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MESSENGER RNA OF RAUSCHER LEUKEMIA VIRUS

IDENTIFICATION AND BIOLOGICAL ACTIVITY

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Promotores: Prof. Dr. H. Bloemendal en Dr. H.P.J. Bloemers

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Voor Miek,
Aljoscha en Anniek

The investigations described in this thesis were carried out at the Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands.

The majority of the experimental work reported in this thesis is covered by the following publications:

Gielkens *et al.*, 1972 (153); Gielkens *et al.*, 1974 (163); Gielkens *et al.*, 1976 (252).

CONTENTS

CHAPTER 1

MOLECULAR BIOLOGY OF RNA TUMOR VIRUSES

1.1. INTRODUCTION

One of the fundamental problems of cancer research is to discover how and why a normal cell becomes a cancer cell. At the present time, overwhelming evidence is accumulating that certain viruses are able to induce the neoplastic transformation of cells. The genome of these tumor inducing viruses consists of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The mechanism of neoplastic transformation of cells after infection with DNA or RNA tumor viruses is still largely unknown.

The Rauscher murine leukemia virus belongs to the group of RNA tumor viruses and causes an erythroid leukemia in mice. The aim of our studies was to investigate the synthesis of virus-specific proteins in cells infected with the Rauscher leukemia virus. It is obvious, that detailed studies on this step in the replication cycle, contribute to our knowledge on the expression of viral information in cells infected with RNA tumor viruses.

We were able to identify the virus-specific mRNA species associated with polyribosomes isolated from cells infected with Rauscher murine leukemia virus. In addition, definite proof for their biological function as messenger RNA was provided by experiments showing the cell-free synthesis of virus-specific polypeptides under the direction of isolated virus-specific messenger RNA fractions.

1.2. RNA TUMOR VIRUSES

As an introduction to the experimental work presented in this thesis, we will provide a comprehensive review on some aspects of the biochemistry and the replication mechanism of RNA tumor viruses. For more detailed studies the reader is referred to some excellent reviews on various aspects of RNA tumor virology, namely on the history (1,2), its morphology and biochemistry (2-9), its biology (10), the interaction between virus and host (2,10,11), the DNA provirus hypothesis (12) and the origin and evolution of RNA tumor viruses (13-16).

All RNA tumor virus particles have common structural features: the virions have a roughly spherical shape with a diameter of 60 to 150 nm; they

consist of a spherical nucleoid complex covered by an outer envelope made of a unit membrane (17,18). According to the fine structural organization of mature virus particles, RNA tumor viruses are classified in two distinct groups: (1) B-type particles of mouse mammary tumor virus with an eccentric nucleoid enclosed within a unit membrane envelope, containing large surface spikes, and (2) C-type particles of the leukosis-sarcoma viruses with a centrally located spherical nucleoid, enclosed within a unit membrane envelope containing knob like structures (17,18). The viral envelope is acquired during the budding of the virions from the cell surface (1).

The genome is a single stranded RNA molecule of 60-70S, which is dissociated at elevated temperatures or in the presence of dimethylsulfoxide into subunits of approximately $3x10^6$ molecular weight (19-22). The replication of the RNA genome involves a DNA intermediate (DNA provirus) (12) and the virions themselves contain a 'DNA polymerase' system (reverse transcriptase) capable of transcribing the viral genome into small pieces of DNA (23,24).

Virus particles with the biochemical features (i.e. virions containing 60-70S RNA and a 'DNA polymerase' system) and morphological properties of RNA tumor viruses have been isolated from a wide variety of species including reptiles, birds, mice, rats, hamsters, cats, pigs, cows, sheep, monkeys, apes and also from man. Some of these viruses produce tumors, particularly leukemias, lymphomas, sarcomas or mammary carcinomas, whereas the oncogenic potential of many other isolates has still not been established.

Several lines of evidence suggest that the chromosomal DNA of cells of most or all avian and mammalian species contains multiple copies of nucleic acid sequences that can code for the production of RNA tumor virus particles. The early findings of Gross (25), suggesting the transmission of murine leukemia virus (MuLV) by germ cells, have now been confirmed and extended to other type-C viruses by numerous groups of investigators. All strains of mice have the capacity to produce MuLV or certain viral antigens (26-29) and genetic studies show that the capacity to produce MuLV, seggregates according to Mendelian laws (28,30-33). By hybridization experiments, sequences complementary to type C-type viral RNA were detected in the cellular DNA of all normal avian and mammalian cells tested (34-40). Virus-free cell lines from chickens and different mammalian species can begin to release C-type viruses (endogenous viruses) either spontaneously or after treatment with chemical inducers (41-47). Together, these findings are strong arguments for

the genetic transmission of C-type RNA viruses within the high molecular weight DNA of normal cells of mammals and birds.

Inbred mouse strains have been shown to contain three biological distinguishable classes of inducible endogenous viruses (48-51). At least one class of these endogenous C-type viruses has been shown to be oncogenic in mice (52,53). It is, however, possible that other biological distinct endogenous viruses are not tumorigenic for their natural host. An obvious feature of the endogenous viruses is the close relation, as measured by hybridization, to the DNA of the progenitor cells (16). The RNA genome of exogenous viruses also hybridizes, although to a limited extent, with the DNA genome of uninfected host cells indicating that the RNA genome of the latter group of type-C viruses is more distantly related to the host DNA genome (16). Thus, speculation on the origin of the genome of exogenous RNA tumor viruses leaves two possibilities: Either an independent piece of genetic information acquired a virus-host relationship with a vertebrate at some stage of the evolution and picked up certain host sequences in the process, or a host sequence was released at some time from the vertebrate genome and has developed the semi-autonomous virus-host relation of present-day RNA tumor viruses (16).

The presence of genetic information for type-C viruses in cellular genes of numerous species suggests that these 'virogenes' are related to each other in the same way as their natural hosts are related, based upon geological or anatomical criteria. Evidence in support of this suggestion has been provided by Benveniste and Todaro (54), showing that sequences related to those of endogenous baboon type-C virus were present in the cellular DNA from tissues of other Old World monkeys, of apes and man. Besides, at some point during the evolution endogenous type-C viruses have also been transferred between species that are only distantly related, such as mouse and pig or primate and cat (37,55). This was established by hybridization experiments, showing for example that type-C viral sequences present in pig cellular DNA are partially homologous to those found in the mouse cellular DNA (37).

Since oncogenic type-C viruses [simian sarcoma virus (Si-SV) (56) and gibbon ape leukemia virus (Ga-LV) (57)] have been isolated from naturally occurring tumors of subhuman primates, it may be suspected that similar viruses are involved in the etiology of tumors in man. Electron microscopic investigations, however, of biopsies of human tumors failed to provide

convincing evidence for the existence of human type-C viruses. Other, more succesfully, attempts have been made to identify human type-C viruses by biochemical methods. Several groups were able to demonstrate that neoplastic cells, obtained from human leukemias, lymphomas, sarcomas and breast or skin cancers contained viral-related RNA and particles containing a 'DNA polymerase' system complexed to high molecular weight RNA (58-65). The 'DNA polymerase' detected in human leukemic cells was shown to be immunologically related to the 'DNA polymerase' from oncogenic RNA viruses from subhuman primates (66,67). An even more interesting finding is the presence of proteins specifically related to structural antigens of type-C viruses from subhuman primates and a endogenous cat virus in normal human tissues and in neoplasias of human origin (68,69). Recently, several investigators have succeeded in isolating type-C viruses from human tumor cells grown *in vitro* (70-73). Initial results indicate that these isolates are related to oncogenic type-C viruses of subhuman primates (Ga-LV and Si-SV) (70-73).

These findings support the proposition that RNA tumor virus related components exist in human neoplastic cells. However, the role of these particles in human oncogenesis has still to be resolved.

1.3. MURINE LEUKEMIA AND SARCOMA VIRUSES

The type-C viruses can be classified into two groups: (1) the nontransforming leukemia or helper viruses which productively infect fibroblast cell cultures and (2) the transforming or sarcoma-producing viruses which produce a morphological transformation of fibroblast cultures.

Gross (74) demonstrated in 1951 that cell-free filtrates from leukemic mouse tissue could induce leukemia and lymphosarcomas after inoculation into newborn mice of inbred strains with a low incidence of spontaneous leukemia. The isolation of the Gross leukemia virus was followed within a few years by other murine leukemia virus strains: the Graffi strain, a myeloid leukemia virus; the Friend and Rauscher leukemia viruses, inducing a erythroblastosis-like disease and the Moloney leukemia virus, inducing lymphatic leukemia (74-78).

Murine sarcoma viruses have been isolated from mice or rats infected with extracts of either mice or rats with a virus-induced leukemia. The first murine sarcoma virus was isolated by Harvey (79). After prolonged passage of the Moloney strain of murine leukemia virus in rats, a virus isolate was obtained

from leukemic rats, that induced pleomorphic sarcomas and erythroblastosis when inoculated into newborn mice. Subsequently, Moloney (80) reported the isolation of a murine sarcoma virus (Moloney strain) from a rhabdomyosarcoma, which developed in newborn mice after inoculation with high doses of the Moloney strain of murine leukemia virus. The Kirsten strain of murine sarcoma virus was isolated by passage of the Kirsten murine erythroblastosis virus in rats (81). The isolation of murine sarcoma viruses from extracts of naturally occurring sarcomas has also been reported (82,83).

Infectious murine sarcoma virus isolates are mixtures of two viruses, the original leukemia virus and the transforming sarcoma virus. Attempts to separate the sarcoma inducing capacity from the ability to induce leukemias were unsuccessful, leading to the recognition that the mouse sarcoma viruses are defective for replication. They can only replicate in the presence of a helper leukemia virus, to yield MSV-(MLV) pseudotypes (84,85). However, it has been possible to infect fibroblast cells with sarcoma virus particles containing the genetic information for the production of cellular transformation but not for complete viral replication (85,86). These cells, which are isolated at terminal dilutions of sarcoma virus stocks, do not produce infectious sarcoma virus particles. When the MSV transformed nonproducer cells are superinfected with a helper virus, the cells release a mixture of transforming and nontransforming virus particles (85,86).

1.4. THE NUCLEIC ACID COMPONENTS OF RNA TUMOR VIRUSES

Most biochemical studies on the nucleic acid components of RNA tumor viruses are concerned with the RNA genome of avian type-C viruses. Since the genome of the avian viruses shares many structural properties with that of the murine RNA tumor viruses, we decided to discuss the nucleic acid components of both virus groups.

1.4.1. *The fast sedimenting RNA component*

The purified RNA of RNA tumor viruses consists of a class of high molecular weight RNA with a sedimentation coefficient of 60-70S (corresponding to a molecular weight of 10×10^6) and a second class of RNA with a sedimentation value of 4-7S (19,20,88-90). RNA preparations of purified avian

myeloblastosis virus contain also minor amounts of 18S and 28S RNA (91-93). Since the presence of ribosomes within virions has been reported, these are most likely the source of the 18S and 28S RNA species (94).

When the 60-70S RNA component is denatured by heat or dimethylsulfoxide, the sedimentation coefficient decreases to 30-40S (3 x 10^6 M_r) (21,22,95-97). Therefore, it was suggested that the 60-70S RNA was segmented and contained 3-4 30-40S RNA subunits linked together by hydrogen bound regions (21). The alternative possibility, decrease of the sedimentation value of denatured 60-70S RNA due to an extreme conformational change, could not be excluded (97). However, evidence in favor of the postulated subunit structure of the 60-70S RNA was recently provided by several groups of investigators. They employed length measurements of the denatured RNA in the electron microscope, a method independent of the hydrodynamic properties of the RNA. The results indicate that the 60-70S RNA consists of at least two subunits of 30-40S RNA with a molecular weight of about 3 x 10^6 (98-102). The 60-70S RNA is less suitable for length measurements, because of its extensive secondary structure. However, by utilizing the gene-32 procedure of Delius (103) for spreading of RNA molecules, length measurements on the 60-70S RNA became possible (98). By this method the 60-70S RNA is mildly denatured and extended. The RNA-gene-32 protein complexes exhibited a network structure, consisting of several linear molecules held together at many different points. An average length was estimated corresponding with a molecular weight of 6 x 10^6 (98). This molecular weight value is about 3 x 10^6 lower than the molecular weight based on sedimentation velocity studies. The difference may be due to partial dissociation of the 60-70S RNA under the conditions chosen for incubation of the viral RNA and the bacteriophage T4 gene-32 protein.

Two interesting questions, why the two to four 30-40S RNA subunits are linked together in a 60-70S RNA complex and how these subunits are linked together, still require an answer. Concerning the latter problem indirect evidence suggests that several low molecular weight (4-7S) RNA molecules may be involved (see below). The assembly process of the subunit RNA molecules in a 60-70S RNA aggregate probably starts during the budding process at the plasma membrane and is completed within 30 min after release of the virions (104,105). Cheung *et al* (104) found that the RNA of Rous sarcoma virus particles, harvested at 5 min intervals sedimented in a rather broad peak at 55-60S, with a shoulder at 30-40S, whereas virus harvested at hourly

intervals contained 60-70S RNA. The presence of 30-40S RNA in immature (rapid harvest) virus was confirmed by Canaani *et al* (105). They detected predominantly 30-40S RNA in virus particles collected within 3 min intervals. Moreover, when freshly harvested virus was incubated *in vitro* at 40° for 5 to 15 min, most of the 30-40S RNA was converted to 60-70S RNA. Therefore, it seems likely that the 30-40S RNA subunits are precursors of the 60-70S RNA.

1.4.1.1. *Presence of poly(A) in the viral RNA*

Most messenger RNAs from eukaryotic cells contain poly(A)-rich sequences of various lengths (60-200 nucleotides) at their 3' ends (106-110). The precise role of poly(A) in the transport of mRNA from nucleus to cytoplasm and the translation of mRNA is still unknown. Poly(A) stretches of about 200 nucleotides long (60,000 daltons) have also been found in the 60-70S RNA of RNA tumor viruses (111-114). The number of these poly(A) stretches in the high molecular weight RNA has been calculated from the poly(A) content of the 60-70S RNA. Assuming that the 60-70S RNA consists of 3-4 30-40S RNA subunits, it followed that there is about one large poly(A) track for each viral 30-40S RNA subunit (111,112,115).Determination, however, of the binding capacity of native and denatured viral RNA to oligo(dT)-cellulose or poly(U)-sepharose revealed that 90% of the 60-70S RNA but only 65% of the 30-40S RNA was bound to the columns at high ionic strength (116,117). This indicates that about one out of three 30-40S RNA subunits contains no or very short segments of poly(A). The presence of large poly(A) tracks within the 30-40S RNA chains seems unlikely, since the hydrodynamic and electrophoretic properties of the 30-40S RNA were not affected by digestion of poly(A) sequences with RNAase H in the presence of poly(dT) (117). The experimental approach, however, leaves the possiblity that internal oligo(A) sequences are present, which resist digestion with RNAase H at low ionic strength and 38°. Indirect experimental evidence indeed suggests that A-rich sequences other than the poly(A) of 200 nucleotides are present within the 30-40S RNA (117). The presence of a few internal oligo(A) sequences, too short to be detected with oligo(dT)-cellulose chromatography, is the most likely explanation for the apparent discrepancy in the number of poly(A) tracks per 30-40S RNA as mentioned above.

Like the poly(A) of eukaryotic messengers and other viral RNAs, the

poly(A) of tumor virus RNA is located at the 3' end of this molecule (115, 117-119). It has been shown by end group analysis, that the viral poly(A) consists of a homogeneous segment containing one adenosine per 180 to 190 AMP residues (115,118). Identification of the nucleotides adjacent to the poly(A) track indicates that most of the 30-40S RNA chains have a common 3'-terminal nucleotide sequence $-G(C,U?)A_{190}A_{OH}$ (115,118). Interesting is that pyrimidine residues also precede the poly(A) track of several mammalian messengers (106,120).

More recently the 5'-terminal structure of many mammalian mRNAs and viral RNAs has been studied (121-128). The 30-40S RNA of RNA tumor viruses (129,130) like mammalian messengers and other viral RNAs, contains a blocked methylated 5'-terminal sequence of the type $m^7G(5')ppp(5')X^n$ (cap structure). The viral RNA subunits contain in addition to the cap structure about ten methylated adenosine nucleotides (m^6A) at internal positions, which is also a characteristic structural feature of mRNA from eukaryotic cells (127,130). The presence in messenger RNAs of methylated nucleotides and in particular a blocked methylated 5'-terminal structure seems to be required for efficient translation *in vitro* and presumably also *in vivo* (123,128).

1.4.1.2. *Genome of transformation defective avian RNA tumor viruses*

Gel electrophoresis of the 30-40S RNA subunits from uncloned avian sarcoma viruses revealed that this RNA could be separated into two classes of RNA species, called *a* and *b* subunits (131). The 30-40S RNA of the size class *a* is 10-15% larger than the corresponding molecules of size class *b* (132). The 30-40S RNA isolated from transformation defective (td) viruses and all avian leukosis viruses consists only of *b* subunits (131,133). Whereas both *a* and *b* subunits were detected in uncloned isolates of avian sarcoma viruses, only RNA molecules of size class *a* were found in cloned avian sarcoma virus (134). The presence of *a* and *b* subunits in uncloned stocks of avian sarcoma viruses has been found to originate from nontransforming variants, which arise spontaneously during replication of avian sarcoma viruses (134). This spontaneous loss of transforming ability correlates with the loss of the larger *a* subunits (133,134). Consequently,Duesberg *et al* (134) suggested that the *a* subunit of avian sarcoma viruses contains the genetic information necessary for transformation of chicken fibroblast cells, whereas the

b subunit of transformation defective viruses lacks this information. In a recent study Wang *et al* (351), reported the location of sarcoma-specific oligonucleotides on the RNA of three nondefective (nd) avian sarcoma viruses. The sarcoma-specific oligonucleotides were identified by comparison with the RNA of corresponding td viruses. Based on the map position it was estimated that the nucleotide sequences concerned with transformation were located between 6.6 and 20% from the poly(A) end of the viral RNA. It follows that the sarcoma-specific nucleotide sequence comprises 300,000 to 450,000 daltons. This calculation is in accordance with the size difference between the *a* and *b* subunits. In addition they detected a heteropolymeric sequence of 140,000 molecular weight, which starts at the poly(A) end and is partially or completely shared by transforming and transformation defective viruses.

Competitive hybridization studies of the sequence relationship between RNA of avian sarcoma, transformation defective and avian leukosis viruses indicate that these viruses have the majority of nucleotide sequences in common (135,136). All sequences of the nontransforming viruses were found in RNA of avian sarcoma virus, whereas only 70-90% of sarcoma virus sequences were represented in the RNA of transformation defective and leukosis viruses.

1.4.1.3. *RNA of murine sarcoma viruses*

As already mentioned, most murine sarcoma viruses were isolated after prolonged animal to animal passage of several stocks of murine leukemia viruses. Moloney sarcoma virus (Mo-SV) arose on mouse passage of Moloney leukemia virus (Mo-MuLV), whereas the Harvey (Ha-SV) and Kirsten (Ki-SV) sarcoma viruses arose on rat passage of Mo-MuLV and Ki-MuLV, respectively. These murine sarcoma viruses are replication defective and synthesis of infectious progeny virus is dependent upon the presence of an associated helper leukemia virus.

Based on hybridization experiments it could be established that: (1) Ki-SV and Ha-SV contain distinct sets of nucleic acid sequences, one set is contained in the corresponding murine helper virus (Ki-MuLV and Mo-MuLV), a second set in rat type-C helper virus and a set of extra sequences, which are not found in mouse or rat type-C viruses; (2) Mo-SV contains sequences of the homologous leukemia virus of mouse origin (Mo-MuLV) and large sequences not represented in the Mo-MuLV RNA (137-139). These hybridization data suggest that Ki-SV and Ha-SV arose by a recombination event between Ki-MuLV and Mo-MuLV,

respectively and genetic information in rat cells, during passage of these viruses in rats (138).

As in the avian system a size difference has been observed between non-defective and defective viruses. Maisel *et al* (140) have shown that the size of the RNA of the replication defective murine sarcoma viruses (2.3 x 10^6 M_r) is approximately 2 x 10^5 daltons smaller than of their respective helper leukemia viruses (2.5 x 10^6 M_r). These results have been confirmed by Tsuchida *et al* (141,142), showing the presence of 30S RNA (2.3 x 10^6 M_r) in virus isolates containing an excess of Mo-SV over Mo-MuLV.

The [^{3}H]-DNA product of the Ki-SV(Ki-MuLV) RNA-dependent DNA polymerase, can be used as probe to study the virus-specific RNA species in cells infected with both Ki-SV and Ki-MuLV. However, by eliminating from this DNA probe all sequences related to the mouse helper virus (Ki-MuLV), a probe of sarcoma-specific (Ki-SV) DNA sequences is obtained (143). With such transcripts Tsuchida *et al* (143) found a complementary 30S RNA species only in mouse cells nonproductively transformed by the Ki-SV, whereas no RNA was found related to the helper MuLV. After 5'-bromodeoxyuridine treatment these cells were triggered to release both sarcoma and helper type-C viruses [Ki-SV(Ki-MuLV)]. With the MuLV-specific DNA probe a major 35S RNA component and a minor 30S RNA component were detected in the induced cells. The Ki-SV-specific DNA probe hybridized with the 30S RNA only.

We already mentioned that Ki-SV has specific sequences in common with rat type-C virus. These sequences have presumably been acquired during the rat to rat passage of the original Ki-MuLV. All experimental evidence suggests that these common sequences reside in the 30S RNA. RNA molecules of this size were detected, utilizing the Ki-SV-specific DNA probe not only in spontaneously rat type-C virus producing rat cells, but more surprisingly also in normal 'virus free' rat cells (144). Thus, strongly suggesting that the murine sarcoma virus-specific 30S RNA arises by a stable recombination between the original leukemia virus and genetic information of rat or mouse.

1.4.1.4. *Sequence complexity of the 60-70S RNA*

Since the 60-70S RNA genome of RNA tumor viruses consists of 2-4 subunits of 30-40S RNA it is important to establish whether these subunits are identical (polyploid genome) or different (haploid genome) with regard to their

nucleotide sequence. A sequence complexity of about 9 x 10^6 M_r was found by hybridization of excess 60-70S RNA to the complementary DNA probe (145,146) and thus argues for a haploid genome. However, fingerprint analysis of unique oligonucleotides obtained after nuclease digestion of 60-70S RNA, yields a unique sequence molecular weight of 3 x 10^6 (147-150). A molecular weight of 3 x 10^6 is also consistent with the results of studies on defective avian type-C viruses with deletions in genes coding for the transformation product(s) and the envelope glycoprotein (151). Most data, therefore, suggest that the nucleotide sequences of the 30-40S RNA are repeated two to four times and appear to rule out the possibility that each RNA subunit is completely different. The reason for the discrepancy between the kinetic and physical complexity analysis is still unknown.

1.4.1.5. *Evidence for the messenger properties of the genome of RNA tumor viruses*

Like most eukaryotic messenger RNAs the genome of RNA tumor viruses contains 3'-terminal tracts of poly(A) and a blocked methylated 5'-terminus. These properties already suggest that the viral RNA genome functions as messenger RNA. This notion is supported by several lines of experimental evidence: (1) The 60-70S RNA and the 30-40S RNA subunits of avian and murine RNA tumor viruses direct the synthesis of virus-specific polypeptides of low molecular weight in an *E.coli* cell-free system (152-154, this thesis); (2) More recently, native and denatured 60-70S RNA have been shown to serve as messenger in eukaryotic cell-free systems (155-158) and in *Xenopus* oocytes (159); (3) Virus-specific RNA sequences were detected in polyribosomes of virus producing cells, by hybridization with labeled DNA complementary to the viral genome. One of the virus-specific RNA species from polyribosomes corresponds in size to the 30-40S subunit RNA (160-163, this thesis) and contains poly(A) (this thesis); (4) Both the 30-40S viral RNA subunits of Rauscher murine leukemia virus (R-MuLV) and the 30-40S mRNA of R-MuLV producing cells, direct the synthesis of a similar set of virus-specific polypeptides in mammalian cell-free systems (157, this thesis).

