussion (Chapter 6).

Persistent infection by reovirus

A particular case of interaction between cell and virus is observed during the establishment of persistent infection. Under particular conditions it is possible to obtain cell lines producing virus without being killed (reviewed in: Fields, 1983; Joklik, 1985). The virus obtained from these cells is somewhat modified but there is also selection of certain subpopulation of cells (Ahmed et al., 1981). Infection with virus stocks obtained by growth at high multiplicity of infection is known to more readily generate persistent infection. Such virus stocks generally contain viral mutants especially in the S4 gene. Study of viral reassortants between serotype 2 and 3 has also shown that there is a selection against the S4 gene of serotype 2 in persistently infected cell lines (Ahmed and Fields, 1982). Altogether the data suggested that the S4 gene is important for the

"establishment" of persistent infection. The S1 gene also has a role in the "maintenance" of persistent infection (Kauffman, Ahmed and Fields, 1983). However it is important to realize that the situation leading to persistent infection is very complex and it is difficult to dissociate random mutations and rearrangements from significant changes (Ahmed, Kauffman and Fields, 1983).

OTHER EXAMPLES OF VIRAL-INDUCED ALTERATION OF PROTEIN SYNTHESIS

Reovirus infection is just one example of modification of the translational machinery following viral infection. It is thus of interest to compare the situation occurring during reovirus infection with other viral infections. I will describe here the most salient features of some typical situations where viral-induced alterations of protein synthesis occurs. I do not intend to review in detail each of the situations but I will mention the most recent and significant findings.

Picornaviruses

Among the best-known example of translational control during infection is the situation occurring during infection by picornavirus (a positive-strand RNA virus), especially poliovirus infection of HeLa cells (reviewed in: Ehrenfeld, 1982; Ehrenfeld, 1984). Poliovirus mRNA is uncapped (Nomoto, Lee and Wimmer, 1976; Hewlett, Rose and Baltimore, 1976) and its translation is not affected by antibody against cap binding proteins (Sonenberg et al., 1981a). As a result of poliovirus infection there is a rapid inhibition of cellular mRNA translation apparently due to inhibition of cap binding proteins activity (Trachsel et al., 1980; Lee and Sonenberg, 1982). This inhibition is probably caused by cleavage of the 220 kDa component of the cap binding protein complex (Etchison et al., 1982; Lee, Edery and Sonenberg, 1985). It was also shown that upon infection of HeLa cells with human rhinovirus (another picornavirus), there is cleavage of the same protein (Etchison and Fout, 1985). However, infection with another well-known picornavirus (encephalomyocarditis virus)

apparently does not result in cleavage of this protein (Mosenkis et al., 1985). The inhibition of host cell protein synthesis by this virus apparently occurs by a different but still unclear mechanism (Jen, Detjen and Thach, 1980; Jen and Thach, 1982). The exact nature of the protease involved in cleavage of cap binding protein by poliovirus is unknown (Lloyd, Etchison and Ehrenfeld, 1985; Lee et al., 1985; Lloyd et al., 1986), however the study of poliovirus mutants unable to specifically inhibit host cell protein synthesis clearly indicates that a virus-encoded protein is somehow involved in the inhibition of translation and cleavage of p220 (Steiner-Pryor and Cooper, 1973; Bernstein, Sonenberg and Baltimore, 1985). Furthermore, a study involving viral mutants suggested that wild type poliovirus not only actively inhibits translation of cellular mRNAs, but also avoids early inhibition of its own protein synthesis; the mechanism is however unknown (Bernstein, Sonenberg and Baltimore, 1985). Other authors still contest that cleavage of cap binding proteins is the essential phenomenon in host cell protein synthesis inhibition. They suggest an alternative mechanism involving membrane damage by viral proteins thus altering the intracellular ionic composition and as a consequence the relative translational efficiency of viral and cellular mRNA (Carrasco, 1977; Lacal and Carrasco, 1982). This model definitely needs further support, especially to explain some of the results of in vitro translation experiments. It is not a generally accepted model (Ehrenfeld, 1984). However we cannot disprove that this other mechanism may affect translation to a certain extent in vivo and in some virus/cell systems.

Semliki Forest virus

The situation is different in Semliki Forest virus infection (a positive-strand RNA virus). There is a gradual transition from early to late viral mRNA translation during infection of neuroblastoma cells by this virus. Late in infection there is inhibition of both early viral mRNA and cellular mRNA translation even if these mRNAs are still present (van Steeg et al., 1981a). This inhibition is due to the presence of a major viral capsid protein that apparently interferes with the activity of cap-binding proteins (van Steeg et al., 1984). As a result, binding of mRNA (other than late viral mRNA) to initiation complexes is prevented. The viral protein is present in the crude initiation factors fraction prepared from infected cells and can be purified from this fraction (van Steeg et al., 1984). However late viral mRNA is capped but apparently requires lower amount of cap binding proteins for optimal translation (van Steeg et al., 1981b) as also indicated by its lack of sensitivity to cap analogs (van Steeg et al., 1981a).

Vaccinia virus

The vaccinia virus is a very complex DNA virus and it is not surprising that the exact events leading to inhibition of host cell protein synthesis, concomitant with efficient viral-specific protein synthesis, are still controversial. Two different viral proteins have been recently implicated in the inhibition of host cell protein synthesis [(Mbuy, Morris and Bubel, 1982); (Ben-Hamida, Person and Beaud, 1983; Person-Fernandez and Beaud, 1986)]. Either of the two proteins can apparently inhibit initiation of protein synthesis in in vitro translation systems, but the experiments do not explain the

discrimination between viral and cellular mRNA translation. Alternatively, a completely different mechanism was suggested that might explain the discrimination between viral and cellular mRNAs. It was shown that small poly(A)-rich RNA transcripts synthesized by the virus can selectively inhibit translation of mRNA other than vaccinia virus mRNA when added to in vitro translation systems (Coppola and Bablanian, 1983; Bablanian et al., 1986). It was also shown that free polyadenylic acid can inhibit translation of cellular mRNA while vaccinia virus is relatively resistant to this inhibition (Bablanian and Banerjee, 1986). It is quite possible that the inhibitory proteins and short poly(A) transcripts act synergistically to achieve a perfect control on the protein synthesis in infected cells; however, the details are still lacking.

Frog virus 3

In Frog virus 3 (a DNA virus related to vaccinia virus), different subpopulations of mRNA are translated at different times during infection. Late in infection, only late viral mRNA is translated despite the fact that early viral mRNA is present. A factor present among the crude initiation factors from infected cells is apparently responsible, and absolutely required, for efficient translation of late viral mRNA (Raghow and Granoff, 1983). This is an example of positive effect on translation by a viral product. The nature of the factor and if it is really encoded by the viral genome is unknown, but genetic evidence has indicated a role of viral proteins in the posttranscriptional controls in this system (Goorma, Willis and Granoff, 1979). The structural difference that are responsible for the discrimination between early and late viral mRNA

is unknown. The complexity of this virus suggests that the mechanisms of translational controls might be very intricate.

Human T-lymphotropic virus type III (HTLV-III)

In this virus (a retrovirus) it has been recently suggested that two genes (the "tat" and "art" genes) that appear to exert a positive control on the expression of viral mRNA, might act at the level of translation (Rosen et al., 1986; Sodroski et al., 1986). The "tat" gene encodes a protein essential for viral replication (Fisher et al., 1986; Goh et al., 1986). In the case of "tat", it seems that sequences in the 5'-untranslated region of viral mRNA are also necessary for the stimulation of translation, the exact mechanism of action of the "tat" gene product is however unknown (Rosen et al., 1986). The steady-state level of mRNA is also possibly affected by "tat" (Cullen, 1986). The "art" gene seems to derepress the translation of certain viral mRNAs; the exact mechanism is still not clear but is possibly at the level of splicing of mRNA and not directly at the translational level (Feinberg et al., 1986). It has been postulated that the "tat" and "art" functions might control the latent and lytic phases of the virus life cycle by allowing efficient expression of different subpopulations of viral mRNA (Sodroski et al., 1986; Cullen, 1986; Feinberg et al., 1986; reviewed by: Chen, 1986). This point is currently under study in many laboratories.

Adenovirus

In adenovirus (a DNA virus) infection it is a nucleic acid molecule, the small VAI RNA coded by the virus, that is necessary for normal growth of the virus and for efficient translation in infected

cells. This viral product is apparently required for efficient translation of both cellular and viral mRNAs late in infection (Thimmappaya et al., 1982; Schneider, Weinberger and Shenk, 1984; Svensson and Akusjarvi, 1984; Reichel et al., 1985). The presence of this RNA apparently prevents the activation by interferon, upon viral infection, of a kinase activity able to block the recycling of an initiation factor necessary for protein synthesis (Siekierka et al., 1985; O'Malley et al., 1986; Kitajewski et al., 1986). This whole mechanism can be viewed as a viral mechanism to counteract the effect of interferon on translation. Related phenomena were also reported for influenza virus (Katze, Chen and Krug, 1984) and vaccinia virus (Whitaker-Dowling and Youngner, 1984). Some authors claimed that the VAI RNA activity is somewhat specific to adenovirus mRNA recognizable by its characteristic leader sequence at the 5'-end (Katze, Chen and Krug, 1984; Svensson and Akusjarvi, 1984, 1985; Kaufman, 1985), this matter is still controversial.

Summary

Some of the modifications of the host translational machinery following viral infection result in inhibition of translation of cellular mRNA thus generally favoring viral mRNA translation (poliovirus, vaccinia, Semliki Forest virus); in other situations the translation of viral mRNA itself (especially late viral mRNA) is directly stimulated (Frog virus 3, adenovirus, HTLV-III). Other viruses probably also exert a control on translation but the results in these systems are too preliminary or too controversial to be discussed here. It is quite logical to suppose that most cytolytic viruses interact with the protein synthesis machinery at one point or

another. Further studies will probably reveal further diversity and subtlety of the translational controls in virus-infected cells.

FIGURE. 1

Organization of the reovirus particle

The general organization of the reovirus particle, as we presently understand it, is schematized in this figure. (▼) lambda 2; (■) outer capsid capsomers: the dark portion represents sigma 3 and the light one the mulC protein; (▽) sigma 1; (□) inner capsid capsomers: probably made of lambda 1 and sigma 2; (S, M, L) represent segments of double-stranded RNA of the small, medium and large size classes; the dotted region is the space between the two capsids.

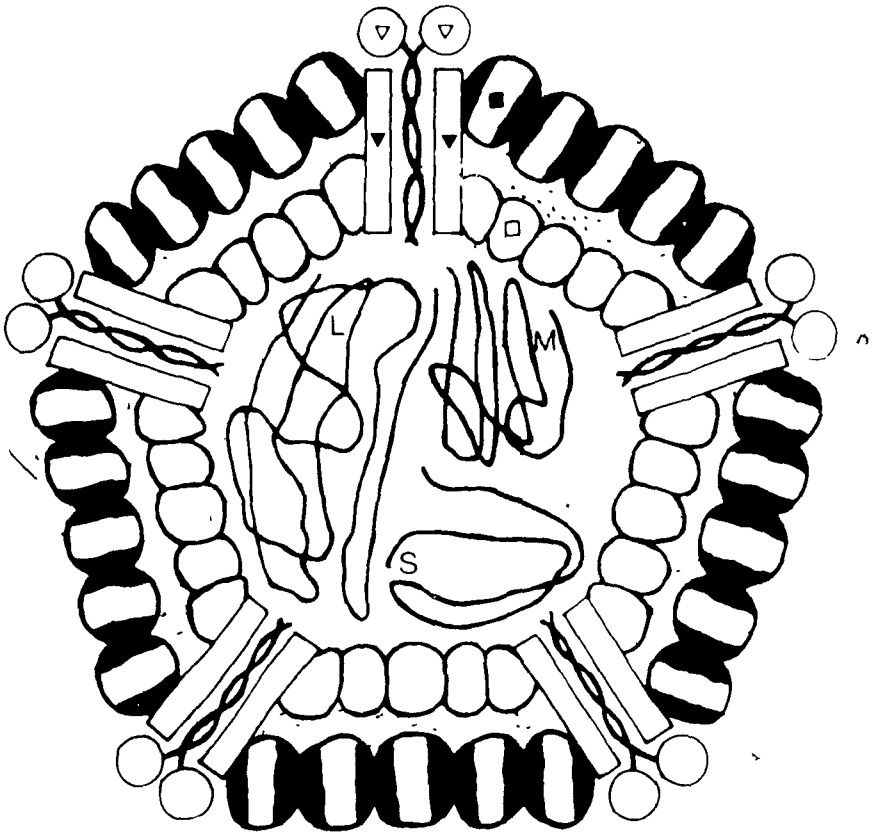

L
M
S

TABLE. I

Protein composition of reovirus serotype 3 (Dearing strain)

Gene	Protein	Molecular weight (kDa)	Approximate number of molecules per virion (% in weight)	Location
L1	Lambda 3	135	12 (2%)	core
L2	Lambda 2	140	90 (11%)	core
	Lambda 2C	125	--------	----
L3	Lambda 1	155	105 (15%)	core
M1	Mu 2	70	12 (2%)	core
M2	Mu 1	80	20 (2%)	core
	Mu 1C	72	550 (35%)	outer shell
M3	Mu NS	75	---------	----
	Mu NSC	70	---------	----
S1	Sigma 1	42 (49*)	24 (1%)	outer shell
	Sigma 1bNS	14 (14)	---------	----
S2	Sigma 2	38 (37)	200 (7%)	core
S3	Sigma NS	36 (41)	---------	----
S4	Sigma 3	34 (41)	900 (28%)	outer shell

* In parentheses is indicated the molecular weight calculated from the amino acid sequence deduced from DNA sequencing of the cloned gene.

TABLE. II

Relative transcriptional and translational efficiencies of the different reovirus genes

Gene	Transcription frequency in vitro	Transcription frequency in vivo	Translation frequency in vivo
L1	0.275	0.05	0.03 (0.02*)
L2	0.275	0.05	0.15 (0.29)
L3	0.275	0.05	0.10 (0.18)
M1	0.500	0.15	0.03 (0.03)
M2	0.500	0.30	1.00 (0.42)
M3	0.500	0.50	0.50 (0.25)
S1	0.880	0.50	0.05 (0.12)
S2	0.880	0.50	0.20 (0.42)
S3	1.000	1.00	0.30 (0.55)
S4	1.000	1.00	0.70 (1.00)

* More recent data (Gaillard and Joklik, 1985) are indicated in parentheses.

FIGURE. 2

Main events occurring during the reovirus replicative cycle

This figure briefly summarizes the main events occurring during infection of L cells by the human reovirus serotype 3. This figure is not intended to describe all the events occurring in the infected cell. A more detailed description is given in the text. The event mentioned as "modification" is the main topic of this thesis. (Reprinted from Skup, Zarbl and Millward, 1981)

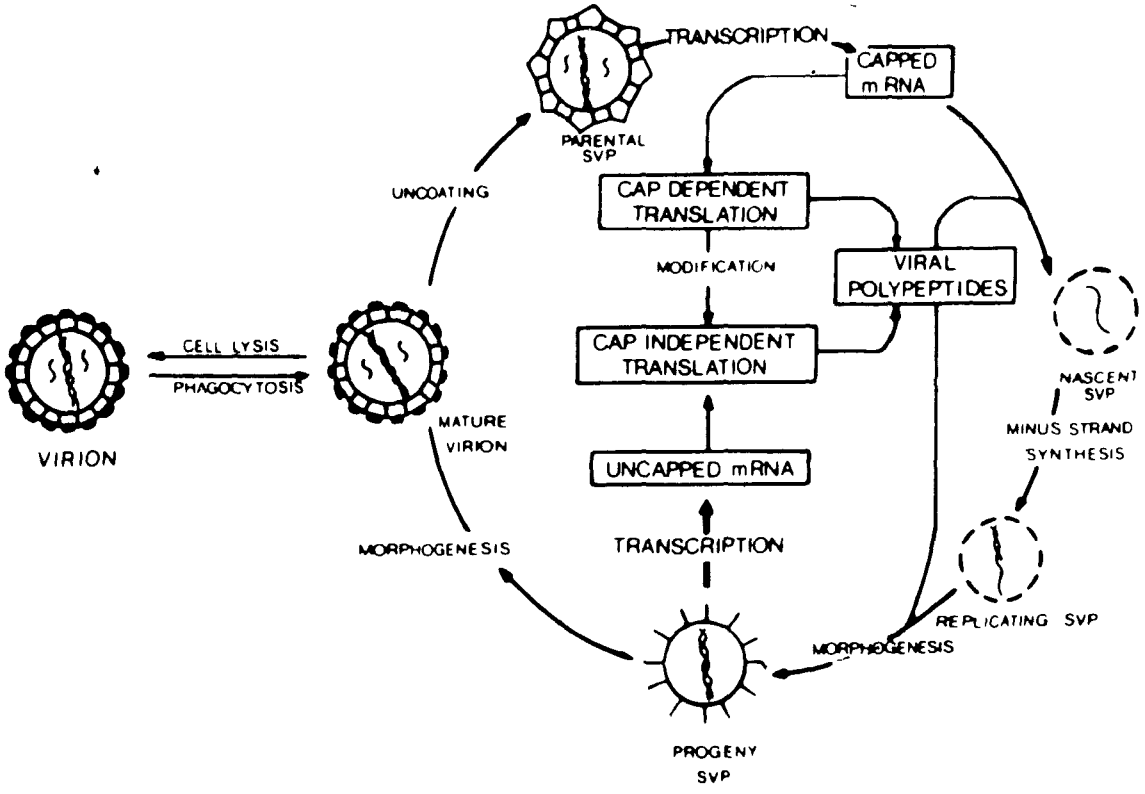
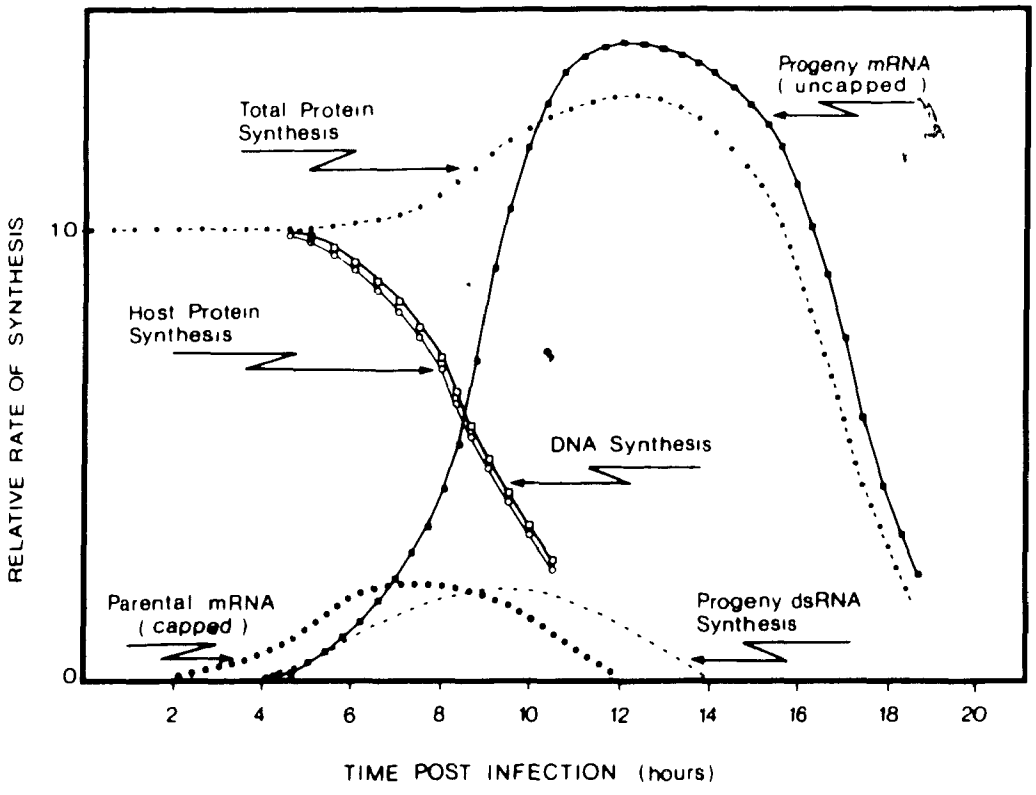

TRANSCRIPTION
CAPPED
m RNA
PARENTAL
SVP
UNCOATING
CAP DEPENDENT
TRANSLATION
MODIFICATION
VIRAL
POLYPEPTIDES
CELL LYSIS
PHAGOCYTOSIS
CAP INDEPENDENT
TRANSLATION
NASCENT
SVP
MINUS STRAND
SYNTHESIS
VIRION
MATURE
VIRION
UNCAPPED mRNA
MORPHOGENESIS
TRANSCRIPTION
REPLICATING SVP
MORPHOGENESIS
PROGENY
SVP

FIGURE. 3

Time course of the events occurring in reovirus-infected cells

This figure indicates at which time post-infection each of the events described in the text occurs. This time course refers to infection of mouse L cells with the reovirus serotype 3 (Dearing strain) at 37 °C. At 30 °C each event takes about twice as long to occur. Infection with the reovirus serotype 1 is also about twice as long as it is with the serotype 3 at the same temperature. (Reprinted from Zarbl and Millward, 1983)


Progeny mRNA
(uncapped)
Total Protein
Synthesis
10
Host Protein
Synthesis
DNA Synthesis
Parental mRNA
(capped)
Progeny dsRNA
Synthesis
0
RELATIVE RATE OF SYNTHESIS
2
4
6
8
10
12
14
16
18
20
TIME POST INFECTION (hours)

CHAPTER 2

Inhibition of translation in L cell lysates by free polyadenylic acid: Differences in sensitivity among different mRNAs and possible involvement of an initiation factor

FOREWORD

This chapter presents data concerning the inhibition of *in vitro* translation by free polyadenylic acid [poly(A)]. These observations suggest a possible role of the poly(A) tract of mRNA in the translation process. This point is of possible interest for the control of translation in reovirus-infected cells because reovirus mRNA lacks the 3'-poly(A) tail present on most eucaryotic mRNAs. A possible role of poly(A) in the control of protein synthesis during infection by vaccinia virus has been suggested (see chapter 1) giving another incentive to the study of poly(A) effect on translation in the L cell/reovirus system.

The results presented in this chapter are all my own results and are mostly original. Where the experiments presented are confirmation of results already obtained in other systems it is clearly stated in the text. This chapter has been published [Lemay, G., and S. Millward (1986) Arch. Biochem. Biophys. 249, 191-198] and is presented here in the same form.

ABSTRACT

Free polyadenylic acid specifically inhibits *in vitro* translation of naturally polyadenylated mRNAs in L cell lysates. The polynucleotide affects the initiation of protein synthesis but has no apparent effect on elongation of polypeptide chains. Reovirus mRNA, naturally devoid of a poly(A) tail, is much less sensitive to this inhibition than are naturally polyadenylated mRNAs. Reovirus mRNA that was polyadenylated *in vitro* is not more sensitive than normal reovirus mRNA. The degree of inhibition of translation varies for the different reovirus mRNA species. The addition of proteins contained in a high salt wash of ribosomes can mitigate the inhibition of translation of naturally polyadenylated mRNAs by free polyadenylic acid. Altogether these results suggest that the inhibition by polyadenylic acid may be mediated by its interaction with a cellular (initiation) factor. The various sensitivities exhibited by different mRNAs may indicate differences in requirement for this factor.