1.4.2. *The low molecular weight viral RNA*

RNA tumor viruses contain in addition to the 60-70S RNA complex 30 to 100 molecules of low molecular weight 4-7S RNA. This latter class consists of distinct RNA species with sedimentation values of 4S, 5S and 7S (21,91, 93,95,352-354).

1.4.2.1. *4S RNA*

The virion 4S RNAs exhibit the structural and functional properties of cellular tRNA. The viral 4S RNA fraction contains RNA species with amino acceptor activity (91,164,352) and fingerprints of the viral 4S RNA indicate the presence of modified pyrimidines and methylated bases characteristic of cellular tRNA (165,166,354). This obvious similarity with host tRNA strongly suggests that the viral tRNAs are host derived and become incorporated within virions during the budding process at the cell membrane. It is, therefore, striking that the viral tRNAs do not represent a random assortment of cellular tRNA species and that different RNA tumor viruses probably contain the various tRNAs in different proportions (167,168,352). In contrast, there exists a clear consistency in the relative amounts of tRNA species associated with virions from different isolates of one particular strain (164,169,170,352). In addition, the 4S RNA fraction of Rous sarcoma virus and Rous associated virus, both grown in chick embryo fibroblasts, have even been reported to contain different populations of tRNA (168). One of the most prevalent tRNA species in virions is methionyl tRNA ($tRNA^{Met}IV$) (168,170-172).

Apart from the class of 4S RNA which occurs free within the virions, avian and probably also murine RNA tumor viruses contain 4S RNA associated with the high molecular weight viral RNA. Most of this 4S RNA is released from the 60-70S RNA by thermal denaturation and this dissociation occurs over a rather broad range of temperatures (165,166,173,354). As the free 4S RNA class, the 60-70S associated 4S RNA exhibits the structural and functional features characteristic of transfer RNA (166,174,354). The 4S RNA constitutes 3-4% of the 60-70S RNA complex or the equivalent of 12 to 20 molecules per 60-70S RNA (175,354).

The transcription of the 60-70S genome of RNA tumor viruses by the endogenous 'DNA polymerase' is a primer dependent reaction (176). The primer

is a low molecular weight RNA molecule, hydrogen bonded to the 60-70S RNA, and provides a free 3'-OH terminus for the initiation of the DNA synthesis (176-183). By thermal denaturation of the viral RNA it could be shown that the primer remains attached to the template at a temperature (65^{o}), which completely dissociates the 60-70S RNA into its subunits (177,181). However, denaturation of the viral genome at higher temperature (80^{o}) results in loss of 80-90% of the template activity (177,180,181). Loss of template activity occurred over an identical temperature range as the release of one low molecular weight RNA molecule (181). This particular RNA species appears to be the principal primer for the DNA polymerase in avian RNA tumor viruses (181,182) and has been shown to be identical to tryptophan tRNA ($tRNA^{Trp}$) (175,182-187). $tRNA^{Trp}$ is the most abundant small RNA associated with the 60-70S RNA complex (175). A tRNA species with similar properties has also been found among the free low molecular weight viral RNA species, in normal chicken cells and in mammalian cells (182,183,185). Further support for the primer function of $tRNA^{Trp}$ has been provided by reassociation studies of either free, 60-70S associated or cellular tRNA and the dissociated 60-70S RNA, lacking template activity. These experiments show that of all tRNA species only $tRNA^{Trp}$ selectively reassociates with a limited number (2-4) of sites on the viral genome (186,187). After reannealing, the template activity for the reverse transcriptase is almost completely restored (186,187). The ability of $tRNA^{Trp}$ to act as a primer is further substantiated by the observation that of all tRNAs of normal chicken cells or avian myeloblastosis virus, only $tRNA^{Trp}$ and $tRNA^{Met}$IV are able to bind with high affinity to the reverse transcriptase, although the latter was bound less efficiently (188). The binding could be prevented after treatment of the enzyme with antibody against the reverse transcriptase, suggesting that highly specific site(s) on the enzyme are involved in the binding of $tRNA^{Trp}$ (188). Thus, at least one of the different tRNAs within virions of avian RNA tumor viruses, and presumably also of RNA tumor viruses of other species, appears to have a specific function in the replication cycle.

Another interesting tRNA selectively associated with the 60-70S RNA of avian RNA tumor viruses is $tRNA_f^{Met}$I, which can be formylated with *E.coli* transformylase (172). All four isoaccepting methionyl tRNAs are present, both free and associated with the 60-70S RNA complex. However, especially $tRNA_f^{Met}$ was found in association with the high molecular weight viral

RNA (172).

The reason for the association of tRNA species, other than $tRNA^{Trp}$, with the 60-70S virus genome, the significance of the inclusion of tRNA species within the virions as free molecules and whether they participate in any step of the life cycle of RNA tumor viruses still requires elucidation.

1.4.2.2. *5S and 7S RNA*

Two additional low molecular weight classes of RNA, 5S and 7S are found free and associated with the 60-70S RNA of RNA tumor viruses (173,175,353,354). Both the free and the 60-70S associated 5S RNA molecules are identical in base composition to 5S RNA isolated from the large ribosomal subunit of eukaryotic cells (354). The number of 5S RNA molecules associated with the 60-70S RNA has been estimated to be 3 to 4 (354). These molecules are released from the 60-70S RNA complex at a Tm value identical to that needed for dissociation of the high molecular weight RNA, which is significantly higher than that for most 4S RNA species (189,354). Therefore, it is not surprising that the 5S RNA can be reannealed to the high molecular weight subunits of the viral RNA (186). These observations suggest that 5S RNA may function as linker of the RNA subunits to form the 60-70S RNA complex.

Likewise, a small number of 7S RNA molecules (5-10) is present within the virions (353). Fingerprints of nuclease digested 7S RNA from avian RNA tumor viruses and 7S RNA from uninfected chick fibroblast cells were identical, suggesting that the viral 7S RNA is also derived from the host (190). The T_m value for release of 7S RNA from the 60-70S RNA of murine RNA tumor viruses is almost identical to that of 5S RNA (189). Consequently, the 7S RNA species is also candidate for the function of linker of the subunits of the 60-70S viral RNA.

1.5. PROTEINS OF MURINE LEUKEMIA VIRUSES

The structural proteins of murine leukemia viruses (MuLV) have been studied by various techniques including polyacrylamide gel electrophoresis (191-198), gel filtration in 6M guanidine hydrochloride (GuHCl) (196,198, 199), isoelectric focusing (193,200) and chromatography on phosphocellulose (197). According to the proposed nomenclature (201) virion proteins are

designated according their molecular weight in thousands with the abbreviation 'p' for protein and 'gp' for glycoprotein. The molecular weights of the smaller proteins are designated by the results of GuHCl gel filtration, whereas the apparent molecular weight of the viral glycoprotein is estimated by sodium dodecyl sulfate electrophoresis.

Whereas the structure and properties of the viral RNAs of avian and mammalian viruses are so related that we have discussed them together, it should be noted that the proteins of avian and mammalian type-C viruses are immunologically different. Although certain avian proteins apparently correspond to certain mammalian counterparts as far as function or localization in the virion is concerned, we confine ourselves in this introduction to the mammalian type-C viruses with a special emphasis on the murine viruses.

Purified virions contain six major proteins, when analysed by gel filtration: gp69/71, gp45, p30, p15, p12 and p10. In addition to these major proteins a large number of minor protein bands can be seen when the purified virus preparations are analysed by polyacrylamide gel electrophoresis (197). Many of these minor bands may well be derived from the host cell and thus may not be coded for by the viral RNA.

Following treatment with detergents MuLV particles can be separated into the viral core particles and the viral membranes (Fig. 1). The major constituents of the viral core preparation are the low molecular weight proteins p30 and p10, whereas p15 is present in low and variable amounts (203). MuLV protein p12 is not found in core preparations and its location in the virion is still uncertain. The major glycoprotein gp69/71 is most probable the main structural component of the knob-like surface projections of the viral envelope (195,216). After further degradation of the viral core structure a ribonucleoprotein complex can be isolated containing p10 and trace amounts of p30 (203).

Based on immunological studies of mammalian RNA tumor viruses, the antigenic determinants of the virion proteins have been classified into three categories: (1) species-specific determinants, shared by polypeptides from different isolates of type-C viruses from one animal species; (2) type-specific determinants of polypeptides, by which strains of type-C viruses within a given species can be distinguished; (3) interspecies-specific determinants of polypeptides, which are common among type-C viruses of different animal species. It should be noted that one viral protein may contain

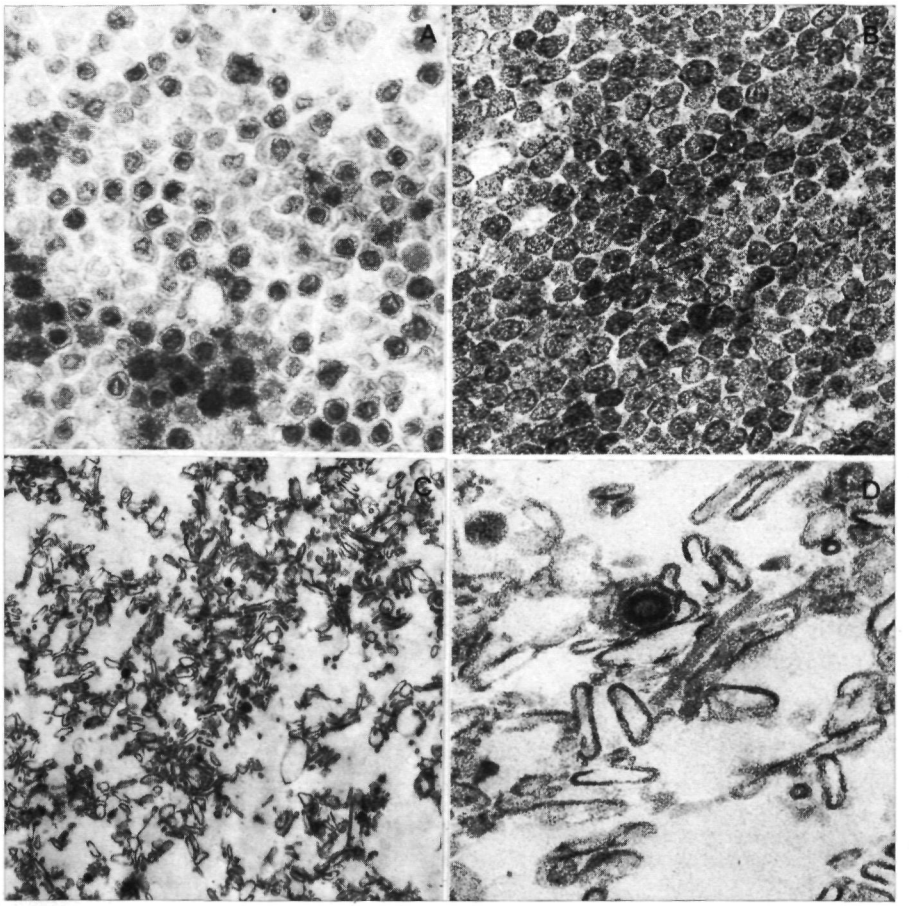


Fig. 1. Electron micrographs of Rauscher leukemia virus particles and the virus substructures. (A) Thin section of a virus pellet prepared from the plasma of Swiss mice infected with Rauscher leukemia virus (magnification 36,400 x.). Thin section of a pellet of virus particles treated with 0.002% sodium dodecyl sulfate and ether; (B) core fraction (magnification 69,600 x.); (C) viral envelope fraction (magnification 22,400 x.); (D) viral envelope fraction (magnification 107,300 x.). Thin sections were stained with uranyl acetate and lead citrate. Electron micrographs prepared by Drs W.J.M. van de Ven, Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands.

different antigenic determinants belonging to different categories. The various antigenic determinants are especially of interest to study the relatedness of mammalian type-C viruses in view of the origin and evolution of these viruses.

1.5.1. *Major structural protein p30*

The major structural protein p30 constitutes approximately 20-30% of the total proteins of MuLV (202). It is a neutral protein (193), which probably constitutes the core shell of the virus (203). The tryptic peptides of p30 from several strains of MuLV show a strong homology, with only 3 to 4 differences per peptide map (204). This strong homology is also reflected in the amino acid sequence of the first 24 residues (205). Only one amino acid difference (position 4) was found between the p30's from different mouse type-C viruses. In addition, mouse p30 contains specific amino acid sequences (highly conserved regions) homologous with p30 from type-C viruses of other mammalian species as cat, rat, apes and Old World monkeys (205). The close relation among p30's from different strains of MuLV and the presence of several highly conserved regions in the p30's of type-C viruses of genetically distant animals is further emphasized by the results from immunological studies. The majority of the antigenic determinants of the MuLV p30's is species-specific (206-208) and thus restricted to this group of viruses. In addition, several interspecies antigenic determinants are present (conserved regions), which are shared in different degree by type-C viruses from other mammalian species (196,197,206,208-211). A third class of antigenic determinants, comprises the type-specific regions in p30 (208,212). The type-specific determinants represent highly variable regions of amino acid sequences in p30, providing the base for differentiation between different strains of murine leukemia viruses.

1.5.2. *Major envelope glycoproteins*

Murine leukemia viruses contain one major glycoprotein component with an apparent molecular weight of 70,000 (192,199,213-215). By high resolution polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, this major glycoprotein could be resolved into two components with

approximate molecular weights of 69,000 and 71,000 (197). Further, several investigators reported the presence of a minor glycoprotein component with a molecular weight around 45,000-60,000 (192,199,213-215). At this time, it has not been examined whether the two larger (gp69/71) and the smaller (gp45-60) viral glycoproteins are related. Based on immunological data it seems likely that the 69,000 and 71,000 daltons proteins have an identical or highly related primary structure (202). The 45,000-60,000 M_r component is either derived from the larger glycoprotein gp69/71 or is a host-specific protein.

The viral glycoproteins have been shown to be the main constituents of the MuLV surface knobs, located on the external membrane (195,216). The efficient neutralization of Rauscher and Friend murine leukemia virus by monospecific antisera prepared against purified gp69/71 confirms the envelope localization of the glycoproteins (217). The antigenic determinants of the major glycoprotein gp69/71, are predominantly type-specific and species-specific determinants, and a small fraction was shown to be interspecies-specific (197,208,218). This indicates that the primary structure of the polypeptide moiety of the glycoprotein, is partially conserved among type-C viruses from different species.

1.5.3. *Protein p15*

The low molecular weight protein p15 is one of the minor structural components of murine leukemia viruses and is considered to be located in the viral core (203). Protein p15 has been found in all murine leukemia viruses studied, with the exception of the Kirsten strain (219). Serological studies have shown that p15 is immunologically distinct from the other viral proteins: gp69/71, p30, p12 and p10 (220,221). Most antigenic determinants in p15 of Rauscher and Moloney murine leukemia virus are type-specific (80-90%), whereas determinants shared with other murine type-C viruses (species-specific) and type-C viruses from other mammalian species are only minor components of the total antigenic specificities of this protein (219,221,222). In addition to these three categories of antigenic determinants, subclasses of species-specific determinants have been detected: one class common to two murine type-C viruses (Rauscher and Friend) and a second class common to three murine type-C viruses (Rauscher, Friend and Moloney) (219,222). Chemical

analysis by tryptic peptide mapping of the purified p15 proteins of Rauscher, Moloney and Gross murine leukemia virus, revealed large differences between the individual p15 proteins (223). This observation is consistent with the immunological finding that p15 is highly type-specific.

Recently, a new murine viral protein has been described, designated as p15(E), which is distinct from p15 as demonstrated by serological and biochemical methods (198). Based on the reactivity of natural antibody of mice to intact murine leukemia virions and the identification by polyacrylamide gel electrophoresis of p15(E) as one of the main proteins in the immunecomplex, this protein has been located in the viral envelope (E) (224).

1.5.4. *Protein p12*

The 12,000 molecular weight protein (p12) of murine leukemia viruses is an acidic protein, which probably contains some carbohydrate (8,196). Protein p12 is not found in viral core preparations and is consequently thought to represent a surface constituent of the virus (203). Antiserum directed against Friend murine leukemia virus p12 does not react with other virus proteins (196). In contrast, for the Kirsten strain as well as for the Moloney strain of murine leukemia virus a cross reaction between the proteins p10 and p12 has been reported (219). Studies on the antigenic properties of p12 indicate that this protein is mainly type-specific. Protein p12 contains also species-specific determinants but no interspecies-specific antigenic reactivity (196, 212,225). The p12 protein of Rauscher murine leukemia virus exhibits a strong cross reactivity with Friend murine leukemia virus but much less with Moloney murine leukemia virus; therefore, a third class of antigenic determinants is likely to be present (196,212,225). The analysis of the tryptic peptides of the individual p12 proteins of Rauscher, Moloney and Gross murine leukemia virus revealed almost no homology between the p12's of the three virus strains (223).

1.5.5. *Protein p10*

The smallest protein of murine leukemia viruses p10 is strongly basic and represents the main protein component of the ribonucleoprotein complex (203, 220). Examination of the antigenic determinants of p10 from Friend murine

leukemia virus has shown that this molecule is strongly species-specific in its serological reactivity (196). This immunological finding is consistent with the high degree of homology among the tryptic peptide maps of p10 from different murine leukemia virus strains (223). No interspecies reactivity could be demonstrated (196).

The serological and chemical analysis of the low molecular weight proteins of different murine leukemia virus strains show a striking homology among the individual p30 and p10 proteins and in contrast, lack of homology among the p15 and p12 proteins of these strains. The results suggest that the genes coding for p30 and p10 tolerate hardly mutations, whereas those coding for p15 and p12 are apparently more accesible to viable mutations. The role of the structural proteins in the replication cycle, the assembly and the structural composition of murine leukemia viruses is still largely unknown. The presence, however, of p30 in the viral core shell, the tight association of the basic protein p10 with the viral RNA and the low tolerance for mutations suggest that these proteins have a specific function in the core architecture.

1.6. THE PROTEINS OF RAUSCHER LEUKEMIA VIRUS AS STUDIED BY SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS: EVIDENCE FOR THE HETEROGENEITY OF SOME LOW MOLECULAR WEIGHT PROTEINS

In this paragraph we shall discuss some of our experiments on the proteins of the Rauscher leukemia virus (R-MuLV). Rat embryo fibroblast cells (REF) productively infected with R-MuLV were labeled with [^{35}S]methionine or [^{3}H]glucosamine and the radioactive virus was purified on density gradients. Fig. 2A shows the separation of the virion proteins on polyacrylamide gels run at neutral pH in the presence of sodium dodecyl sulfate. The gel pattern of [^{35}S]methionine labeled proteins shows that p30 and p15,p12 were the main virion proteins, whereas all other components represented minor constituents of the R-MuLV virion (compare Fig. 2B). One protein component with an apparent molecular weight of 70,000, which contained the highest amount of [^{3}H]glucosamine, corresponds in size with the major viral glycoprotein described by other investigators (192,199,213-215). In addition, two [^{35}S]methionine labeled proteins with approximate molecular weights of 50,000 and 90,000 contained minor amounts of sugar residues.

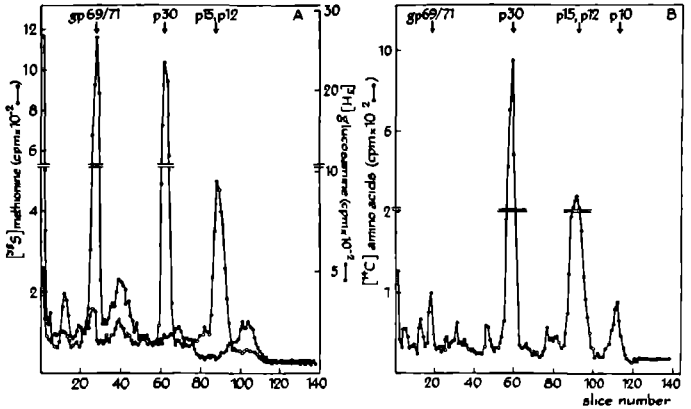


Fig. 2. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of Rauscher murine leukemia virus proteins. Rat embryo fibroblast cells (REF) productively infected with R-MuLV were labeled for 16 h with one of the following radioactive precursors, [^{14}C] amino acids, [^{3}H] glucosamine or [^{35}S] methionine. Virus was isolated from the cell supernatant fluids as described (88). Electrophoresis of the virion proteins was performed in 10% acrylamide gels in the presence of 0.1% sodium dodecyl sulfate, using the neutral buffer system as described by Weber and Osborn (258). Gels were run at a constant current of 8 mA per gel. After electrophoresis gels were fractionated by slicing and the amount of radioactivity was determined as described in section 2.2.5. (A) Proteins of R-MuLV labeled with [^{35}S] methionine or [^{3}H] glucosamine and separated on parallel gels; (B) proteins of R-MuLV labeled with [^{14}C] amino acids.

The [^{3}H] glucosamine label, which did not comigrate with [^{35}S] methionine labeled peaks may represent glycolipids or free carbohydrates. Comparison of the proteins of virus labeled with [^{14}C] amino acids (Fig. 2B) with those labeled with [^{35}S] methionine (Fig. 2A), revealed that almost no [^{35}S] methionine labeled protein was present in the 10,000 M_r region of the gel. Thus, indicating that p10 contained no or a very low number of methionine residues.

Since slight differences in electrophoretic mobility are not visualized when radioactive proteins are localized by slicing of gels, analysis of the separation of radioactive virion proteins was performed by contact exposure

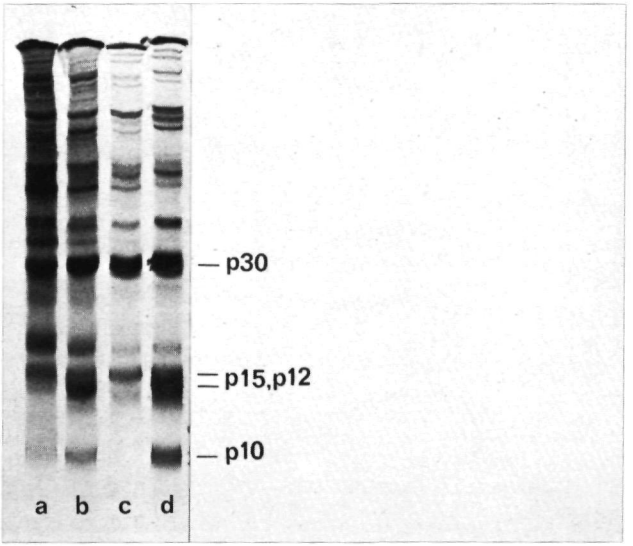


Fig. 3. Polyacrylamide gel electrophoresis of the proteins of Rauscher murine leukemia virus isolated from different cell lines. Rat embryo fibroblast cells (REF) or bone marrow cells from BALB/c mice (JLS-V9) productively infected with R-MuLV were labeled for 16 h with $[^{14}C]$ amino acids or $[^{35}S]$ methionine. Radioactive proteins of purified virus preparations were separated by electrophoresis in 10% acrylamide gels as described in the legend to Fig. 2. After electrophoresis longitudinal slices of the gels were dried and exposed to Kodak X-ray film as described in section 2.2.5. (a) Autoradiogram of the protein pattern of $[^{35}S]$ methionine labeled R-MuLV isolated from JLS-V9 cells; (b) $[^{14}C]$ amino acid labeled R-MuLV from JLS-V9 cells; (c) $[^{35}S]$ methionine labeled R-MuLV from REF cells; (d) $[^{14}C]$ amino acid labeled R-MuLV from REF cells.

of dried gels to X-ray film. Fig. 3 shows the autoradiographic analysis of protein patterns of R-MuLV isolated from different cell lines, namely a bone marrow cell line of BALB/c mice (JLS-V9) and a rat embryo fibroblast cell line (REF). Most virion proteins were present in virus isolates from both cell lines. This observation indicates that, although several proteins may be host derived, they represent characteristic components of R-MuLV particles. Striking is, that in addition to p10, also one of the components designated as p15,p12

was poorly labeled with radioactive methionine.

The resolution of both high and low molecular weight proteins in the same gel is possible when gels are constructed, consisting of linear gradients of increasing acrylamide concentration. In addition, resolution is further improved by using a discontinuous buffer system as described by Laemmli (259), instead of the phosphate buffer system used before. For this experiment virus was isolated from the plasma of leukemic mice and from the supernatant fluids of JLS-V9 or REF cells grown in the presence of radioactive precursors. The protein patterns of these virus preparations are shown in Fig. 4. The most obvious feature of this analysis is the high degree of similarity among the protein patterns of the three R-MuLV isolates. Protein bands, designated x and y, were always more prominent in virus preparations from JLS-V9 cells (Fig. 4b and d) than in those derived from REF cells or plasma of leukemic mice (Fig. 4a,c,e). One of these components, namely protein x, is presumably identical to the precursor protein of the low molecular weight structural components (Van Zaane, personal communication). In the low molecular weight region protein p10, which was previously considered to be a single protein component, appeared to be resolved in two or even three protein molecules with slightly different mobilities. This observation suggests that p10 consists of 2-3 protein molecules with different primary structure or, more likely, that these components were formed by proteolytic cleavage of p10 or inaccurate processing of the precursor protein containing p10. The most rapidly migrating components and a component, which is most likely identical to the viral structural protein p15 (based on gel filtration in the presence of 6M GuHCl), were poorly labeled with radioactive methionine.

1.7. ENZYMES WITHIN RNA TUMOR VIRUS PARTICLES

Purified preparations of RNA tumor viruses contain numerous enzymes, such as ATPase, nuclease, DNA ligase, phosphatases, RNA methylases and amino acyl synthetases (see for review Temin and Baltimore, 5). Since avian myeloblastosis virus core particles are able to synthesize virus-specific polypeptides *in vitro*, it is obvious that all enzymes needed for protein synthesis and even ribosomes are associated with these virions (226). These enzymes are most likely derived from the host cell. However, one virion enzyme, the viral 'DNA polymerase', is coded for by the viral RNA (227,228).

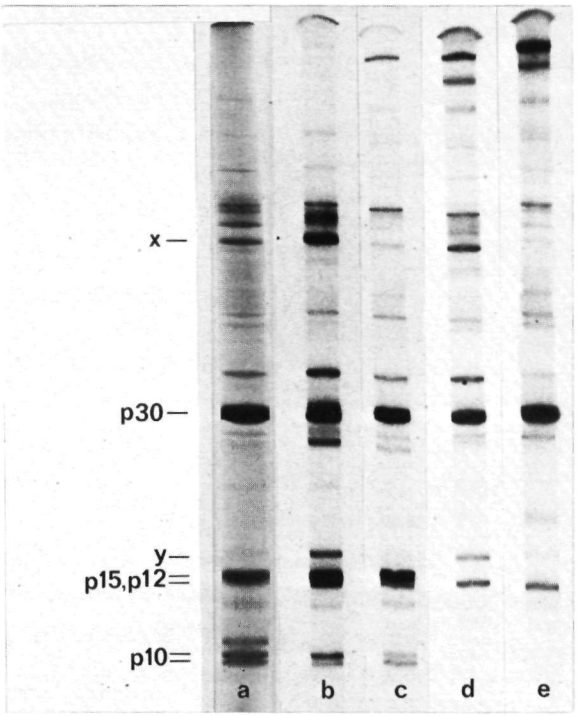


Fig. 4. Proteins of Rauscher murine leukemia virus separated in polyacrylamide gels, constructed with a linear 10.5-16% acrylamide gradient. Proteins of purified virus preparations were separated by polyacrylamide gel electrophoresis in the discontinuous buffer system according to Laemmli (259). Separating gels containing a 10.5-16% linear gradient of acrylamide were constructed as described in section 5.2.8. After electrophoresis, gels were stained with Coomassie Brilliant Blue (section 2.2.5.). Gels containing radioactive proteins were sliced longitudinally, dried and exposed to Kodak X-ray film. (a) Proteins of R-MuLV isolated from the plasma of leukemic mice (stained gel); (b) Autoradiographic analysis of the protein pattern of R-MuLV isolated from JLS-V9 cells grown in the presence of $[^{14}C]$amino acids; (c) $[^{14}C]$amino acid labeled R-MuLV from REF cells; (d) $[^{35}S]$methionine labeled R-MuLV from JLS-V9 cells; (e) $[^{35}S]$methionine labeled R-MuLV from REF cells.

The viral 'DNA polymerase', also called reverse transcriptase, is located in the viral core and utilizes the viral 60-70S RNA as a template to synthesize small DNA fragments [(-) strands], which are covalently bound to a tRNA primer

molecule (23,24,176,179). The purified enzyme also exhibits RNAase H activity, which specifically degrades the RNA moiety of RNA-DNA hybrids (236-238). Although, definitive experimental proof is lacking, it is assumed that RNAase H activity is required to displace the RNA template from the RNA-DNA hybrid, allowing synthesis of double stranded DNA. The next step in reverse transcription of the viral RNA is the synthesis of (+) stranded DNA. The viral 'DNA polymerase' utilizes the (-) DNA strands as template, yielding double stranded DNA molecules (229-231).

The 'DNA polymerase' of murine leukemia virus is a single polypeptide with a molecular weight of 70,000 to 80,000 (232-237). The immunological cross reactions observed between structural proteins of different mammalian type-C viruses is also characteristic for the 'DNA polymerase' of these viruses. Most prominent are those antigenic determinants shared by the individual enzymes of type-C viruses from one mammalian species (211,239,240). Besides, several distinct sets of interspecies antigenic determinants are present on the 'DNA polymerase' molecule of type-C viruses from different mammalian species (211, 239,240).

1.8. REPLICATION CYCLE OF RNA TUMOR VIRUSES

The knowledge on the replication cycle of RNA tumor viruses is largely derived from studies on the virus replication in sensitive cells grown in tissue culture.

(1) The initial step in the replication cycle of RNA tumor viruses is the attachment of virions to specific receptors on the cell membrane. After the virus has penetrated into the cytoplasm the virion is uncoated.

(2) The next event is the cytoplasmic synthesis by the viral 'DNA polymerase' of the provirus DNA (241-248). The first replicative intermediates detected within a few hours after infection are (-)stranded DNA transcripts of the viral RNA genome (244,247). Subsequently, the cytoplasmic synthesis of (+)stranded DNA molecules follows catalyzed by the same enzyme and using the (-)DNA strand as a template (224,247). Analysis of the newly synthesized DNA in the cytoplasm of infected cells, indicates the presence of linear and open circular duplex DNA molecules of the size of the subunits (or smaller) of the viral genome (242,244,246,248). These molecules are partially single stranded consisting of (-)DNA strands complexed with shorter segments of the

subsequently synthesized (+)DNA strands (246). After transport to the nucleus the viral DNA forms closed circular, supercoiled DNA molecules of a molecular weight of 5-6 x 10^6, which is about twice the molecular weight of the viral subunit RNA (244,245,248). The integration of the newly synthesized DNA into the cell genome has been demonstrated by Varmus (241). Whether integration occurs at specific sites of the host genome has still to be resolved.

(3) The next step is the synthesis of virus-specific RNA and proteins. The virus-specific RNA is presumably transcribed from the integrated provirus DNA by a host enzyme. This step is inhibited by α-amanitin (249,250 and Gielkens unpublished observations) an inhibitor of the cell nuclear form II DNA-dependent RNA polymerase (251), which is considered to be responsible for mRNA synthesis. The newly synthesized virus-specific RNA is processed and adenylated in the nucleus and transported to the cytoplasm. The major virus-specific RNA in the cytoplasm is identical in size to the virion 35S subunit RNA, but lower molecular weight RNA has also been found (160-163). The association of these virus-specific RNA species with polyribosomes indicates that they function as messenger RNA (160-163). Both free and membrane-bound polyribosomes are involved in the virus-specific protein synthesis (162,163,252). Although this virus-specific protein synthesis has not been studied in detail, convincing evidence exists that the low molecular weight structural proteins of RNA tumor viruses are made as a part of large precursor polypeptides, which are cleaved by proteolytic enzymes to yield the functional protein molecules (253-257).

Several virus-specific antigens appear on the cell surface as a result of the infection with RNA tumor viruses (see for review, 6). It has been shown that at least some of these cell-surface antigens, induced by murine leukemia viruses are coded for by the viral genome (202).

(4) The assembly process has not yet been studied in detail by biochemical methods. Electron microscopic studies have shown that the assembly of virion proteins and RNA into a nucleoid occurs at the plasma membrane, from which virions bud, thus acquiring their outer envelope.

CHAPTER 2

TRANSLATION OF THE HIGH MOLECULAR WEIGHT RNA OF RNA TUMOR VIRUSES AND EUKARYOTIC MESSENGER RNA IN THE *E.COLI* CELL-FREE SYSTEM

2.1. INTRODUCTION

In 1970, Baltimore (24) and Temin (23), reported that after treatment with nonionic detergents avian and murine RNA tumor viruses were able to incorporate deoxynucleoside triphosphates into DNA. The observations that this reaction was sensitive to RNAase and the synthesized DNA was complementary to the viral 60-70S RNA, led to the conclusion that the viral 'DNA polymerase' utilizes the 60-70S RNA as template (229,269).

Apart from the function of the 60-70S RNA as template for the synthesis of complementary DNA, it was not known, whether the viral RNA was also able to function directly as a messenger in a protein synthesizing system. For that reason we attempted to translate the viral RNA in cell-free systems from mammalian sources. However, under conditions in which globin and α-crystallin mRNAs are very actively translated in their encoded proteins, no evidence was obtained for the synthesis of polypeptides coded for by the viral RNA, when the cell-free products were analyzed by polyacrylamide gel electrophoresis. In order to avoid any misunderstanding at this point, it should be mentioned here that translation of the RNA of RNA tumor viruses in mammalian and other eukaryotic systems is detectable after immunoprecipitation of the synthesized products with antisera against purified virus as is described later in this thesis. The first indication that the RNA of RNA tumor viruses does function as messenger was provided by Siegert *et al* (152). These investigators have shown that the 60-70S RNA of avian myeloblastosis virus is able to direct the synthesis of virus-specific polypeptides in the bacterial cell-free system from *E.coli*. Consequently, we attempted to translate the high molecular weight 60-70S RNA and the 30-40S subunit RNA from Rauscher murine leukemia virus (R-MuLV), mouse mammary tumor virus (MTV) and avian myeloblastosis virus (AMV) in such a prokaryotic system.

In the present chapter evidence will be provided that in the cell-free system of *E.coli* the viral RNA genome of these RNA tumor viruses directs the synthesis of polypeptides, which are specific for each particular viral RNA.

Furthermore, it was found that small mammalian mRNAs as the globin 9S and α-crystallin 14S mRNA are not translated in the prokaryotic system in their encoded polypeptides under the conditions employed.

2.2. MATERIALS AND METHODS

2.2.1. *Cells and virus*

The JLS-V5 cell-line, derived from BALB/c mice spleen and thymus cells (261), infected with and producing Rauscher murine leukemia virus, was grown in monolayer in Eagle's Minimal Essential Medium completed with 10% calf serum, penicillin (100 I.U. x ml^{-1}) and streptomycin (50 μg x ml^{-1}). For the isolation of radioactive virus, cells in logarithmic phase were labeled for 16 h with 2.5 μCi x ml^{-1} of [^{14}C]amino acids (54 mCi x $matom^{-1}$) or 5 μCi x ml^{-1} of [^{35}S]methionine (32-41 Ci x $mmol^{-1}$). Virus was isolated by density gradient centrifugation as described by Duesberg *et al* (88).

2.2.2. *Bacteria and Phage M12 preparation*

Escherichia coli strain K12 AB301 (F^+, λ^+, met^-, his^-, *RNAase* I^-), was used for the cultivation of bacteriophage M12 as well as for the preparation of cell-free extracts. Cells were grown in M-9 medium at 37°, with vigorous aeration until the culture had reached a density of 3 x 10^8 cells x ml^{-1} (262). Preparation and purification of phage M12 was performed by Dr. R.N.H. Konings (Department of Molecular Biology, University of Nijmegen, Nijmegen, The Netherlands).

2.2.3. *Isolation of the viral RNA*

Plasma from leukemic Swiss mice or leukemic chickens (generous gift of Dr. J.W. Beard, Duke University, Durham, N.C., USA) was clarified by centrifugation at 12,000 x g for 10 min at 4° in a Sorvall RC2-B preparative centrifuge. The supernatant fraction was layered on top of a discontinuous gradient consisting of 3 ml 65% (w/v) sucrose in TNE (10 mM Tris.HCl, pH 7.4 at 20°, 100 mM NaCl and 1 mM EDTA) and 10 ml of 20% (w/v) sucrose in TNE (88). After centrifugation at 94,000 x g for 90 min at 4° in the SW 27 rotor, virus

present on the 65% sucrose cushion was collected, diluted by the addition of 2 volumes of TNE and pelleted by centrifugation at 150,000 x g for 45 min at 4^{o} in a Spinco type-Ti 50 rotor.

Purified mouse mammary tumor virus of (C3H/HeA x 020)F_1 mice was a generous gift of Dr. P. Hageman (Antoni van Leeuwenhoekhuis, Amsterdam, The Netherlands).

For the isolation of RNA, pelleted virions were resuspended in 1 ml of 10 mM Tris.HCl, pH 8.0 at 20^{o}, 100 mM NaCl, 1 mM EDTA and 1% 2-mercaptoethanol and subsequently incubated for 30 min at 37^{o} in the presence of 1% sodium dodecyl sulfate and 500 µg x ml^{-1} of RNAase free pronase. After incubation two volumes of extraction buffer (10 mM sodium acetate pH 5.0, 100 mM NaCl and 1 mM EDTA) were added and the viral RNA was deproteinized with two volumes of phenol-chloroform (1:1) saturated with extraction buffer (263). After two extractions with phenol-chloroform, the aqueous layer was reextracted with two volumes of chloroform-isoamylalcohol (100:1) and RNA present in the aqueous phase was precipitated by the addition of 0.1 volume of 1 M sodium acetate pH 5.2 and two volumes of ethanol at -20^{o}.

High molecular weight viral RNA (60S) was isolated by centrifugation in isokinetic glycerol gradients, 15.0-30.2% (w/v), containing TNE. Tubes were spun at 200,000 x g for 4.5 h at 4^{o} in an SB 283 rotor of the IEC centrifuge. RNA profiles were recorded with a continuous flow monitoring system using an LKB optical unit, coupled to a logarithmic recorder. RNA from the 50-70S region of the gradient was recovered by precipitation with ethanol at -20^{o}.

2.2.4. *Cell-free protein synthesis*

Pre-incubated S-30 from the RNAase I^{-} *E.coli* strain and phage M12 RNA (sedimentation value 27S) purified on sucrose gradients was prepared by Dr. R.N.H. Konings as previously described (264,265). Globin 9S and α-crystallin 14S mRNA were a gift of Dr. A.J.M. Berns of our laboratory.

The incubation mixture contained per ml: 50 nmoles of each of the added unlabeled amino acids, 50 µmoles Tris.HCl, pH 7.8 at 20^{o}, 10 µmoles of magnesium acetate, 70 µmoles NH_4Cl, 8 µmoles 2-mercaptoethanol, 0.25 mg *E.coli* tRNA, 5 µmoles ATP, 0.3 µmoles GTP, 5 µmoles PEP, 8 µg pyruvate kinase, 0.24 ml of pre-incubated S-30 extract containing 10 mg x ml^{-1} of ribosomes, 100-125 µCi [^{35}S]methionine (32-41 Ci x $mmol^{-1}$) or 30-80 µCi [^{14}C]amino acids

(54 mCi x matom^{-1}) and 300-700 µg of viral or messenger RNA. Incubation was performed at 37^{0} for 30 min in a volume of 60 µl.

After incubation the reaction was terminated in one of the following ways:

1. For the determination of the amino acid incorporation a 10 µl aliquot of the reaction mixture was diluted with 0.5 ml of 0.1 N KOH, incubated at 37^{0} for 15 min and the radioactive polypeptide chains were precipitated with 5% trichloroacetic acid in the presence of 100 µg of carrier bovine serum albumin. Subsequently, the precipitate was collected on Whatman GF/C filters and washed with 5% trichloroacetic acid. The acid precipitable radioactivity was counted in the Packard Tri-carb Liquid Scintillation Spectrometer using a toluene based scintillator.
2. For the analysis of newly synthesized polypeptides on polyacrylamide gels, the incubation mixture was diluted with 60 µl of 0.2 M Tris-HCl, pH 7.8 at 20^{0} and incubated for an additional 15 min at 37^{0} in the presence of 10 µg of pancreatic RNAase and 10 mM EDTA. Then, trichloroacetic acid was added to a final concentration of 5% and the precipitate was collected by centrifugation at 12,000 x g. The precipitated proteins were subsequently washed three times with 5% trichloroacetic acid and three times with cold aceton at 0^{0}. The final precipitate was dissolved in gel electrophoresis buffer according to Laemmli (259), and heated for 3 min at 95^{0}.
3. For the separation of released polypeptides from ribosome-bound peptidyl-tRNA, the incubation mixture was quickly cooled to 0^{0}, diluted with 200 µl of TMK (10 mM Tris-HCl, pH 7.8 at 20^{0}, 5 mM magnesium acetate, 80 mM KCl and 1 mM 2-mercaptoethanol) and analyzed on a sucrose gradient, consisting of a linear gradient of 10-20% (w/v) sucrose in TMK on top of a linear gradient of 20-30% (w/v) sucrose in 10 mM Tris-HCl, pH 7.8 at 20^{0}, 80 mM KCl, 10 mM EDTA, pH 7.4 at 20^{0} and 1 mM 2-mercaptoethanol. Tubes were spun at 4^{0} for 6 h at 200,000 x g in an SW 41 rotor. After centrifugation, the bottom of the tube was punctured and nine-drop fractions were collected. Trichloroacetic acid precipitable radioactivity was determined from 25 µl aliquots of each fraction as described before. To pooled fractions of released polypeptide chains (top), stripped polypeptide chains in the EDTA part of the gradient and to the resuspended pellet fraction, *E.coli* S-30 was added as carrier and the labeled material was prepared for polyacrylamide gel electrophoresis as described before.

2.2.5. *Polyacrylamide gel electrophoresis*

For sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Laemmli (25), gels were cast in 8.0 x 0.5 cm cylindrical glass tubes. The resolving gels contained 12.5% acrylamide, 0.33% N,N'-methylene-bisacrylamide, 0.025% N,N,N',N'-tetramethylethylenediamine (TEMED), 0.1% ammonium persulfate, 0.1% sodium dodecyl sulfate and 0.275 M Tris.HCl, pH 8.5. Shortly before use a spacer gel was applied containing 4% acrylamide, 0.11% N,N'-methylene-bisacrylamide, 0.05% TEMED, 0.1% ammonium persulfate, 0.1% sodium dodecyl sulfate and 0.125 M Tris.HCl, pH 6.8. Other solutions were: electrode buffer, 0.2 M glycine, 25 mM Tris, 0.1% sodium dodecyl sulfate; final sample buffer, 60 mM Tris.HCl, pH 6.8, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol and 10% glycerol.

Electrophoresis was performed at 2 mA per gel at room temperature, until the bromophenol blue marker reached the bottom of the gel tube. After electrophoresis gels were stained in 0.2% Coomassie Brilliant Blue in methanol: water:acetic acid (50:43:7 v/v) for 1 h at 37^{o} and destained overnight in methanol:water:acetic acid (50:875:75 v/v) at 37^{o}.

The distribution of radioactivity in gels was determined according to one of the following procedures:

(1) Gels were cut transversely in slices of 1 mm. The gel fragments were transferred to vials containing 300 µl NCS solution (NCS:H_2O, 90:10 v/v) and incubated overnight at 50^{o}. Subsequently, 10 ml of a toluene scintillator was added and the amount of radioactivity was determined in the Packard Tri-carb liquid Scintillation Spectrometer.

(2) When $[^{14}C]$ and $[^{3}H]$amino acid labeled proteins were separated in the same gel, 1 mm slices of this gel were dried and processed with the aid of the Tri-carb Sample Oxidizer (Packard Model 305). By this method the $[^{3}H]$ and $[^{14}C]$labels can be measured separately.

(3) Gels were sliced longitudinally, dried under vacuum on a piece of Whatman 3 MM filter paper and radioactive bands were located by autoradiography using Kodak RP-Royal X-omatic film.

2.3. STIMULATION OF AMINO ACID INCORPORATION BY RNA TUMOR VIRUS RNA AND MAMMALIAN MESSENGER RNA

Addition of deproteinized total virus RNA of R-MuLV, MTV or AMV to the cell-free system from *E.coli* resulted in a significant stimulation of labeled amino acids in acid precipitable radioactivity (Table 1).

TABLE 1

Stimulation of amino acid incorporation by viral RNA and eukaryotic RNA preparations in the *E.coli* cell-free system

	RNA added	[^{14}C]amino acid (cpm incorporated)		RNA added	[^{35}S]methionine (cpm incorporated)
Exp I	none	1016	Exp II	none	5820
	Phage M12	25,386		Phage M12	138,866
	R-MuLV	2116 (4020)*		R-MuLV	8784 (14,398)
	R-MuLV 60S	3856 (5498)		Globin 9S	8960 (10,102)
	MTV	4066 (4772)		α-crystallin 14S	6506 (7290)
	Ribosomal 18S	1208	Exp III	none	2898
	Ribosomal 28S	1226		Phage M12	153,622 (143,322)
				AMV	11,322 (17,186)

To each 60 µl incubation mixture 4 µg RNA and 5 µCi (Exp I) [^{14}C]amino acid mixture (54 mCi x matom^{-1}) or 8 µCi (Exp II and Exp III) [^{35}S]methionine [32 Ci x mmole^{-1} (Exp II), 41 Ci x mmole^{-1} (Exp III)] was added. After 30 min incubation at 37^{o} the reaction was terminated and trichloroacetic acid precipitable radioactivity was determined from 5 µl aliquots of the incubation mixture. 1 pmol of incorporated [^{35}S]methionine corresponds to 40,000 cpm.
*Values obtained after denaturation of the RNA at 65^{o} are given in parentheses.

This stimulation of the amino acid incorporation could be enhanced by heat denaturation of the viral RNAs at 65^{o}, prior to addition to the cell-free system. This effect was most pronounced for the denatured RNAs of AMV and R-MuLV.

When the *E.coli* cell-free system was programmed with purified 60-70S RNA of R-MuLV a higher stimulation of amino acid incorporation was observed as that with the total viral RNA. This was probably, at least in part, due to an inhibitory effect of the low molecular weight viral 4-5S viral RNA, present in the total viral RNA preparation, on the translation of the 60S RNA. Evidence for this assumption was derived from the fact that this low molecular weight RNA fraction also inhibited the translation of phage M12 RNA (266).

For comparison we examined the effect of globin and α-crystallin messenger RNA on the amino acid incorporation. Whereas a significant stimulation of amino acid incorporation was observed in response to 9S globin mRNA, which was even enhanced by denaturation, the effect of α-crystallin 14S mRNA, coding for the A_2-chain of α-crystallin, was almost negligible. In a control experiment, no significant stimulation was observed after addition of either 18S or 28S ribosomal RNA to the cell-free system.

2.4. ANALYSIS OF THE PRODUCTS SYNTHESIZED UNDER THE DIRECTION OF RAUSCHER LEUKEMIA VIRUS RNA AND PHAGE M12 RNA

To examine whether the addition of R-MuLV to an *E.coli* cell-free lysate gives rise to the synthesis of specific polypeptides, the *in vitro* synthesized products were subjected to electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate. For comparison products synthesized in the endogenous *E.coli* system were run in a parallel gel.