INTRODUCTION

Eucaryotic mRNAs have a sequence of about 50-250 adenylic acid residues at their 3′ terminus (for review see: Brawerman, 1981; Littauer and Soreq, 1982). This poly(A) tail is generally absent from procaryotic mRNAs. Among the cellular mRNAs only those coding for histones are generally devoid of a poly(A) tail; however a fraction of the molecules belonging to other classes of mRNAs is also devoid of it. The exact significance of these two observations is not known. The majority of animal virus mRNAs also have a poly(A) stretch at their 3′ end (adenoviruses, picornaviruses, retroviruses, etc.). However the mRNAs from the Reoviridae are never polyadenylated (Stoltfus, Shatkin and Banerjee, 1973; Joklik, 1983a) and some other viruses (vaccinia, encephalomyocarditis virus) apparently produce molecules of both types (Littauer and Soreq, 1982; Nevins and Joklik, 1975; Hruby and Roberts, 1977). Despite its widespread occurrence the exact role of the poly(A) tail has never been really clarified. An increase in mRNA stability is the principal suggested role (Brawerman, 1981; Littauer and Soreq, 1982; Soreq et al., 1974; Huez et al., 1974, 1975, 1978; Marbaix et al., 1975; Nudel et al., 1976; Huez, Bruck and Cleuter, 1981).

A direct involvement of the poly(A) tail in protein synthesis has been occasionnally suggested (Doel and Carey, 1976; Hruby and Roberts, 1977; Deshpande, Chatterjee and Roy, 1979; Jacobson and Favreau, 1983) but other experiments failed to demonstrate it (Fernandez Munoz and Darnell, 1974; Soreq et al., 1974; Huez et al., 1974; Nevins and Joklik, 1975; Spector, Villa-Komaroff and Baltimore,

1975). However it was noticed by some authors (Doel and Carey, 1976; Deshpande, Chatterjee and Roy, 1979; Jacobson and Favreau, 1983) that most of the negative results were obtained by using *in vitro* translation systems that reinitiate poorly. These studies generally compare the translation of poly(A)+ to the translation of poly(A)- mRNAs from natural sources, or prepared *in vitro* by deadenylation or occasionally polyadenylation.

More recently a different approach has revealed a possible direct involvement of poly(A) in protein synthesis (Jacobson and Favreau, 1983). These authors observed that free polyadenylic acid inhibits *in vitro* translation of natural poly(A)+ mRNAs in reticulocyte lysates much more than other polyribonucleotides inhibit, moreover, mRNAs naturally devoid of a poly(A) tail are clearly more resistant to this inhibition. In this chapter we first extend these observations to translation in L cell lysates developed in our laboratory (Skup and Millward, 1977) and demonstrate that, as previously suggested by others (Lodish and Nathan, 1972; Jacobson and Favreau, 1983), the observed inhibition is at the level of initiation of protein synthesis. Natural or *in vitro* polyadenylated reovirus mRNA is shown to be resistant to inhibition by free polyadenylic acid. Finally we demonstrate that proteins contained in a high salt wash of ribosomes (crude initiation factors) can mitigate the inhibition of translation of naturally polyadenylated mRNAs. The possibility that the free polyadenylic acid interacts with an initiation factor is discussed.

MATERIALS AND METHODS

Cells and virus. Mouse L cells were kept exponentially growing and reovirus serotype 3 (Dearing strain) was grown in these cells and purified as described (Smith, Zweerink and Joklik, 1969; Lemieux, Zarbl and Millward, 1984).

Synthesis and purification of reovirus mRNA. Reovirus mRNA was made in vitro in conditions resulting in formation of capped mRNA as described previously (Skup and Millward, 1980). The resultant mRNA was purified by phenol extraction and Sephadex G-100 chromatography.

Globin mRNA. Pure globin mRNA was obtained from Bethesda Research Laboratories.

Polyadenylation of reovirus mRNA. Reovirus mRNA was polyadenylated in vitro according to published procedures (Sippel, 1973) but using 1 mM ATP, 100 µg/ml of reovirus mRNA and 80 units/ml of Escherichia coli poly(A) polymerase (Bethesda Research Laboratories). We also included 1 mCi/ml of [alpha-^{32}P]ATP (3000 Ci/mmol, New England Nuclear) when labeling of the poly(A) tail was necessary . After 15 min of incubation at 37 °C, sodium dodecylsulfate (SDS) was added to 0.1% and the mixture extracted with phenol/chloroform (1/1) and RNA precipitated overnight with 3 vol of ethanol at -20 °C. Polyadenylated molecules were selected by oligo-dT cellulose chromatography in 0.7 M NaCl and extensive washing of the column with the same solution, then the polyadenylated mRNA was eluted with sterile distilled-deionized water.

Polynucleotides. Polyadenylic acid and polyguanylic acid were

purchased from Boehringer-Mannheim Corporation or Biopolymers Incorporated. They were dissolved at a concentration of 0.5 mg/ml in sterile distilled-deionized water. Reduction in the length of polynucleotides was obtained, when desired, by alkaline treatment. Briefly, the polynucleotides at a concentration of 1 mg/ml were incubated at 37 °C in 0.2 N KOH for 3 or 7 min; the solution was then neutralized with HCl and the polynucleotides were precipitated with ethanol. All polynucleotide solutions were kept at -20 °C.

Gel electrophoresis of polynucleotides and mRNAs. Polynucleotides and mRNAs were separated on denaturing agarose gels according to Bailey and Davidson (Bailey and Davidson, 1976) and stained with ethidium bromide. For autoradiography the gels were dried under vacuum and exposed at room temperature.

Cell-free translation. Cell-free lysates were prepared from L cells grown in suspension culture as described by Skup and Millward (Skup and Millward, 1977) and modified by Lemieux et al. (Lemieux, Zarbl and Millward, 1984); when necessary the lysate was rendered mRNA-dependent by treatment with micrococcal nuclease as described (Skup and Millward, 1977). The translation reactions in a total volume of 20 µl were allowed to proceed at 30 °C in conditions that have been described (Lemieux, Zarbl and Millward, 1984). The incubation time is indicated in each experiment. Efficiency of the translation reaction was measured by the incorporation of [^{35}S] methionine (1000 Ci/mmol, New England Nuclear Corp.) into hot trichloroacetic acid (TCA) precipitable products as described previously (Skup and Millward, 1977).

Preparation of ribosomal salt wash. Preparation of crude initiation factors by high salt wash of ribosomes recovered from L cell lysates was done by established procedures (Schreier and Staehelin, 1973). Briefly, lysate [postnuclear supernatant prepared as described (Skup and Millward, 1977)] from 2 billion cells (4 liters of exponentially growing cells) was centrifuged (45,000 rpm for 1 h in a Beckman Type 50 rotor, at 4 °C) and the ribosomal pellet was resuspended in 0.6 ml of buffer A [10 mM 4-(2-hydroxyl)-piperazine-ethanesulfonic acid (Hepes)-KOH, pH 7.5, 10 mM potassium acetate and 1.5 mM magnesium acetate]. We then adjusted the KCl concentration to 0.5 M and the ribosomes were pelleted again. The supernatant constitutes the ribosomal salt wash (RSW) and was dialyzed for 3 h against dialysis buffer (10 mM Hepes-KOH pH 7.5, 100 mM KCl, 0.05 mM disodium-EDTA and 6 mM 2-mercaptoethanol) before being frozen in liquid nitrogen. Protein concentration determined by the Bradford procedure (Bradford, 1976) was generally around 10 mg/ml.

Gel electrophoresis of proteins. Proteins were separated on SDS-polyacrylamide slab gels according to Laemmli (Laemmli, 1970). After electrophoresis, gels were stained with Coomassie blue, destained, dried and subjected to autoradiography.

RESULTS

Effect of polyadenylic acid on translation of naturally polyadenylated mRNAs in L cell lysates.

It was demonstrated by Jacobson and Favreau (1983) that free polyadenylic acid inhibits in vitro translation of polyadenylated mRNAs more strongly than other ribopolymers, for example, polyguanylic acid. Their experiments were performed in reticulocyte lysates. Translation in L cell lysates has been developed in our laboratory (Skup and Millward, 1977) and we decided to verify if translation is also inhibited by polyadenylic acid in this system or if it is a peculiarity of the reticulocyte system.

We first compared the effect of addition of free polyadenylic or polyguanylic acid at different concentrations on protein synthesis programmed with cellular mRNAs in lysates untreated with nuclease (endogenous mRNAs). The average of similar results obtained using three different lysates is shown in Fig 4-A.

It is clear that polyadenylic acid is much more inhibitory than polyguanylic acid at the same concentration; the difference is especially evident at low concentration of ribopolymers. Other ribopolymers that were tested behave like polyguanylic acid (data not shown). For the other experiments described in this chapter we generally used only polyguanylic acid as a control when necessary. All these results are consistent with the observations made in reticulocyte lysates. The concentration of polyadenylic acid to obtain 50% inhibition may slightly vary with different lysates. However the inhibitory activity is evident in all lysates. We also

noticed a small stimulation by polyguanylic acid especially at low concentration. This last unexplained observation is also consistent with the previous study (Jacobson and Favreau, 1983). The specific inhibition by polyadenylic acid was also observed during translation of added L cell mRNAs (data not shown) or globin mRNA (Fig. 4-B) to lysates that have been depleted of their endogenous mRNAs by nuclease treatment. The concentration of poly(A) required to achieve inhibition is, however, reduced, possibly due to the fact that the endogenous mRNAs are present as ribonucleoproteins and may be less sensitive to the inhibition (Jacobson and Favreau, 1983). Furthermore the exact concentration of translatable endogenous mRNAs is not known and may be much higher than the concentration of pure mRNA added; notice also that a certain proportion of the incorporation of amino acids due to endogenous mRNAs represents elongation of polypeptide chains already initiated.

It was suggested that the effect of poly(A) on translation is at the level of initiation (Lodish and Nathan, 1972; Jacobson and Favreau, 1983). The translation of endogenous mRNAs in the lysates was particularly well suited to prove this point. If we blocked the initiation of translation by addition of aurintricarboxylic acid (ATA) to the reaction (Goldstein, Reichmann and Penman, 1974; Skup and Millward, 1977) we observed a residual protein synthesis representing the completion of polypeptide chains already initiated before incubation. This residual synthesis is almost totally resistant to polyadenylic acid (Table III) demonstrating clearly that free poly(A) affects only the initiation of translation.

We further checked the concentration of each polynucleotide

solution by optical density, and the length of each was also determined by gel electrophoresis and shown to be somewhat heterogeneous and around 300-1000 nucleotides long (data not shown). We also used two different sources for each polynucleotide (purchased from Boehringer-Mannheim Corporation and Biopolymers Incorporated) and obtained similar results with both (data not shown). Finally, degradation of polynucleotides by alkaline hydrolysis did not change the polynucleotides' activity as long as their size was 150 nucleotides or longer. The difference in activity between poly(A) and poly(G) thus can not be due to differences in size or concentration.

The translation products of endogenous mRNAs were analyzed by gel electrophoresis (data not shown). We noticed that the pattern of proteins produced is apparently the same in the presence or absence of free poly(A). However the gel system allowed the comparison of only some well-resolved protein species and did not really eliminate the possibility that the synthesis of some proteins was preferentially affected. There was no apparent accumulation of premature termination products, consistent with the inhibitory effect being only at the initiation of synthesis.

Effect of free polyadenylic acid on the translation of reovirus mRNA in L cell lysates.

According to Jacobson and Favreau (1983), the translation of mRNAs naturally devoid of a poly(A) tail [poly(A)-] is much less sensitive to inhibition by polyadenylic acid. Reovirus mRNA is a good example of such mRNA lacking a poly(A) tail (Stoltfus, Shatkin and Banerjee, 1973): it is easy to obtain pure and in good yields, it is very active in the L cell lysates (Skup and Millward, 1977) and it has

been used routinely in our laboratory (Skup and Millward, 1977; Skup and Millward, 1980; Lemieux, Zarbl and Millward, 1984). Finally it consists of a mixture of ten different mRNAs coding for different protein species resolvable by gel electrophoresis.

Using the nuclease-treated L cell lysates our results showed that at the same concentration of mRNA, the translation of naturally poly(A)- reovirus mRNA is much less sensitive to inhibition by polyadenylic acid than poly(A)+ mRNAs such as globin mRNA (Fig. 5-A). In fact, translation of reovirus mRNA was stimulated at low concentrations of poly(A), as described for other poly(A)- mRNAs in reticulocyte lysates (Jacobson and Favreau, 1983), and was inhibited only by much higher concentration of free poly(A). Kinetic analysis has revealed that the stimulatory effect is more important in the second half of the incubation period (data not shown). This clear stimulation was not seen with poly(G) which showed a small inhibition of translation of reovirus mRNA. This resistance to polyadenylic acid was also observed when we studied translation of endogenous mRNAs (mostly viral) in lysates prepared from reovirus-infected L cells (data not shown). This last result was, however, more difficult to interpret because we ignore the relative concentration of mRNAs in infected cells compared to uninfected cells. Also it should be noted that the nature of the modifications to the protein synthesis machinery of reovirus-infected cells is not completely known.

The analysis of translation products obtained with reovirus mRNA in nuclease-treated lysates revealed that the synthesis of the different viral proteins was not affected to the same extent by the presence of polyadenylic acid in the translation reaction mixture. It

is clear on the autoradiogram (Fig. 5-B) that the synthesis of the upper protein band at 150 kDa is inhibited but that synthesis of the lower band of this doublet is increased. We also observed a decrease of the bands at around 88 and 70 kDa but the two bands at around 80 kDa did not look affected. This point is examined further under Discussion.

Effect of polyadenylation on translation of reovirus mRNA

Reovirus mRNA is naturally devoid of a poly(A) tail; however it is possible to polyadenylate it with Escherichia coli poly(A) polymerase in vitro. Preliminary data indicated that a reaction time of 15 min followed by selection of the polyadenylated mRNA on oligo-dT cellulose does not result in significant degradation of the mRNA as judged by gel electrophoresis (data not shown). The different species of mRNA were all polyadenylated as judged by the incorporation of radioactivity from [alpha- ^{32}P]ATP in each part of the electrophoretic profile of mRNA (data not shown). The length of added poly(A) was estimated by adsorption of labeled molecules on oligo-dT cellulose or nitrocellulose filters. It has been shown (reviewed by Brawerman, 1981) that only molecules having a poly(A) tail of more than 15 bases bind to oligo-dT cellulose; these mRNAs are generally considered as poly(A)+ mRNAs. Furthermore, molecules having a 3′ end of more than 50 adenylic acid residues bind to nitrocellulose under high salt conditions (0.5 M NaCl). About 50% of the polyadenylated reovirus mRNA that we purified on oligo-dT cellulose also binds to nitrocellulose filters (data not shown), an amount comparable to the amount we measured for L cell mRNA. It thus seems that the in vitro synthesized poly(A) tail is about the same length as the poly(A) tail

of naturally poly(A)+ cellular mRNAs.

The translation of this artificially polyadenylated mRNA examined in the presence or absence of free polyadenylic acid revealed that it behaves like normal reovirus mRNA (Fig. 6-A), being as resistant to the inhibition by polyadenylic acid as is normal poly(A)- reovirus mRNA. The translation products did not seem to be different from those obtained after translation of normal reovirus mRNA (Fig. 6-B) except for the proteins at 150 kDa, which decreased. This decrease is probably due to degradation of the longer mRNAs coding for these proteins; degradation of these long mRNAs was more difficult to judge by gel electrophoresis.

Effect of ribosomal salt wash proteins on the inhibition of translation by polyadenylic acid.

The previous results together with the published observation that the concentration of free poly(A) needed to achieve 50% inhibition increases with the concentration of mRNA (Jacobson and Favreau, 1983) suggested that the free poly(A) probably inhibits translation by competing with mRNA for a limited supply of a cellular factor. Since the inhibition was at the level of initiation only, we tried to release this effect by adding an excess of proteins prepared by high salt wash of ribosomes (crude initiation factors). At the concentrations used we never observed an inhibitory effect of RSW proteins. In some lysates we observed a certain increase of translation upon addition of RSW (see Table IV lysate 2). Those lysates were generally less active lysates where the most sensitive components of RSW were presumably in limiting amount; in many lysates we did not observe such an effect. In every lysate tested and with

different RSW preparations we noticed that the RSW decreases the inhibition by free poly(A). Typical results are presented in Table IV. Ribosomal salt wash proteins prepared as described under Materials and Methods contain a negligible amount of translatable mRNAs. However, to eliminate completely the possibility that mRNAs could account for the observed effect of RSW, we treated the RSW with micrococcal nuclease at five times the concentration normally used to treat lysates. This treatment has essentially no effect on the activity of the ribosomal salt wash (data not shown). Incubation of RSW with radiolabeled poly(A) did not detect nuclease activity. Other experiments using fractionated systems have also indicated that the RSW do not contain ribosomes (data not shown). Furthermore addition of dialyzed and nuclease-treated ribosomes or postribosomal supernatant has no effect on the inhibition by poly(A) (data not shown).

DISCUSSION

The use of *in vitro* translation allowed us to study translation occurring during a short period of time. The stability of mRNA has probably a negligible role in this system, as suggested by the linear incorporation of amino acids during the short incubation period (Skup and Millward, 1977), allowing us to reveal small differences in the efficiency of translation, independently of indirect effects on the stability of mRNA. Furthermore, the use of lysates rendered completely mRNA-dependent by treatment with micrococcal nuclease allowed us to study easily the translation of a particular mRNA in the absence of other competing mRNAs.

The reticulocyte lysate translation system was used by Jacobson and Favreau (Jacobson and Favreau, 1983) to study, in greater detail, the already-known inhibition of *in vitro* translation by ribopolymers (Lodish and Nathan, 1972). They showed that poly(A) is a much better inhibitor than any other ribopolymer and that it is specific for translation of naturally polyadenylated mRNAs. They suggest that free poly(A) competes with the poly(A) tail of mRNA. These results obtained in reticulocyte lysates were confirmed in this study by means of the L cell lysate system developed in our laboratory (Skup and Millward, 1977).

It was suggested that ribopolymers in general (Lodish and Nathan, 1972) and especially polyadenylic acid (Jacobson and Favreau, 1983) affect the initiation of translation. We have shown this much more clearly by using an inhibitor of translation initiation (ATA) as described under Results. Using reovirus mRNA that is naturally devoid

of a poly(A) tail we confirmed the resistance of poly(A)- mRNA to the inhibition by polyadenylic acid. Furthermore, we demonstrated that reovirus mRNA polyadenylated in vitro is still active and is not more susceptible to inhibition by polyadenylic acid. Its resistance to polyadenylic acid-induced inhibition does not reside in the absence of a poly(A) tail in itself. It thus seems that polyadenylic acid does not compete with mRNAs, such as reovirus, that are naturally devoid of a poly(A) tail. One possible explanation is that translation of reovirus mRNA has greatly reduced dependence on the hypothetical factor that recognizes poly(A). The different reovirus mRNA segments may also have different requirements or different affinities for the factor, explaining their different sensitivities to inhibition. The alternative explanation, by a general non-specific action, as proposed by Lodish and Nathan (Lodish and Nathan, 1972) in their study of globin mRNA, is doubtful because we did not observe a difference of sensitivity to poly(G) among different reovirus mRNA segments. What is the feature that causes this difference among the reovirus mRNA segments? What are the features of reovirus and histone mRNAs that explain their lack of poly(A) tail? These two points are not clear but certainly merit further studies.

Addition of a ribosomal salt wash decreased the inhibition by free poly(A). Different tests have eliminated different trivial explanations for this activity. We interpret this effect as meaning that the hypothetical factor involved in the poly(A) effect is present in the RSW. As this fraction (generally referred to as "crude initiation factors") is the only active fraction we suggest that such an initiation factor, not necessarily a known factor, is responsible

for the effect. We have tried to characterize further this factor but it is apparently difficult to fractionate the RSW without losing the activity. A fraction obtained by ammonium sulfate fractionation (25 to 40% saturation) seems to be the most active. However this fraction has a complex composition and attempts to further purify the factor have been so far unsuccessful. Clearly much more work will be necessary to devise a procedure allowing the purification of this interesting component (or components) and to determine its exact role in translation.

FIGURE. 4

Effect of polynucleotides on translation in L cell lysates

In vitro translation in L cell lysates was done as described under Materials and Methods, in the presence or absence of polynucleotide; in each case the percentage of control was calculated by comparison with the reaction in absence of polynucleotide. Each reaction was incubated for 30 min before measuring hot TCA-precipitable material in a 5 µl aliquot of each reaction. (A) The control reaction without polynucleotide gave an incorporation of around 200,000 cpm, (B) around 100,000 cpm. (●) Reaction in the presence of polyadenylic acid; (■) reaction in the presence of polyguanylic acid. (A) Effect of polynucleotide on translation of endogenous mRNAs, average of results obtained with three different lysates. (B) Average of the results obtained in two similar experiments with two different nuclease-treated lysates with 0.3 µg of globin mRNA per reaction.

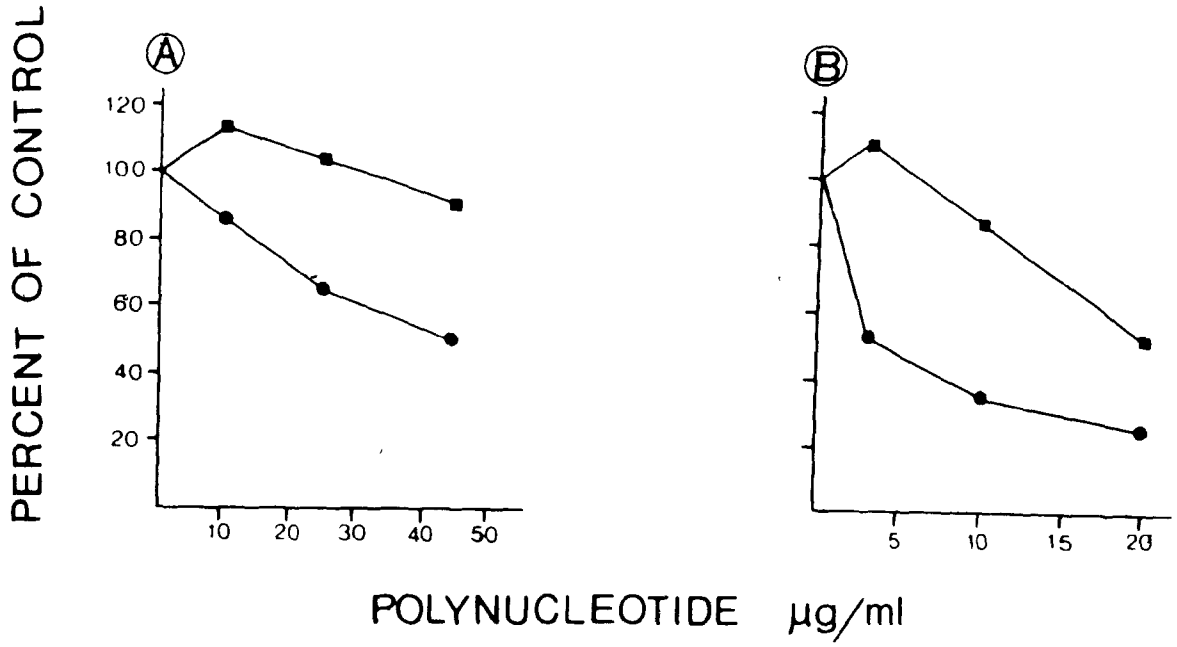

PERCENT OF CONTROL
A
120
100
80
60
40
20
10
20
30
40
50
B
5
10
15
20
POLYNUCLEOTIDE μg/ml

TABLE III

Effect of free poly(A) on initiation of protein synthesis

ATA added (mM)	Lysate number *	Absence of free poly(A) (counts X 10^3) **	Presence of free poly(A) (40 µg/ml)	% of inhibition by free poly(A)
0	1	64	35	45
0	2	316	150	53
0.1	1	14	13	7
0.1	2	65	64	2

* Translation of endogenous RNA, absence of micrococcal nuclease treatment.