From the results presented in Fig. 5A it can be concluded that several distinct polypeptides were made in response to the native R-MuLV RNA, with molecular weights ranging from 10,000 to 45,000. Most of these products were absent in the gel pattern of polypeptides synthesized in the endogenous system. Co-electrophoresis of the *in vitro* products, directed by the denatured viral RNA, with radioactive proteins from purified R-MuLV virions, indicated that at least two polypeptides in the low molecular weight region of the gel (slice no. 52-71) coincided with native viral proteins (Fig. 5B). Furthermore additional polypeptides were synthesized which migrated to the 30,000 M_r region (slice no. 32-40). The effect of denaturation of R-MuLV RNA on the translation is striking. It resulted in the appearance of a polypeptide of approximately 45,000 M_r (slice no. 24-27) which was present in trace amounts only among the cell-free products directed by the native R-MuLV RNA

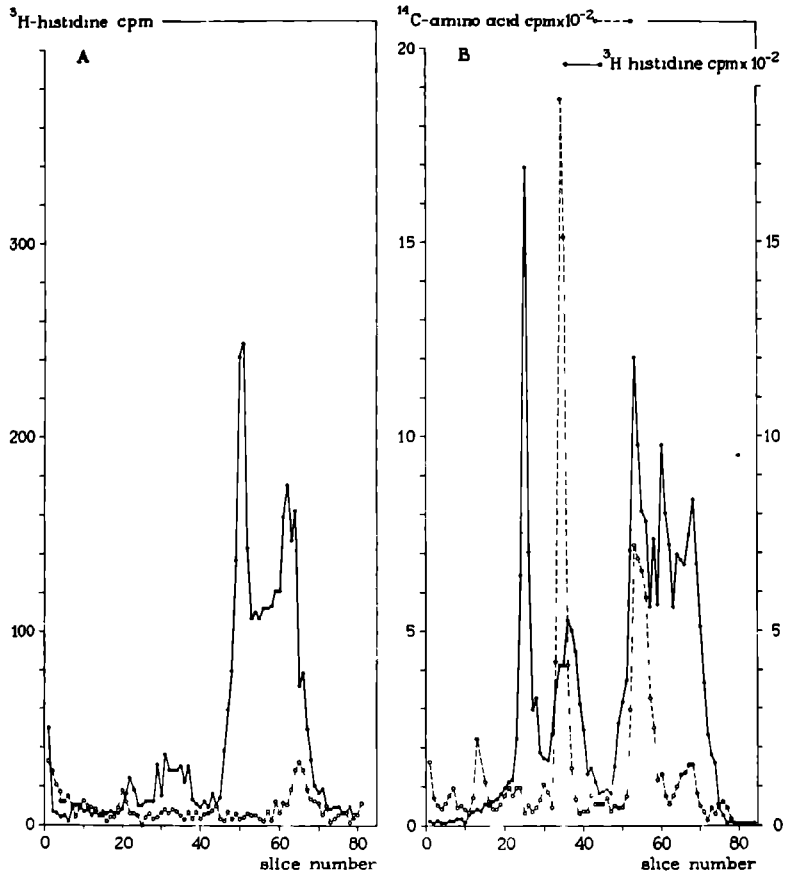


Fig. 5. Analysis of the polypeptides synthesized under the direction of R-MuLV RNA by polyacrylamide gel electrophoresis. (A) Polypeptides synthesized in the absence (o -- o) and presence (● — ●) of R-MuLV RNA. (B) Comparison by co-electrophoresis of the polypeptides synthesized *in vitro* under the direction of denatured R-MuLV RNA (● — ●) with the viral proteins labeled with [^{14}C]amino acids (o -- o). Incubation in the presence of [^{3}H] histidine. After electrophoresis the gels were sliced in 1 mm sections and the amount of radioactivity was determined as described in section 2.2.5.

(Fig. 5A and B).

In order to obtain more accurate data about the size and number of the

synthesized products, autoradiographic analysis of dried gels was performed. Translation of denatured R-MuLV RNA in the presence of either $|^{14}C|$amino acids or $|^{35}S|$methionine led to the synthesis of mainly two polypeptides, with molecular weights of 15,000 and 45,000 (Fig. 6b and e.). For comparison

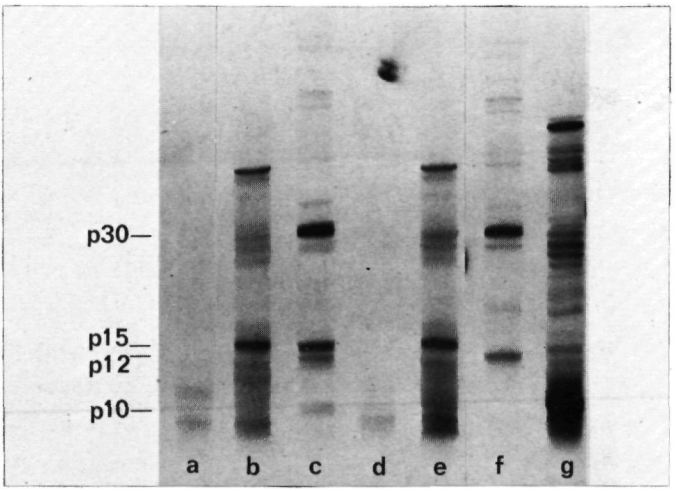


Fig. 6. Autoradiogram of the polypeptides synthesized under the direction of R-MuLV RNA and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Products of the endogenous *E.coli* system (a) and (d); with denatured R-MuLV RNA (b) and (e); with phage M12 RNA (g). Virion proteins of R-MuLV labeled with $[^{14}C]$amino acids (c) or $[^{35}S]$methionine (f). Incubation in the presence of $[^{14}C]$amino acids (a) and (b), in the presence of $[^{35}S]$methionine (d), (e) and (g).

the gel patterns of viral proteins labeled with $[^{14}C]$amino acids (Fig. 6c) and $[^{35}S]$methionine (Fig. 6f) are included. It is obvious that from those polypeptides which were made *in vitro* only the 15,000 M_r polypeptide has an identical electrophoretic mobility as one of the proteins, i.e. p15, of mature virus, labeled with $[^{14}C]$amino acids (Fig. 6c). From previously performed experiments it was known that $[^{35}S]$methionine was present at most in trace amounts in the virion protein p15 (Fig. 6f). Therefore, it was surprising that the 15,000 M_r polypeptide, synthesized *in vitro* was labeled with $[^{35}S]$methionine. In first instance, this finding seemed to be in contrast to the

assumption that both polypeptides were identical. Since it is known, however, that all phage specific polypeptides, which are synthesized in cell-free systems from *E.coli*, start with the amino acid N-formyl methionine (267-270), it is plausible to assume that [^{35}S]methionine was introduced as N-terminal amino acid in the *in vitro* synthesized 15,000 M_r polypeptide, by the initiator $tRNA^{fMet}$.

Addition of M12 RNA to the *E.coli* lysate led to the synthesis of two major polypeptides with molecular weights of approximately 12,000 (coat) and 60,000 (RNA synthetase) (Fig. 6g). Moreover a substantial number of polypeptides of intermediate molecular weights were discernible, which presumably arose by incomplete translation of the synthetase cistron (262). Therefore, it is likely that the R-MuLV RNA directed polypeptides migrating to the 30,000 M_r region and faster than 15,000 M_r resulted from premature polypeptide chain termination.

Since the RNA present in the R-MuLV virion, consists of high and low molecular weight RNA species, experiments were performed to investigate whether any difference could be established between the polypeptides synthesized under the direction of total RNA and the purified 60S RNA component of the virion. Analysis of the cell-free products revealed that both RNA preparations directed the synthesis of the same set of polypeptides (Fig. 7a and c).

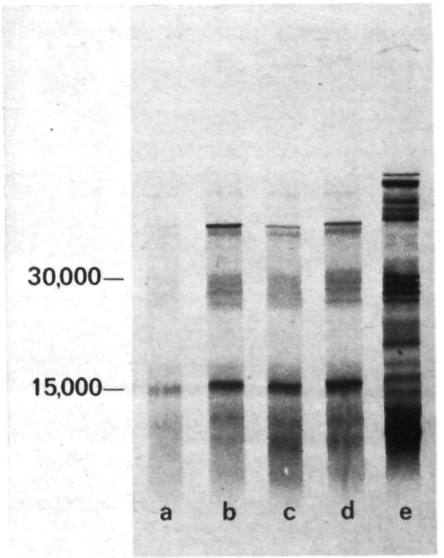


Fig. 7. Comparison of the polypeptides synthesized in the *E.coli* cell-free system under the direction of the high molecular weight RNA component or a total RNA isolate from R-MuLV. (a) Products of the system supplemented with R-MuLV RNA; (b) with denatured R-MuLV RNA; (c) with native 60S R-MuLV RNA; (d) with denatured 60S R-MuLV RNA; (e) with M12 RNA. Incubation in the presence of [^{14}C]amino acids.

Moreover the cell-free synthesis of the 45,000 M_r polypeptide was enhanced after heat denaturation of both RNA preparations (Fig. 7b and d).

Finally, to establish whether the newly synthesized polypeptides mediated by the viral RNA were released from polyribosomes, we analyzed the cell-free incubation mixtures programmed with R-MuLV RNA on a "strip"-gradient. Fig. 8 exhibits that about 50% of trichloroacetic acid precipitable radioactivity remained at the top of the gradient, representing released polypeptide chains. Twentyfive percent was stripped from ribosomes in the EDTA-containing part of the gradient and 25 percent sedimented together with ribosomes to the bottom of the gradient. A similar result was obtained with M12 RNA directed polypeptides (Fig. 8). The gel analysis of the polypeptides

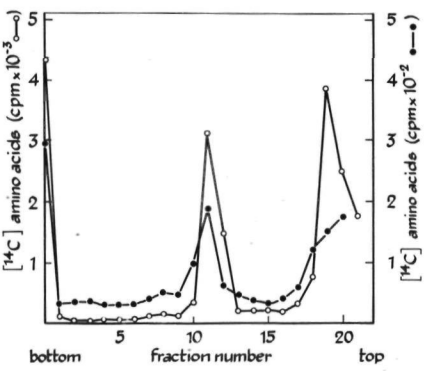


Fig. 8. Analysis of polypeptides synthesized under the direction of phage M12 RNA and R-MuLV RNA on 'strip' gradients. Preparation of the samples from cell-free incubation mixtures and the 'strip' gradients was described in section 2.2.4. Distribution of radioactive products synthesized under the direction of phage M12 RNA (o — o) and R-MuLV RNA (• — •).

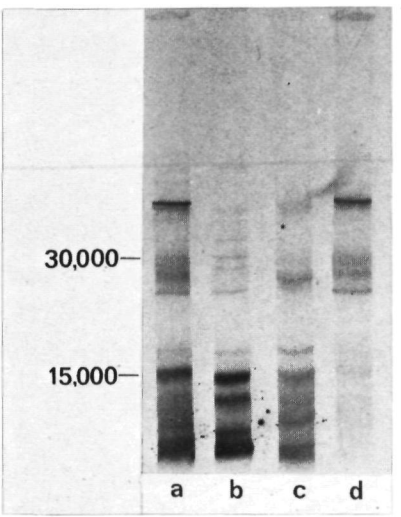


Fig. 9. Autoradiograph of polyacrylamide gels showing the polypeptide patterns of different fractions of the 'strip' gradient. (a) Polypeptides synthesized under the direction of R-MuLV RNA; (b) R-MuLV RNA directed products in the top fraction of the 'strip' gradient; (c) in the strip fraction (c); in the bottom fraction (d).

which were released during protein synthesis, stripped from ribosomes by EDTA or which remained attached to ribosomes is shown in Fig. 9. It is evident that a large proportion of the 15,000 M_r polypeptide was recovered in the gradient fractions containing released polypeptide chains, whereas the 45,000 M_r polypeptide was virtually absent in this fraction (Fig. 9b). The latter polypeptide was only recovered from the pellet fraction of the gradient (Fig. 9d).

From the results presented in this section we may conclude that addition of R-MuLV RNA to an *E.coli* lysate gave rise to the synthesis of several polypeptides. One major *in vitro* product of 15,000 molecular weight comigrates with the R-MuLV virion protein p15. A second prominent polypeptide with a molecular weight of 45,000 was revealed after denaturation of the viral RNA. Finally, the 60S viral RNA contains all the genetic information necessary to code for the polypeptides made in the *in vitro* system.

2.5. MAGNESIUM ION DEPENDENCE OF THE CELL-FREE PRODUCTS MADE IN RESPONSE TO RAUSCHER LEUKEMIA VIRUS RNA AND PHAGE M12

The effect of the magnesium ion concentration on the incorporation by the *E.coli* lysate of [^{35}S]methionine into acid precipitable products in response to the addition of R-MuLV RNA and phage M12 RNA is shown in Fig. 10.

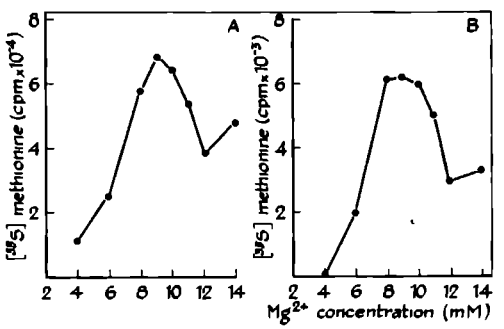


Fig. 10. Effect of Mg^{2+} concentration on R-MuLV RNA (A) and phage M12 RNA (B) directed incorporation of [^{35}S]methionine. Conditions for incubation were as described in section 2.2.4., with the exception of the Mg^{2+} concentration.

The Mg^{2+} ion dependence of the incorporation was similar for both viral messengers and optimal stimulation of amino acid incorporation occurred at Mg^{2+}

concentrations between 8 and 11 mM. This indicates that optimal ionic conditions for the translation of the mammalian viral RNA were employed. This conclusion was confirmed by the analysis of polypeptides made in response to the R-MuLV RNA at different Mg^{2+} ion concentrations, on polyacrylamide gels (Fig. 11, 1-8). It is obvious that both the 15,000 and 45,000 M_r polypeptides were

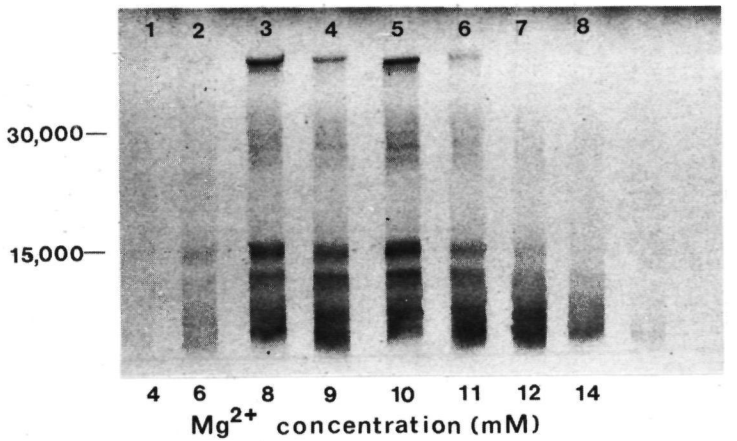


Fig. 11. Polyacrylamide gel electrophoresis of the R-MuLV-specific products synthesized at different Mg^{2+} concentrations.

only synthesized at Mg^{2+} ion concentrations of 8 to 11 mM. When a similar analysis was performed with the phage M12 RNA directed polypeptides a differential effect of Mg^{2+} on the synthesis of coat protein and RNA synthetase was observed (Fig. 12, 1-8). Altering of the Mg^{2+} ion concentration between 6 and 14 mM did not significantly affect the synthesis of coat protein, whereas a high rate of formation of RNA synthetase was observed only at 8 to 9 mM Mg^{2+}. This finding is essentially identical to the observation of Zagórski *et al* (271) that alteration of the Mg^{2+} ion concentration could determine which gene of f2 RNA was translated in the *in vitro* system. We have not examined which step of protein synthesis was affected at suboptimal Mg^{2+} concentrations.

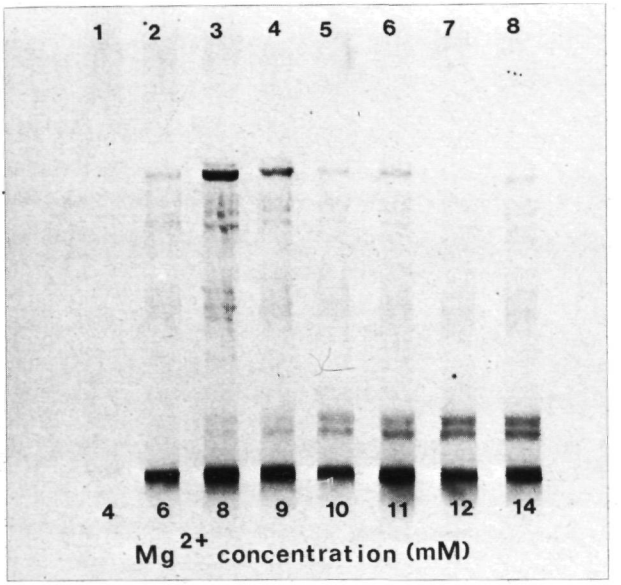


Fig. 12. Polyacrylamide gel electrophoresis of the phage M12-specific products synthesized at different Mg^{2+} concentrations.

2.6. SYNTHESIS OF SPECIFIC POLYPEPTIDES DIRECTED BY AVIAN MYELOBLASTOSIS VIRUS RNA AND MOUSE MAMMARY TUMOR VIRUS RNA

Additional evidence for the reliability of the *in vitro* translation of R-MuLV RNA could be obtained if we were able to demonstrate that addition of RNA from other RNA tumor viruses to the *E.coli* system led to the synthesis of polypeptides which differed in molecular size from the R-MuLV specific products. Therefore, we compared the cell-free products mediated by native or denatured AMV or MTV RNA with those synthesized in response to R-MuLV RNA.

Native MTV RNA gave rise to the synthesis of a substantial number of polypeptides in the molecular weight range of 8000 up to 60,000 (Fig. 13b). The distribution of radioactive bands in the gel shows that at least some of the MTV RNA directed polypeptides did not coincide with those mediated by R-MuLV RNA (Fig. 13f). Denaturation of MTV RNA did not result in a strong enhancement of the synthesis of a particular polypeptide (Fig. 13c), as was

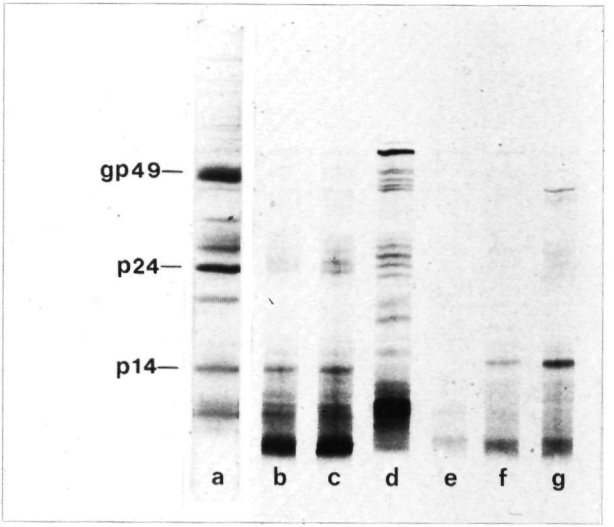


Fig. 13. Comparison of the polypeptides synthesized under the direction of MTV RNA with those of R-MuLV RNA by polyacrylamide gel electrophoresis. (a) Virion proteins of MTV (stained gel); (b) *E.coli* system supplemented with MTV RNA; (c) with denatured MTV RNA; (d) with M12 RNA; (e) endogenous products; (f) with R-MuLV RNA; (g) with denatured R-MuLV RNA. Incubation in the presence of $[^{14}C]$amino acids.

observed in case of R-MuLV RNA (Fig. 13g). Among the *in vitro* products made in response to MTV RNA at least one major polypeptide (14,000 M_r) was detected (Fig. 13b and c), which corresponded in molecular size to protein p14 of purified MTV virions (Fig. 13a). This latter polypeptide migrated always slightly faster than the R-MuLV RNA directed polypeptide of 15,000 molecular weight. Finally, among the polypeptides synthesized *in vitro* in response to MTV RNA, another prominent band was always visible comigrating with the buffer front, and was perhaps the result of early artificial termination.

Fig. 14c shows the electrophoretic profile of the products synthesized in the *in vitro* protein-synthesizing system programmed by AMV RNA. One distinct polypeptide was synthesized with a molecular weight of approximately 19,000, which was absent in the gel analysis of the endogenous products

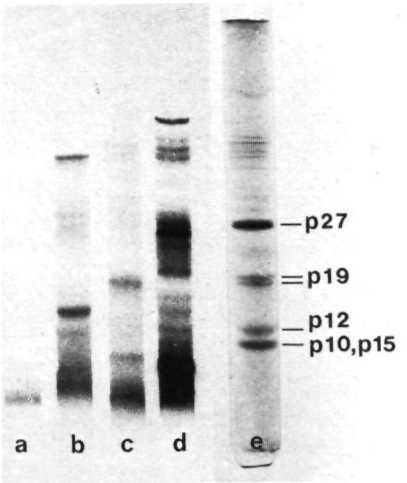


Fig. 14. Comparison of the polypeptides synthesized under the direction of AMV RNA with those of R-MuLV RNA by polyacrylamide gel electrophoresis. (a) Endogenous products of the *E.coli* cell-free system; (b) with denatured R-MuLV RNA; (c) with denatured AMV RNA; (d) with phage M12 RNA. (e) Virion proteins of AMV (stained gel). Incubation in the presence of [^{35}S] methionine.

(Fig. 14a) and the R-MuLV RNA directed polypeptides (Fig. 14b). This 19,000 M_r polypeptide is most probably identical in size to the AMV virion protein p19 (Fig. 14e). Denatured AMV RNA directed the synthesis of a similar set of polypeptides (not shown).

From these results, one may conclude that the cell-free system from *E.coli* gives rise to different sets of newly synthesized polypeptides when programmed with the RNAs of different RNA tumor viruses.

2.7. TRANSLATION OF EUKARYOTIC mRNA IN THE *E.COLI* CELL-FREE SYSTEM

The *E.coli* system has also been used to test the cell-free translation of mammalian messengers. Although a specific stimulation of protein synthesis by mammalian mRNA fractions was observed, in most reports no convincing evidence was provided that these messengers were faithfully translated (272-275). Actually, the results of only one report suggest that in response to an 8S messenger RNA fraction from rabbit reticulocytes, material was synthesized having properties of rabbit globin (275).

Since the purified globin 9S and α-crystallin 14S mRNA were available, we were interested to investigate whether both messenger fractions were faithfully translated in the *E.coli* system. In section 2.3. we established

that addition of globin 9S mRNA to the *E.coli* lysate resulted in a stimulation of the amino acid incorporation, whereas this stimulation was negligible in a lysate programmed with the α-crystallin 14S mRNA. Sodium dodecyl sulfate polyacrylamide gel analysis of the products synthesized *in vitro* in response to both messengers, revealed that no polypeptides were made which correspond in size to globin or the A_2-chain of α-crystallin (Fig. 15a-d, i and j). However, we cannot exclude that in response to both messengers small polypeptides were made, which were lost during preparation of the synthesized products for gel analysis.