** Hot TCA-precipitable counts in 5 µl aliquots after 30 min of incubation as described under Materials and Methods.

FIGURE. 5

Effect of polyadenylic acid on translation of globin and reovirus mRNA

(A) L cell lysates were rendered mRNA-dependent by treatment with micrococcal nuclease and translations were done as described under Materials and Methods. The same experiment was done using two different lysates and the average of the similar results (less than 10% differences) are presented here. We used either 0.3 μg of globin mRNA (●) or 0.3 μg of in vitro synthesized reovirus mRNA (■). Each translation was done in the presence of the indicated concentration of free poly(A) and hot TCA-precipitable counts in 5 μl aliquots were counted after 45 min of incubation. The percentage of inhibition by poly(A) was calculated after the background of incorporation without mRNA was substracted. In each case the incorporation was around 100,000 cpm in the control reaction, with a background around 5,000. (B) The translation products obtained with the reovirus mRNA in the absence of added polynucleotides (C) or in the presence of 15 μg/ml of free poly(A) (+A) or poly(G) (+G) were analyzed by 7.5% SDS-polyacrylamide gel; -, a reaction without added RNA. Incorporation results: (C) 100,000 cpm; (+A) 120,000 cpm; (+G) 80,000 cpm per 5 μl aliquot after 30 min of incubation.

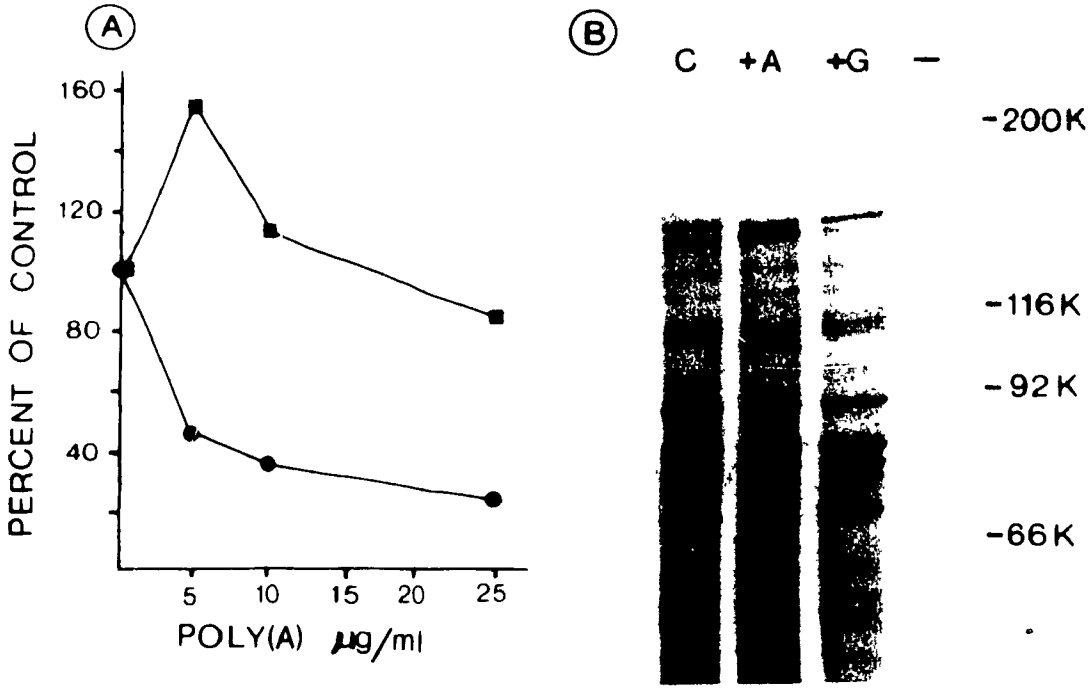

A
PERCENT OF CONTROL
160
120
80
40
5
10
15
20
25
POLY(A) µg/ml
B
C
+A
+G
—
-200K
-116K
-92K
-66K

FIGURE. 6

Effect of polyadenylation on translation of reovirus mRNA

Reovirus mRNA was synthesized in vitro and polyadenylated as described under Materials and Methods. Translation of 0.3 µg of each mRNA was done in the presence or absence of free poly(A). After 30 min of incubation hot TCA-precipitable material in 5 µl was counted and 5 µl was analyzed by 7.5% SDS-polyacrylamide gel electrophoresis. (A)●, reovirus mRNA; ■, in vitro polyadenylated reovirus mRNA. (B) Proteins synthesized in the absence (-) or presence (+) of 3 µg/ml of free poly(A) using reovirus mRNA (a) or in vitro polyadenylated reovirus mRNA (b).

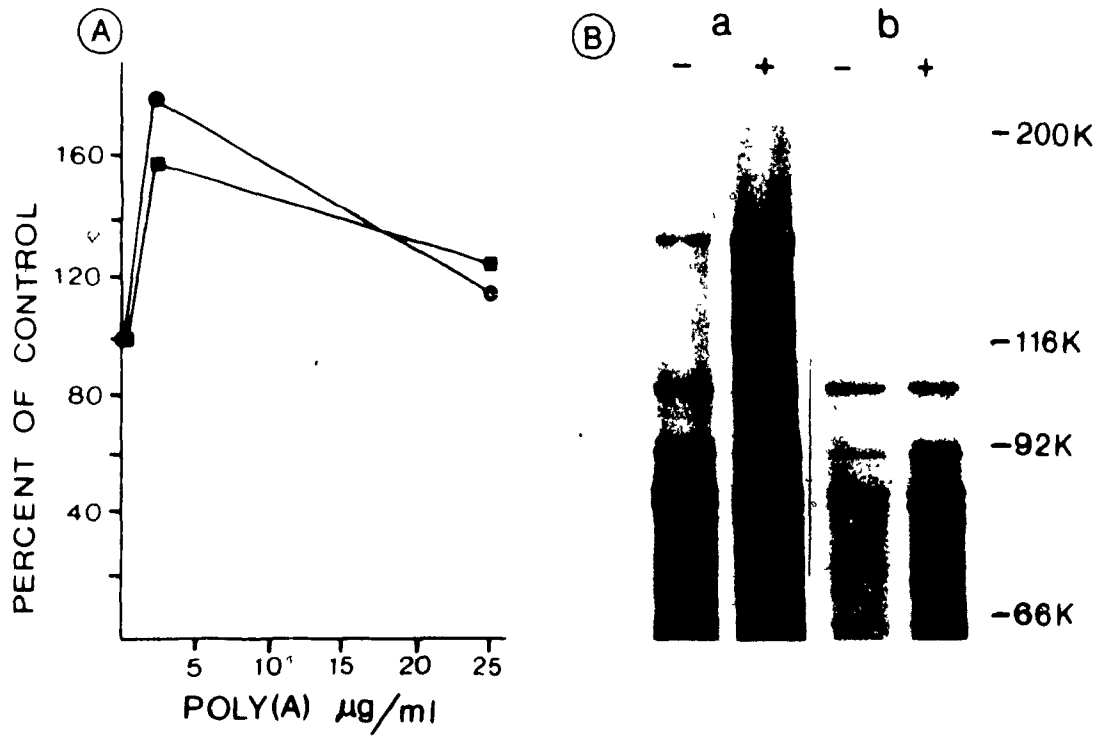

A
PERCENT OF CONTROL
160
120
80
40
5
10
15
20
25
POLY(A) µg/ml
B
a
b
–
+
–
+
–200K
–116K
–92K
–66K

TABLE IV

Effect of the addition of ribosomal salt wash proteins on the inhibition of translation by free poly(A)

RSW added *	Lysate number **	Absence of free poly(A) (counts X 10^3) ***	Presence of free poly(A) (70 μg/ml)	% of inhibition by free poly(A)
None	1	121	55	55
No 1	1	134	107	20
None	2	64	35	45
No 2	2	103	77	25

* Four microliters of ribosomal salt wash proteins (two different preparations No 1 and No 2) prepared as described under Materials and Methods

** Translation of endogenous RNA, absence of micrococcal nuclease treatment.

*** Hot TCA-precipitable counts in 5 μl aliquots after 30 min of incubation

CHAPTER 3

The viral protein sigma 3 participates in the translation of late viral mRNA in reovirus-infected L cells

FOREWORD

This chapter is the first of three chapters concerning the characterization of a factor involved in the translation of late viral mRNA. This late reovirus mRNA was previously shown to differ from cellular mRNA by the absence of a cap structure at the 5'-end.

Some of the data presented in this chapter were used as confirmations of, then unpublished, results obtained by Real Lemieux in our laboratory. These results, together with some original results also presented in this chapter and previous results obtained by Réal Lemieux, have been submitted for publication (Lemieux, R., G. Lemay, and S. Millward., J. Virol. submitted). Some of these results, mentioned as "data not shown" in the manuscript, are however described in greater details in this chapter. This chapter is thus different from the manuscript but the general conclusions are the same, some sections are also directly taken from the manuscript. The actual data presented in this chapter are all the results of my own experiments.

ABSTRACT

Reovirus late (uncapped) mRNA is more efficiently translated *in vitro* using lysates prepared from infected than from uninfected L cells. Reovirus serotype 3 also inhibits host cell protein synthesis during infection but such an inhibition does not occur with the serotype 1. Lysates prepared from cells infected with either serotype are more efficient than lysates prepared from uninfected cells in translating late viral mRNA. This observation suggests that the two phenomena (inhibition of host cell protein synthesis and stimulation of late viral mRNA translation) are not directly correlated. Preliminary data (obtained by Réal Lemieux in our laboratory) indicated that different fractions from infected cells can stimulate translation of late viral mRNA when added to uninfected lysates; the ribosomal salt wash protein fraction is however the most active. The sigma 3 viral protein is also enriched in this fraction. Purification of this protein reveals that it can stimulate translation of late viral mRNA without effect on other mRNAs. The protein binds to ribosomes; this binding apparently occurs during initiation of protein synthesis. The protein does not bind directly to mRNA. Altogether the results suggest that the sigma 3 viral protein has properties and may have a role similar to an initiation factor of protein synthesis.

INTRODUCTION

Infection of mouse L cells with the widely studied serotype 3 (Dearing strain) of human reovirus results in a gradual decrease of host cell specific protein synthesis with a concomitant increase in viral specific protein synthesis (Zweerink and Joklik, 1970). At times when viral protein synthesis is maximal there is virtually no synthesis of cellular proteins.

We have previously shown that in reovirus serotype 3 infected cells there occurs a gradual transition in the translational machinery. This change results in very efficient translation of late viral mRNA (Skup and Millward, 1980a; Skup, Zarbl and Millward, 1981; Lemieux, Zarbl and Millward, 1984). This mRNA is synthesized by progeny subviral particles and lacks a cap structure (uncapped mRNA) (Zarbl, Skup and Millward, 1980). At the same time there is inhibition of translation of both early viral mRNA (capped mRNA) and cellular mRNA (also capped) (Skup and Millward, 1980; Skup, Zarbl and Millward, 1981).

These findings suggested that the modification of the translational machinery may consist in the inactivation of the CBP's (cap-binding proteins) by the reovirus, a precedent already established for poliovirus infection of HeLa cells (Trachsel et al., 1980; Lee and Sonenberg, 1982). However, in the case of reovirus, several lines of evidence suggested that the inactivation of the CBP's could not account fully for the mRNA discrimination observed in in vitro translation systems. First, unlike the poliovirus RNA, the late reovirus mRNA is not translated efficiently in lysates prepared from

uninfected cells, even in presence of antibodies directed against the CBP's (Sonenberg et al., 1981a). Furthermore, the translation of late viral mRNA in lysates prepared from infected cells is sensitive to inhibition by the cap analog m^7GTP (Lemieux, Zarbl and Millward, 1984). These differences between the poliovirus and reovirus situations will be further discussed in chapter 6.

In this chapter we first compare the situation occurring during infection with the closely related serotype 1 of reovirus with the situation during serotype 3 infection. The results indicate that the stimulation of late viral mRNA translation occurs in cell infected with the serotype 1 despite the lack of inhibition of host cell protein synthesis, indicating that the two phenomena are distinct. These observations, plus other results of competition experiments between cellular and late viral mRNA showing that the two types of mRNA do not compete with each other in lysates prepared from late-infected cells (Lemieux, Zarbl and Millward, 1984), prompted previous investigators to suppose the presence of a discriminatory factor in infected cell lysates (Zarbl and Millward, 1983; Lemieux, Zarbl and Millward, 1984). The reduced efficiency of translation of capped mRNA was caused only by a reduced activity of the crude initiation factor preparations from infected cells (Zarbl, H., R. Lemieux, and S. Millward. unpublished data). In contrast, in fractionated cell-free systems, the factor that stimulated the translation of reovirus uncapped mRNA was present in all fractions from infected cells (Lemieux, R., unpublished data).

Analysis of the proteins present in the fractions prepared from infected cell lysates has shown that the distribution of the factor

necessary for efficient translation of uncapped reovirus mRNA was similar to the one of the viral capsid protein sigma 3. The fraction prepared by high salt wash of ribosomes or ribosomal salt wash proteins (RSW), and containing crude initiation factors, is enriched in the stimulatory factor and in sigma 3 viral protein. Furthermore, preincubation of the RSW fraction from infected cells with a monospecific anti-sigma 3 antiserum inhibited the activity that normally stimulates the translation of the late viral mRNA. In this chapter we used purified sigma 3 protein to further proves its ability to stimulate translation of late viral mRNA without effect on the translation of other mRNAs.

The properties of the binding of sigma 3 to the ribosomes in the presence of different inhibitors of protein synthesis or following different treatments to dissociate polysomes or remove mRNA, were also examined. Those results demonstrate that sigma 3 is associated with the ribosomes and not directly with the mRNA on polysomes. They also suggest that it acts during the initiation of protein synthesis and has properties consistent with a role of viral specific discriminatory initiation factor.

MATERIALS AND METHODS

Cells.

Mouse L cells were grown in monolayer in MEM (Flow: MEM modified Earles salts) containing 5% of fetal calf serum and 50 μg/ml of gentamicin sulfate. For suspension culture, MEM modified for suspension culture was substituted and cells were grown in Erlenmeyer flasks and gently mixed with magnetic stirring bars. Cells in suspension were held at 37 °C in a warm room; monolayers were held in a 37 °C incubator in an humidified atmosphere containing 5% CO_2.

Virus.

Human reovirus serotype 3 (Dearing strain) was obtained from American Type Culture Collection and serotype 1 (Lang strain) was a generous gift from Dr. B.N. Fields. Both viruses were propagated at low multiplicity of infection in mouse L cells. For virus purification and translation studies, infections of L cells were done at 10 PFU/cell as previously described (Lemieux, Zarbl and Millward, 1984). The virus was purified by freon extraction and isopycnic banding in CsCl essentially as described (Smith, Zweerink and Joklik, 1969).

mRNAs.

Uninfected cell mRNA and late-infected cell mRNA (30 h at 30 °C) were prepared by streptomycin sulfate precipitation of polysomes (Kaulenas et al., 1977) essentially as described (Skup, Zarbl and Millward, 1981), TMV (tobacco mosaic virus) RNA was prepared as described previously (Lemieux, Zarbl and Millward, 1984).

Labeling of cellular proteins.

For in vivo labeling of proteins, the cells (uninfected or infected) were centrifuged and resuspended at a concentration of 5 X 10^6 cells/ml in MEM (minus methionine) containing 2% heat-inactivated dialyzed fetal calf serum, 50 uCi/ml of [^{35}S]methionine (NEN, 1000 Ci/mmol) was then added and the incubation continued for 1 h at 37 °C.

Preparation and fractionation of cell-free lysates

The S10 lysates were prepared from uninfected or reovirus late-infected cells by a modification of the initially described procedure (Skup and Millward, 1977). Cells were first pelleted and resuspended at a density of 5 X 10^6 cells/ml in MEM (minus methionine) and incubated 2.5 h at 37 °C. Cells were then pelleted again, washed 4 times in PBS and disrupted with a Dounce homogenizer in hypotonic buffer (10 mM Hepes-KOH pH 8.0, 10 mM potassium acetate, 1.5 mM magnesium acetate) containing 1 mM DTT and 40 μM of hemin. After removal of the nuclei (10,000 g for 10 min) the S10 supernatant was frozen in liquid nitrogen. Lysates were rendered mRNA-dependent by addition of 1 mM $CaCl_2$ and 75 units/ml of micrococcal nuclease. After 10 min at 20 °C the reaction was stopped by addition of 3 mM EGTA (pH 8.0). Rabbit reticulocyte lysates were prepared and rendered mRNA-dependent by standard procedures (Pelham and Jackson, 1976). These lysates were obtained from Karl Hasel in our laboratory. For the preparation of the cell fractions from L cells, S10 lysates were fractionated by ultracentrifugation into the 200,000 g supernatant (S200), the ribosomal salt-washed proteins (RSW), and the salt-washed ribosomes essentially as described (Schreier and Staehelin, 1973). Briefly, the S10 lysate was ultracentrifuged (45,000 rpm, 1 h at 4 °C

in a Type 50 rotor); the supernatant is designated the S200 or postribosomal supernatant. The ribosomes in the pellet were then resuspended in hypotonic buffer (one fifth of the original S10 volume) and 0.5 M KCl was added; after 20 min on ice the ribosomes were pelleted again. The supernatant constitutes the crude initiation factor fraction or ribosomal salt wash proteins (RSW). This fraction was then dialyzed for 3 h against 10 mM Hepes-KOH pH 7.5, 100 mM KCl, 0.05 mM EDTA, 6 mM 2-mercaptoethanol. The fractions were stored in liquid nitrogen. The protein concentrations of the fractions were determined by the Bradford procedure (Bradford, 1976) using BSA as the standard protein.

In vitro translation systems.

The conditions for in vitro translation of mRNA have been described (Skup and Millward, 1977). The incubation mixture contained in a final volume of 20 µl: 6 µl of nuclease-treated lysate prepared from uninfected cells, 4 µl of a 5 times concentrated master mix solution, 4 µl of RNA solution, and 6 µl of the RSW preparation containing 2 µg of proteins or the same volume of purified sigma 3 containing 20 ng of the protein. The concentrated master mix solution contained 50 mM Hepes-KOH pH 7.5, 600 mM potassium acetate, 2.5 mM magnesium acetate, 250 µM spermidine, 5 mM dithiothreitol, 50 mM creatine phosphate, 2 mg/ml creatine phosphokinase, 2.5 mM GTP pH 7.0, and 2 mCi/ml of [^{35}S]methionine (NEN, 1000 Ci/mmol). The conditions of translation in reticulocyte lysates were essentially the same except for the concentration of magnesium acetate which was 12 mM in the concentrated master mix to obtain optimum activity for these lysates. Transfer RNA at 1 mg/ml and ATP at 5 mM were also added to

the master mix for translation in reticulocyte lysates. The final polysomal RNA concentration added was 200 µg/ml. The translation mixtures were incubated for 1 h at 30 °C at which time hot trichloroacetic acid (TCA) precipitable radioactivity was determined on 5 µl aliquots as described previously (Skup and Millward, 1977).

SDS-Polyacrylamide gel electrophoresis.

Proteins were analyzed by electrophoresis on 10% SDS-polyacrylamide slab gels using the Laemmli gel buffer system (Laemmli, 1970). Samples were boiled in sample buffer containing 1% SDS and 5% 2-mercaptoethanol for 5 min just before loading. After the electrophoresis, the gels were fixed, stained with Coomassie Brilliant Blue R-250 (0.2% in 40% methanol and 10% acetic acid), destained in 40% methanol, dried on Whatman 3MM paper and exposed against Kodak X-Omat AR-5 films. Some gels were treated for fluorography (mentioned in the legends) using En^{3}Hance (New England Nuclear) as described by the manufacturer. Silver staining was also occasionnally necessary to detect low amounts of protein, in these cases the BioRad silver stain kit was used as described by the manufacturer. Standard proteins of known molecular weights were obtained from BioRad.

Preparation of poly(I)-poly(C) cellulose.

The procedure was described by Huismans and Joklik (1976). Two grams of CF11 cellulose (Whatman) were washed extensively, first in ethanol, then in sterile distilled-deionized water. Poly(I)-poly(C) (15 mg) obtained from Boehringer Mannheim Corporation was resuspended at a concentration of 2 mg/ml, precipitated with 2 vol of ethanol and resuspended in 3 ml of 10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 10 mM NaCl.

The solution was then added to the cellulose in a petri dish. After complete drying under vacuum the cellulose was resuspended in 20 ml of ethanol and irradiated with a 25 watt General Electric germicidal ultraviolet light at 15 cm for 30 min. The cellulose was kept in ethanol at 4 °C and washed extensively with 10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 0.2 M NaCl before using it.

Purification of the sigma 3 viral protein.

The sigma 3 viral protein was purified from reovirus serotype 3 infected cells by a modification of a published procedure (Huismans and Joklik, 1976). Ribosomal salt wash proteins (labeled or not) prepared at 22 h post-infection (infection at 30 °C) were prepared as described above. The protein solution was then mixed with an equal volume of poly(I)-poly(C) cellulose prepared as described previously. After one hour on ice with occasional mixing the mixture was poured into a column and the flowthrough recovered. The column was then washed extensively with 10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 0.2 M NaCl. The sigma 3 protein was eluted sequentially with the same buffer containing 0.4 or 1.0 M NaCl. The protein was then dialyzed extensively against the standard buffer used for RSW. The purification scheme is schematized in Fig. 10.

Immunoprecipitation.

Proteins were diluted in 0.5 ml of immunoprecipitation buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM methionine, 1 mM PMSF, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.1% BSA, 100 KIU/ml of aprotinin) and subjected to quantitative immunoprecipitation using anti-reovirus antiserum from goat (M.A. Bioproducts). After overnight

agitation at 4 °C, the antigen-antibody complexes were precipitated using the affinity of Staphylococcus aureus protein A for immunoglobulin (Kessler, 1975). A volume of 100 µl of protein A Sepharose CL-4B was added and left for 1 h at 4 °C with gentle agitation. The beads of sepharose were then collected by centrifugation in an Eppendorf centrifuge for 1 min at 4 °C and washed extensively in immunoprecipitation buffer and PBS. The immunoprecipitated material was then eluted from the beads by boiling for 5 min in gel sample buffer containing 3% SDS and 300 mM 2-mercaptoethanol. The beads were then removed by centrifugation and the supernatant analyzed by SDS-polyacrylamide gel electrophoresis.

RESULTS

Translation of uninfected and infected cell mRNA in L cell lysates.

The principal basis for the investigations reported in this thesis is the already published observation (Skup, Zarbl and Millward, 1981; Lemieux, Zarbl and Millward, 1984) that the late viral mRNA (uncapped) is more efficiently translated in lysates prepared from infected than from uninfected L cells. The polysomal RNAs extracted from uninfected or reovirus serotype 3 late-infected cells, as described under Materials and Methods, were thus translated in lysates prepared from uninfected or reovirus serotype 3 late-infected cells. The results shown in Fig. 7 are consistent with the already published results. Since oligo-dT cellulose chromatography cannot be used to purify the non-polyadenylated reovirus mRNAs, polysomes from late-infected cells which are rich in late viral mRNA were used as a source of RNA.

Protein synthesis in cells infected with reovirus serotype 1 or 3.

All the work previously done in our laboratory concerns the serotype 3 of reovirus. Most of the studies concerning translation in reovirus-infected cells were also done with this serotype. However it was recently reported that, with the closely related serotype 1, the inhibition of host cell protein synthesis generally observed with the serotype 3 is lacking (Munemitsu and Samuel, 1984). The situation occurring with the serotype 1 virus was thus briefly studied.

We have first confirmed that there is no inhibition of cellular protein synthesis, even at late times post-infection (60 h at 30 °C or 30 h at 37 °C), in cells infected with the serotype 1. In cells

infected with the serotype 3 essentially all the proteins synthesized at late times post-infection (30 h at 30 °C or 16 h at 37 °C) are viral-specific (Fig. 8). This result confirms the detailed description of this difference in property that was already published by other investigators (Mumemitsu and Samuel, 1984).

Translation in lysates prepared from cells infected with reovirus serotype 1 or 3.

The stimulation of late viral mRNA translation occurring in infected cells can be demonstrated in vitro by using translation lysates prepared from late-infected cells (Skup and Millward, 1980; Skup, Zarbl and Millward, 1981; Lemieux, Zarbl and Millward, 1984; see also Fig. 7). We wanted to verify if this modification also takes place during infection by the serotype 1. Lysates were thus prepared on the same day from cells infected with either serotype using the method already described under Materials and Methods. We used an higher temperature of incubation during infection by serotype 1 because of the slower multiplication rate of this virus. Lysates were prepared after incubation period corresponding to about 60% of the time necessary for lysis of the infected cells. To obtain these conditions cells infected with the serotype 3 were incubated for 30 h at 30 °C and cells infected with the serotype 1 were incubated also for 30 h but at 37 °C. Lysates prepared from cells infected with the serotype 1 were less active probably because of the use of this higher temperature. These lysates were however clearly more efficient (3 to 3.5-fold) than uninfected cell lysates for translation of late viral mRNA when compared to translation of uninfected cell mRNA. Lysates prepared from cells infected with the serotype 3 were about 4.5 to 5-

fold more efficient than uninfected cell lysates in this particular experiment; results are presented in Table V. It thus seems that the stimulation of translation of late viral mRNA also takes place during infection by reovirus serotype 1 despite the lack of inhibition of host protein synthesis.

Effect of addition of RSW proteins on late viral mRNA translation.

To shed light on the molecular basis of the stimulation of translation of late viral mRNA observed in serotype 3 infection, the infected cell lysate was fractionated and the effect of addition of each fraction to uninfected cell lysates was examined. It was shown that the fraction prepared by high-salt wash of ribosomes (ribosomal salt wash proteins; RSW) and containing crude initiation factors is the most active fraction (Lemieux, R., unpublished data) Results of a typical experiment using ribosomal salt wash are shown in Fig. 9; the stimulatory effect is clearly seen on late viral mRNA translation. Other mRNAs, like uninfected cell polysomal RNA, are not affected suggesting again that the factor necessary for efficient translation of late viral mRNA is not directly involved in inhibition of host cell protein synthesis. Another observation is that the stimulatory effect is somewhat dependent on the type of translation system because no effect was observed in rabbit reticulocyte lysate. This experiment may indicate that the factor responsible for the stimulation interacts with an L cell lysate component but that this interaction is prevented in the reticulocyte lysate.

Effect of purified sigma 3 on late viral mRNA translation.

The RSW fraction that is enriched in the factor activity was

shown to be enriched in the sigma 3 viral protein. Results of preincubation of the RSW with different anti-reovirus antibodies also indicated the participation of a viral protein, possibly sigma 3, in the stimulatory activity (Lemieux, R., unpublished data). However the interpretation of these results was still ambiguous because of the strong affinity between sigma 3 and other viral proteins that are co-immunoprecipitated. To demonstrate more definitely the involvement of this protein in the stimulation of late viral mRNA translation, sigma 3 was purified from RSW of infected cells using its affinity for double-stranded polyribonucleotides as described under Materials and Methods and schematized in Fig. 10. Results of the purification are shown in Fig. 11. In the first panel is shown a silver-stained pattern of the flowthrough as well as 0.4 and 1 M eluates from the affinity column. In the second panel is shown the autoradiogram obtained with the same three fractions obtained from the RSW of infected cells that have been labeled with [^{35}S] methionine prior to preparation of RSW. The 1 M (and probably also the 0.4 M) fraction contain a pure protein comigrating, as expected, with sigma 3. Analysis of the purified protein by two-dimensional gel electrophoresis also confirmed identity of this protein with the sigma 3 protein of the viral capsid (see chapter 4). The yield in the 1 M fraction is a maximum of about 0.5 µg of protein from 2 liters of infected cells (2 X 10^9 cells). There is still some sigma 3 in the flowthrough partially because of formation of the already described complexes between sigma 3 and mul/mulc (Huismans and Joklik, 1976; Lee, Hayes and Joklik, 1981b). The fact that these complexes do not bind on the column allowed us to obtain purified free sigma 3 protein.

The protein contained in the 1 M salt eluate fraction was then added to uninfected cell lysates to study its effect on translation. Results of radioactive methionine incorporation (Table VI) showed that the purified sigma 3 stimulates translation of late viral mRNA. The specific activity of purified sigma 3 is about 20 to 40-fold higher than total RSW proteins in different experiments using different preparations of sigma 3. Translation products obtained in one of these experiments were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography of the dried gel confirms that the synthesis of viral proteins is stimulated by sigma 3 (Fig. 12). The translation of uninfected cell mRNA or TMV RNA is not affected by the presence of sigma 3 (Fig. 12 and Table VI). These last experiments provide strong evidence that the sigma 3 viral protein is the factor responsible for the stimulation of translation of late (uncapped) viral mRNA.

Properties of sigma 3 association with ribosomes.

The exact mechanism of action of sigma 3 is still unknown. The specificity of the effect for late viral mRNA might suggest that the protein binds specifically to mRNA to facilitate initiation of protein synthesis. A previous study (Huismans and Joklik, 1976), however, indicated that the sigma 3 protein has no apparent affinity for single-stranded RNA. To study the remaining possibility that sigma 3 binds to viral mRNA under in vivo conditions, thus explaining its sedimentation with polysomes, we examined the effect of different conditions on the partition of sigma 3 between ribosomal pellet and proteins released into the supernatant. Polysomes were pelleted from infected cells and different treatments were applied in attempts to release sigma 3 (Fig. 13). As already described, high-salt treatment

releases crude initiation factors from the ribosomes along with sigma 3. Treatment with EDTA disrupts the polysomes releasing mRNA and the two ribosomal subunits (see for example: Hamilton, 1982), in this case the sigma 3 protein remains associated with the pelleted ribosomal subunits. After degradation of mRNA with nuclease, sigma 3 also remains associated with ribosomes. Altogether these results indicate that the association of the sigma 3 protein with ribosomes does not occur through the mRNA but rather that the protein binds directly to a component of the translational machinery (ribosomes or initiation factors).

Effect of protein synthesis inhibitors on sigma 3 association with ribosomes.

If the sigma 3 protein is playing a role in the initiation of protein synthesis, it should probably associate with ribosomes during the initiation process and be released from the ribosomes at the end of the initiation step of protein synthesis. To study this point we incubated lysates from infected cells in conditions of protein synthesis in the presence of different inhibitors of protein synthesis (Table VII). As expected an inhibitor of elongation [anisomycin (described by: Pestka, 1974)] produces accumulation of sigma 3 on the ribosomes presumably because of accumulation of initiation complexes. In contrast, addition of inhibitors of initiation of protein synthesis [m^7GTP (used in this system by: Lemieux, Zarbl and Millward, 1984) or ATA (Goldstein, Reichmann and Penman, 1974)] results in a net release of sigma 3 presumably by blocking its binding occurring during initiation while still allowing elongation during which sigma 3 is released.

DISCUSSION

The modification(s) occurring to the translational machinery during reovirus infection result in the inhibition of host protein synthesis and in the efficient translation of late viral (uncapped) mRNA. The inhibition of translation of host and early viral capped mRNAs observed in late-infected cells, and in the corresponding cell-free translation systems, previously led us to postulate that an inactivation of the cap-binding proteins could be responsible for the mRNA discrimination observed. Indeed a decrease in the amount of active cap-binding proteins has been observed in lysates prepared from late-infected cells (Sonenberg et al., 1981a; Zarbl, H., R. Lemieux, and S. Millward, unpublished data). Although such a modification could be responsible for the inhibition of host and early viral capped mRNAs translation, it could not completely explain the efficient translation of late viral uncapped mRNA in late-infected cells since this type of mRNA was not efficiently translated in lysates from uninfected cells, even in presence of antibodies against cap-binding proteins (Sonenberg et al., 1981a). The finding that the late viral mRNA can easily outcompete the cellular mRNA for translation in late-infected cell lysates (Lemieux, Zarbl and Millward, 1984) suggested that an hypothetical factor stimulating the translation of late viral mRNA might play a predominant role in the regulation of translation at late times post-infection (Lemieux, Zarbl and Millward, 1984; Zarbl and Millward, 1983).

In contrast with the serotype 3 that we are currently studying the serotype 1 of human reovirus does not significantly inhibit host

cell specific protein synthesis during infection of L cells. The serotype 1 is very similar to the serotype 3 but has a slower multiplication rate even if the final yield of virus is essentially the same (Munemitsu and Samuel, 1984). We showed that the stimulation of late viral mRNA translation described during infection with serotype 3 also occurs in lysates prepared from serotype 1 infected cells. The stimulation of translation of late viral mRNA and the inhibition of host cell protein synthesis are thus two distinguishable phenomena. This is also indicated by the demonstration that addition of fractions from cells infected with the serotype 3, and especially RSW proteins, stimulates translation of late viral mRNA without affecting other mRNAs like cellular mRNA.

Previous experiments concerning the distribution of viral proteins and factor activity in different subcellular fractions, and the effect of antibodies on the stimulation, suggested that the sigma 3 viral protein might be involved in late viral mRNA translation. Better evidence for a participation of the sigma 3 protein in the translation of late viral mRNA was obtained in this chapter by using the purified protein. The results showed that the activity of 1 µg of the pure protein is 15 to 20-fold higher than the activity of 1 µg of total RSW proteins. Sigma 3 is thus apparently responsible for the efficient translation of late viral mRNA.

The stimulation of late viral mRNA translation may be absolutely necessary for efficient translation and growth of the virus, in contrast the inhibition of host mRNA translation seems dispensable as demonstrated by its absence during serotype 1 infection. However this inhibition may be partially responsible for the faster growth of

reovirus serotype 3 by removing cellular mRNA from the ribosomes thus facilitating translation of reovirus mRNA. Alternatively it is possible that the inhibition of cap-dependent translation, shown to be more stringent on viral early (capped) mRNA, helps to free early mRNA as suggested previously (Zarbl and Millward, 1983). The early mRNA has a dual role as it is also used as the plus strand of genomic RNA and template for synthesis of the second strand of this double-stranded RNA (Schonberg et al., 1971). Inhibition of its translation may facilitate this process and as a result the growth of the virus.

The mechanism by which the sigma 3 protein can specifically stimulate the translation of late viral mRNA remains unclear. It is reasonable to postulate that the uncapped 5'-end structure of late viral mRNA is important for this effect. However, the sigma 3 protein has no apparent affinity for single-stranded RNA (Huismans and Joklik, 1976). This absence of direct interaction of sigma 3 with mRNA was further substantiated by the experiment showing that two different treatments resulting in dissociation of polysomes (EDTA) or degradation of mRNA (nuclease) do not result in dissociation of sigma 3 from the ribosomes. The protein most likely interacts with another component of the translational machinery.

We suggest that the sigma 3 protein might play the role of a late-viral-mRNA-specific discriminatory initiation factor. This idea is further supported by the observation that the presence of an inhibitor of elongation (anisomycin) tends to increase the amount of sigma 3 on ribosomes while inhibitors of initiation (m^7GTP, ATA) tend to decrease this amount. This observation is consistent with a binding of sigma 3 during initiation followed by release in the later stages of protein

synthesis.

Recent results obtained in other systems suggest that the direct participation of viral protein(s) in the mechanisms of translational control might be a strategy used by several other viruses, either to confer the viral mRNA a translational advantage over host mRNA, or to regulate the virus multiplication cycle (see chapter 1).

Finally notice that, in the case of reovirus, genetic recombination experiments have shown that the S4 gene coding for the sigma 3 protein is responsible for the inhibition of host-specific protein synthesis following infection with reovirus serotype 2 (Sharpe and Fields, 1982). This is another piece of evidence that the sigma 3 protein has a central role in the control of protein synthesis during reovirus infection; the exact situation occurring in serotype 2 infection is, however, incompletely understood. Also it has been shown that high-passage reovirus stocks contain a mutated S4 gene (Ahmed, Chakraborty and Fields, 1980) and that this gene is apparently involved in the establishment of persistent reovirus infections (Ahmed and Fields, 1982). The finding that the sigma 3 protein is essential for the efficient translation of viral mRNA in late-infected cells provides new perspectives concerning the mechanism by which the sigma 3 protein might be involved in these phenomena and suggests that its role might be crucial for the normal virus multiplication cycle.

FIGURE. 7

Stimulation of late viral mRNA translation in infected cell lysates

Uninfected or late-infected cells were used to prepare cell-free translation lysates (S10) and these lysates were used for in vitro translation as described under Materials and Methods. Polysomal RNA was extracted and used as described: -, absence of added RNA; U, RNA extracted from uninfected L cells; I, RNA extracted from late-infected L cells. After 60 min of incubation at 30 °C aliquots of 5 µl were analyzed by SDS-polyacrylamide gel electrophoresis and radiolabeled products detected by autoradiography as described under Materials and Methods. Positions of molecular weight markers are indicated at left.

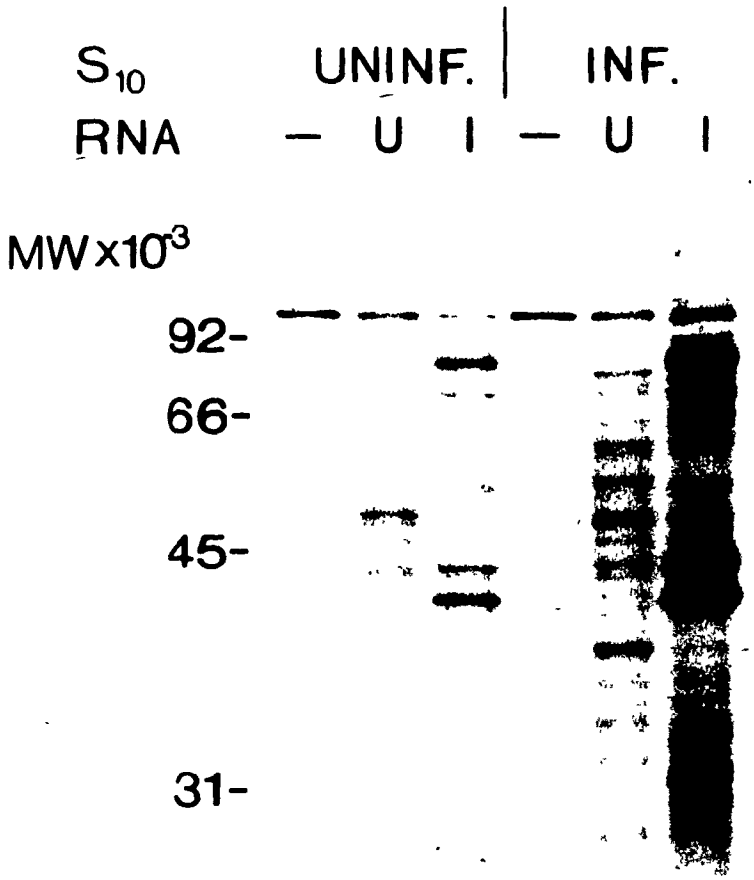

S10
UNINF.
INF.
RNA
—
U
I
—
U
I
MW x10^3
92-
66-
45-
31-

FIGURE. 8

Protein synthesis in cells infected with reovirus serotype 1 or 3

Cells were infected and proteins labeled as described under Materials and Methods. The same amount of hot TCA-precipitable counts of each sample was then analyzed by SDS-polyacrylamide gel electrophoresis. U, proteins synthesized in uninfected cells; 1, proteins synthesized in cells infected by reovirus serotype 1 (30 h at 37 °C); 3, proteins synthesized in cells infected by reovirus serotype 3 (30 h at 30 °C). The positions of the three classes of viral proteins (lambda, mu, sigma) are indicated.

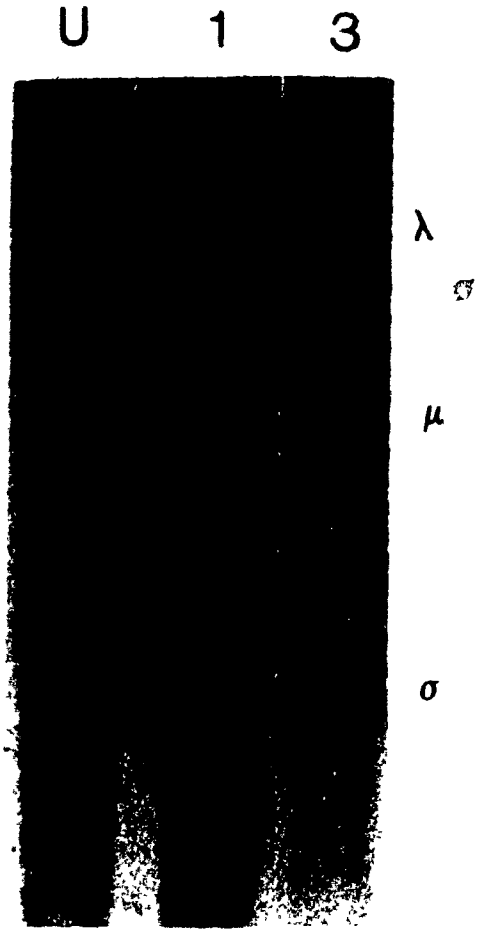

U 1 3
λ
μ
σ

TABLE V

Translation in lysates from cells infected with reovirus serotype 1 or serotype 3

Lysate *	Ratio I/U **	Fold stimulation ***
U	1.37	---
I (3)	6.46	4.7
I (1)	4.67	3.4

* The lysates used were prepared from: U, uninfected L cells; I(3), cells infected with reovirus serotype 3 (30 h at 30 °C); I(1), cells infected with reovirus serotype 1 (30 h at 37 °C)

** Ratio of hot TCA-precipitable counts obtained by translation of RNA extracted from infected cells compared to RNA extracted from uninfected cells

*** Stimulation of late viral mRNA translation obtained by comparing the ratio I/U observed in infected lysates with the ratio observed in the uninfected cell lysate

FIGURE. 9

Effect of RSW from infected cells in L cell and reticulocyte lysates

Crude initiation factors were prepared from late-infected cells by high salt wash of ribosomes as described under Materials and Methods and added to uninfected L cell or reticulocyte lysate as indicated on the figure. -, absence of added RNA; U, RNA extracted from unifected L cells; I, RNA extracted from late-infected L cells. Translation was done in the presence (+) of crude initiation factors (RSW) or of the same volume (2 µl) of buffer (-). After incubation, aliquots of 5 µl of L cell translation reaction or 2 µl of reticulocyte translation reaction were analyzed by gel electrophoresis and autoradiography. Positions of molecular weight markers are indicated.

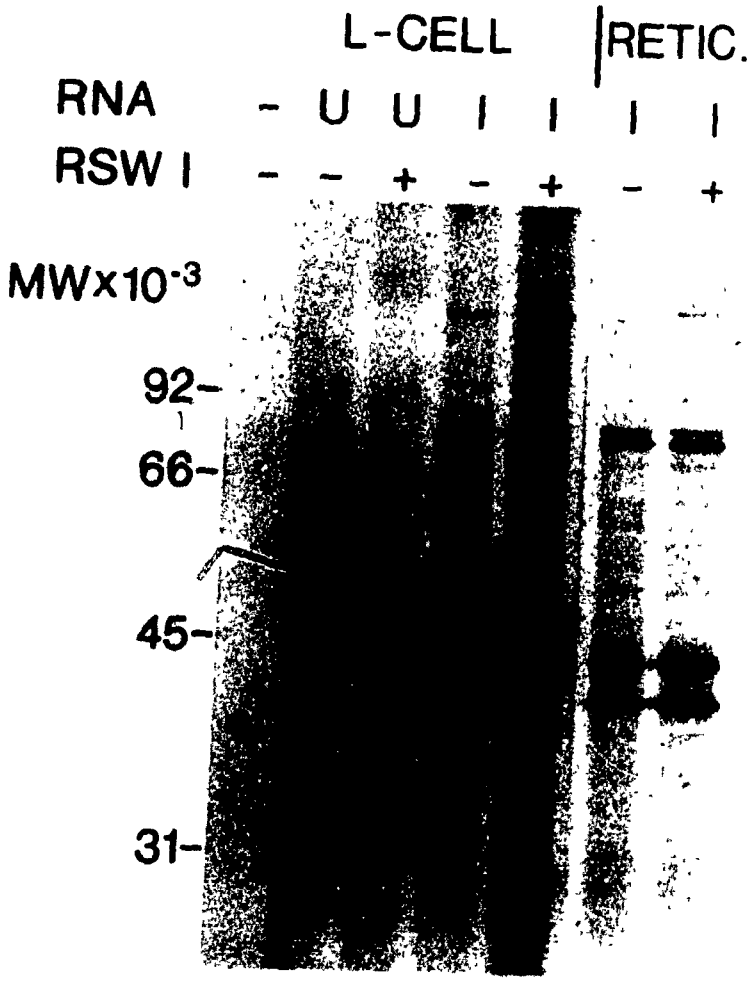

L-CELL
RETIC.
RNA - U U I I I I
RSW I - - + - + - +
MWx10-3
92-
66-
45-
31-

FIGURE. 10

Purification scheme of the sigma 3 viral protein

This figure summarizes the procedure used for purification of sigma 3 from infected cells. The procedure is described in greater details under Materials and Methods.