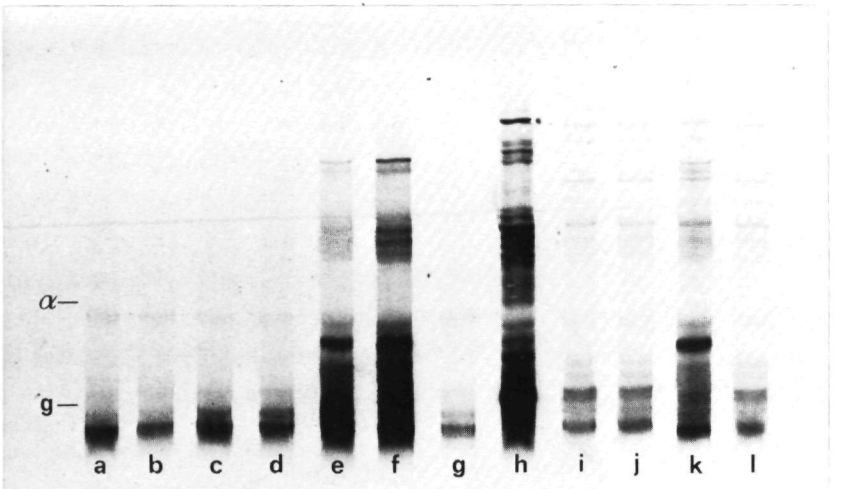


Fig. 15. Autoradiogram of the polypeptides synthesized under the direction of globin 9S and α-crystallin 14S mRNA. (a) Products synthesized under the direction of globin 9S mRNA; (b) with denatured globin 9S mRNA; (c) with α-crystallin 14S mRNA; (d) with denatured α-crystallin 14S mRNA; (e) with R-MuLV RNA; (f) with denatured R-MuLV RNA; (g) endogenous *E.coli* products; (h) with phage M12 RNA. Incubations in the presence of [^{35}S]methionine. (i) Products synthesized under the direction of globin 9S mRNA; (j) with α-crystallin 14S mRNA; (k) with R-MuLV RNA; (l) endogenous *E.coli* products. Incubations in the presence of [^{14}C]amino acids. α stands for α-crystallin, g stands for globin.

2.8. DISCUSSION

Evidence was provided, that the RNA isolated from three nonrelated RNA tumor viruses was able to funtion as messenger RNA in a cell-free lysate from *E.coli*. In response to the individual viral RNA preparations of R-MuLV, MTV and AMV specific sets of products were synthesized. Moreover, comparison of the gel patterns of the *in vitro* products with the virion proteins suggest, that in response to each particular viral RNA at least one polypeptide was made, which corresponds in size to one of the virion proteins. Additional evidence that the 15,000 molecular weight polypeptide, mediated by R-MuLV RNA is coded for by the viral RNA has been provided by Salden (personal communication). He was able to demonstrate that the latter polypeptide could be precipitated with a rabbit antiserum directed against purified R-MuLV. More recently Twardzik *et al* (154) reported the translation of AKR-murine leukemia virus (AKR-MuLV) RNA in cell-free extracts from *E.coli*. These authors described the synthesis of one major polypeptide of 30,000 M_r and two minor products of about 9000 and 12,000 M_r. Furthermore, employing antiserum directed against MuLV they showed that about 40% of the newly synthesized polypeptides could be precipitated. However, no further analysis was performed on the precipitated proteins. Since both the RNA from AKR-MuLV and R-MuLV, code for antigenic homologous proteins it is conceivable that a similar set of group-specific polypeptides was synthesized under the direction of both viral RNAs. On the other hand, we have never observed the synthesis of a polypeptide which corresponded in size with the major group-specific antigen p30. This discrepancy is most probably due to differences in the analysis of the newly formed products. Whereas the location of the radioactive polypeptides as determined by slicing of the gels suggests that part of the products made *in vitro* comigrated with the virion protein p30 (Fig. 5B), autoradiographic analysis (Fig. 6b and e) clearly shows that the slices in question actually comprised three to four bands with slightly different electrophoretic mobilities. For the present time it is, therefore, extremely hard to compare the results of this group with our data. We are concerned with a similar problem, if we try to compare our findings on the translation of AMV RNA with those of Siegert *et al* (152). These authors claimed that addition of AMV RNA to the *E.coli* lysate gave rise to the synthesis of polypeptides, which corresponded in size and relative amount to the AMV virion antigens p27, p19, p15 and p12.

The radioactive pattern of the polypeptides synthesized *in vitro* was determined by slicing of the gels. Employing immunodiffusion analysis, it could further be demonstrated that one of these proteins synthesized *in vitro* was antigenically related with p12. Our electropherograms reveal that we can confirm the synthesis of a polypeptide with the approximate size of p19; however, we cannot support their finding that p27, p15 and p12 were also synthesized. As mentioned before, this discrepancy is most probably due to differences in the procedure by which radioactive protein bands were localized in the gel. Our results clearly show that location and resolution of radioactive bands in polyacrylamide gels are greatly improved, by employing autoradiographic analysis in stead of gel slicing, and subsequent counting.

More recent studies on the synthesis of RNA tumor virus-specific proteins in cells labeled *in vivo*, showed that mature virion proteins arise by proteolytic cleavage of high molecular weight precursor polypeptides (253-257). Moreover, by employing specific antisera it became possible to detect the virus-specific polypeptides synthesized under the direction of purified RNA of several RNA tumor viruses in cell-free extracts of mammalian origin (155-158). With respect to the synthesis of R-MuLV-specific polypeptides Naso *et al* (155) claimed that R-MuLV RNA mediated the synthesis of two classes of polypeptides (140,000-185,000 and 50,000-75,000 M_r) in a S-30 system derived from R-MuLV infected JLS-V5 cells. Both classes of polypeptides were larger than the group-specific antigens of mature virions. On the other hand,Salden *et al* (157) demonstrated that in response to R-MuLV RNA mainly two polypeptides (65,000 and 72,000 M_r) were synthesized, sharing common amino acid sequences with the virion protein p30. No mature virion proteins were detected after cell-free incubation of the R-MuLV RNA. Cell-free translation of the 60-70S RNA of an avian RNA tumor virus (Rous sarcoma virus) also led to the formation of a high molecular weight precursor (76,000 M_r) of the mature virion proteins (156).

In view of these results we feel that, although the *E.coli* system is capable to translate the viral messenger RNA with fidelity, the newly synthesized polypeptides were most probably only fragments of the virion precursor polypeptides. The reason for this may be:

1. Initiation of polypeptide synthesis occurred at internal sites of the message, resulting in the synthesis of products of limited size.
2. Small fragments arose by premature termination of protein synthesis caused

by degradation of the viral RNA by nucleases.
3. Newly synthesized polypeptides of high molecular weight were rapidly processed by proteolytic enzymes, present in the lysate.
Evidence for the first two alternatives was provided by experiments of Rekosh *et al* (276) demonstrating the synthesis of a heterogeneous mixture of virus-specific polypeptides in the molecular weight range up to 60,000 when the *E.coli* cell-free system was programmed with poliovirus RNA. Initiation of polypeptide synthesis started on at least eight distinct sites on the viral RNA and gradient analysis of the viral RNA after incubation in the cell-free system indicated that the RNA was rapidly degraded. We have not examined which of these alternatives is applicable to the translation of the RNA of RNA tumor viruses. However, additional support for our notion that at least one of the polypeptides directed by AMV RNA, may be identical to the virion protein p19 is derived from the observation of Eisenman *et al* (256) that p19 is located closest to the N-terminal end of the precursor polypeptide (76,000 M_r) of the mature virion proteins. The location of p19 nearest to the N-terminal side, makes it likely that the efficiency of translation of this part of the genome is highest. This supposition is consistent with our observation that p19 was the major product mediated by the RNA of AMV.

CHAPTER 3

VIRUS-SPECIFIC MESSENGER RNA IN FREE AND MEMBRANE-BOUND POLYRIBOSOMES OF CELLS INFECTED WITH RAUSCHER MURINE LEUKEMIA VIRUS

3.1. INTRODUCTION

In cells infected with RNA tumor viruses several virus-specific proteins are synthesized, including the internal capsid proteins, virion envelope proteins, the RNA-dependent DNA polymerase and, presumably, proteins responsible for the transformation of the infected cells. It is important to characterize the virus-specific mRNA(s) involved in the synthesis of these virus-specific proteins. Moreover, in view of the fact that we did not observe the translation of native or denatured 60-70S RNA of Rauscher murine leukemia virus (R-MuLV) in mammalian cell-free systems, we were interested to examine whether the 60-70S RNA or the 35S subunit RNA of R-MuLV function as messenger, and whether mRNAs exist in sizes other than that of the viral genome.

RNA tumor viruses contain an enzyme capable of transcribing DNA from the 60-70S viral RNA. The DNA products synthesized by the endogenous 'DNA polymerase' appear to be a mixture of single-stranded DNA and double-stranded DNA (229). However, when the synthesis of double-stranded DNA is inhibited by incubation of detergent-treated virus in the presence of actinomycin D, preferentially single-stranded DNA complementary to the viral RNA is synthesized (231, 277,278). This single-stranded DNA represents a complete and relatively uniform copy of the entire viral genome (160,279) and, therefore, constitutes an extremely sensitive hybridization probe for the detection of virus-specific RNA in cells infected with RNA tumor viruses (280-284).

Hybridization of unlabeled RNA, fractionated according to size, from cells tranformed by the Moloney strain of murine sarcoma virus with the labeled viral DNA probe indicated the presence of two size classes of virus-specific RNA, 35S and 20S (285). A similar analysis of avian sarcoma virus (RSV) infected cells revealed that the intracellular virus-specific RNA was heterogeneous in size sedimenting between 4S and 50S (286). However, evidence for virus-specific RNA species in association with functioning polyribosomes was still lacking.

In this chapter we report the presence of Rauscher murine leukemia virus-specific RNA sequences in free and membrane-bound polyribosomes from cells

chronically infected with this virus. In addition, we examined the size of the virus-specific mRNAs associated with the various polyribosomal preparations.

3.2. MATERIALS AND METHODS

3.2.1. *Cells and virus*

The JLS-V9 cell line, derived from bone marrow cells of BALB/c mice infected with and producing Rauscher leukemia virus (R-MuLV) was grown as monolayer in Eagle's Minimal Essential Medium completed with 10% calf serum, penicillin (100 I.U. x ml^{-1}) and streptomycin (50 µg x ml^{-1}) (287).

3.2.2. *Experimental conditions*

In order to minimize the possibility of RNAase contamination, all glassware was sterilized by dry heat and all buffers, sucrose and glycerol solutions used during handling and isolation of polysomes and RNA were sterilized by autoclaving. All solutions were handled with sterile glass pipettes.

Buffers to be used at 4^{o} were standardized to the desired pH with concentrated HCl at 4^{o}. All manipulations were performed at 0-4^{o} unless otherwise stated. Centrifugation gravity values quoted are the average g-values generated at particular speeds of rotation.

3.2.3. *Cell fractionation and isolation of free and membrane-bound polyribosomes*

In order to obtain a maximal yield of polyribosomes, fresh growth medium and cycloheximide (0.1 µg x ml^{-1}) were added 180 and 20 min, respectively, before harvesting of the cells (288,289). Cells were trypsinized at 37^{o} and cooled rapidly with crushed frozen isotonic medium (35 mM Tris-HCl, pH 7.5, 146 mM NaCl).

Cells were collected by 5 min centrifugation at 1000 x g and washed three times with isotonic medium. Two ml of pelleted cells were suspended in 13 ml of hypotonic medium containing 10 mM Tris-HCl, pH 7.4, 15 mM KCl, 1.5 mM magnesium acetate and 2 mM 1,4 dithioerythritol. All media used in this and further isolation steps contained 100 µg x ml^{-1} of RNAase inhibitor from

bovine eye-lens, purified on DEAE-cellulose (kindly provided by Dr. W.G.M. van de Broek of our laboratory). After 15 min swelling, cells were disrupted by 20 strokes of a Dounce homogenizer (B pestle). Nuclei and unbroken cells were removed by 10 min centrifugation at 700 x g.

Polyribosomes associated with membranes were isolated by flotation in discontinuous sucrose gradients. Ten ml of the postnuclear supernatant was made 2.2 M in sucrose by the addition of 90% (w/v) sucrose and transferred to tubes of the Spinco type-SW 27 rotor. Eight ml of 1.8 M sucrose in TMK (50 mM Tris-HCl, pH 7.8, 80 mM KCl, 5 mM magnesium acetate and 1 mM 1,4 dithioerythritol) and 8 ml of 1 M sucrose in TMK were layered on top of the 2.2 M sucrose cushion. Centrifugation was performed at 95,000 x g for 3 h. The membrane layer on top of the 1.8 M sucrose cushion was removed and the membranes were solubilized by addition of a mixture of nonidet P-40/deoxycholate (1:1) to a final concentration of 0.5%. The released polyribosomes were further purified by centrifugation through a layer of 4 ml of 2 M sucrose in TMK for 18-24 h at 95,000 x g in a SW 27 rotor.

For the isolation of free polyribosomes, 2 ml of the postnuclear supernatant was diluted with 6 ml TMK and centrifuged at 13,000 x g for 5 min in a Spinco type-Ti 50 rotor. The supernatant was layered on a discontinuous sucrose gradient consisting of 8 ml 1 M sucrose in TMK on top of 4 ml 2M sucrose in TMK and centrifuged for 18-24 h at 95,000 x g in a SW 27 rotor.

3.2.4. *Isolation of total polyribosomes*

A mixture of free and membrane-bound polyribosomes (total polyribosomes) were isolated as described previously (288). Cells were collected and washed as described in section 3.2.3. To the pelleted cells 2% nonidet P-40 in high salt buffer (50 mM Tris-HCl, pH 8.5, 225 mM KCl, 8 mM magnesium acetate, 2 mM 1,4 dithioerythritol and 100 $\mu g \times ml^{-1}$ of RNAase inhibitor) was added and the cells were lysed by 10 strokes of a tight fitting Dounce homogenizer (B pestle). Nuclei and cell debris were removed by centrifugation at 12,000 x g for 5 min in a Spinco type-Ti 50 rotor. The isolation of polyribosomes from the supernatant was performed as described for free polyribosomes in section 3.2.3.

3.2.5. *Sucrose-gradient centrifugation of purified polyribosomes*

Polyribosomal pellets were resuspended in TMK, layered on 20-30.7% (w/v) isokinetic sucrose gradients in TMK and centrifuged at 5^{o} in an SB 283 rotor of an IEC centrifuge for 60 min at 200,000 x g. Polyribosomal profiles were recorded with a continuous flow monitoring system using an LKB optical unit, coupled to a logarithmic recorder. To each fraction of the gradient 20 µg of *E.coli* tRNA was added as carrier and the polyribosomes were precipitated with 0.1 volume of sodium acetate pH 5.2 and 2 volumes of ethanol at -20^{o}.

Messenger RNP was released from purified polyribosomes, resuspended in TMK, by the addition of neutralized EDTA to a final concentration of 15 mM. Polyribosomes treated with EDTA were analyzed in 20-30.7% (w/v) isokinetic sucrose gradients in 50 mM Tris-HCl, pH 7.8, 80 mM KCl and 1 mM 1,4 dithioerythritol. Centrifugation conditions were as mentioned before.

3.2.6. *Extraction and analysis of polyribosomal RNA*

Nucleic acid from the resuspended polyribosomal pellets or from gradient fractions was extracted with phenol-chloroform according to Perry *et al* (263). Polyribosomal suspensions in extraction buffer (10 mM Tris-HCl, pH 8.0 at 20^{o}, 100 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulfate) were rapidly deproteinized after addition of 2 vol. of phenol-chloroform (1:1) saturated with extraction buffer. The water phase was reextracted twice with chloroform-isoamylalcohol (100:1). After centrifugation, RNA was precipitated from the aqueous phase with 0.1 vol. of 2M sodium acetate pH 5.2 and 2 vol. of ethanol at -20^{o}.

The extracted RNA was dissolved in TNE 7.4 (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA) containing 0.5% sodium dodecyl sulfate, layered onto a 15-30.2% (w/v) isokinetic glycerol gradient in TNE and centrifuged at 5^{o} in an SB 283 rotor at 200,000 x g for 9 h. RNA profiles were monitored with the aid of an LKB optical unit.

3.2.7. *Synthesis of the virus-specific cDNA probe*

The viral cDNA probe used to detect intracellular viral RNA was prepared by the endogenous reaction of R-MuLV virus isolated from plasma of virus

infected Swiss mice. Transcription of the viral RNA was performed in the presence of actinomycin D, to inhibit synthesis of double-stranded DNA (231, 277,278).

Purified R-MuLV 200-300 μg x ml^{-1}, isolated as described in section 2.2.3., was added to an 1 ml incubation mixture containing 50 mM Tris-HCl, pH 8.3 at 20^{o}, 6 mM $MgCl_2$, 60 mM NaCl, 10 mM 1,4 dithioerythritol, 0.2 mM each of dATP and dTTP, 0.015% (v/v) nonidet P-40, 100 μg x ml^{-1} actinomycin D and 0.02 mM of [^{3}H]dCTP and [^{3}H]dGTP. Enzyme reactions were incubated at 37^{o} for 4-6 h. The reaction was stopped by addition of 3 M KOH to a final concentration of 0.3 M, the solution was kept at 37^{o} for 18 h and finally neutralized with 0.1 vol. of 3 M HCl. After addition of sodium dodecyl sulfate to a final concentration of 0.5%, the reaction mixture was passed over a Sephadex G-50 column equilibrated with 50 mM triethylammonium bicarbonate buffer, pH 7.0. Samples from the eluted fractions were assayed for trichloroacetic acid precipitable radioactivity and those fractions which contained labeled DNA were pooled and lyophilized. The lyophilized material was resuspended in distilled water, 200 μg *E.coli* tRNA was added as carrier and nucleic acids were precipitated by addition of 0.1 vol. 2 M NaCl and 2 vol. of ethanol at -20^{o}. The specific activity of the cDNA was about 7500 cpm x ng^{-1}.

It is of importance to mention that the synthesis of cDNA by the endogenous reverse transcriptase reaction, is strongly dependent on the nonidet P-40 concentration (290). Therefore, we determined in test experiments the amount of virion protein optimal at a particular nonidet P-40 concentration.

3.2.8. *Purification of Aspergillus oryzae single-strand specific nuclease*

Aspergillus oryzae S_1 single-strand specific nuclease was purified from crude α-amylase powder essentially as described (291,292). Five g of crude amylase was dissolved in 200 ml column buffer (0.02 M KPO_4, pH 6.9 and 5% glycerol) and the insoluble material was removed by low speed centrifugation. The supernatant was applied to a DE-52 column equilibrated in column buffer. After rinsing the column with buffer until the effluent was colorless, nuclease was eluted with a linear 0-0.4 M NaCl gradient in column buffer, with 400 ml total volume. Those fractions with high single-strand specific nuclease activity were diluted with glycerol to a final concentration of 50% and stored at -20^{o}.

3.2.9. *Nucleic acid hybridization*

Annealing of viral and polyribosomal RNA with [^{3}H]cDNA (750 cpm) was performed at 65^o in 0.2 M phosphate buffer, pH 7.0 and 0.5% sodium dodecyl sulfate. Reactions were carried out in volumes of 10 µl in sealed microcapillary tubes. The extent of hybridization was assessed by measuring resistance to *Aspergillus oryzae* single-strand specific nuclease. The reaction mixture contained in a volume of 1 ml, 100 mM sodium acetate buffer, pH 4.5, 1 mM $ZnSO_4$, 10 µg denatured calf thymus DNA and sufficient S_1 nuclease to degrade this amount of DNA. The samples were incubated for 30 min at 45^o, followed by addition of 40 µg yeast tRNA and precipitated with 5% trichloroacetic acid. Precipitates were collected on millipore filters, dried and counted in the Packard Tri-carb Liquid Scintillation Spectrometer.

3.3. ASSOCIATION OF VIRUS-SPECIFIC RNA WITH FREE AND MEMBRANE-BOUND POLYRIBOSOMES

To study the presence of virus-specific RNA sequences in free and membrane-bound polyribosomes, an isolation procedure was chosen, by which cross contamination of both types of polyribosomes was prevented. Contamination of free polyribosomes with membrane-bound polyribosomes was precluded by centrifugation of the 13,000 x g supernatant of the cell-lysate through a 2 M sucrose layer. In addition, by flotation of the polyribosomes associated with membranes through a layer of 1.8 M sucrose any contamination with free polyribosomes was eliminated.

The optical profiles of both classes of polyribosomes, monitored after centrifugation in isokinetic sucrose gradients, were practically identical (Fig. 16 A and B). From these gradients, fractions were collected and the polyribosomes were deproteinized with phenol-chloroform. The content of virus-specific RNA in each fraction was determined by hybridization with the labeled single-stranded DNA copy of the viral RNA (cDNA). For that purpose the RNA from each fraction was dissolved in the same volume of distilled water and samples of equal volume from these RNA solutions were taken for the hybridization reactions. When the two hybridization patterns of viral cDNA, RNA from free and membrane-bound polyribosomes were compared, it appeared that approximately six times as much of the former RNA had to be used to match the level

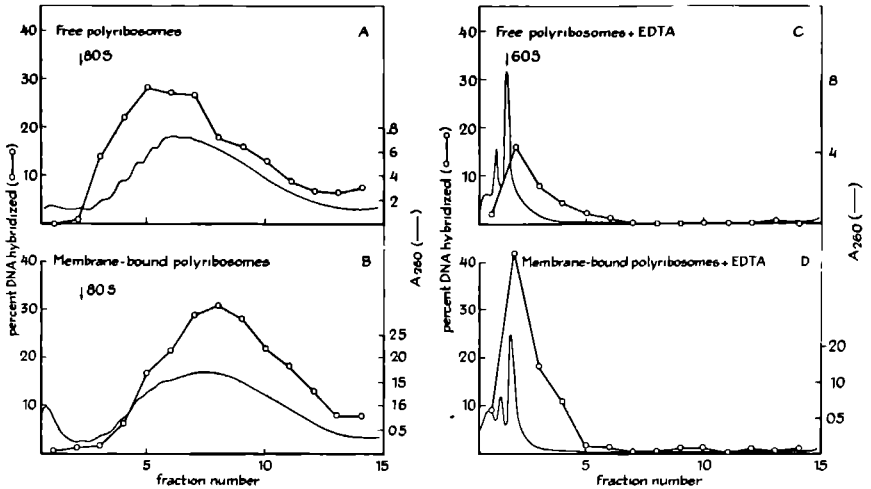


Fig. 16. Virus-specific RNA in free and membrane-bound polyribsomes of R-MuLV infected cells. Free and membrane-bound polyribosomes or ribonucleoprotein particles released by EDTA treatment of these polyribosomes were analyzed in isokinetic sucrose gradients (section 3.2.5.). Fractions of 0.8 ml were collected and the polyribosomes were precipitated with ethanol. After extraction of the RNA, aliquots of each fraction were hybridized with R-MuLV cDNA. (A) Free polyribosomes; (B) membrane-bound polyribosomes; (C) free polyribosomes dissociated by EDTA; (D) membrane-bound polyribosomes dissociated by EDTA.

of hybridization with the latter RNA. This observation indicates that of both types of polyribosomes membrane-bound polyribosomes were especially enriched with virus-specific sequences. Hybridization of cDNA occurred with RNA from free and membrane-bound polyribosomes of all size classes and followed almost the optical profile of the ribosomes (Fig. 16 A and B). The highest absolute amount of virus-specific RNA was associated with polyribosomes with sedimentation values of 300-400S. However, when the same amount of RNA from each polyribosomal fraction was used in the hybridization reaction maximal hybridization, the highest relative amount, was found in polyribosomes with sedimentation values of 450-550S (Fig. 17). These sedimentation values correspond with polyribosomes containing 13-18 ribosomes per message.