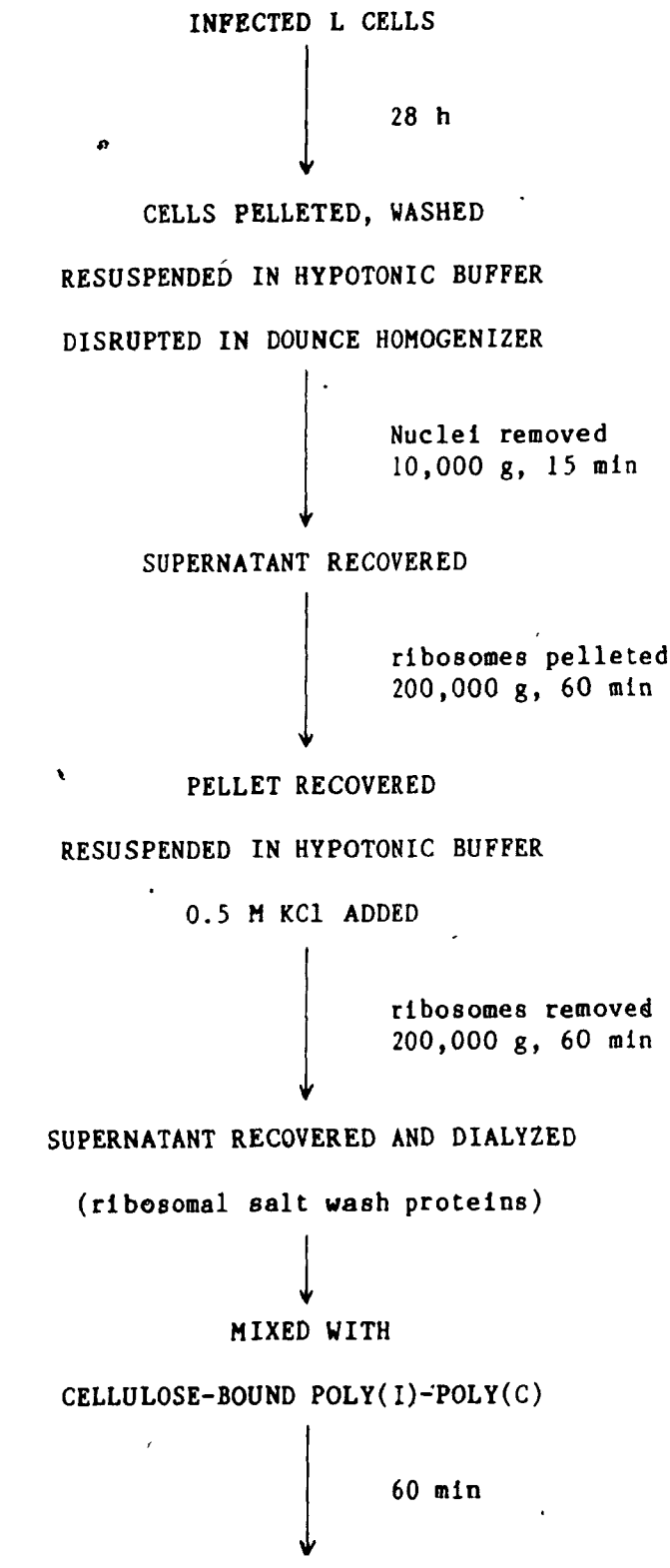

INFECTED L CELLS
28 h
CELLS PELLETED, WASHED
RESUSPENDED IN HYPOTONIC BUFFER
DISRUPTED IN DOUNCE HOMOGENIZER
Nuclei removed
10,000 g, 15 min
SUPERNATANT RECOVERED
ribosomes pelleted
200,000 g, 60 min
PELLET RECOVERED
RESUSPENDED IN HYPOTONIC BUFFER
0.5 M KCl ADDED
ribosomes removed
200,000 g, 60 min
SUPERNATANT RECOVERED AND DIALYZED
(ribosomal salt wash proteins)
MIXED WITH
CELLULOSE-BOUND POLY(I)-POLY(C)
60 min
CELLULOSE WASHED WITH 0.2 M NaCl
SIGMA 3 ELUTED WITH 0.4 OR 1.0 M NaCl
PURIFIED SIGMA 3 DIALYZED

FIGURE. 11

Results of purification of sigma 3

The viral protein was purified from late-infected cells as described in Fig. 10 and under Materials and Methods. Aliquots of the 0.1 M flowthrough and of both the 0.4 M and 1.0 M salt eluates have been analyzed by SDS-polyacrylamide gel electrophoresis. In the first panel aliquots corresponding to 10^7 cells (flowthrough) or 10^8 cells (eluates) have been analyzed by electrophoresis followed by silver staining of the gel. The second panel shows the results obtained when the proteins were labeled in vivo during 1 h before the beginning of the purification procedure. In this case one-tenth of each fraction was loaded on the gel and the autoradiogram obtained is shown here. Positions of molecular weight markers are indicated and the arrow indicates the position of sigma 3 determined by comigration of proteins from purified virus.

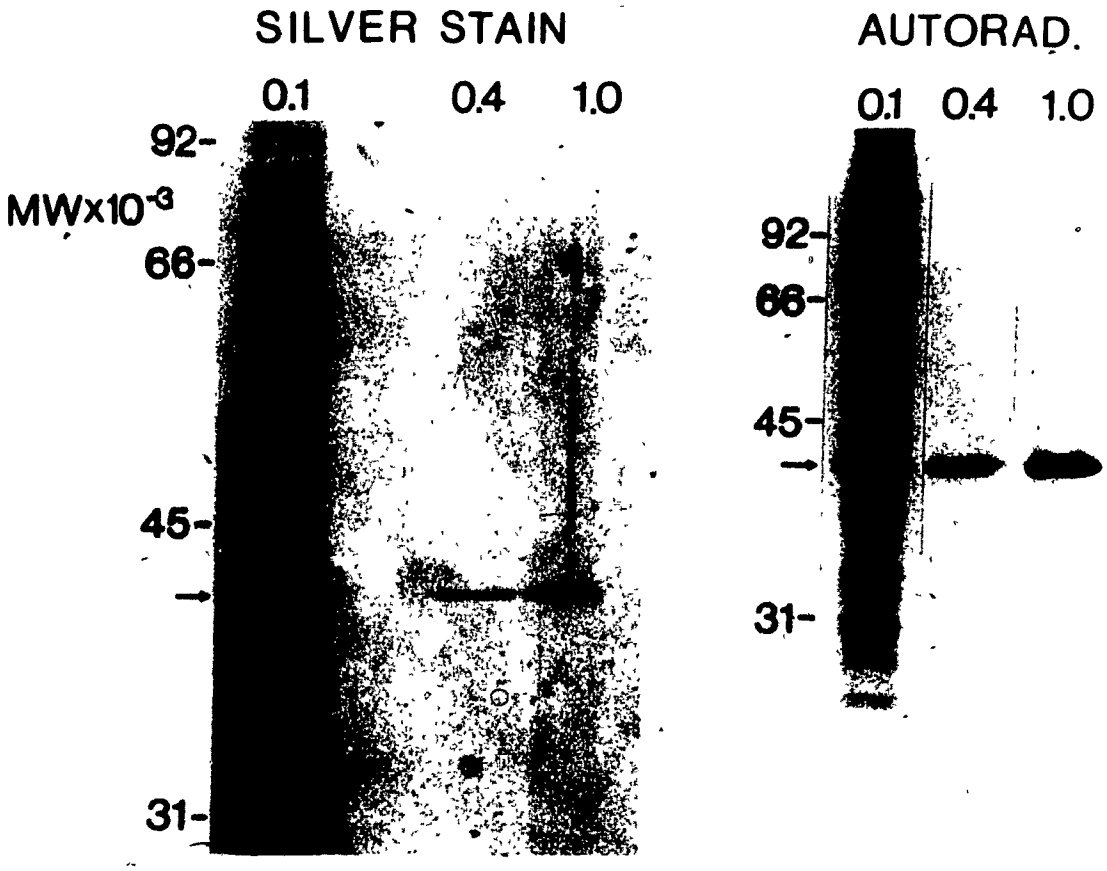

SILVER STAIN
AUTORAD.
0.1
0.4
1.0
0.1
0.4
1.0
MWx10-3
92-
66-
45-
31-
92-
66-
45-
31-

TABLE VI

Effect of purified sigma 3 on translation

RNA	Control (counts X 10^3) *	+Sigma 3 (1 µg/ml) **	Fold-stimulation of late viral mRNA translation
TMV	607	628	--
Late reovirus	70	287	4
L-cell	25	23	--
Late reovirus	20	40	2

* Hot TCA-precipitable counts in 5 µl aliquots after 1 h of incubation as described under Materials and Methods

** Two different preparations of purified sigma 3 were used for these two sets of data.

FIGURE. 12

Effect of purified sigma 3 on translation

Sigma 3 protein purified to homogeneity as described under Materials and Methods and in Fig. 10 and 11 was added at a concentration of 1 µg/ml to uninfected L cell lysate. -, absence of exogenous RNA; U, uninfected cell RNA; I, late-infected cell RNA. Translation was done in the presence (+) or absence (-) of added sigma 3. Aliquots of 10 µl of each translation reaction were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Positions of molecular weight markers are indicated.

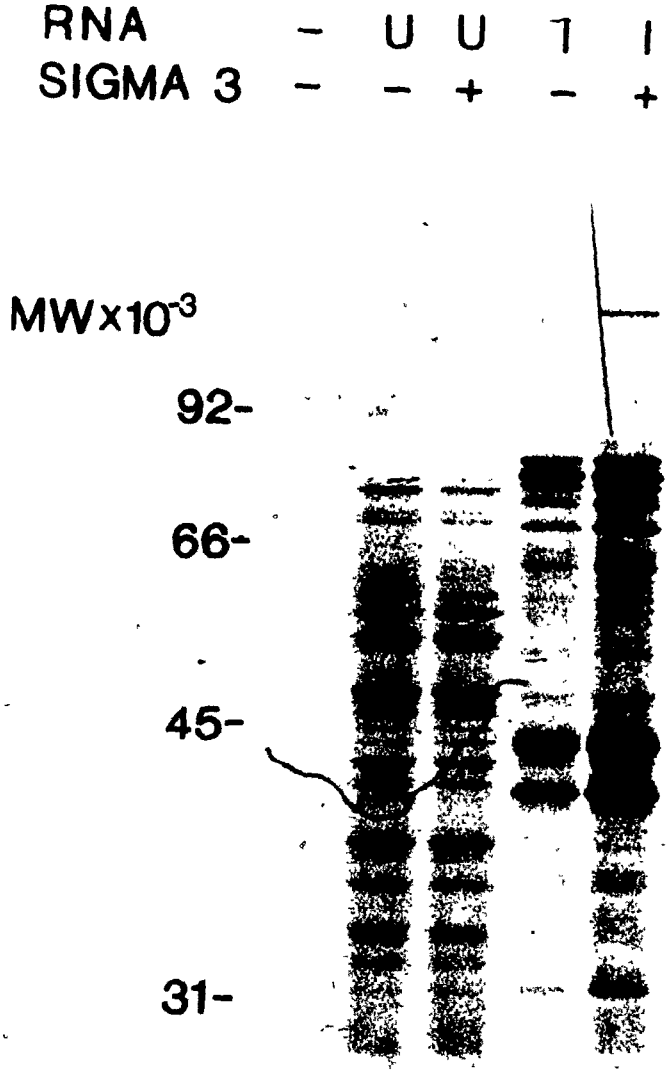

RNA – U U T I
SIGMA 3 – – + – +
MW x 10-3
92-
66-
45-
31-

FIGURE. 13

Properties of sigma 3 association with ribosomes

Cells were infected, proteins labeled with [^{35}S] methionine 4 h after infection, lysate prepared and ribosomes pelleted by centrifugation as described under Materials and Methods. Ribosomes were then resuspended in hypotonic buffer (10 mM Hepes-KOH pH 8.0, 10 mM potassium acetate, 1.5 mM magnesium acetate, 1 mM DTT). One aliquot was left on ice without any addition (Cont.), to another aliquot 0.5 M KCl was added and the ribosomes left on ice 20 min (KCl), to another aliquot 20 mM EDTA pH 8.0 was added and the ribosomes left on ice 20 min (EDTA), to the last aliquot 1 mM $CaCl_2$ and 75 units/ml of micrococcal nuclease was added and incubated 20 min at room temperature (Nucl.). Ribosomes in each sample were then pelleted, resuspended and both the supernatant and pellet dialyzed against 10 mM Tris-HCl pH 8.0. Each sample was then submitted to quantitative immunoprecipitation as described under Materials and Methods. Immunoprecipitated material was then analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography and autoradiography. Position of sigma 3 as determined by comigration with viral proteins is indicated by the arrow.

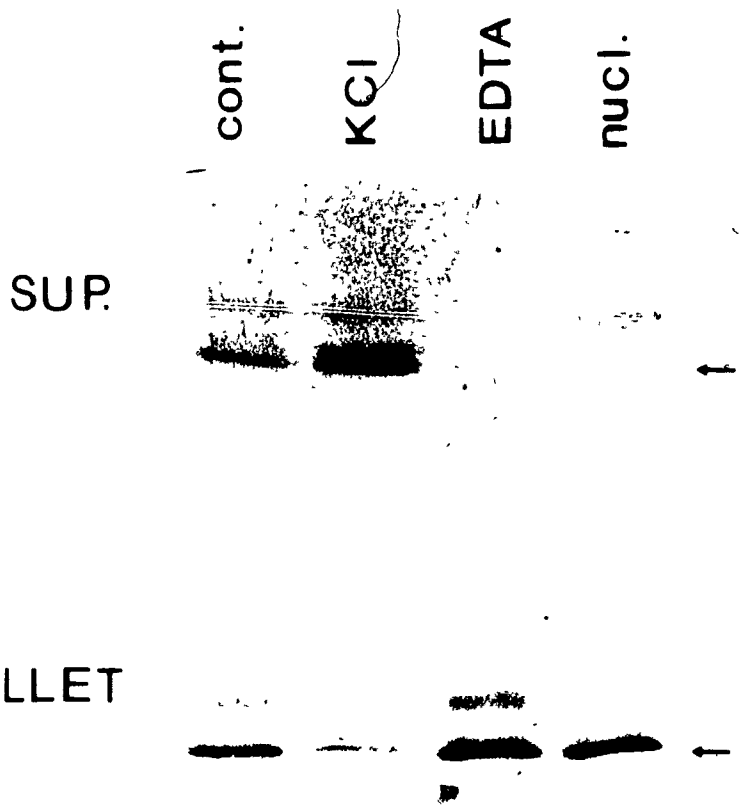

cont.
KCl
EDTA
nucl.
SUP.
PELLET

TABLE VII

Effect of protein synthesis inhibitors on sigma 3 association with ribosomes

Treatment *	Ratio pellet/supernatant **
Control	23
ATA (0.1mM)	15
m^7GTP (1 mM)	11
Anisomycin (1 mM)	40

* Incubation of labeled lysate from infected cells in conditions of *in vitro* translation in the presence of the different inhibitors

** Ratio obtained by densitometric scanning of the gel obtained by immunoprecipitation of sigma 3 from the ribosomal pellet and the supernatant of each reaction

CHAPTER 4

Multiple forms of the sigma 3 protein of reovirus: occurrence and binding properties

FOREWORD

In the preceding chapter, the sigma 3 protein was shown to be responsible for efficient translation of late viral mRNA. This protein is also a structural protein of the viral capsid. We were interested to determine if the presence of different forms of sigma 3, previously noticed by some authors, may be responsible for the different properties associated with this same protein: structural role, binding to double-stranded RNA, binding to ribosomes and stimulation of translation of late viral mRNA. The properties of different forms of sigma 3 resolvable by two-dimensional gel electrophoresis were thus analyzed.

The results presented in this chapter are original results except for the demonstration of multiple forms of sigma 3 in serotype 3 virus that was previously observed by other investigators. I have to mention the collaboration of Wendy Hauck for the experiment involving *in vitro* transcription with the SP6 transcription system. This chapter has been submitted for publication as a short communication (submitted to Virology) and is presented here in the same form.

ABSTRACT

The sigma 3 protein of reovirus serotype 3 in infected L cells and in the purified virus is present in different forms differing in isoelectric point, as already described by other authors. Proteolytic digestion of each of these forms generates similar peptides. The different forms are present in the mature virus and all have affinity for double-stranded RNA and for ribosomes. The observations that the different forms have the same functional properties and are absent in reovirus serotype 1 suggest that the presence of multiple forms is dispensable. The different forms can be synthesized by *in vitro* translation of mRNA prepared from cells infected with reovirus serotype 3. However, only one form is clearly made with mRNA synthesized *in vitro*, using the cloned S4 gene (which encodes sigma 3) as a template. Similarly, the cloned S4 gene expressed *in vivo* produces only one form of the protein. These results indicate that the other forms are not derived by posttranslational modification. The different forms are possibly due to the presence, in cells infected with reovirus, of mutant viruses with variations in the S4 gene.

Each of the ten double-stranded RNA segments composing the human reovirus genome encodes one primary translation product, the only known exception being the S1 segment which encodes two proteins in two different reading frames. Some of these primary translation products are later cleaved to generate smaller proteins, the best known being the mu1C component of the outer capsid (For a recent review see: Joklik, 1985). Another possible level of diversity of the reovirus proteins has been demonstrated using two-dimensional gel electrophoresis. At least two different groups have noticed the presence of multiple forms of some reovirus proteins, these forms having the same (or very similar) molecular weight but different isoelectric points (Samuel, 1983; Ewing, Sargent and Borsa, 1985).

Our laboratory is currently investigating the role of the sigma 3 protein of reovirus serotype 3 in the control of translation during reovirus infection. Sigma 3 is one of the proteins apparently present in multiple forms differing in isoelectric point (Samuel, 1983; Ewing, Sargent and Borsa, 1985). We were thus interested to determine if different properties are associated with the different forms observed.

The proteins synthesized in reovirus-infected L cells were first labeled *in vivo* using [^{35}S]methionine and analyzed by two-dimensional gel electrophoresis using the O'Farrell procedure (O'Farrell, 1975); a 10% SDS-polyacrylamide gel using the Laemmli buffer system (Laemmli, 1970) was used for the second dimension. In cells infected with reovirus serotype 3 (Dearing strain, obtained from American type culture collection) multiple forms of sigma 3 were detected, as already noted above. We detected 3 different forms present around

the neutral region of the isoelectric focusing gel (Fig. 14a). A fourth form with a lower isoelectric point was sometimes difficult to detect unless the autoradiograms were overexposed. Throughout the rest of this chapter the four forms will be called A, B, C, and D, in order of decreasing isoelectric point.

After infection with reovirus serotype 1 (Lang strain, obtained from Dr. B.N. Fields) the situation observed was, however, different. Only two forms were detected (Fig. 14b) that comigrated (with a slightly higher molecular weight) with forms B and C detected in serotype 3. The presence of four different forms, as observed in serotype 3 is thus apparently dispensable since they are not present in the closely related serotype 1. One form of sigma 3 is quite probably sufficient.

It has been reported that different forms of sigma 3 are retained in the mature virus (Samuel, 1983; Ewing, Sargent and Borsa, 1985). We analyzed proteins from virus that was purified by freon extraction and cesium chloride gradient centrifugation essentially as described (Smith, Zweerink and Joklik, 1969). Two-dimensional gel electrophoresis, followed by staining of the proteins with Coomassie blue, revealed the presence of the same proportion of the different forms in the mature virus as in the infected cells when either serotype 3 (Fig. 14c) or serotype 1 (Fig. 14d) were examined. The different forms of sigma 3 thus have apparently the same capability of being incorporated into the virion, an observation consistent with their dispensable role if they have equivalent functional properties.

It has been assumed that these different components observed in serotype 3 are different forms of the same protein, namely sigma 3.

The different forms are all immunoprecipitated (data not shown) with a monospecific anti-sigma 3 antiserum (a generous gift from Dr. P.W.K. Lee). To further prove that these different forms are indeed related proteins, the gel fragments containing the four radioactive spots were excised from dried gels, loaded into the wells of a second gel, and the proteins analyzed by proteolytic digestion in the gel, using the procedure originally described by Cleveland et al. (Cleveland et al., 1977). Three different endopeptidases produce distinctive patterns of proteolytic fragments that were identical with each of the four forms (Fig. 15) clearly indicating they are different forms of the same sigma 3 protein.

Properties of sigma 3 other than incorporation into the virion were examined to determine if the different forms also share these properties. It was described by Huismans and Joklik that the sigma 3 protein has affinity for double-stranded polyribonucleotides [for example poly(I)-poly(C)] and that this property can be used to purify the protein from infected cells (Huismans and Joklik, 1976). We also recently used this same procedure for the purification of sigma 3 (see Chapter 3). This purified, radiolabeled, protein obtained from infected cells (Fig. 16a) was then analyzed by two-dimensional gel electrophoresis. The result clearly indicates that the four forms are purified together (Fig. 16b) and in the same proportion as they occur in infected cells or in the virion. These four forms of sigma 3 thus have a similar affinity for double-stranded RNA.

We recently observed another property of sigma 3: this protein is enriched in the ribosomal pellet obtained from infected cells and can be released from it by a 0.5 M KCl wash (see chapter 3 and 5). We

therefore also analyzed, by two-dimensional gel electrophoresis, the proteins present in the ribosomal salt wash prepared from infected cells. Again the four forms of sigma 3 were detected in the same relative proportion (data not shown).

We cannot at this point completely exclude a difference in properties among the different forms of sigma 3, but all the functional properties, that we know of, are shared by the four different forms detectable using two-dimensional gel electrophoresis.

We wanted to examine the possible mechanisms involved in the generation of the multiple forms of sigma 3. Among the mu proteins different forms differing in isoelectric point might be due to different posttranslational modifications (for example glycosylation or phosphorylation) that have been detected by different groups (Krystal et al., 1975; Krystal, Perrault and Graham, 1976; Lee, 1983). In contrast the only known posttranslational modification of sigma 3 is a low, and still unconfirmed, level of O-glycosylation (Lee, 1983).

In an effort to get some information concerning the synthesis of the different forms of sigma 3 we used two-dimensional gel electrophoresis to analyze the protein synthesized in vitro using rabbit reticulocyte lysates (Pelham and Jackson, 1976) or L cell lysates (Skup and Millward, 1977). Messenger RNA was first prepared by streptomycin sulfate precipitation of polysomes (Kaulenas et al., 1977) from late-infected cells, as described (Skup and Millward, 1981). This mRNA was then used for in vitro translation and the translation products analyzed by two-dimensional gel electrophoresis. The different forms were generated in the same proportion during in vitro translation as they are in vivo; similar results were obtained

using either L cell (data not shown) or reticulocyte lysate (Fig. 17a). We then prepared mRNA in vitro using the SP6 transcription system. The complete coding region of the cloned S4 gene encoding sigma 3 [(Giantini et al., 1984); a generous gift from Dr. A.J. Shatkin] was introduced into the SP64 transcription vector using standard molecular cloning techniques (Maniatis, Fritsch and Sambrook,, 1982). The plasmid was then linearized and used as a template for the synthesis of mRNA as described (Krieg and Melton, 1984). When this mRNA was translated in vitro and translation products analyzed by SDS-polyacrylamide gel electrophoresis, we always observed a reproducible pattern of translation products smaller than sigma 3 but the major translation product always comigrated with authentic sigma 3 (data not shown). However, when analyzed by two-dimensional gel electrophoresis the only clearly detectable form of full-length sigma 3 was form A (Fig. 17b). The sigma 3 protein, possessing the exact amino acid composition deduced from the DNA sequence of the cloned gene, thus corresponds to form A. The cloned S4 gene was also recently expressed in vivo in L cells using eucaryotic promoters (see chapter 5). Analysis of this protein by two-dimensional gel electrophoresis again revealed the presence of form A only (data not shown).

These observations suggested that forms B, C and D are not derived from form A by posttranslational modifications but are independently synthesized. A logical explanation is that the different forms have a slightly different amino acid composition because they are synthesized from different mRNAs. This situation might be due to the generation of mutant viruses in the viral stocks. Analysis of different virus stocks revealed that some stocks produce

different amounts of the different forms (data not shown). These observations also explain that different groups have reported the presence of multiple forms of sigma 3 but with apparent variations in the exact pattern of distribution of these forms (Samuel, 1983; Ewing, Sargent and Borsa, 1985). A frequent occurrence of mutant S4 gene is also consistent with the observation that the S4 gene is among the most divergent genes between the different serotypes (Gaillard and Joklik, 1982). It thus seems that the sigma 3 protein is allowed to vary to an appreciable extent while still retaining the same apparent properties.

FIGURE. 14

Analysis of sigma 3 in reovirus serotype 1 or 3 by two-dimensional gel electrophoresis

Autoradiogram of a two-dimensional gel electrophoresis analysis of radiolabeled proteins (similar amounts of hot TCA-precipitable material) from reovirus serotype 3 (a) or serotype 1 (b) infected cells; labeling was done at respectively 14 and 24 h post-infection (37 °C). Proteins (20 µg) from purified reovirus serotype 3 (c) or serotype 1 (d) analyzed by two-dimensional gel electrophoresis followed by Coomassie blue staining; virus was purified at respectively 40 and 70 h post-infection (31 °C). Migration direction of the first dimension (isoelectric focusing; IEF) is indicated, migration was from the basic to the acidic end; migration direction of the second (SDS) dimension is also indicated. The small arrow indicates the position of sigma 3 in the second dimension and the asterisk that of sigma NS. The positions in the first dimension of the three major forms of sigma 3 (A, B, and C) are also indicated. Only the gel portion comprising sigma 3 is shown on the figure.

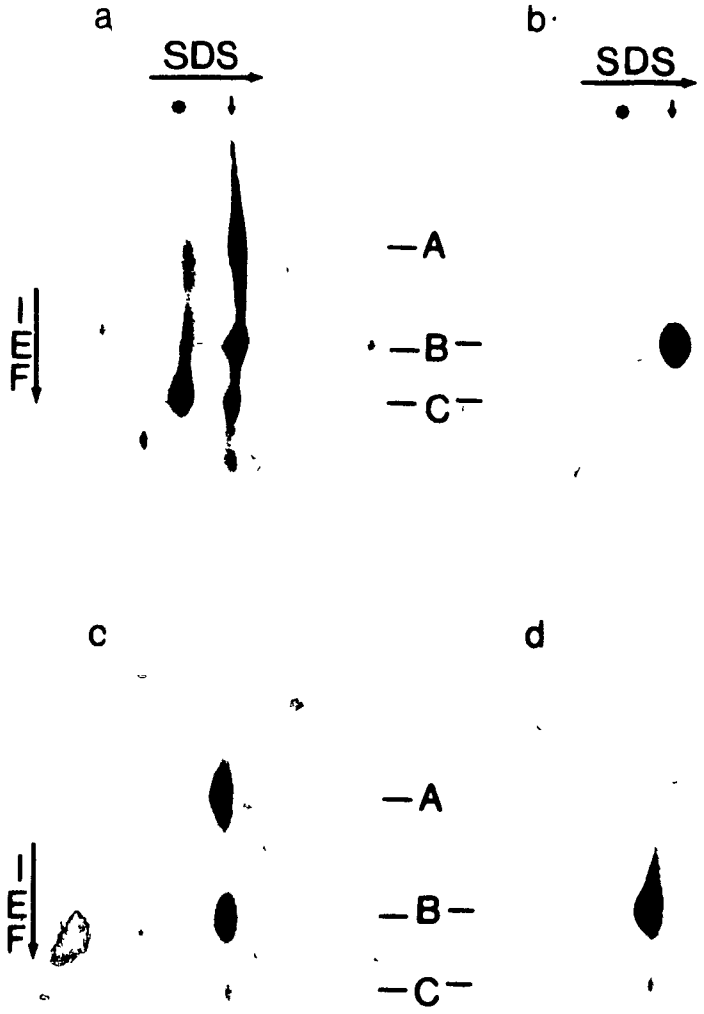

a
SDS
b
SDS
IEF
A
B
C
c
d
IEF
A
B
C

FIGURE. 15

Proteolytic digestion of the different forms of sigma 3

Virus was purified from infected cells labeled with [^{35}S] methionine. Viral proteins were resolved by two-dimensional gel electrophoresis and the gel fragments containing each of the forms (A, B, C, D) of sigma 3 were excised from the dried gels, rehydrated, loaded into the wells of a second polyacrylamide gel (3% stacking gel-16% resolving gel). The protein in each fragment was then digested, using standard procedures (Cleveland et al., 1977), with 2.5 μg of Staphylococcus aureus V8 protease, chymotrypsin A4, or papain. The figure is a composite of autoradiograms obtained after 3 days of exposure (forms A and B) or 12 days of exposure (forms C and D).

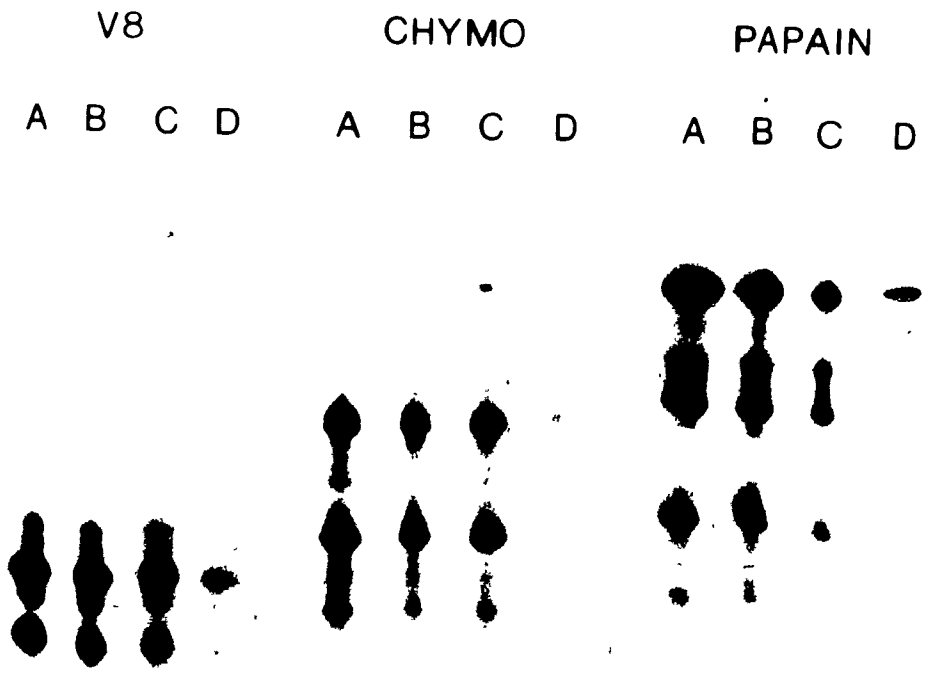

V8
CHYMO
PAPAIN
A B C D
A B C D
A B C D

FIGURE. 16

Analysis of purified sigma 3 by two-dimensional gel electrophoresis

Autoradiogram obtained after the sigma 3 protein from reovirus serotype 3 labeled with [^{35}S]methionine and purified from infected cells by poly(I)-poly(C) cellulose chromatography was analyzed by SDS-polyacrylamide gel electrophoresis (a) or two-dimensional gel electrophoresis (b). The migration directions of the two-dimensional gel are indicated, the IEF direction being from the basic to the acidic end.

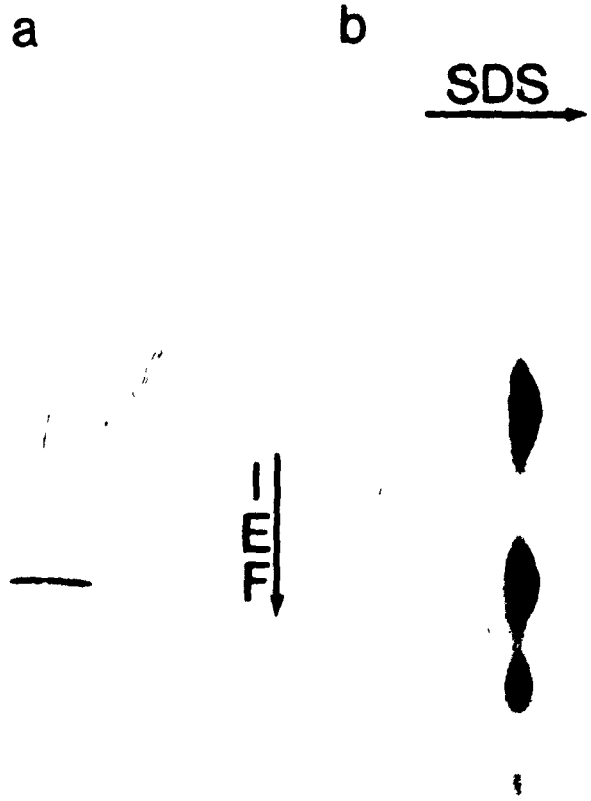
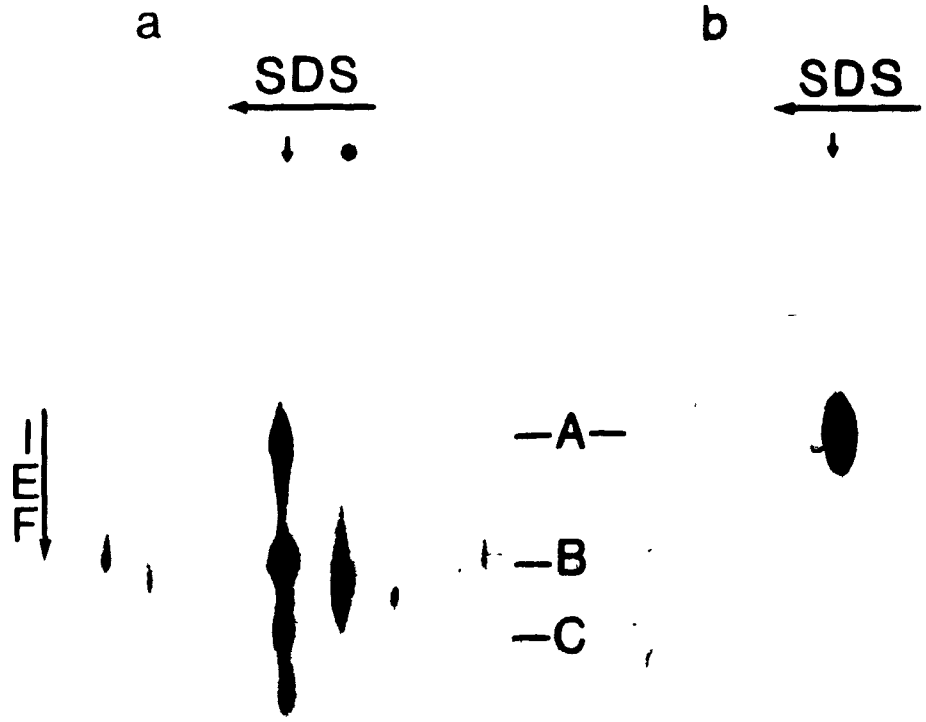

a
b
SDS
IEF

FIGURE. 17

Analysis by two-dimensional gel electrophoresis of sigma 3 obtained by in vitro translation

Autoradiogram of a two-dimensional gel analysis of proteins synthesized in vitro in rabbit reticulocyte lysate. Messenger RNA was prepared from late-infected cells (a) or by in vitro transcription (Krieg and Melton, 1984) using SP6 RNA polymerase (b). Migration direction of the first dimension (isoelectric focusing; IEF) is indicated, migration was from the basic to the acidic end; migration direction of the second (SDS) dimension is also indicated. The small arrow indicates the position of sigma 3 in the second dimension and the asterisk that of sigma NS. The positions in the first dimension of the three major forms of sigma 3 (A, B, and C) are also indicated. Only the gel portion comprising sigma 3 is shown on the figure.


a
SDS
b
SDS
IEF
—A—
—B
—C

CHAPTER 5

Expression of the cloned S4 gene of reovirus serotype 3 in transformed eucaryotic cells: Enrichment of the viral protein in the crude initiation factor fraction

FOREWORD

Following the demonstration that the viral protein sigma 3 can apparently stimulate translation of late viral mRNA we decided to express, in eucaryotic cells, the sigma 3 protein encoded by the cloned S4 gene. The possibility of obtaining authentic sigma 3 from the cloned gene was already indicated in chapter 4. In this chapter, the isolation of a L cell line expressing the protein, as well as some studies concerning the properties of the protein, are described. This is the first example of expression of a cloned reovirus gene in eucaryotic cells. The use of such an approach permits confirmation of some of the conclusions presented in chapter 3 and yields more information concerning the interaction of sigma 3 with the translational machinery. Furthermore, such a cell line is an useful tool for future studies.

All the data presented in this chapter are original and are the results of my own experiments. A shorter version of this chapter has been published [Lemay, G., and S. Millward (1986) Virus Res. 6, 133-140]. This chapter is a longer version describing in greater details the techniques and including some results presented in the manuscript as "data not shown". Some more experiments are also included.

ABSTRACT

The sigma 3 protein of reovirus is believed to play a role in the control of protein synthesis in reovirus-infected cells. In this chapter we describe the establishment of L cell lines expressing the sigma 3 protein from the cloned S4 gene of reovirus serotype 3 placed under the control of eucaryotic promoters. Lysates prepared from these cells are more efficient than normal L cell lysates for translation of late viral mRNA. The protein was enriched in the crude initiation factor fraction prepared by a high salt wash of ribosomes. There was no apparent detrimental effect on the cell lines. This approach confirms, in a better defined system, the effect of sigma 3 on viral protein synthesis.

INTRODUCTION

During the course of reovirus serotype 3 (Dearing strain) infection of mouse L cells there is a gradual decrease of host-specific protein synthesis concomitant with an increase in viral protein synthesis (Zweerink and Joklik, 1970). Genetic experiments using viral reassortants have shown that the more rapid protein synthesis inhibition observed with the reovirus serotype 2 compared with the serotype 3 is linked with the S4 gene coding for the major outer capsid protein sigma 3 (Sharpe and Fields, 1982). However these experiments did not show directly the effect of sigma 3 in cells infected with reovirus serotype 3.

Studies performed in our laboratory have shown the occurrence of a transition from cap-dependent to cap-independent translation during the course of reovirus serotype 3 infection of L cells (Skup and Millward, 1980a; Skup, Zarbl and Millward, 1981). At late times after infection there is a partial inhibition of cellular and viral cap-dependent translation (early viral mRNA) and very efficient translation of viral uncapped mRNA (late viral mRNA) synthesized by progeny subviral particles (Zarbl, Skup and Millward, 1980; Skup and Millward, 1980b; Skup, Zarbl and Millward, 1981). Further studies suggested the presence of a viral-specific discriminatory factor in infected cells (Lemieux, Zarbl and Millward, 1984). This factor, stimulating only the translation of reovirus uncapped mRNA, was shown to be most probably sigma 3 (see chapter 3).

A cloned cDNA copy of the S4 gene of reovirus serotype 3 was recently obtained in another laboratory (Giantini et al., 1984). In

this chapter the cDNA clone was used to express the "cloned" protein to facilitate further study of its role during translation. We describe the isolation and some properties of a permanent L cell line obtained after transformation into L cells of the S4 cDNA placed under the transcriptional control of the SV40 early promoter (Southern and Berg, 1982). Alternatively the metallothionein promoter was also used (Hamer et al., 1982; Karin et al., 1984). The transient expression using the SV40 promoter in COS-1 cells (Gluzman, 1981; Mellon et al., 1981) was also achieved. All these approaches allowed expression of the sigma 3 protein detectable by immunoprecipitation.

There is apparently no detrimental effect of the sigma 3 protein in the cell lines obtained. The synthesis of viral protein following infection of these cells is also identical to normal cells. However lysates prepared from these cells are more efficient for translation of late viral mRNA than lysates from uninfected cells. This approach has thus confirmed the effect of sigma 3 on late viral mRNA translation in the absence of any other viral component. Furthermore it allows to demonstrate unequivocally that sigma 3 can bind on ribosomes by direct interaction with the translational machinery rather than by indirect interaction depending on the presence of late viral mRNA.

MATERIALS AND METHODS

DNAs.

Vector DNA pKSV-10 and BglII linkers were obtained from Pharmacia. A cloned cDNA copy of S4 was a generous gift from Dr. A.J. Shatkin (Giantini et al., 1984). Vector containing the metallothionein promoter was constructed and obtained from Karl Hasel in our laboratory. The plasmid pSVtk-neo (Nicolas and Berg, 1983) was a generous gift from Dr. C.P. Stanners. DNA fragments were isolated from low melting agarose (Seaplaque) using elutip-D columns (Schleicher & Schuell) as recommended by the manufacturer. Small-scale and large-scale isolation of plasmid DNA were done according to standard procedures (Maniatis, Fritsch and Sambrook, 1982).

Enzymes.

Restriction enzymes were purchased from Boehringer Mannheim except Tth 111 type I (Pharmacia) and used in the conditions recommended by the supplier. DNA polymerase I, DNA polymerase I Klenow fragment, deoxyribonuclease I, calf intestine phosphatase and T4 DNA ligase were also obtained from Boehringer Mannheim and used essentially according to standard procedures (Maniatis, Fritsch and Sambrook, 1982).

Transformation of bacterial cells.

Competent Escherichia coli RR1 bacterial cells were prepared and transformed by plasmid DNA using the calcium chloride procedure described by Maniatis (Maniatis, Fritsch and Sambrook, 1982).

Cells and virus.

Mouse LTA cells [L tk- aprt- (Kit and Dubbs, 1977)] or COS-1 cells (Gluzman, 1981) were grown in monolayer using MEM (Flow Laboratories) supplemented with 10% fetal calf serum and 50 µg/ml of gentamicin. Cells were kept in a 5% CO_2 humidified atmosphere. The LTA cells were also grown in suspension culture in MEM for suspension (Flow Laboratories) containing the same amount of serum and gentamicin. The cell lines obtained after transformation were grown under the same conditions. Reovirus serotype 3 (Dearing strain) was obtained from American Type Culture Collection and propagated at low multiplicity of infection.

Transformation of eucaryotic cells.

Introduction of plasmid DNA into cells was done essentially by the calcium phosphate coprecipitation method (Graham and van der Eb, 1973). LTA cells were seeded the day before at 10^6 cells per 50 mm diameter plastic petri dish. Medium was replaced with fresh medium 3 h before transfection. DNA was diluted in 0.2 ml of 1 mM Tris-HCl pH 7.9, 0.1 mM EDTA and mixed with 0.2 ml of 0.5 M $CaCl_2$. This mixture was then slowly added to 0.4 ml of HBS (50 mM Hepes, 280 mM NaCl, 1.5 mM sodium phosphate dibasic, final pH adjusted to 7.1 with NaOH), air bubbles were slowly injected into the solution at the same time. The final solution was left 30 min at room temperature and added to the medium (5 ml of medium) of the petri dish. After 4 h at 37 °C in an humidified atmosphere of 5% CO_2 the medium was replaced with fresh medium and then replaced again after 24 h. After another 24 h the cells were feeded with the same medium containing in addition 400 µg/ml of G418 sulfate (geneticin) from Gibco laboratories. The medium

was then replaced every 3 days with medium containing G418 for 2 weeks until colonies of resistant cells were clearly visible. The colonies were then trypsinized individually using glass cloning cylinders (Bellco) and grown in individual petri dishes. Transformation of COS-1 cells was done essentially by the same procedure except that after 4 h the media was removed and the cells exposed to sterile glycerol diluted to 20% in media. After 1 min at room temperature the glycerol solution was removed and the cells washed carefully with media before incubation in normal media (Mellon et al., 1981).

Extraction of chromosomal DNA.

Cells grown in monolayer were trypsinized, resuspended in phosphate-buffered saline and pelleted in an Eppendorf centrifuge at 4 °C. They were then resuspended in 0.5% NP-40 and the nuclei pelleted in an Eppendorf centrifuge. Nuclei were resuspended in DNA extraction buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl). After addition of 0.5% SDS and 200 µg/ml of proteinase K (Boehringer Mannheim) the mixture was incubated overnight at 37 °C, extracted with phenol-chloroform (1/1) and the DNA precipitated with ethanol and resuspended in distilled-deionized water.

Agarose gel electrophoresis and DNA blot hybridization.

DNA fragments obtained by digestion with restriction enzymes were resolved on 1% horizontal agarose gel using the Tris-acetate buffer system (described in: Maniatis, Fritsch and Sambrook, 1982). Resolved DNA fragments were transferred by capillarity (Southern, 1975) onto Gene screen plus membranes (New England Nuclear) and hybridized using the dextran sulfate method suggested by the manufacturer. DNA

fragments used as probes were labeled with [^{32}P] by nick translation essentially as described (Rigby et al., 1977). In some experiments the undigested DNA was bound directly onto nitrocellulose filter by the dot-blot procedure (Thomas, 1980) and hybridized following the same procedure.

Immunoprecipitation.

Proteins were labeled in vivo with [^{35}S]-methionine (1000 Ci/mmol, New England Nuclear) in medium lacking methionine essentially as described previously. Cells were lysed in 0.5% NP-40 and the nuclei removed by centrifugation (10,000 x g, 10 min). Adequate amounts of proteins (as indicated in the figures' legends) were diluted in 0.5 ml of immunoprecipitation buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM methionine, 1 mM PMSF, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.1% BSA, 100 KIU/ml of Aprotinin) and subjected to quantitative immunoprecipitation using either 20 µl of anti-reovirus antiserum from goat (M.A. Bioproducts) or rabbit monospecific anti-sigma 3 antiserum (a generous gift of Dr. P.W.K. Lee). After overnight agitation at 4 °C, the antigen-antibody complexes were precipitated using the affinity of protein A for immunoglobulin (Kessler, 1975). A volume of 100 µl of protein-A Sepharose CL-4B was added and left for 1 h at 4 °C with gentle agitation. The beads of Sepharose were then collected by centrifugation in an Eppendorf centrifuge for 1 min at 4 °C and washed extensively in immunoprecipitation buffer and PBS. The immunoprecipitated material was then eluted from the beads by boiling for 5 min in gel sample buffer containing 3% SDS and 300 mM 2-mercaptoethanol. The beads were then removed by centrifugation and

the supernatant analyzed by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis.

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis using the procedure of Laemmli (Laemmli, 1970). After electrophoresis the gels were stained with Coomassie blue, destained, dried, and subjected to direct autoradiography or fluorography.

In vitro translation.

Cell-free translation lysates were prepared from cells grown in suspension and rendered mRNA-dependent as described (Skup and Millward, 1977). Translation reactions were done and incorporation into hot TCA-precipitable material measured by filter binding assay as described previously. To study the effect of anti-reovirus antiserum the serum was dialyzed against 10 mM Hepes-KOH pH 8.0, 100 mM KCl and 2 µl of antiserum was added to a 20 µl translation reaction.

Fractionation of cell-free lysates.

Proteins were labeled in vivo and S10 lysate prepared as described previously. The lysate was then fractionated essentially as described (Schreier and Staehelin, 1973). Briefly, the S10 lysate was ultracentrifuged (45,000 rpm, 1 h at 4 °C in a Type 50 rotor); the supernatant is the S200 or postribosomal supernatant. The ribosomes in the pellet were then resuspended in hypotonic buffer (one fifth of the original S10 volume) and a final concentration of 0.5 M KCl was added. After 20 min on ice the ribosomes were again pelleted and the supernatant constitutes the ribosomal salt wash proteins (RSW). The salt-washed ribosomes were then resuspended in hypotonic buffer and each fraction dialyzed for 3 h against 10 mM Hepes-KOH pH 7.5, 100 mM

KCl, 0.05 mM EDTA, 6 mM 2-mercaptoethanol. The fractions were then stored in liquid nitrogen until needed.

RESULTS

Subcloning of the S4 gene in expression vectors.