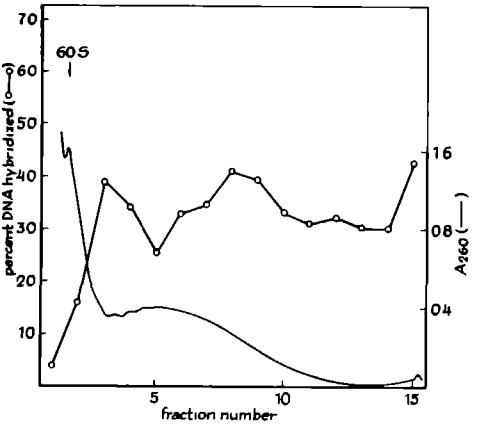


Fig. 17. Distribution of virus-specific RNA sequences in polyribosomes isolated from R-MuLV infected cells by detergent treatment. A mixture of free and membrane-bound polyribosomes was prepared by lysis of cells in the presence of nonidet P-40 as described in section 3.2.4. After lysis of the cells, nuclei and cell debris were removed by low speed centrifugation at 750 x g for 5 min. The supernatant was directly layered onto an 20.0-30.7% (w/v) isokinetic sucrose gradient in TMK buffer and centrifuged at 5^{o} in an SB 283 rotor of the IEC centrifuge for 45 min at 200,000 x g. Fractions of 0.8 ml were collected and polyribosomes were precipitated with ethanol. From each fraction an equal amount of RNA was hybridized with the R-MuLV specific cDNA probe.

To provide evidence that the virus-specific RNA sedimenting with polyribosomes represented virus-specific mRNA in functioning polyribosomes, aliquots of the free and membrane-bound polyribosomes were treated with 15 mM EDTA. By this procedure, polyribosomes are dissociated and the mRNA is released as mRNP particle. Fig. 16 C and D show that no virus-specific RNA was recovered from the original polyribosomal region of the gradient. All hybridizable RNA was released and displaced towards the top of the gradient. Therefore, it is unlikely that the virus-specific RNA associated with polyribosomes is due to virion (core) particles co-sedimenting with polyribosomes. From these experiments we conclude that the virus-specific RNA

associated with free and membrane-bound polyribosomes of R-MuLV infected cells, is identical to the viral mRNA fraction.

3.4. CHARACTERIZATION OF THE VIRUS-SPECIFIC mRNA

In order to determine the size of the virus-specific mRNA species associated with polyribosomes, RNA was extracted from total polyribosomes and the purified free and membrane-bound polyribosomes. Following centrifugation of the extracted RNA through isokinetic glycerol gradients, the separate fractions were assayed for virus-specific RNA by hybridization with the labeled R-MuLV DNA probe. In a preliminary experiment we established that no significant hybridization occurred in the 60-70S region of gradients resolving particles with sedimentation values up to 100S (not shown).

Fig 18A illustrates the size distribution of hybridizable RNA extracted from a mixture of free and membrane-bound polyribosomes (total polyribosomes) A rather heterogeneous distribution of virus-specific RNA was observed, with two predominant peaks in the 35S and 20-22S regions of the gradient. In addition a shoulder of hybridizable RNA was detected in the 14S region. The 35S RNA associated with polyribosomes corresponds in size with the virion RNA subunit, visualized by denaturation of the virion 60-70S RNA (22,97). The same three classes, 35S, 20-22S and 14S, of virus-specific RNA were found in purified free and membrane-bound polyribosomes (Fig. 18B and C) Whereas the 20-22S and 14S RNAs species were minor components only of the virus-specific RNA in free polyribosomes, they constituted the major part of the hybridizable RNA of membrane-bound polyribosomes.

The presence of similar virus-specific mRNA species in free and membrane-bound polyribosomes allows several explanations:

1. Virus-specific mRNA from either type of polyribosomes may code for different polypeptides, despite the similarity of the S values in both populations.
2. The same viral polypeptides are synthesized on both types of polyribosomes; Polypeptide synthesis starts on free polyribosomes and after the nascent chains have been elongated to a certain length polyribosomes become membrane-bound.

The first explanation is unlikely, since virus-specific RNA from both classes of ribosomes hybridized to 100% with the labeled DNA probe In order to get some evidence for the second possibility, RNA from free and

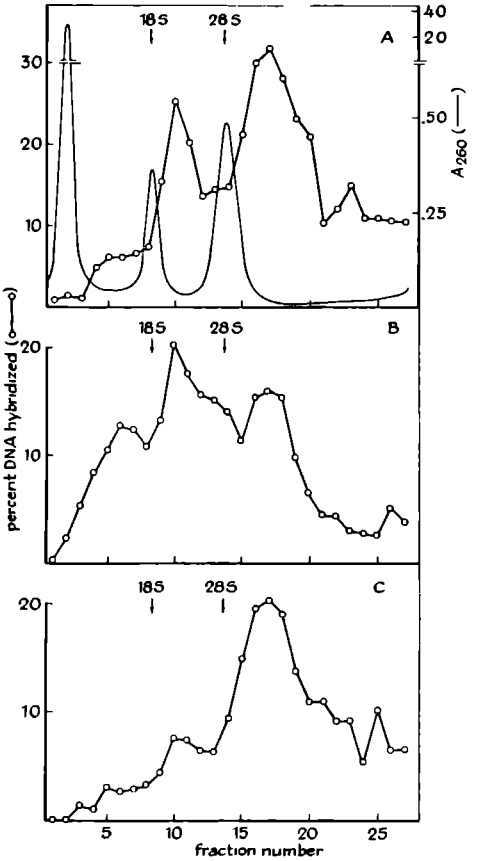


Fig. 18. Size distribution of R-MuLV specific RNA associated with polyribosomes. Free, membrane-bound and total polyribosomes were deproteinized with phenol. The extracted RNA was dissolved in TNE buffer containing 0.5% sodium dodecyl sulfate and analyzed in isokinetic glycerol gradients (section 3.2.6). Fractions of 0.4 ml were collected and precipitated with ethanol after addition of 20 µg *E.coli* tRNA as carrier. The relative amount of virus-specific RNA in each fraction was determined by hybridization with R-MuLV cDNA.
(A) RNA from total polyribosomes; (B) RNA from membrane-bound polyribosomes; (C) RNA from free polyribosomes.

membrane-bound polyribosomes of different gradient fractions (see insets of Fig. 19A, B and C and Fig. 19D, E and F) was isolated and analyzed in glycerol gradients. In all size classes of free polyribosomes three discrete RNA

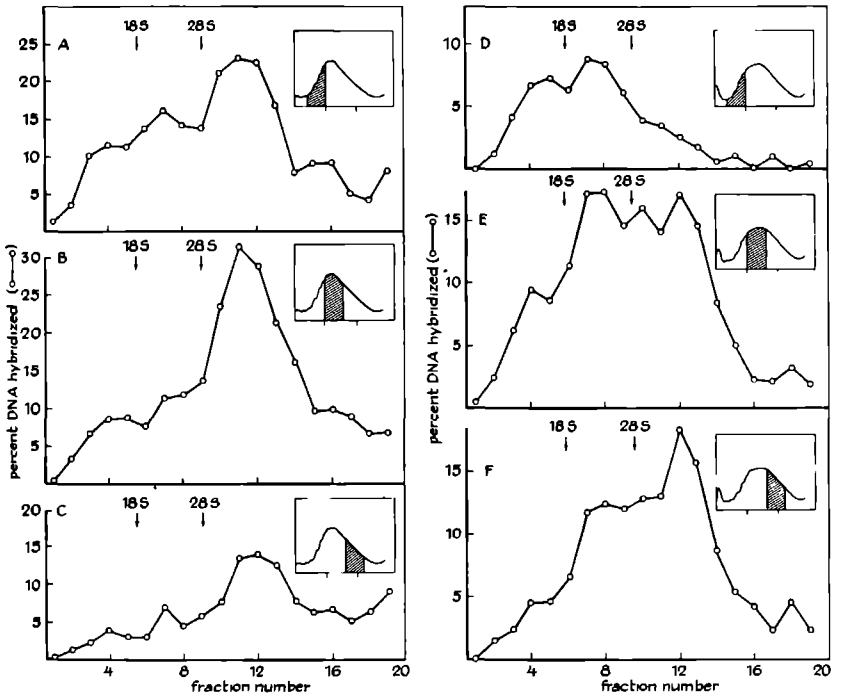


Fig. 19. Size distribution of R-MuLV-specific RNA associated with free and membrane-bound polyribosomes of different size classes. The sedimentation profiles of isolated free and membrane-bound polyribosomes in 20-30.7% (w/v) isokinetic sucrose gradients are depicted in the insets. Fractions from these gradients were pooled as indicated by the *hatched areas*. After deproteinization of the polyribosomes, the purified RNA was analyzed on isokinetic glycerol gradients (section 3.2.6.). Fractions of 0.6 ml were collected, and the RNA was precipitated with ethanol after addition of 20 µg *E.coli* tRNA as carrier. The relative amount of virus-specific RNA in each fraction was determined by hybridization with the R-MuLV-specific cDNA.

species with sedimentation values of 35S, 20-22S and 14S were present

(Fig. 19A, B and C). However, the lower molecular weight RNA species were present in a higher content in small polyribosomes (1-6 ribosomes) than in large polyribosomes (more than 6 ribosomes). This is in agreement with the theoretical expectation that small messengers are associated with less ribosomes than larger messengers. The picture obtained from material derived from membrane-bound polyribosomes was quite different (Fig. 19D, E and F). Almost no hybridizable RNA was found in the 35S region of that gradient, when polyribosomes up to six monomers were analyzed (Fig. 19D). Furthermore, it was found that the ratio of virus-specific 35S RNA to the smaller virus-specific RNAs of 14S and 20-22S was higher in the larger size classes of the polyribosomes (Fig. 19B and C). This effect was particularly pronounced in membrane-bound polyribosomes with more than 13 monomers, whereas for free polyribosomes an almost similar distribution was observed for polyribosomes with 6-13 and larger than 13 monomers.

If the distribution of virus-specific RNA species in different size classes of membrane-bound polyribosomes is representative for the situation *in vivo*, these results suggest that the viral polypeptide synthesis directed by the 35S messenger may start on free polyribosomes, which thereafter, become attached to membranes. Our results do not allow any conclusion concerning the mechanism which may be operative for the 14S and 20-22S RNA species. The results presented were not significantly affected if the pretreatment of the cells with 0.1 µg/ml of cycloheximide was omitted.

3.5. RELATIVE AMOUNTS OF VIRUS-SPECIFIC MESSENGER RNA IN POLYRIBOSOMES

The percentage of polyribosomal RNA representing virus-specific sequences can be computed by comparison of the half-C_rt values of polyribosomal and viral RNA (Table 2). The values indicated that about 0.2 percent of the RNA of total polyribosomes was virus-specific. Fan and Baltimore (160) found that in uninfected JLS-V9 cells approximately 0.02 percent of the cytoplasmic RNA was hybridizable with the R-MuLV DNA probe, whereas approximately 0.25 percent of the cytoplasmic RNA of Moloney murine leukemia virus infeced JLS-V9 cells was virus-specific. Thus, hybridization data for Moloney leukemia virus infected JLS-V9 cells agree well with our calculations of the virus-specific RNA content of polyribosomes from R-MuLV infected JLS-V9 cells. Finally, our results show that the relative content of virus-specific

RNA was 5 to 10 fold higher in membrane-bound polyribosomes than in free polyribosomes.

TABLE 2

VIRUS-SPECIFIC RNA IN POLYRIBOSOMES

RNA	Half-C_rt (mole-sec/liter)	% virus-specific RNA
60S R-MuLV	1.58×10^{-2}	100
Free polyribosomes	3.16×10^{1} (3.98×10^{1}) *	0.05 (0.04)*
Membrane-bound polyribosomes	5.63 (3.98)*	0.30 (0.40)*
Total polyribosomes	7.95	0.20

RNA was isolated from free, membrane-bound and total polyribosomes. Increasing amounts of each RNA fraction were hybridized to 800 cpm of ^{3}H -labeled R-MuLV-specific cDNA. The percentage of DNA hybridized was plotted in a semi-logarithmic graph against the product of RNA concentration x time (C_rt). The proportion of virus-specific sequences in the polyribosomal RNA was calculated by comparison of the C_rt values at which 50% of the maximal hybridization was obtained with the half C_rt of the 60S RNA of R-MuLV.

* RNA isolated from polyribosomes of cells pretreated with 0.1 µg/ml of cycloheximide.

3.6. DISCUSSION

Most cell fractionation procedures for the isolation of free and membrane-bound polyribosomes from tissue culture cells have the disadvantage of giving low yields of membrane-bound polyribosomes. A substantial proportion of polyribosomes is removed together with nuclei and cell debris at 750 x g. From the JLS-V9 cell line only 5 to 10 percent of the polyribosomes could be recovered as membrane-bound polyribosomes. Nevertheless, our results obtained after cell lysis in the presence of detergents (Fig. 18A) indicate that

we have isolated a representative sample of the total population of membrane-bound polyribosomes, at least as far as the total content of hybridizable sequences in mRNA is concerned. Moreover, in chapter 5 a different technique was employed for the isolation of both types of polyribosomes, yielding 20-30 percent of the polyribosomes as membrane-bound polyribosomes and no result obtained with this technique was in conflict with the conclusion arrived in the present chapter.

While this work was in progress Vecchio *et al* (293) reported the finding of virus-specific RNA and nascent polypeptides in free and membrane-bound polyribosomes from cells replicating the Moloney strain of murine sarcoma-leukemia virus [Mo-SV (Mo-MuLV)]. Moreover, they calculated that polyribosomes bound to membranes contained between 4 and 5 times more virus-specific RNA than free polyribosomes. Their results are in agreement with our data presented in this chapter.

Sedimentation analysis of the RNA associated with total polyribosomes derived from R-MuLV infected JLS-V9 cells indicated the presence of three discrete size classes of virus-specific RNA with estimated molecular weights of 3×10^6 (35S), $1.0\text{-}1.5 \times 10^6$ (20-22S) and 0.4×10^6 (14S). However, after separation of cellular ribosomes in free and membrane-bound polyribosomes a striking difference in the size distribution of hybridizable RNA associated with both types of polyribosomes was observed. Whereas one major virus-specific RNA species (35S) was detected in free polyribosomes, three major size classes (35S, 20-22S and 14S) were found in membrane-bound polyribosomes.

It should be mentioned that the hybridization profile does not necessarily reflects the actual distribution of virus-specific RNA sequences and allows only a qualitative interpretation. A quantitative analysis is obtained if two criteria are fulfilled: all of the RNA sequences detectable with the cDNA must be present in each fraction and these sequences must be in excess of the DNA probe (160). We were able to demonstrate that 100% hybridization was possible with RNA sedimenting in the 35S and 20-22S region. Moreover, the concentration of RNA in the hybridization reaction was always in excess of the DNA probe. The above criteria were, therefore, at least partially met.

After this work was finished, several reports on the identification of virus-specific mRNA in cells infected with murine and avian RNA tumor viruses were published (160-162). The most prominent virus-specific mRNA species

associated with polyribosomes was identical in size to the viral RNA subunit (30-40S). Furthermore, all investigators reported that isolated polyribosomes contained a second fraction of virus-specific RNA with sedimentation values of 20-30S. The distribution of virus-specific mRNA species between free and membrane-bound polyribosomes has been studied by Shanmugan *et al* (162). They found that free polyribosomes from Mo-SV (Mo-MuLV) transformed cells contained only virus-specific mRNA with a sedimentation value of 35S. In contrast, two species of virus-specific mRNA, 35S and approximately 20S, were found in association with membrane-bound polyribosomes. These findings are in accordance with our data on the R-MuLV-specific mRNA in different types of polyribosomes.

The finding of 35S mRNA in association with polyribosomes is not surprising, since this mRNA is identical in size to the 35S subunits of the virion 60-70S RNA. Reliable experimental evidence exists for the assumption that the 35S RNA subunits of the virion RNA are identical (147,150). For that reason we may suggest that the virion 35S subunits and the virus-specific 35S mRNA encode the same polypeptides. This notion is substantiated by our results from studies concerning the cell-free translation of virus-specific RNA from polyribosomes (252, Chapter 5) and by those of Salden *et al* (157) on the translation of native and denatured RNA of R-MuLV.

The physiological function of the virus-specific 20-22S and 14S RNA is more difficult to understand. It is important to find out whether the RNA population sedimenting slower than 35S consists of functional virus-specific mRNAs or is a trivial cleavage product of the 35S messenger. Several possibilities to explain degradation of 35S mRNA may be considered:

1. The 20-22S and 14S RNA is an *in vivo* degradation product of the 35S RNA.
2. RNA associated with polyribosomes is degraded by nucleases during the isolation of the polyribosomes.
3. The 35S RNA is degraded during the deproteinization of the polyribosomes.

If the third possibility is correct the extent of degradation should be the same in each polyribosomal fraction. The results presented in Fig. 18 and Fig. 19 show that this is not the case. In this moment no definite evidence is available to exclude possibility 2. We can only mention that the presence of purified RNAase inhibitor or of the nuclease inhibitors heparin and diethylpyrocarbonate during homogenization of the cells did not affect the results. In addition, Tsuchida *et al* (294) reported that Mo-SV(Mo-MuLV)

transformed cells contained two discrete species of virus-specific RNA with sedimentation coefficients of 35S and 20S, and almost no lower molecular weight RNA was detected. In contrast, virus-specific 33S RNA but no 20S RNA was found in hamster cells nonproductively transformed by Mo-SV (295). Finally, Fan and Besmer (295) detected two virus-specific RNA species, 38S and 27S, in uninfected JLS-V9 cells by hybridization of the cellular RNA with cDNA complementary to the RNA of endogenous virus, liberated from these cells by iododeoxyuridine treatment. Presumably, these RNA species correspond to the 35S and 20-22S RNAs of cells productively infected with R-MuLV. The above observations do not support the possibility that the 14S and 20-22S RNA fractions arose by degradation of the 35S RNA during the isolation procedure, but more likely are a reflection of the *in vivo* situation. No experimental evidence is available to exclude the first alternative. Two approaches may be considered to study this possibility, first by examining *in vivo* whether the 14S and 20-22S RNA fractions function as messenger for virus-specific proteins and second, a more obvious approach, by demonstrating the cell-free synthesis of virus-specific proteins under the direction of these RNA fractions.

It is generally accepted that attachment of ribosomes to membranes facilitates the synthesis and sequencial secretion of proteins destined for export or may even be required for that function (296-298). However, in cells which do not secrete proteins membrane-bound polyribosomes are present as well (299, 300). Furthermore, we could demonstrate the synthesis of almost the same set of polypeptides by free and membrane-bound polyribosomes from Ehrlich ascites tumor cells or JLS-V9 cells (299, chapter 5). Thus, although binding of polyribosomes appears to be a more general phenomenon, these observations do not exclude that certain messengers may be preferentially associated with free or membrane-bound polyribosomes. Our hybridization data indicated that both types of polyribosomes contained the same size classes of virus-specific RNA. However, the relative amount of virus-specific RNA was 4-10 times less on free polyribosomes than on membrane-bound polyribosomes, suggesting the preferential synthesis of virus-specific polypeptides on the latter class of polyribosomes.

If the virus-specific RNA species associated with free and membrane-bound polyribosomes are representative for the situation *in vivo*, the presence of 35S mRNA in both types of polyribosomes, together with our

observation that this RNA is almost absent in membrane-bound polyribosomes up to 230S, suggest that the virus-specific protein synthesis directed by this mRNA starts on free polyribosomes. That such a mechanism may be operative has been proposed by several authors (293,302,303). The synthesis of virus-specific proteins on membrane-associated polyribosomes may be necessary to facilitate the maturation process of virus-specific precursor polypeptides, involving proteolytic cleavage and glycosylation. Additional evidence for the role of membranes in the synthesis of virus-specific polypeptides is presented in chapter 5.

CHAPTER 4

VIRUS-SPECIFIC RIBONUCLEIC ACID IN CELLS INFECTED WITH MURINE LEUKEMIA AND SARCOMA VIRUSES: EFFECT OF DENATURATION AND ISOLATION OF MESSENGER RNA

4.1. INTRODUCTION

Denaturation of the 60-70S RNA of RNA tumor viruses by heat or organic solvents gives rise to 30-40S RNA subunits (21,22,95), a discrete population of 4S, 5S and 7S RNA and heterogeneous RNA sedimenting between 10S and 30S (88-93,95,173,177,304,352-354). Avian sarcoma virions harvested from infected cells at 3 min intervals, yield a class of 60-70S RNA, a major class of 30-40S RNA and 4-12S RNA (105). Most of the 30-40S RNA in early harvest virus was converted to 60-70S RNA by incubation of the virus preparation at 40^{o}. Upon denaturation, the RNA from early harvest virus is converted into a rather uniform population of 30-40S RNA and a heterogeneous population of 10-30S RNA molecules. The proportion of the latter fraction was strongly increased in RNA preparations derived from late harvest virus (104). Consequently, the heterogeneous population of small RNA is thought to be produced as a result of random nicking of the 35S RNA species, by nucleases present within the virion (305,306).

When the polyribosomal RNA of Rauscher murine leukemia virus (R-MuLV) infected JLS-V9 cells was sedimented in sucrose gradients, peaks of virus-specific RNA were detected sedimenting at 35S, 20-22S and in the 14S region (chapter 3). We could not exclude the possibility that the virus-specific RNAs, sedimenting slower than 30S, were cleavage products of the virus-specific 35S RNA. Therefore, and because probably also aggregation of virus-specific RNA had occurred, we examined whether a different distribution of hybridizable RNA was obtained after denaturation of polyribosomal RNA, prior to centrifugation. In addition, we compared the size distribution of intracellular virus-specific RNA of R-MuLV infected JLS-V9 cells and murine sarcoma virus [Mo-SV (Mo-MuLV)] transformed rat embryo cells.

The 60-70S RNA genome of RNA tumor viruses contains adenylic acid-rich sequences (111-114), which are covalently linked to the 3' end of the viral RNA (115,117-119). The molecular weight of each poly(A)-rich track was estimated to be about 60,000, whereas the total molecular weight of the adenylic

acid-rich segments was about 250,000. Assuming that each 60-70S RNA consists of 3 to 4 30-40S subunits it was concluded that each subunit contains about one large poly(A) track (111,112,115). However, nothing is known about the actual presence of internal oligo(A) sequences. Scolnick *et al* (307) have reported the presence of poly(A)-rich sequences in the virus-specific RNA isolated from rat cells nonproductively transformed by murine sarcoma virus. For these reasons, it seems likely that the virus-specific RNA associated with polyribosomes contains poly(A) tracks as well.

The association of various virus-specific RNA species with polyribosomes strongly suggests that these RNA molecules are involved in the synthesis of virus-specific polypeptides. However, more convincing evidence for their messenger function would be provided, if we were able to demonstrate the synthesis of virus-specific polypeptides under the direction of these RNA fractions. In addition, analysis of the products formed by the various species of virus-specific mRNAs would provide valuable information on a phase of the 'life-cycle' of RNA tumor viruses that is still poorly understood. In order to achieve this purpose, we attempted to purify the poly(A)-containing RNA from infected cells by oligo(dT)-cellulose affinity chromatography.

4.2. MATERIALS AND METHODS

4.2.1. *Cells and virus*

R-MuLV infected cells were grown in monolayer as described in section 3.2.1. The $78A_1$ cell line, chronically infected with the Moloney strain of murine sarcoma virus [Mo-SV(Mo-MuLV)] was grown in monolayer in Eagle's Minimal Essential Medium, completed with 10% foetal calf serum, penicillin (100 I.U. x ml^{-1}) and streptomycin (50 µg x ml^{-1}).

4.2.2. *Isolation of total polyribosomes and purification of RNA*

Conditions for optimal growth of cells were chosen to obtain a maximal yield of polyribosomes. Thus, cells in logarithmic phase were replenished with fresh growth medium 16 h and 3 h, before harvesting. In addition 0.1 µg x ml^{-1} of cycloheximide was added 20 min before trypsinization of the cells. The isolation of total polyribosomes was described in section 3.2.4.

For the isolation of RNA, polyribosomal suspensions were deproteinized as described in section 3.2.6. In order to minimize processing of RNA chains in some experiments (as indicated in the legends to the figures) polyribosomes were dissociated with 2% sodium dodecyl sulfate in TNE (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA) for 5 min at 37^{o} and directly analyzed at 5^{o} in a 15-43.5% (w/w) isokinetic glycerol gradient containing TNE by centrifugation at 95,000 x g for 14 h in an SW 27 rotor. Fractions of 2 ml were collected from the bottom of the centrifuge tubes with continuous u.v. monitoring with an LKB optical unit.

4.2.3. *Isolation of cytoplasmic RNA*

Optimal growth conditions were as described before (section 4.2.2.). All operations up to swelling of cells in hypotonic buffer were carried out as described in section 3.2.3. After swelling, the cells were collected by centrifugation for 5 min at 700 x g. Packed cells (0.5 ml) were resuspended in 6 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 150 mM NaCl and 2 mM 1,4 dithioerythritol). Subsequently 0.7 ml of a solution of 5% nonidet P-40 and 2.5% sodium deoxycholate was added to the cell suspension and cells were lysed by 10 strokes of a tight fitting Dounce homogenizer (B pestle). When indicated, the RNAase inhibitor diethylpyrocarbonate was added to a final concentration of 0.1%, prior to disruption of the cells. Nuclei and cell debris were removed by 5 min centrifugation at 700 x g. Because of the high amount of protein, the cytoplasmic supernatant was diluted to 30 ml with phenol extraction buffer (50 mM Tris-HCl, pH 8.0 at 20^{o}, 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulfate) and deproteinized with phenol-chloroform (section 3.2.6.).

4.2.4. *Oligo(dT)-cellulose chromatography*

Method 1

Oligo(dT)-cellulose chromatography of phenol extracted polysomal RNA was performed according to Aviv and Leder (308). All operations were performed at room temperature. Fifty A_{260} units of deproteinized polysomal RNA, dissolved in buffer A (10 mM Tris-HCl, pH 7.5 at 20^{o}, 500 mM NaCl, and 0.05% of sodium

dodecyl sulfate) were applied onto a 2 ml oligo(dT)-cellulose column, prepared according to Gilham (309), in buffer A and elution was started at a flow rate of 4 ml x h^{-1}. To remove all non-adsorbed material the elution was continued until the A_{260} level of the eluate had dropped below 0.025. The material retained to the column was eluted in two steps: In the first step RNA, weekly bound to the oligo(dT)-cellulose column was eluted with 10 ml of buffer B (10 mM Tris-HCl, pH 7.5 at 20° and 100 mM NaCl), whereas in the second step poly(A)-rich RNA was eluted with 3 ml of buffer C (10 mM Tris-HCl, pH 7.5 at 20°). RNA-containing fractions eluted with buffer A, B and C were adjusted to 0.2 M in sodium acetate pH 5.2 and the RNA was precipitated with two volumes of ethanol at -20°. The amount of RNA retained and eluted with buffer C was 1-2% of the total RNA applied.

Method 2

Poly(A)-rich RNA used in cell-free systems was isolated according a slightly modified procedure. Fifty A_{260} units of polyribosomes were dissociated in 4 ml buffer A (10 mM Tris-HCl, pH 7.5 at 20°, 500 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulfate) at 37° for 5 min. The dissociated polyribosomes were directly applied onto a 2 ml oligo(dT)-cellulose column in buffer A. Elution with buffer A and buffer B (10 mM Tris-HCl, pH 7.5 at 20°, 100 mM NaCl, 1 mM EDTA and 0.05% sodium dodecyl sulfate) was performed as described for method 1. Poly(A)-rich RNA specifically bound to the column was eluted with buffer C and precipitated with ethanol at -20°. Routinely about 60-80 µg of poly(A)-containing RNA was obtained by this procedure.

4.2.5. *Poly(dT)-cellulose chromatography*

Poly(dT)-cellulose was a kind gift of Dr. U. Bertazzoni (Laboratoire de Génétique Moléculaire, Institut de Biologie Moléculaire, Paris, France) and was prepared as described by Bertazzoni *et al* (310). The approximate length of the poly(dT) chain was 50-70 nucleotides. 500-750 µg of phenol extracted polysomal RNA in 500 µl of buffer 1 (10 mM Tris-HCl, pH 7.5 at 20°, 100 mM NaCl, 1 mM EDTA and 0.05% sodium dodecyl sulfate) was applied at 20° to a 200 µl column of poly(dT)-cellulose. Unadsorbed material was washed out with 3 ml of buffer 1. Subsequently, RNA weekly bound to the column was eluted

with 1 ml of buffer 2 (10 mM Tris-HCl, pH 7.5 at 20^{o} and 16 µg x ml^{-1} of wheat germ tRNA). Poly(A)-rich RNA was eluted at 50^{o} with 0.75 ml of buffer 2. The eluted material was precipitated at -20^{o} by addition of 0.1 volume of 2 M sodium acetate pH 5.2 and 2 volumes of ethanol. In this way 15-20 µg poly(A)-containing RNA was recovered.

4.3. FORMAMIDE AND HEAT DENATURATION OF POLYRIBOSOMAL RNA

Total polyribosomes from R-MuLV infected JLS-V9 cells were isolated and deproteinized with phenol-chloroform. When denaturation of polyribosomal RNA was omitted, the hybridization profile shown in Fig. 2OC was obtained. As expected, the predominant RNA species that annealed with the R-MuLV-specific DNA probe sedimented in the 30-40S region of the gradient. A drastic change in the hybridization profile was revealed after pretreatment of the polyribosomal RNA with 85% formamide at 37^{o} for 5 min prior to centrifugation. As shown in Fig. 2OA, hybridizable RNA had accumulated in the 14S and 20-22S regions at the expense of the 30-40S region. Moreover, virtually no hybridizable RNA was detected in the bottom fractions of this gradient (compare fractions 16, 17, 18 and 19 in Figs. 2OA and C) indicating the effectiveness of the denaturation procedure. For comparison polyribosomal RNA was denatured at 55^{o} for 5 min in 10 mM Tris-HCl, pH 7.5 at 20^{o} (Fig. 1 B). Heat denaturation, prior to centrifugation established a similar shift of hybridizable RNA from the 30-40S region of the gradient to the 10-30S region as the one observed after formamide denaturation. These results indicate aggregate formation of virus-specific mRNA on sucrose gradients, which could be prevented by thermal or formamide denaturation, prior to centrifugation. It is hard to exclude the possibility, however, that hidden nicks are visualized through denaturation of intramolecular H-bonds. Some of the following experiments suggest that hidden nicks caused at least part of the observed shift.

In a second experiment polyribosomes were dissociated with 2% sodium dodecyl sulfate in TNE buffer for 5 min at 37^{o}, and directly applied onto isokinetic gradients. RNA sedimenting faster than 28S (fraction 1), between 28S and 18S (fraction 2) and slower than 18S (fraction 3), was pooled and precipitated with ethanol. The RNA of these pooled fractions was treated with 85% formamide at 37^{o} and separately analyzed in isokinetic gradients. Although cross contamination of the pooled fractions occurred, it is evident

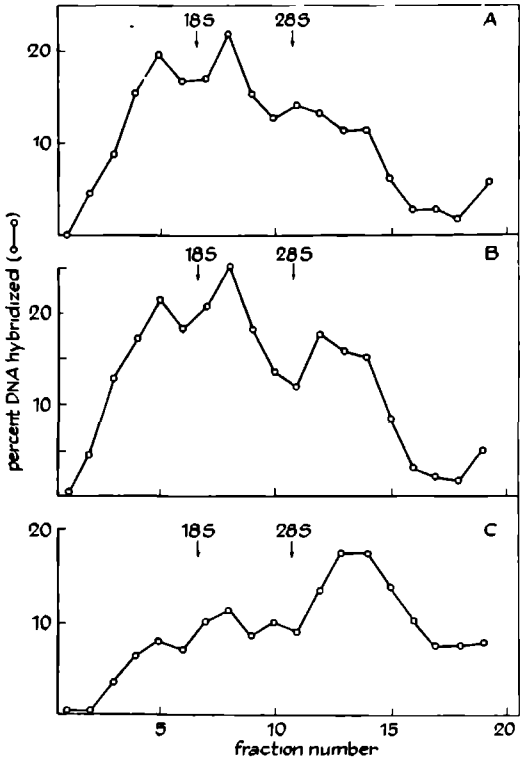


Fig. 20. Size distribution of R-MuLV specific mRNA after denaturation by heat of formamide. (A) Polyribosomal RNA was denatured at 37° for 5 min in 85% formamide, 10 mM Tris-HCl, pH 7.5 at 20° and 0.5% sodium dodecyl sulfate. After dilution with 3 volumes of TNE buffer the sample was directly applied onto an isokinetic glycerol gradient; (B) Polyribosomal RNA after thermal denaturation at 50° for 5 min in 10 mM Tris-HCl, pH 7.5 at 20° and 0.5% sodium dodecyl sulfate. After dilution with TNE buffer the sample was directly analyzed in a glycerol gradient; (C) Polyribosomal RNA analyzed in a glycerol gradient without prior denaturation. The 15-30.2% (w/v) isokinetic glycerol gradients in TNE buffer were centrifuged at 5° in a Spinco type Ti-SW 41 rotor for 13 h at 150,000 x g. Fractions of 0.6 ml were collected and the relative amount of virus-specific RNA was determined as described before (Fig. 18).

that virus-specific RNA from the 14S and 20-22S regions did not aggregate

under these conditions (Fig. 21B and C). Recentrifugation of the denatured RNA from fraction 1 revealed a broad heterogeneous distribution of virus-specific RNA molecules and hybridization peaks were detectable in the 20-22S and 30-40S region of the gradient (Fig. 21A).

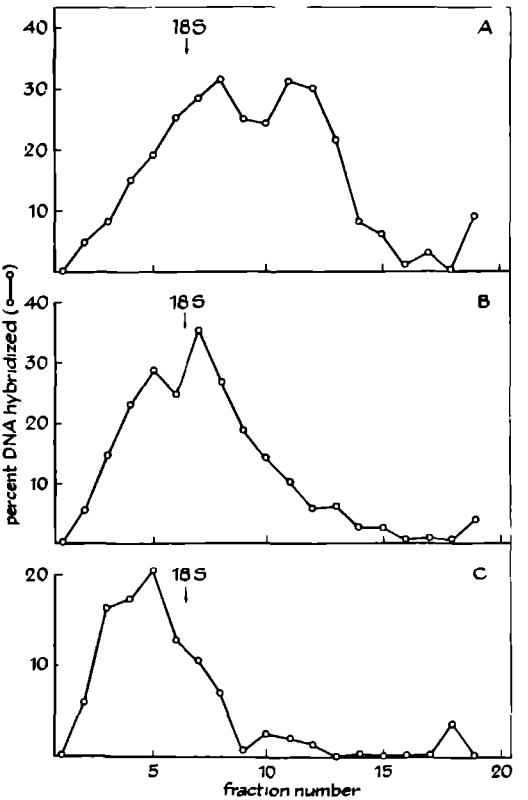


Fig. 21. Effect of formamide denaturation on the sedimentation pattern of different size classes of R-MuLV-specific mRNA.. Purified polyribosomes were dissociated with sodium dodecyl sulfate and the polyribosomal RNA was analyzed in glycerol gradients as described in section 4.2.2. RNA fractions sedimenting faster than 28S (1), between 18S and 28S (2) or slower than 18S (3) were pooled and precipitated with ethanol. RNA of the pooled fractions was denatured with 85% formamide (Fig. 20) and analyzed in an isokinetic gradient (section 3.2.6.). The relative amount of R-MuLV-specific RNA in each fraction was determined as described before (Fig. 18). (A) Size distribution of denatured RNA from fraction 1; (B) from fraction 2; (C) from fraction 3.

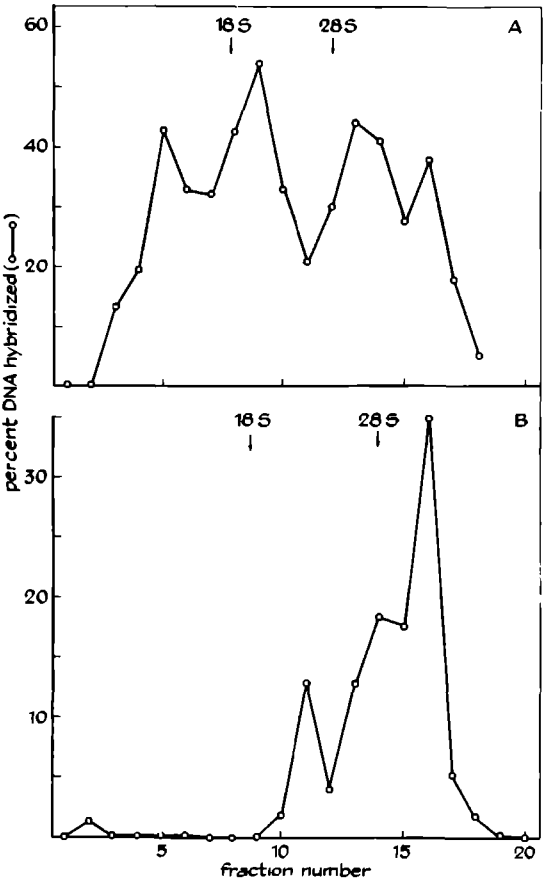


Fig. 22. (A) Size distribution of R-MuLV-specific RNA isolated from the cytoplasm of JLS-V9 cells. Cytoplasmic RNA was denatured with 85% formamide (Fig. 20) and layered onto a 25-38.8% (w/v) isokinetic sucrose gradient in TNE buffer. Gradient centrifugation was performed at 5^{0} in a Spinco type Ti-SW 41 rotor for 15 h at 160,000 x g. RNA sedimenting in the 30-40S region was pooled and precipitated with ethanol after addition of 20 µg 28S RNA from rabbit reticulocyte ribosomes. (B) Sedimentation of purified virus-specific 30-40S RNA in a sucrose gradient containing formamide. In order to remove contaminating RNA the 30-40S RNA fraction, isolated as described in the legend to Fig. 22A, was recentrifuged in sucrose; the 30-40S region of this gradient was pooled and the RNA was precipitated with ethanol after addition of 20 µg of 28S retic RNA. Pooled RNA was denatured with 85% formamide (Fig. 20) and applied onto

a 5-22.7% (w/v) isokinetic sucrose gradient in TNE buffer, and containing 50% (v/v) formamide. Centrifugation was performed at 4^{0} in an Ti-SW 41 rotor for 15 h at 200,000 x g. The relative amount of R-MuLV-specific RNA was determined as described before (Fig. 18).

Similar results were obtained when cytoplasmic RNA isolated from R-MuLV infected JLS-V9 cells was treated with 85% formamide and analyzed in a sucrose gradient. Fig. 22A shows that three distinct virus-specific RNA species were resolved, sedimenting in the 30-40S, 20-22S and 14S regions of the gradient The hybridization profile has roughly the same shape as that shown in Fig. 20A for denatured polyribosomal RNA. A similar result was also obtained when the cytoplasmic RNA was isolated in the presence of the nuclease inhibitor diethylpyrocarbonate (not shown), suggesting that de-aggregation rather than exposure of hidden nicks, introduced during isolation, explains the shift in the centrifugation profile seen after formamide treatment In order to substantiate further that the virus-specific RNA in the 30-40S region consisted of physically intact RNA molecules, the following experiment was performed RNA from the 30-40S region of the gradient shown in Fig 22A was pooled and recentrifuged in a sucrose gradient to remove contaminating material. The 30-40S region of the second gradient was pooled and analyzed in a gradient containing 50% formamide. The profile of the hybridizable RNA shows that at least part of the virus-specific RNA, isolated from the 30-40S region of the first gradient, consisted of physically intact molecules (Fig. 22B). Since no detectable hybridization up to fraction 10 of the formamide containing gradient was observed, a random degradation of the virus-specific RNA during the handling of this preparation could be excluded. It should be noted that, although enough rRNA was present as carrier during all operations, we observed a substantial loss of virus-specific 30-40S RNA during handling.

In summary, these results indicate a similar size distribution for the virus-specific RNA isolated either from polyribosomes or from the cytoplasm of JLS-V9 cells. Thermal or solvent denaturation resulted in a transition of part of the RNA from the 30-40S region to slower sedimenting fractions, mainly due to dissociation of noncovalent RNA aggregates.

4.4. INTRACELLULAR VIRUS-SPECIFIC RNA FROM CELLS TRANSFORMED BY THE MOLONEY MURINE SARCOMA VIRUS

All preceding experiments were performed with material derived from the JLS-V9 cell line productively infected with R-MuLV. As an extension of these studies, we decided to examine the virus-specific RNAs of a rat embryo cell line ($78A_1$) transformed by the Moloney strain of murine sarcoma virus and producing both Mo-SV and Mo-MuLV. Cytoplasmic RNA from these cells was prepared and the size distribution of the virus-specific RNA in sucrose gradients was determined with the labeled R-MuLV DNA probe. Fig. 23A shows the hybridization profile of the cytoplasmic RNA. A sharp peak of virus-specific RNA was observed in the 30-40S region. In addition, a rather heterogeneous population of hybridizable RNA sedimenting between 10S and 28S was detected. It is obvious that there was only a low background of hybridization due to aggregation of RNA (Fig. 23A, fractions 15 and higher). The effect of heat and formamide denaturation on the size distribution of hybridizable RNA is shown in Fig. 23B and C. Most striking is the shift of virus-specific RNA from the 30-40S region to the 14S and 20-22S region of the gradient. The latter hybridization profiles strongly ressemble those obtained after denaturation of intracellular or polyribosomal RNA from the R-MuLV infected JLS-V9 cell line.

Considering these results we may conclude that the experiments described for the JLS-V9 cell line infected with R-MuLV do not reveal a situation unique for mammalian RNA tumor viruses.

4.5. ISOLATION OF POLY(A)-CONTAINING VIRUS-SPECIFIC mRNA

Separation of virus-specific RNA from the vast excess of ribosomal 18S and 28S is important if one wants to study the translation of viral messengers in cell-free systems. The most straightforward method for purification of mRNA is based on the observation that the RNA of RNA tumor viruses as well as many mammalian messengers contain poly(A)-rich regions which hybridize with oligo(dT) covalently bound to cellulose. Consequently, we attempted to purify virus-specific RNA from R-MuLV infected cells by oligo(dT)-cellulose (average chain length 10 nucleotides) and poly(dT)-cellulose (average chain length 60 nucleotides) chromatography. Phenol extracted polyribosomal RNA was applied

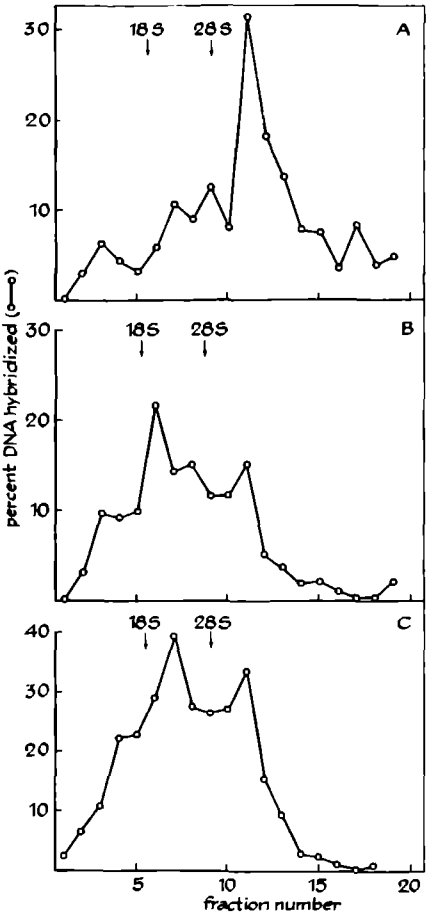


Fig. 23. Size distribution of intracellular MuLV-specific RNA isolated from rat embryo fibroblast cells ($78A_1$) transformed by the Moloney murine sarcoma virus. Rate zonal centrifugation of the cytoplasmic RNA, isolated in the presence of dietylpyrocarbonate, was performed in isokinetic sucrose gradients as described in the legend to Fig. 22A. The relative amount of virus-specific RNA was determined by hybridization with R-MuLV cDNA. (A) Size distribution of undenatured cytoplasmic RNA; (B) after heat denaturation at 55° for 3 min in 10 mM Tris-HCl, pH 8.0 and 0.5% sodium dodecyl sulfate; (C) after denaturation with 85% formamide in 10 mM Tris-HCl, pH 8.0 and 0.5% sodium dodecyl sulfate at 37° for 5 min.

onto the columns and the amount of virus-specific RNA in each eluted fraction was calculated from hybridization data. In table 3 the results of these experiments are summarized. About 50-60 percent of the virus-specific RNA was bound to the oligo(dT)-cellulose column and represents the poly(A)-rich RNA fraction. Since poly(dT)-cellulose has a higher affinity for poly(A), it was not surprising that almost 70 percent of the virus-specific RNA could bind to this column. Assuming that the hybridizable RNA in total polyribosomes is about 0.2 percent (section 3.5., table 2), it can be calculated that a 17 fold purification of virus-specific RNA was obtained. Thus, these experiments indicate that 50 to 70 percent of the virus-specific RNA associated

with polyribosomes contained poly(A)-rich sequences.

TABLE 3

Distribution of virus-specific RNA in fractions of polyribosomal RNA separated by oligo(dT)-cellulose and poly(dT)-cellulose affinity chromatography

	column fraction	NaCl concentration (M)	temp °C	Quantity of virus-specific RNA (%)	Virus-specific RNA (% of total RNA in fraction)
oligo(dT)-cellulose	A	0.5	20	37	0.06
	B	0.1	20	6	1.20
	C	0.0	20	57	3.50
poly(dT)-cellulose	I	0.1	20	23	N.D.
	II	0.0	20	9	N.D.
	III	0.0	50	68	N.D.

Phenol extracted polyribosomal RNA was fractionated on oligo(dT)-cellulose (section 4.2.4., method 1) or poly(dT)-cellulose columns (section 4.2.5.). A fixed amount of RNA from each fraction was hybridized with 800 cpm of [^{3}H]-labeled R-MuLV specific cDNA for varying lengths of time. The percentage of virus-specific sequences was calculated as described in Table 2.

For the preparative isolation of poly(A)-containing mRNA, we applied polyribosomes, dissociated in the presence of sodium dodecyl sulfate, directly onto the oligo(dT)-cellulose column. Since omission of deproteinization of polyribosomes by means of phenol did not affect the yield of poly(A)-containing RNA, it seems likely that in the presence of sodium dodecyl sulfate proteins do not interfere with the binding of the poly(A) tracks to the oligo(dT).

4.6. SIZE DISTRIBUTION OF THE POLY(A)-CONTAINING VIRUS-SPECIFIC mRNA

When the poly(A)-rich RNA fraction of the polyribosomal RNA was analyzed in a sucrose gradient, hybridizable RNA could be detected as a broad heterogeneous population throughout the gradient (not shown) Since this was possibly due to aggregation of RNA, the poly(A)-containing material was denatured with 85% formamide prior to centrifugation in a sucrose gradient Fig. 24 shows that two distinct peaks of hybridizable RNA were detected in the 30-40S and 20-22S region of the gradient In contrast to our earlier observations, formamide denaturation of the poly(A)-containing RNA did not result in a complete dissociation of noncovalent aggregates (Fig 24, fractions 15-20). Therefore, we tried to prevent aggregate formation of mRNA by using sucrose gradients containing 50% v/v of formamide The result of this experiment is shown in Fig 25. The size distribution of hybridizable RNA in this figure already indicates that aggregation of the poly(A)-rich RNA was precluded. Translation of the individual RNA containing fractions and analysis of the cell-free products supports this conclusion (chapter 5) The hybridization profile revealed two major classes of virus-specific RNA in the 30-40S and 20-22S region of the gradient containing formamide. In addition, it is striking that less hybridizable RNA was detected in the 14S and 20-22S region of this gradient than in gradients of formamide denatured polyribosomal RNA (Fig. 20A). This result suggests that part of the 14S and 20-22S virus-specific RNA contained no poly(A)-rich sequences.

Finally, these results indicate that oligo(dT)-cellulose chromatography is a useful tool for the isolation of poly(A)-containing virus-specific mRNA.