Construction of the first plasmid used for expression is schematized in Fig. 18. The cDNA copy of the S4 gene of reovirus serotype 3 cloned in pBR322 was digested with both restriction endonucleases Tth111 type I (T) and EcoRI (R). The first enzyme has a recognition site in the 5′-untranslated region of S4 shortly before the initiation codon and EcoRI has a recognition site in the pBR322 sequence leaving a piece of pBR322 at the 3′-end of the S4 gene. These enzymes do not have recognition sites in the coding region of S4, the fragment obtained thus encompassed the whole coding sequence for the sigma 3 protein. This fragment was gel-purified, the ends filled-in with the Klenow fragment of DNA polymerase I and BglII linkers added. After digestion of the excess linker with BglII the fragment was ligated to the plasmid vector pKSV-10 [previously linearized with BglII (B) and phosphatase-treated]. The plasmid pKSV-10 contains a unique BglII site located between the SV40 early promoter with enhancer and the splice site with termination and polyadenylation signals of the same transcriptional unit. Transformation of Escherichia coli RR1 bacterial cells and screening for the correct orientation were done by standard procedures (Maniatis, Fritsch and Sambrook, 1982) using the presence of the PstI (P) site to determine the orientation.

The same S4-containing fragment was also cloned at the unique BglII site of another expression vector. In this vector the SV40 promoter and enhancer were replaced by the 5′-flanking region of the

human metallothionein II-A gene. This region contains the promoter and control regions of the metallothionein gene responsible for increased transcription in the presence of heavy metals (Hamer et al., 1982; Karin et al., 1984).

Introduction of plasmid DNA into L cells.

The plasmid obtained (pSV-S4) was introduced into LTA cells by the calcium-phosphate coprecipitation procedure (Graham and van der Eb, 1973) as described under Materials and Methods. The plasmid (15 μg) was cotransformed with another plasmid (0.15 μg) containing the neomycin resistance gene under the control of an hybrid SV40tk promoter (Nicolas and Berg, 1983). The mixture of the two plasmid was applied on 400,000 LTA cells growing in a 5 cm diameter petri dish. The neomycin resistance gene can transform eucaryotic cells to resistance to G418 sulfate (Davies and Jiminez, 1980; Colbere-Garapin et al., 1981; Southern and Berg, 1982). By using a very small amount of the resistance plasmid compared to pSV-S4 most of the resistant colonies should contain at least one integrated copy of pSV-S4 (Wigler et al., 1979). Twelve independent colonies were grown and screened by DNA dot-blot hybridization (Thomas, 1980) using as a probe the nick-translated S4 DNA obtained by gel purification of the PstI fragment from the cloned S4 cDNA. Ten colonies out of twelve were positive to different extents by this assay. The colony giving the strongest signal was kept for further analysis and the cell line obtained will be called LS-S4 in the rest of this chapter. The same procedure was also used with the plasmid using the metallothionein promoter and the cell line obtained is called LM-S4.

Analysis of the S4 DNA integrated to the chromosomal DNA in the cell lines.

Chromosomal DNA analyzed by Southern blot analysis (Southern, 1975) with an S4 probe revealed the presence of many (probably between 5 and 10) different integrations of S4 DNA in the chromosomal DNA of both cell lines (LS-S4 and LM-S4) by using a restriction enzyme (HindIII) having restriction site in the plasmid DNA but outside the S4 gene (Fig. 19). A darker fragment observed in both cell lines corresponds to the total length of the plasmid and probably results from tandem integrations of plasmid DNA. Using an enzyme having a restriction site at both ends of the S4 insert (BglII) we detected only one fragment of the predicted length (around 2 kbp.) (Fig. 19), the longer fragment results from partial digestion and its length is also predictable. The intensity of the 2 kbp fragment is consistent with the presence of many identical copies integrated at different chromosomal sites. However we do not know if each of these copies are transcriptionally active. It was impossible to detect hybridization to DNA extracted from non-transformed cells, as expected.

Detection of sigma 3 in the cell lines.

The presence of sigma 3 in the transformed cell lines was detected by quantitative immunoprecipitation of in vivo labeled cell proteins using either anti-reovirus antiserum or monospecific anti-sigma 3, as described under Materials and Methods. Results using the anti-sigma 3 antibody are presented in Fig. 20. In late-infected cell lysate (I), the antibody brought down not only the major band of sigma 3 protein but also lower amounts of other proteins (mul, mulC) that are known to be associated with sigma 3 (Huismans and Joklik, 1976;

Lee, Hayes and Joklik, 1981b). In normal L or LTA cells (U) we occasionally detected a background of non-specific precipitation corresponding to the major protein species present in the lysate. In the cell lines LS-S4 (S) and LM-S4 (M) we detected clearly an extra band corresponding in molecular weight to the sigma 3 protein detectable in infected cells (Fig. 20); this protein was not detectable without immunoprecipitation. Peptide mapping of the sigma 3 in LS-S4 by the Cleveland method (Cleveland et al., 1977) revealed the same pattern of peptides as authentic sigma 3 extracted from infected cells (data not shown). Analysis of some of the other positive colonies revealed that most of them (8 out of 10) expressed sigma 3. There is only a small variation in the amount of sigma 3 expressed, suggesting that some of the integrated copies of S4 are transcriptionally inactive. The LS-S4 line chosen for further analysis apparently produces the highest level of sigma 3. Expression obtained using the metallothionein promoter is apparently about the same; the expression can however be stimulated about 3-fold by the addition of zinc sulfate (50 μM) to the media (Fig. 20 compare M to M+zn).

Immunoprecipitation did not allow exact comparison of the production of sigma 3 in the cell lines compared with that in late-infected cells because of the different specific activity of labeling due to host-cell protein synthesis inhibition in late-infected cells. However, densitometric scanning of the autoradiogram presented in Fig. 20 indicated that labeled sigma 3 in LS-S4 lysate is produced at about 5% of the rate observed in late-infected cells when viral protein synthesis is maximal (30 h post-infection at 31 °C). This amount

corresponds approximately to the amount observed by immunoprecipitation of early-infected cells (5 h at 37 °C or 9 h at 31 °C). Transient expression using COS-1 cells (Gluzman, 1981; Mellon et al., 1981) that allow replication of the plasmid containing the origin of replication of SV40 was also tested. Immunoprecipitation of proteins labeled 48 h after transformation showed a clear expression of sigma 3 (Fig. 21). A band of lower molecular weight is occasionally detected in the experiments; its exact nature is unknown but it possibly results from proteolytic cleavage of sigma 3 during immunoprecipitation procedure. The amount of sigma 3 observed decreased thereafter but a small production was still detectable after 6 days (data not shown). The amount observed at 48 h is about the same as in LS-S4.

Translation in cell-free lysates prepared from LS-S4.

Cell-free lysates were prepared from the LS-S4 cell line and their efficiencies for translation of uninfected or late-infected cell mRNA was examined. The sigma 3 protein purified to apparent homogeneity has been previously shown to stimulate the translation of late viral mRNA. The protein expressed in LS-S4 has retained this activity (Fig. 22). The lysate prepared from LS-S4 was about 4-fold more efficient at translating late viral mRNA than is normal L cell lysate. The addition of the anti-reovirus antiserum reduces considerably this viral-specific stimulation of translation as expected if the difference is really due to the presence of sigma 3 and not to other unexpected differences between the two lysates. Similar results were obtained with a lysate prepared from the LM-S4 cell line.

Enrichment of sigma 3 in the crude initiation factor fraction.

Experiments performed in our laboratory have previously indicated that the sigma 3 protein is enriched in the crude initiation factor fraction prepared by a high salt wash of ribosomes from late-infected cells. This binding to ribosomes is probably necessary for stimulation of translation of late (uncapped) viral mRNA. Using immunoprecipitation we have found that the protein is also strongly enriched in the crude initiation factor fraction prepared from early-infected cells (Fig. 23-A) even if at this stage essentially all the viral mRNA synthesized is capped (Skup, Zarbl and Millward, 1981). The same analysis was performed on fractions prepared from LS-S4 cells (Fig. 23-B) and showed clearly that the sigma 3 protein can bind to the ribosomes in the absence of any other viral protein and RNA.

DISCUSSION

We reported here the first example of expression of a cloned reovirus gene in eucaryotic cells. The fact that we obtained a functional protein by at least two different criteria, namely the stimulation of late viral mRNA translation and the properties of association-dissociation from the ribosomes, proved that the procedure used in the cloning of the double-stranded RNA genome can produce faithful copies of the genes. It was also reported recently (Richardson and Furuichi, 1985; Masri et al., 1986) that other cloned genes of reovirus, respectively the S3 and S1 genes, expressed in bacterial cells produce apparently functional protein products. Since most of the reovirus genes have been cloned (Cashdollar et al., 1984), these studies open the possibility of studying the effects of each of those genes on the host cell.

Some authors have suggested, based on genetic experiments, that the sigma 3 protein inhibits host cell protein synthesis (Sharpe and Fields, 1982; Ahmed and Fields, 1982); however their results apply better to the rapid inhibition of total protein synthesis observed with the serotype 2 than to the slower host-specific inhibition resulting from serotype 3 infection. Further work is needed to reconcile the results obtained in these two systems.

The amount of sigma 3 produced in the cell lines is lower than observed in late-infected cells, but the protein is continuously produced in the cell and did not seem to affect the rate of growth of these cells. The amount of hot TCA-precipitable material obtained after a one-hour labeling of these cells with radioactive methionine

was identical to control LTA cells and analysis of labeled proteins by gel electrophoresis did not reveal differences between LS-S4, LM-S4 and normal LTA cells (data not shown). Similarly, analysis of viral proteins produced early and late during reovirus infection of these cells was identical to the situation in LTA cells (data not shown). The expression in COS-1 cells is another indication that cellular protein synthesis is not affected by the presence of sigma 3, even though in this case the level of sigma 3 in the cells containing a high copy number of plasmid is probably much higher. If we consider that about 10% of the cells acquired the plasmid (Mellon et al., 1981), then the production of sigma 3 in these cells has to be at least 10 to 20-fold higher than in the permanent cell line to obtain the amount of sigma 3 observed. Moreover, in experiments comparing the frequency of transformation obtained with the plamid vector and with the S4-containing vector, the same numbers of colonies were obtained; such a result is incompatible with a negative impact of the presence of the viral protein. We thus have no evidence that sigma 3 directly affects host cell protein synthesis.

Experiments performed in our laboratory previously indicated that the sigma 3 protein is involved in translational regulation in another way. This protein was shown to stimulate translation of late (uncapped) viral mRNA (see chapter 3). This stimulation is reovirus-specific and can be shown by comparing translational efficiency of RNA extracted from uninfected or late-infected cells. This viral-specific stimulation of translation can be demonstrated in the LS-S4 and LM-S4 lysates even if the amount of sigma 3 present is very low compared to late-infected cells. The absence of other viral proteins may

explain why we obtain such an effect with such a relatively low production of sigma 3. This stimulation is also inhibited by an antibody against reovirus protein, as expected.

The protein sigma 3 is enriched in the crude initiation factor fraction prepared by high-salt wash of ribosomes from late-infected cells. This binding is probably important for the stimulation of translation and it was previously shown that this binding does not likely involve direct interaction with the mRNA (see chapter 3). Analysis of the sigma 3 distribution in fractions of LS-S4 cells demonstrates that the binding of sigma 3 to the ribosomes is completely independent of the presence of late viral mRNA, a conclusion also supported by a similar experiment performed with early-infected cells. However, with early-infected cells it was still possible that the presence of small amount of uncapped reovirus mRNA may explain this observation. The fractionation of the LS-S4 lysate definitely confirms the binding in the absence of late (or even early) viral mRNA and also proves that the binding is independent of the presence of any other viral protein. The isolation of these cell lines expressing sigma 3 has thus further substantiated our conclusions concerning the effect of sigma 3 on the translational machinery and should facilitate further studies concerning the mechanism of action of this protein during translation of late viral mRNA.

FIGURE. 18

Construction of S4 expression clone

The construction described in the text is schematized in this figure. Black boxes indicate the S4 gene, the open boxes are SV40 sequences and the thin lines are pBR322 sequences. Restriction sites are as follow:

T: Tth 111 type I; R: EcoRI; P: Pst I; B: Bgl II

The 5′ end of the S4 gene is on the end closer to the Tth 111 site.

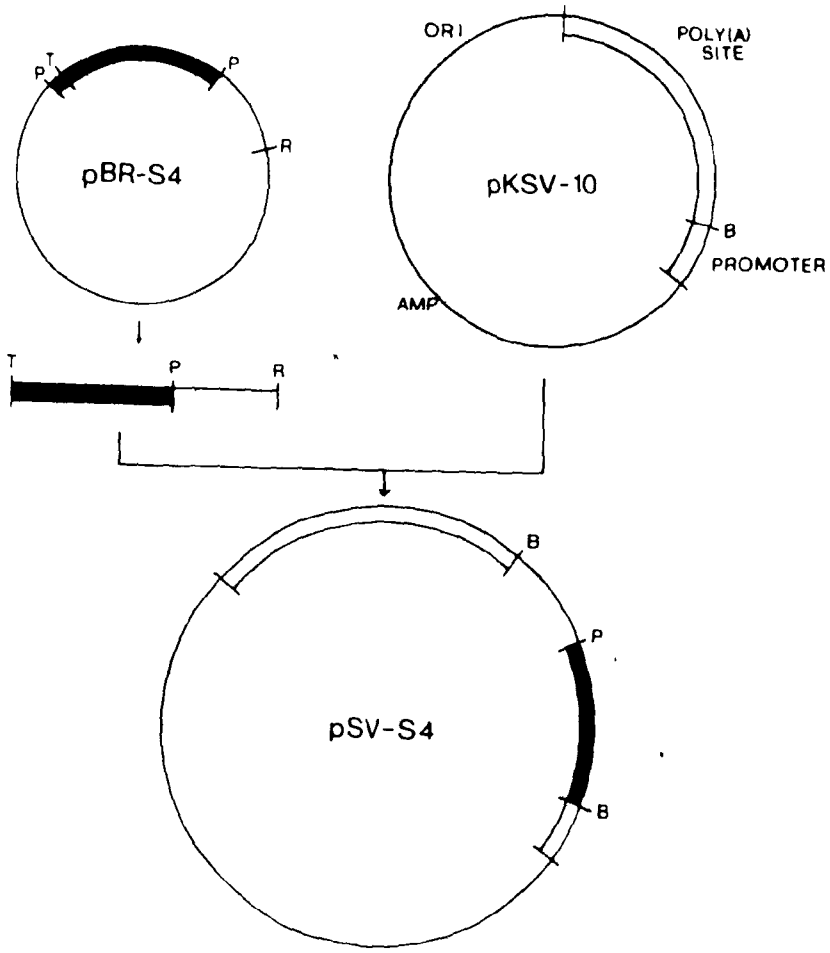

T
P
P
R
pBR-S4
ORI
POLY(A)
SITE
pKSV-10
B
PROMOTER
AMP
T
P
R
B
P
pSV-S4
B

FIGURE. 19

Analysis of S4 DNA in the transformed cells

Chromosomal DNA was extracted as described under Materials and Methods and 10 μg of each was digested with HindIII or BglII as indicated. DNA fragments were resolved on 1% agarose gels, transferred to Gene Screen Plus and hybridized with radiolabeled S4 DNA as described under Materials and Methods. DNA analyzed were extracted from: L, untransformed LTA cells; S, LS-S4 cell line; M, LM-S4 cell line. Positions of DNA fragments of known lengths that were electrophoresed on the same gel are indicated and their lengths expressed in thousands of base pairs (kbp.).

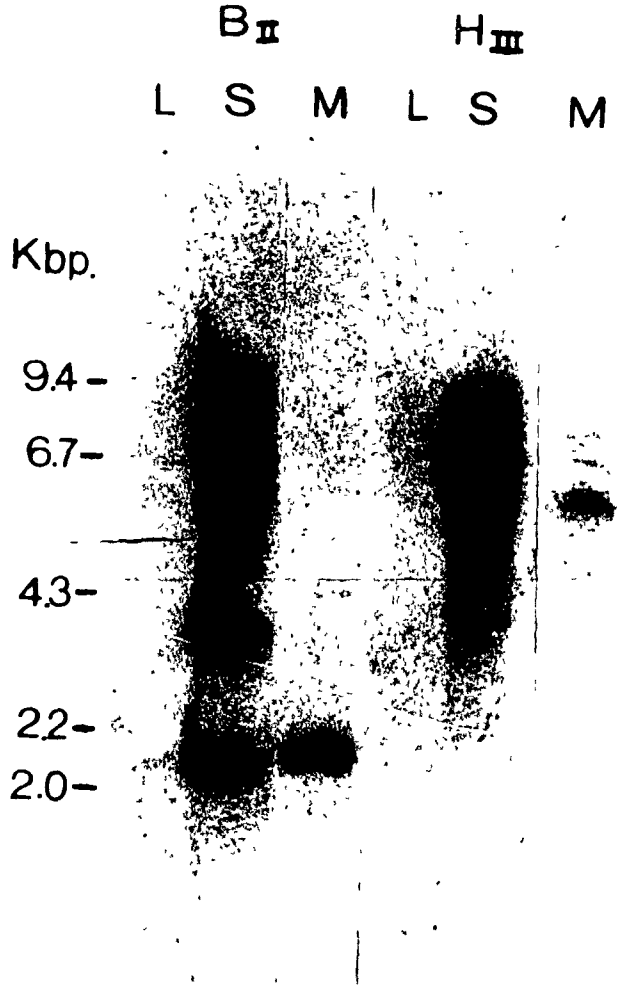

B II
H III
L S M L S M
Kbp.
9.4
6.7
4.3
2.2
2.0

FIGURE. 20

Immunoprecipitation of sigma 3 in transformed cell lines

Proteins were radiolabeled in vivo, cells lysed and the same amount of hot TCA-precipitable material for each sample was subjected to quantitative immunoprecipitation as described under Materials and Methods. Labeling of each cell type was done in the same conditions. A monospecific anti-sigma 3 antiserum was used for immunoprecipitation. The immunoprecipitated material was subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography and finally autoradiography as described under Materials and Methods. Proteins were from: I, late-infected cells; M, LM-S4 cells; S, LS-S4 cells; U, LTA cells. LM-S4 cells were also labeled in normal conditions (M) or after preincubation for 16 h in media containing 50 µM of zinc sulfate (M+zn). The arrow indicates the position of sigma 3 as determined by the comigration of proteins extracted from purified virus.

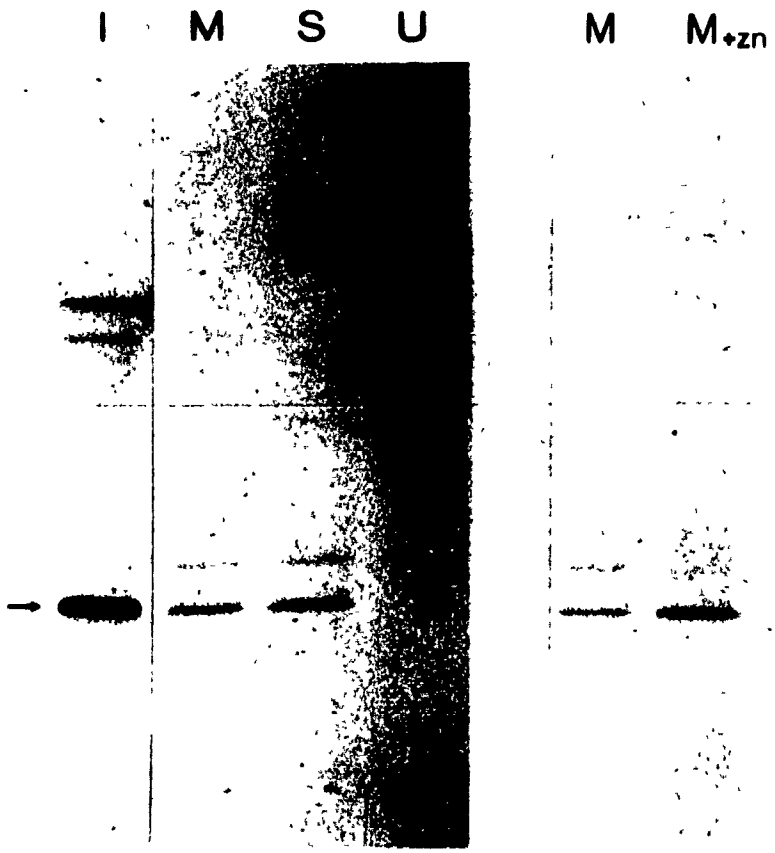

I
M
S
U
M
M+zn

FIGURE. 21

Expression of sigma 3 in COS-1 cells

COS-1 cells were transformed with pSV-S4 and proteins labeled after 48 h and subjected to immunoprecipitation as described under Materials and Methods and in the preceding figure. Proteins were from: I, late infected L cells; U, untransformed COS-1 cells; T, COS-1 cells 48 h after transformation. The position of the sigma 3 marker protein is indicated by the arrow.

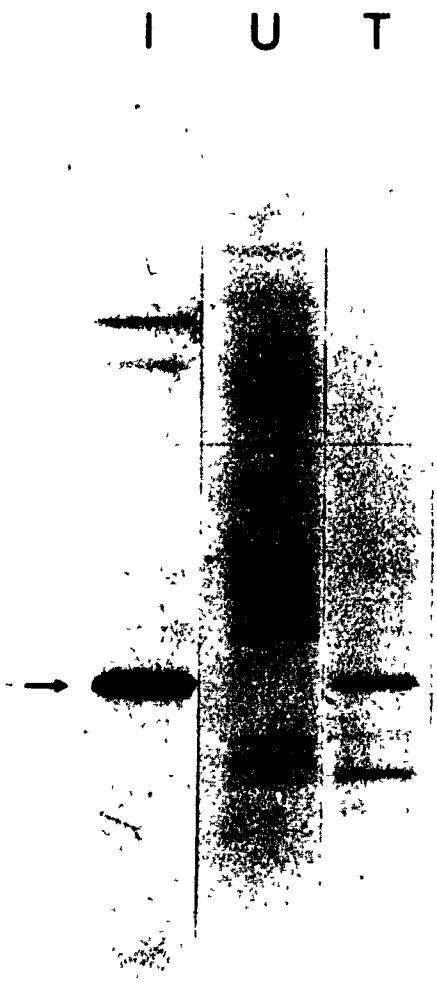

I U T

FIGURE. 22

Stimulation of translation of late viral mRNA in LS-S4

Cell-free translation lysates were prepared from untransformed L cells or LS-S4 cells as described under Materials and Methods. -, absence of added RNA; U, uninfected L cell RNA; I, late-infected cell RNA. Translation reactions were done in the absence (-) or presence (+) of anti-reovirus antiserum. The same volumes of each translation reaction were loaded in the first panel (L). Larger volumes of each sample were loaded in the second panel (LS-S4) to compensate for lower activity of this particular lysate.

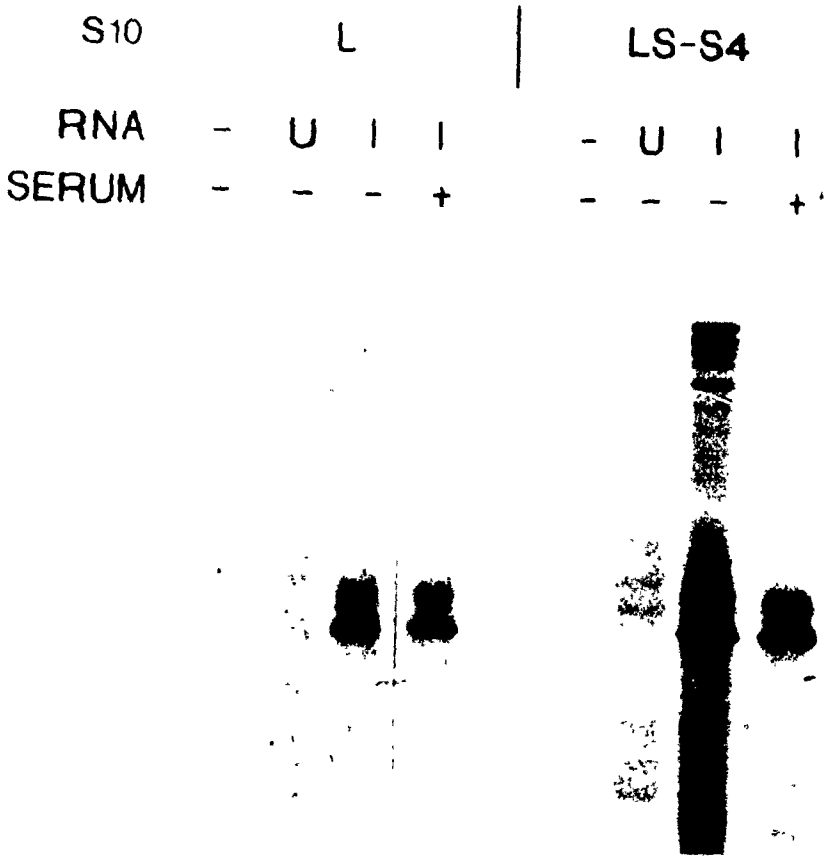
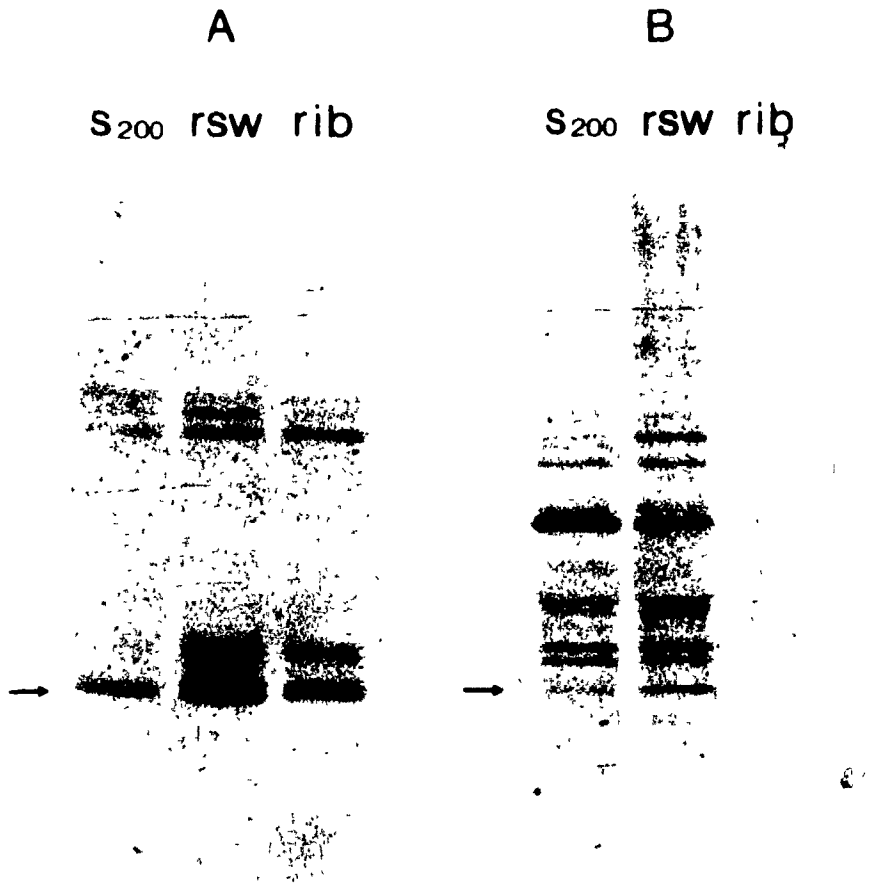

S10
L
LS-S4
RNA - U I I - U I I
SERUM - - - + - - - +

FIGURE. 23

Enrichment of sigma 3 in the crude initiation factor fraction

Labeling of cellular proteins, preparation and fractionation of lysates were described under Materials and Methods. S200, postribosomal supernatant; RSW, ribosomal salt wash or crude initiation factor fraction; RIB, ribosomal fraction after salt wash. After dialysis the same amount of hot TCA-precipitable material of each fraction was subjected to immunoprecipitation as described under Materials and Methods. In panel A fractions were prepared from early-infected cells (5 h at 37 °C) and in panel B fractions were prepared from LS-S4 cells. The position of sigma 3 is indicated by the arrow.


A
B
S_{200} rsw rib
S_{200} rsw rib

CHAPTER 6

General discussion: Conclusions and perspectives

By definition viruses are obligate intracellular parasites that need to use some, or even many, of the host cell functions for their multiplication. The interactions between viruses and host cells have been extensively investigated for many years. Work on bacteriophage infection of bacterial cells has shown that there are different and possibly very complex types of interactions involved.

During the viral multiplication, different host cell processes may be affected to different extents. The extent to which viral multiplication interferes with the normal processes of the host cell varies with the virus and host cell system. The virus itself codes for a certain number of proteins having different functions. Some viruses code for very few proteins, relying essentially on the host cell for most of the enzymes and factors needed for their multiplication. Other viruses, possessing larger genomes, produce many proteins involved in different enzymatic activities and thus use cellular functions to a lesser extent.

A very interesting situation occurs when viral products are used to modify a cellular component for its own use, or even its exclusive use. A very significant, and widely studied example in this regard, is the bacteriophage T4 infection where different viral proteins associate with bacterial RNA polymerase and alter its specificity in transcription to favor transcription of viral genes (described in Luria et al., 1978).

It was expected that the interactions between viruses and eucaryotic cells will be much more varied and complex than they are with the relatively simple bacterial cells. This prediction proved to be true as the study of different animal cell viruses has revealed

intriguing strategies of viral multiplication within the host cell (extensively described in: Luria et al., 1978; Fields and Knipe, 1986).

All viruses are dependent on the host cell translational machinery for the synthesis of their proteins. In many cases viral infection results in preferential synthesis of viral proteins with a more or less extensive inhibition of the synthesis of cellular proteins. The phenomena of modifications of the host protein synthesis machinery following viral infection with cytolytic viruses is a major area of study. Some of the most interesting examples have been briefly described in the introduction of this thesis (see chapter 1). Study of these phenomena is not only important to understand viral multiplication itself, but may well reveal important aspects of the normal functions of the cell.

Despite the extensive use of reovirus as a model system, there is still much to understand concerning the effect of reovirus on the translational machinery. The gradual shift to a preferential synthesis of viral proteins in reovirus-infected L cells was observed a relatively long time ago (Zweerink and Joklik, 1970); however, no hypothesis to explain this phenomenon was proposed until more recently. In this thesis, I have described some of the work that I have done to further understand the complex translational controls present during reovirus infection.

There are some differences of structure between cellular mRNA and reovirus mRNA that may have a role in the translational discrimination between these mRNAs. The first difference is the absence of a poly(A) tract at the 3'-end of reovirus mRNA (Stoltfus, Shatkin and Banerjee,

1973); such a structure is present on most cellular mRNA (reviewed in: Brawerman, 1981). Results presented in chapter 2 confirmed a probable involvement of this structure in initiation of protein synthesis, as suggested by the inhibitory effect of free poly(A) on in vitro protein synthesis (previously suggested in: Jacobson and Favreau, 1983). The absence of inhibition of reovirus mRNA translation by free poly(A), in contrast to cellular mRNA translation, is reminiscent of the situation observed in vaccinia virus where the translation of viral mRNA is also resistant to free poly(A) (Bablanian and Banerjee, 1986). In vaccinia virus it has been suggested that the production by the virus of small poly(A) transcripts is responsible for the preferential inhibition of cellular mRNA translation during viral infection (Coppola and Bablanian, 1983; Bablanian et al., 1986). Reovirus also produces short A-rich oligonucleotides (reviewed in: Zarbl and Millward, 1983) but their role in inhibition of protein synthesis is still in question. They are synthesized very late in infection and retained in the mature virus (as described in the introduction of this thesis) suggesting that they do not have a predominant role in translational control at late times in infection. They are released from the virus early in infection and it is plausible that at this time they might have a role. However, there is no apparent inhibition of host-specific protein synthesis early in infection. A localized effect is still plausible, thus facilitating translation of the scarce molecules of early viral mRNA without significant alteration of the global protein synthesis in the cell; however, with the presently available data such an effect is still hypothetical.

Another difference between reovirus and cellular mRNA was

observed in our laboratory a few years ago. It was previously shown that the bulk of viral mRNA is synthesized relatively late in infection (reviewed in: Zarbl and Millward, 1983). This "late" viral mRNA is synthesized by the so-called progeny subviral particles that contain newly replicated viral genomes; this mRNA synthesized after replication of the viral genome is thus in accordance with the definition of late viral mRNA in other viral systems.

The progeny subviral particles, in contrast to the virus uncoated in vivo (parental subviral particles) or in vitro (virus cores), synthesized uncapped mRNA (Zarbl, Skup and Millward, 1980) thus differing from early viral mRNA and cellular mRNA that have cap structures at their 5′-ends. The enzymatic activities necessary for the synthesis of the cap structure are either latent or absent in the progeny subviral particles (Skup and Millward, 1980b). The demonstration that the guanylyltransferase activity is associated with the lambda 2 spikes (Shatkin et al., 1983; Cleveland, Zarbl and Millward, 1986) is a further evidence that this activity is absent, as the progeny subviral particles have no morphologically detectable spikes and greatly reduced amount of lambda 2 (Morgan and Zweerink, 1974; 1975). The mRNA associated with polysomes late in infection is mostly uncapped viral mRNA and there is thus good evidences that the bulk of the viral proteins is synthesized by translation of uncapped mRNA. This synthesis of uncapped mRNA has been contested by two different groups of investigators (Detjen, Walden and Thach, 1982; Munoz, Alonso and Carrasco, 1985). However, these investigators have presented only indirect evidence for the absence of active uncapped mRNA. None of these groups have actually examined the 5′-terminus of

the late polysomal-associated mRNA or purified the progeny subviral particles to look at their transcription products. Furthermore they used other cell types than the widely used L cells for the reovirus infection. As a result, it is not clear that they are looking at a time point that really corresponds to late times in infection. In fact, one of these groups recently admitted that they were probably looking at phenomena occurring earlier in infection (Ray et al., 1985). It seems doubtful that the morphogenesis of the virion is different in different cell types. It is thus highly probable that the late viral mRNA synthesized by progeny subviral particles is also uncapped in these other cell types. In the remaining part of this discussion I will thus assume that the late viral mRNA is indeed uncapped; however, for some of the main conclusions drawn in this thesis, the nature of the structural difference between cellular mRNA and late polysomal-associated viral mRNA is relatively unimportant.

The demonstration that the late reovirus mRNA is uncapped suggested, by analogy with the better-known poliovirus infection, that there is a transition from cap-dependent to cap-independent translation in infected cells. Poliovirus infection is the most widely studied example of alteration of protein synthesis by viral infection, as already described in chapter 1. It is thus of interest to compare the two systems. It is now clear that the modifications to the translational machinery are quite different in reovirus infection. In reovirus infection, the inhibition of protein synthesis is gradual; host cell protein synthesis persists for a relatively long time after reovirus infection. Furthermore experiments with different reovirus serotypes revealed that the inhibition is not essential for efficient

growth of the virus (Munemitsu and Samuel, 1984; see chapter 3 of this thesis). In contrast, inhibition by poliovirus infection is rapid and mutant viruses that fail to produce this inhibition grow poorly (Bernstein, Sonenberg and Baltimore, 1985). Different approaches have also indicated that the two viruses use different strategies to inhibit host cell protein synthesis. It was shown that as a consequence of poliovirus infection, but not reovirus infection, there is a release of the cellular mRNA from the cytoskeleton (Bonneau, Darveau and Sonenberg, 1985); this event is probably somehow linked to the inhibition of translation. The results of double infection also suggest that the two viruses use at least partially different strategies for the inhibition (Munoz, Alonso and Carrasco, 1985). On the other hand analysis of cap binding proteins by chemical crosslinking revealed a decrease of these proteins late in infection by reovirus similar to that seen in poliovirus-infected cells (Zarbl, Lemieux and Millward, unpublished data). It is possible to observe in vitro a decrease of cap binding proteins by incubating initiation factors with lysates of poliovirus-infected cells (Lee and Sonenberg, 1982); however, all my attempts to repeat these types of experiments with lysates from reovirus-infected cells have been unsuccesful (data not shown). The mechanism altering cap binding proteins in reovirus infection is likely different, and may be more subtle, than seen in poliovirus infection. In fact, it was recently described by another group that the cleavage of the 220 kDa component of the cap binding protein complex, occurring during poliovirus infection, does not occur during reovirus infection (Etchison and Fout, 1985). It is also known that other viruses, like Semliki Forest virus, can affect the cap

binding proteins activity by a mechanism different from poliovirus (van Steeg et al., 1984; see chapter 1).

It rapidly became apparent that alterations in the cap binding proteins cannot completely explain the translational properties of late viral mRNA in reovirus-infected cells. Another mechanism was thus proposed and most of this thesis concerned evidence for this second mechanism. It was first noticed that, while reovirus late (uncapped) viral mRNA is very efficiently translated in lysates prepared from reovirus-infected cells, other naturally uncapped mRNAs are even less efficient in these lysates than they are in uninfected cell lysates (Lemieux, Zarbl and Millward, 1984). The nature of the sequences responsible for "tagging" the different mRNA segments as "reovirus-specific" is still unknown. Obviously the conserved nucleotides at the 5′- and 3′-ends are interesting candidates (see chapter 1). By using the new techniques of site-directed mutagenesis it should be eventually possible to clarify this point. In Semliki Forest virus and Frog virus 3, the nature of the structural difference between the early and late viral mRNA responsible for their different translational properties is unknown. In the reovirus system, however, it is quite clear that this difference resides in the absence of the cap structure on the late viral mRNA. The specific stimulation of late viral mRNA translation is observed also in lysates prepared from serotype 1 infected cells (see chapter 3) and is thus independent of inhibition of protein synthesis. The fact that the stimulation of late viral mRNA translation is more conserved among the different serotypes than the inhibition of protein synthesis suggests that the first phenomenon is more important for the growth of the virus. The

relatively unimportant role of the inhibition is also suggested by the lower extent of inhibition observed when other cell types are infected. However, when present, the inhibition might allow faster growth of the virus as previously discussed (see chapter 3).

Experiments performed in our laboratory have shown that the addition of proteins from infected cells to uninfected cell lysates stimulates translation of the late viral mRNA (see chapter 3). These experiments prompted us to suggest the presence of a viral-encoded or viral-induced factor responsible for this phenomenon. Alternative explanations have been proposed by other investigators to explain the almost exclusive synthesis of viral polypeptides late in infection. One of the proposed mechanism involves competition, between cellular and an excess of viral mRNA, for a limited pool of initiation factors (Walden, Godefroy-Colburn and Thach, 1981; Detjen, Walden and Thach, 1982; Ray et al., 1983). Their use of SC1 cells, that are less widely used for reovirus infection, does not facilitate comparison of their results with the situation occurring in L cells. Furthermore in SC1 cells, as well as HeLa cells, the inhibition of protein synthesis observed by these investigators is much lower than in L cells, therefore complicating further the comparison between the different systems. Competition probably also occurs in early-infected L cells, but experiments in lysates from late-infected L cells revealed that late viral mRNA does not directly compete with cellular mRNA (Lemieux, Zarbl and Millward, 1984), suggesting once again the presence of a factor responsible for the increased translatability of late viral mRNA in these lysates. Furthermore a competition model does not easily explain, in the absence of competing mRNAs, the specific

stimulation observed upon addition of proteins from infected cells to uninfected cell lysates.

Another model was recently proposed involving a viral-induced modification of membrane permeability to explain the change in relative efficiency of viral and cellular mRNA translation (Munoz, Alonso and Carrasco, 1985). The same investigators have previously proposed a similar model for poliovirus infection and also suggested that their model can apply to most viral infections (Carrasco, 1977). Their model is not generally accepted for poliovirus infection but may be valid for other picornavirus infection (see chapter 1). In their investigations of reovirus, their experiments suffer from two serious drawbacks. Most of their experiments used HeLa cells in which the growth characteristics of reovirus are not well known; furthermore, their conclusions are mostly based on experiments using super-infection (double infection) with different viruses that also affect the translational machinery. These experiments are obviously very difficult to interpret. Furthermore the mechanism that they proposed should affect different mRNAs to different extents but we observed that only the reovirus late mRNA is more efficiently translated in lysates from infected cells; all the segments of reovirus mRNA are also similarly affected. In addition, their hypothesis fails to explain the specificity of the stimulation of late viral mRNA translation, without a change in the salt concentration, observed by addition of dialyzed proteins from infected cells to uninfected cell lysates. Clearly this hypothesis cannot explain the translational controls that exist in infected L cells but we cannot, with the data presently available, completely exclude a secondary role of such a

mechanism, especially in cell types other than L cells.

Most of this thesis concerns the unambiguous identification of the factor responsible for the stimulation of late viral mRNA translation. This factor was previously called "discriminatory initiation factor" based on the results of *in vitro* competition experiments (Lemieux, Zarbl and Millward, 1984). It was subsequently determined that this factor is the viral-encoded sigma 3 protein; the different lines of evidence used for the identification have been already discussed in chapters 3 and 5. Also mentioned is the conclusion that there is no evidence that the protein has a direct negative effect on the host cell. However, it is still possible that *in vivo*, in a situation of competition among mRNAs, the protein might have a certain inhibitory effect. The situation in serotype 1 infection suggests however that the stimulatory effect of sigma 3 is not sufficient by itself to drastically inhibit host cell protein synthesis (see chapter 3). It is interesting to note that the sigma 3 protein has been implicated in the rapid inhibition of protein synthesis that occurs during reovirus serotype 2 infection (Sharpe and Fields, 1982). Serotype 2 is quite different from the other two serotypes (see chapter 1) and is less widely studied; the rapid inhibition observed with this virus is quite different from the slower host-specific inhibition observed with the serotype 3. It is not clear how these observations correlate with our own data; however it is certainly interesting that these other investigators, using a completely different approach, also discovered an involvement of sigma 3 in the translational controls of reovirus-infected cells.

For the past few years, viral proteins have been suspected of

playing positive or negative regulatory roles in translation in host cells (see chapter 1). In the case of the sigma 3 protein of reovirus, the situation is especially interesting. It is one of the few clear examples of positive effect on translation; furthermore, the sigma 3 protein is also a structural protein of the virus. The fact that a major capsid protein is used to control translation is an interesting case of optimal use of genetic information in a viral system with a limited amount of genetic material. There is also good evidence that in Semliki Forest virus a capsid protein is involved in negative translational control (van Steeg et al., 1984); capsid proteins with a role in translational control may be also present in other viral systems. The dual role of sigma 3, structural role and translational control, also provides a rationale for the fact that the S4 gene is expressed very early in infection (pre-early mRNA) even if sigma 3 is part of the outer capsid and thus needed for its structural role only late during virus morphogenesis. It also provides a role for the already noted excess of this protein relative to the amount needed for virus morphogenesis; this excess is easy to explain by the high transcriptional as well as translational efficiencies of this gene (see chapter 1).

The detailed mechanism of action of sigma 3 is still incompletely known. The initiation step of protein synthesis, and especially the entry of mRNA into initiation complexes, is the most plausible step to affect the rate of translation as already amply documented (reviewed in: Revel and Groner, 1978; Jagus, Anderson and Safer, 1981). Previous experiments in our laboratory have, in fact, indicated that the formation of initiation complexes with the late viral mRNA is

stimulated in infected cell lysates (Skup, Zarbl and Millward, 1981). In preliminary experiments, I have also observed the same effect upon addition of proteins from infected cells to uninfected cell lysates (data not shown). Interestingly the sigma 3 protein is enriched in the subcellular fraction containing the crude initiation factors (see chapter 3 and 5); a property expected from a protein playing the role of an initiation factor. Viral proteins that are known to affect the initiation of protein synthesis in other viral systems, Semliki Forest virus (van Steeg et al., 1984) and Frog virus 3 (Raghow and Granoff, 1983), are also known to be present in this fraction. It is quite clear that the sigma 3 protein does not bind directly to mRNA (see chapter 3 and 5) but rather interacts with the translational machinery of the host cell to modify its affinity toward late reovirus mRNA. An attractive hypothesis to explain this effect is that sigma 3 replaces or modifies a normal component of the translational machinery, most probably an initiation factor, recalling the situation of bacteriophage T4 proteins modifying the RNA polymerase of the host cell to its own advantage. Preliminary experiments, using affinity chromatography with purified sigma 3, indicated that some proteins, present among crude initiation factors, have an affinity for sigma 3 (data not shown); more work is however needed to confirm this binding and to identify the proteins involved. Such an approach might be eventually facilitated by the production of larger amount of the protein encoded by the cloned gene; we now know that this gene can encode an authentic functional protein product (see chapter 4 and 5).

It is tempting to speculate that the cap binding proteins are the target of the sigma 3 protein, the modification allowing them to

efficiently recognize the uncapped reovirus mRNA. An indication that the cap binding proteins may still be involved in translation of late (uncapped) viral mRNA is the sensitivity of late viral mRNA to cap analogs (Lemieux, Zarbl and Millward, 1984). This sensitivity partially explains that some authors, as already mentioned, claim that the late viral mRNA is capped (Detjen, Walden and Thach, 1982). An opposite situation is observed in Semliki Forest virus where capped mRNA is insensitive to cap analogs (van Steeg et al., 1981a). The sensitivity to cap analogs apparently indicates the involvement of cap binding proteins more than the actual structure of the mRNA. The proposed interaction between sigma 3 and cap binding proteins is only a speculation, but it will be interesting for future investigators to analyze in details the cap binding protein complex isolated from late-infected cells as well as from the cell line expressing sigma 3.

Eventually the sequence of the genes encoding different initiation factors will certainly be obtained in different laboratories; comparison with the already-known sequence of sigma 3 (Giantini et al., 1984) might also give interesting lead to the role of sigma 3.

More work will be needed to completely understand the mechanism of action of sigma 3 as well as the complex events occurring in reovirus-infected cells. The experiments described in this thesis have increased our understanding of this system and, together with previous work, constitute a good starting point. Cell lines and DNA clones have also been obtained that will be possibly helpful to future investigators. There is no doubt that future work will further elucidate the mechanisms of translational controls in reovirus-

infected cells. At the same time, such studies might well reveal interesting aspects of normal translational processes occurring in eucaryotic cells.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE*

1. It has been demonstrated that the initiation of protein synthesis is inhibited by free poly(A) in L cell lysates.
2. The translation of natural or polyadenylated reovirus mRNA is shown to be resistant to inhibition by poly(A).
3. The presence of sigma 3 viral protein is shown to account for efficient translation of late (uncapped) viral mRNA in infected cells.
4. Since the stimulation of late viral mRNA translation by sigma 3 protein occurs even in the absence of inhibition of host protein synthesis, the two phenomena are shown to be distinct.
5. Studies of the properties of sigma 3 show that it does not bind directly to mRNA, but rather interacts with the translational machinery itself, consistent with a role as a viral-specific initiation factor of protein synthesis.
6. The presence of different forms of sigma 3 having different isoelectric points is shown to be dispensable. Each form possesses all the different properties of sigma 3: a structural role in the capsid, affinity for double-stranded RNA, binding to the translational machinery.

7. Eucaryotic cell lines expressing the sigma 3 protein encoded by the cloned gene have been isolated.

8. The cloned gene has been shown to encode an authentic, functional sigma 3 protein. This is the first example of expression of a cloned reovirus gene in eucaryotic cells.

* This section is a mandatory requirement of the McGill faculty of graduate studies.