DISCUSSION

The change in size distribution of the virus-specific intracellular and polyribosomal RNA may be the result of mainly two effects: first, a change in size of the virus-specific RNA species due to dissociation of noncovalent RNA aggregates and second, the appearance of smaller virus-specific RNA molecules due to hidden nicks in the 30-40S viral mRNA. In section 4.3. we described that heat or formamide denaturation of the polyribosomal RNA of

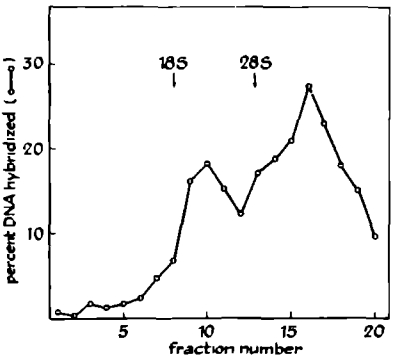


Fig 24 Rate zonal sedimentation of poly(A)-containing RNA isolated from polyribosomes of R-MuLV infected cells Poly(A)-containing RNA isolated by oligo(dT)-cellulose chromatography was denatured with formamide (Fig 20) and applied onto a 25-38 8% (w/v) isokinetic sucrose gradient (Fig 22A) (section 4.2.4., method 1) Centrifugation was performed at 5° in an Spinco type-SW 41 rotor for 17 h at 175,000 x g The relative amount of virus-specific RNA was determined as described before (Fig. 18).

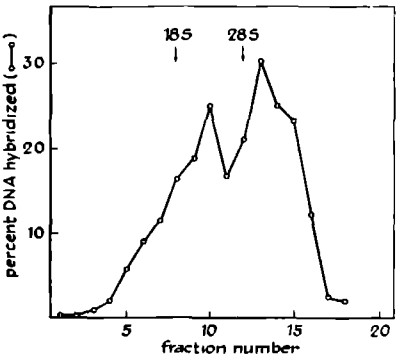


Fig. 25. Sedimentation analysis of poly(A)-containing RNA in sucrose gradients containing 50% formamide Poly(A)-containing RNA isolated by oligo(dT)-cellulose chromatography (section 4 2 4 , method 2) was denatured with formamide (Fig 20) and layered onto an isokinetic sucrose gradient containing 50% formamide (Fig 22B) Centrifugation was carried out for 15 h at 200,000 x g in an Spinco type-SW 41 rotor The relative amount of virus-specific RNA was determined as described before (Fig 18)

R-MuLV infected cells dissociates aggregates of virus-specific RNA (Fig 20A and B; Fig. 21B and C). Since, however, in the gradient analysis of undenatured polyribosomal RNA aggregate formation was observed, it is not possible to make conclusions about the presence of hidden chain breaks, manifested by dissociation of intramolecular hydrogen bonds, in the mRNA fraction sedimenting in the 30-40S region In the hybridization profile of the cytoplasmic RNA of Mo-SV(Mo-MuLV) transformed rat cells ($78A_1$), less noncovalent aggregates of virus-specific RNA are seen (Fig. 23A). Therefore, the size

distribution of Mo-SV(Mo-MuLV)-specific intracellular RNA after denaturation suggests in this case the presence of hidden nicks in part of the 30-40S RNA.

When the intracellular RNA of the $78A_1$ cell line was denatured with dimethylsulfoxide and then allowed to renature prior to polyacrylamide gel electrophoresis, two distinct RNA species of 35S and 20S were detected (294). The hybridization profile of $78A_1$ intracellular RNA we obtained after gradient centrifugation is rather heterogeneous as compared to the electrophoresis profile in that report. This may be due to the difference in denaturation conditions or the technique used for the analysis of the size of the virus-specific RNA species. Moreover, the size distribution of intracellular dimethylsulfoxide denatured RNA of mammalian sarcoma virus producer and nonproducer cells was more or less heterogeneous, dependent on the cell line studied and on whether zonal sedimentation or gel electrophoresis was used for size analysis (141,143,355). The results presented by these authors as well as those obtained by our group do not exclude the possibility that the 10-30S RNA in virus producing cells is simply a cleavage product of the 30-40S RNA, caused by cleavage of this RNA at specific site(s).

The presence of poly(A)-rich sequences in the virus-specific RNA associated with polyribosomes was demonstrated by affinity chromatography on oligo(dT)-cellulose and poly(dT)-cellulose columns. Our data show that about 50-70 percent of the virus-specific polyribosomal RNA in productively infected cells contained poly(A). This finding is consistent with the observation of Scolnick *et al* (307) that the virus-specific RNA of mammalian sarcoma virus transformed nonproducer cells contained poly(A) and the presence of large poly(A) chains covalently bound to the virion subunit RNA molecules. Haines *et al* (311) reported aggregation of ovalbumine mRNA on both sucrose gradients and polyacrylamide gels and a similar observation has been described by Carter (312) for mRNA from Adenovirus-associated virus infected cells. Some preparations of conalbumine mRNA exist even for 70 percent as an aggregate complex (311). These examples indicate that aggregation of mRNA is not unusual. From our experiments it is evident that even formamide dissociation of noncovalent aggregates, prior to centrifugation, may be insufficient. Complete dissociation of aggregates is apparently obtained when RNA dissociated with formamide is analyzed under denaturing conditions. The distribution of virus-specific RNA present in the poly(A)-rich fraction of the polyribosomal RNA, shows that hybridizable RNA was present as a shoulder

in the 14S region and as two peaks in the 20-22S and 30-40S region. A comparable size distribution with peaks at 38S and 27S was obtained for the undenatured intracellular poly(A)-containing RNA in uninfected JLS-V9 cells (295). With regard to our results it has to be noted, that the distribution of virus-specific poly(A)-containing RNA strongly suggests but does not prove that hybridizable RNA in the 14S and 20-22S region contains poly(A). It remains possible, although in view of the results of Fan and Besmer (295) not likely, to argue that the latter RNA species are cleavage products of the poly(A)-containing 30-40S RNA, liberated from nicked molecules under denaturing conditions.

CHAPTER 5

CELL-FREE SYNTHESIS OF RAUSCHER LEUKEMIA VIRUS-SPECIFIC POLYPEPTIDES

5.1. INTRODUCTION

At the present time, there is still uncertainty about the complexity of the 60-70S RNA genome of RNA tumor viruses. A sequence complexity of 8-9 x 10^6 daltons has been determined by measuring the annealing rate of labeled virus-specific DNA with an excess of viral RNA (145,146). In contrast, determination of the annealing rate of the viral RNA to cell DNA indicated a sequence complexity of 3 x 10^6 daltons. This discrepancy between these two results from hybridization studies is not well understood. However, a sequence molecular weight of 3 x 10^6 was also found by fingerprint analysis of unique oligonucleotides obtained after nuclease digestion of the viral 60-70S RNA (147-150). In addition, in favor of a sequence complexity of 3 x 10^6 daltons are results from studies of the minimum size of the integrated viral DNA which is infectious (313,314) and those from studies of deletions and recombinants (150,151,315). Consequently, most experimental evidence suggests that the genome of RNA tumor viruses consists of 2-4 identical subunits with a molecular weight of about 3 x 10^6. In that case the total information is sufficient to encode about 300,000 daltons of protein. It has been proposed that at least 4 genes constitute most of the genome (316). These genes are the envelope (env) gene, encoding the viral envelope proteins; a gene (gag) encoding all of the major internal antigens of the virion; the polymerase (pol) gene, which specifies the reverse transcriptase and a gene (onc) that encodes for a protein able to transform the growth property of cells.

Some of the major proteins of RNA tumor viruses are derived from high molecular weight precursor polypeptides. Vogt and Eisenman (253) reported that the major internal antigens (p27, p19, p15 and p12) of avian RNA tumor viruses are formed by cleavage of a common precursor polypeptide (76,000 M_r). The second high molecular weight polypeptide synthesized in cells infected with these viruses is the incompletely glycosylated precursor (70,000 M_r) of the major viral glycoprotein gp85. Recently, van Zaane *et al* (255) and Strand and Shapiro (257) reported the biosynthesis of two virus-specific precursor

polypeptides in cells infected with Rauscher leukemia virus. One polypeptide (65,000 M_r) is the precursor of the low molecular weight virion proteins p30, p15 and presumably p12 and p10 (257, van Zaane, personal communication), whereas the other polypeptide (82,000-85,000 M_r) is the precursor of the viral glycoproteins gp69/71 (257, van Zaane, personal communication) and p15(E) (van Zaane, personal communication). Since the virus-specific polypeptides constitute only 1-3% of the polypeptides synthesized in infected cells, immuno-precipitation techniques using antisera against disrupted virions or monospecific antisera were applied to visualize the newly formed virus-specific polypeptides.

The virus-specific mRNA species associated with polyribosomes of RSV, Mo-SV(Mo-MuLV) and R-MuLV infected cells, which are most likely involved in the biosynthesis of virus-specific polypeptides, have been identified (160-163). The major virus-specific messenger in productively infected cells is 35S mRNA but evidence for virus-specific RNA molecules of smaller size (20-22S and 14S) has been found. The latter mRNA species appeared to be preferentially associated with membrane-bound polyribosomes (162,163).

In this chapter we describe our investigations on the synthesis of R-MuLV-specific polypeptides *in vitro*. We were able to identify the virus-specific products synthesized either in cell-free systems supplemented with purified free and membrane-bound polyribosomes or in crude cell-free lysates, fractionated in a membrane-free and membrane-rich fraction, from cells productively infected with R-MuLV. Evidence was obtained that virus-specific polypeptides were preferentially synthesized in association with membranes. In addition, we examined the biosynthesis of virus-specific polypeptides in lysates from rabbit reticulocytes, supplemented with poly(A)-rich mRNA isolated by affinity chromatography on oligo(dT)-cellulose columns. It could be demonstrated that addition of 35S mRNA or 20-22S mRNA to the subcellular system resulted in the synthesis of virus-specific polypeptides.

5.2. METHODS

5.2.1. *Experimental conditions*

To minimize RNAase contamination, sterile conditions were used as previously described (section 3.2.2.). All buffers were standardized at a given

pH with HCl at 4^{0} and all manipulations were performed at 0 - 4^{0} unless otherwise stated. Centrifugation gravity values quoted were the average g-values generated at particular speed of rotation.

5.2.2. *Isolation of polyribosomes*

For the isolation of polyribosomes, cells (3 x 10^{8}) grown in spinner culture or monolayer cells, trypsinized at 37^{0}, were cooled rapidly by pouring them on crushed frozen isotonic buffer (35 mM Tris-HCl, pH 7.5, 146 mM NaCl). Cells were harvested by centrifugation at 700 x g for 5 min and washed three times with isotonic buffer. One ml of pelleted cells were suspended in 8 ml of hypotonic buffer (10 mM Tris-HCl, pH 7.4, 15 mM KCl and 1.5 mM magnesium acetate). After swelling for 10 min, the cells were collected by centrifugation, resuspended in 8 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol, 0.1% Triton X-100 and 0,25 M sucrose) and disrupted by 20-30 strokes of a tight fitting Dounce homogenizer (B pestle). Nuclei and unbroken cells were removed by centrifugation at 125 x g for 5 min. The 125 x g supernatant was centrifuged in a Spinco type-Ti 50 rotor at 20,000 x g for 10 min to pellet *membrane-bound polyribosomes*. To exclude contamination with free polyribosomes, the 20,000 x g pellet fraction was washed once in 5 ml of lysis buffer. The 20,000 x g supernatant fractions were used as a source of *free polyribosomes*. For the isolation of *nuclei-associated* and *membrane-bound polyribosomes*, the 125 x g and 20,000 x g pellets were resuspended in 5 ml lysis buffer and 0.5 ml of a solution of 5% sodium deoxycholate and 3% nonidet P-40 was added to solubilize the membranes These fractions were clarified by centrifugation at 12,000 x g for 5 min in a Spinco type-Ti 50 rotor. Polyribosomes were purified from the latter supernatants and the 20,000 x g supernatant, by centrifugation through a 0.7 ml cushion of 2 M sucrose in 20 mM Tris-HCl, pH 7.4, 50 mM potassium acetate, 3 mM magnesium acetate and 1 mM 1,4 dithioerythritol in a Spinco type-SW 41 rotor at 200,000 x g for 4 h.

A mixture of free and membrane-bound polyribosomes (*total polyribosomes*) was isolated essentially as described previously (288): Cells were lysed in the presence of 2% nonidet P-40 in high salt buffer (50 mM Tris-HCl, pH 8.5, 225 mM KCl, 8 mM magnesium acetate and 2 mM 1,4 dithioerythritol) by 10 strokes of a tight fitting Dounce homogenizer (B pestle). Nuclei were removed by

centrifugation at 750 x g for 5 min and the supernatant was centrifuged in a Spinco type-Ti 50 rotor at 12,000 x g for 5 min. Polyribosomes were isolated through a layer of 2 M sucrose as described before. Total polyribosomes to be used for the isolation of poly(A)-containing RNA, were prepared from cells pretreated with cycloheximide (0.1 μg x ml^{-1}) as described in section 3.2.3.

5.2.3. *Preparation of cell-free extracts*

Monolayer cells (JLS-V9) were harvested and washed as described in the preceding section. The packed cells were suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.5 at 20°, 15 mM KCl, 1.5 mM magnesium acetate and 2 mM 1,4 dithioerythritol), allowed to swell for 10 min and collected by centrifugation at 750 x g for 5 min. Cells (1.5 ml) were lysed in 2 volumes of hypotonic buffer by 30 strokes of a tight fitting Dounce homogenizer (B pestle). Nuclei were removed by centrifugation at 125 x g for 5 min. Three ml of the supernatant fluid was further separated into a cytoplasmic supernatant and a pellet by centrifugation at 20,000 x g for 6 min in a Spinco type-Ti 50 rotor. The membrane-rich 20,000 x g pellet fraction was resuspended in 1 ml of hypotonic buffer. In this way, three fractions were obtained, a *125 x g supernatant* containing free and membrane-bound polyribosomes, a *20,000 x g supernatant* enriched with free polyribosomes and a *20,000 x g pellet* fraction containing mainly membrane-bound polyribosomes. Since especially the synthesis of the virus-specific polypeptide of 82,000 M_r was reduced after freezing and thawing of the 125 x g and 20,000 x g pellet fractions, they were used immediately to test their protein synthesizing capacity.

5.2.4. *Components of the cell-free protein synthesizing systems*

5.2.4.1. *Preparation of cell-lysates and S-100*

Lysates of rabbit reticulocytes were prepared according to Berns (318). A ribosome-free supernatant, designated as S-100, was prepared from the 30,000 x g supernatant of lysed reticulocytes by centrifugation in a Spinco type-Ti 50 rotor at 150,000 x g for 2 h.

Postribosomal supernatants of JLS-V9 cells and Ehrlich ascites tumor cells were prepared by the following procedure. Cells were collected and

washed as described in section 5.2.2. Subsequently, the cells were disrupted by 30 strokes of a tight fitting Dounce homogenizer (B pestle) after suspension in 5 volumes of hypotonic buffer and swelling for 10 min. The extract was centrifuged at 750 x g for 5 min to remove the nuclear fraction. In some experiments one volume of 10 mM Tris-HCl, pH 7.4, 20 mM KCl, 2 mM magnesium acetate, 1 mM 1,4 dithioerythritol and 0.5 M sucrose was added prior to low speed centrifugation of the cell-lysate. A ribosomal supernatant was prepared from the 750 x g supernatant by centrifugation in a Spinco type-Ti 50 rotor at 150,000 x g for 2 h. S-100 prepared by either method was suitable for preparing an active pH 5 enzyme fraction. Aliquots of the supernatant were frozen in liquid nitrogen and stored at -70^{o}. For the preparation of pH 5 enzymes from the ribosome-free supernatant unfrozen S-100 was used.

5.2.4.2. *Preparation of pH 5 enzymes*

pH 5 enzymes were prepared from the ribosome-free supernatants from reticulocytes, Ehrlich ascites cells or JLS-V9 cells. To the S-100 supernatants, on ice, 1 M acetic acid was added dropwise until a pH of 5.2 was reached. The precipitate was collected by centrifugation at 1000 x g for 15 min and dissolved in 1/10 of the original volume of buffer containing 10 mM Tris-HCl, pH 7.5 at 20^{o}, 1 mM magnesium acetate, 20 mM KCl, 10% glycerol, 1 mM 1,4 dithioerythritol and 100 µM phenol red. During resuspending, the pH was adjusted to about 7 by addition of 1 M KOH. Subsequently, insoluble material was removed by centrifugation at 1000 x g for 10 min and small aliquots of the supernatant were frozen in liquid nitrogen and stored at -70^{o}.

The optimal concentration of pH 5 enzymes to be used in cell-free systems supplemented with polyribosomes was determined for each batch of pH 5 enzymes.

5.2.4.3. *Preparation of initiation factors*

Crude ribosomes of rabbit reticulocytes were prepared as described by Miller and Schweet (319). Polyribosomes were suspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM magnesium acetate and 1 mM 1,4 dithioerythritol at a concentration of about 450 A_{260} units x ml^{-1}. Thereafter, 4 M KCl was added dropwise with stirring to a final concentration of

0.5 M and stirring was continued at 0^{o} for 60 min. The ribosomes were removed by centrifugation at 150,000 x g for 3 h in a Spinco type-Ti 50 rotor. Small aliquots of the supernatant were frozen in liquid nitrogen and stored at -70^{o}.

5.2.5. *Cell-free protein synthesis*

5.2.5.1. *Assay conditions for cell-free systems supplemented with polyribosomes*

Assays were performed in a standard reaction mixture of 25 µl and contained, 0.6 A_{260} units of polyribosomes, 8 µl of S-100 from reticulocytes or about 10 µg of pH 5 enzymes, 4 µl energy and amino acid mixture (final concentrations in the incubation mixture were, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 0.2 mg x ml^{-1} of creatine kinase, 19 unlabeled amino acids in concentrations used by Palmiter (320) and 50 to 200 µCi x ml^{-1} of [^{35}S]methionine)and, unless otherwise stated 10 mM Tris-HCl, pH 7.5 at 20^{o}, 2.0 mM magnesium acetate, 60 mM KCl, 80 mM potassium acetate and 1 mM 1,4 dithioerythritol. Reaction mixtures were incubated for 150 min at 26^{o}. After incubation a 1 µl aliquot was diluted in 0.5 ml of 0.1 M KOH and incubated for an additional 15 min at 37^{o}. Trichloroacetic acid precipitable radioactivity was determined as described before (section 2.2.4.).

5.2.5.2. *Assay conditions for subcellular fractions of JLS-V9 cells*

A standard reaction mixture contained in a final volume of 25 µl, 15 mM Tris-HCl, pH 7.5 at 20^{o}, 2.5 mM magnesium acetate, 75 mM KCl, 70 mM potassium acetate, 1 mM 1,4 dithioerythritol, 4 µl energy and amino acid mixture as described above; 3 µl (75 µg) of crude initiation factors, 10 µl S-100 from JLS-V9 cells, 3 µl (10 µg) of pH 5 enzymes and crude cell fractions (15 µl of the 125 x g supernatant, 5 µl of the resuspended 20,000 x g pellet or 15 µl of the 20,000 x g supernatant) were added as indicated in the legends to the figures. Incubation was performed at 30^{o} for 150 min.

5.2.5.3. *Assay conditions for mRNA*

Cell-free translation of mRNA was routinely performed in a volume of 25 µl, containing 12 µl of a rabbit reticulocyte lysate, 4 µl of energy and amino acid mixture as described above, 2 mM magnesium acetate, 65 mM KCl, 60 mM potassium acetate and the test solutions as stated in the legends to the figures. Reaction mixtures were incubated at 26^{0} for 150 min.

5.2.6. *Immunoprecipitation of virus-specific polypeptides labeled in vivo*

Precipitation of virus-specific products synthesized *in vivo* was performed by the method of van Zaane *et al.* (255). To label polypeptides newly synthesized *in vivo*, 2 x 10^{6} cells in logarithmic growth were incubated in labeling medium (Hanks basic salt solution supplemented with 10% dialysed calf serum and amino acids except methionine). After 10 min the cells were replenished with fresh labeling medium containing 10 µCi x ml^{-1} of [^{35}S]-methionine and incubated for 15 to 60 min at 37^{0}. Cells were collected by rapid trypsinization at 37^{0} and cooled to 0^{0} by pouring them into a centrifuge tube containing crushed frozen isotonic solution. Alternatively, monolayers were rapidly cooled by washing with ice-cold isotonic buffer and the cells were scraped from the glass with a rubber policeman. Cells were harvested by centrifugation at 750 x g for 5 min and washed three times with isotonic buffer. Cells were lysed by addition of 1 ml of immunoprecipitation buffer (10 mM phosphate, pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.1% sodium deoxycholate and 0.1% sodium dodecyl sulfate). The lysate was clarified by centrifugation at 220,000 x g for 10 min in a Spinco type-Ti 50 rotor. The supernatant fraction was used for immunoprecipitation.

Virus-specific proteins in samples from these lysates were precipitated by the indirect method: appropriate portions of the lysates were incubated at 4^{0} for 16 h with 2 µl rabbit anti-R-MuLV serum, then 50 µl goat anti-rabbit immunoglobulin serum was added, and the incubation was continued at 4^{0} for 2 h. As a blank, 1 µl rabbit anti-BSA serum was added to the same volume of cell lysate, followed by incubation with 25 µl goat anti-rabbit immunoglobulin serum. With both rabbit sera, precipitates containing 100 µg of protein were obtained. In pilot experiments the optimal concentration of rabbit anti-R-MuLV serum was determined.

Precipitates were collected by centrifugation through a layer of 10% (w/v) sucrose in immunoprecipitation buffer for 5 min at 12,000 x g, washed three times with immunoprecipitation buffer and finally dissolved in sample buffer [60 mM Tris-HCl, pH 6.8 at 20^{o}, 2% sodium dodecyl sulfate, 5% mercaptoethanol and 10% glycerol, Laemmli (259)] containing 6 M urea by heating for 2 h at 37^{o} and 2 min at 95^{o}.

5.2.7. *Immunoprecipitation of virus-specific polypeptides synthesized in vitro*

To the cell-free incubation mixtures immunoprecipitation buffer was added and the resulting solution was clarified by centrifugation at 220,000 x g for 10 min in a Spinco type-Ti 50 rotor.

Indirect immunoprecipitation of virus-specific polypeptides was performed as described above for proteins labeled *in vivo*. Since this latter method is only applicable to test samples containing very low concentrations of virus-specific antigens, in some experiments the direct immunoprecipitation technique was applied. For direct immunoprecipitation on cell-free incubation mixtures, 60 µg of an extract of JLS-V9 cells (prepared in immunoprecipitation buffer) and 75 µl of rabbit anti-R-MuLV serum (in immunoprecipitation buffer) was added and the reaction mixture was incubated for 16 h at 4^{o}. As a blank, 0.8 µg of BSA and 25 µl of rabbit anti-BSA serum (in immunoprecipitation buffer) was added to the test sample. After incubation, 20 µg of a suspension of carrier BSA anti-BSA precipitate was added and the immunoprecipitate was collected, washed and dissolved as described above.

Our initial attempts to characterize virus-specific polypeptides synthesized in the *in vitro* subcellular system, by specific immunoprecipitation with antisera directed against R-MuLV did not succeed. Centrifugation of the incubation mixtures after RNAase and EDTA treatment, resulted in loss of 50 to 80 percent of the acid-precipitable radioactivity, and immunoprecipitation of the supernatant by the direct or indirect method showed no specificity. Conditions to solubilize the proteins in the *in vitro* reaction mixtures and to maintain them in solution during incubation with antiserum were therefore developed and established as described above. Thus, 70 to 90 percent of the acid precipitable radioactivity was left in the supernatant after

centrifugation for 10 min at 220,000 x g. In addition, the specificity of the precipitation was strongly improved by the latter high speed centrifugation step and collection of the immunoprecipitate by centrifugation through a layer of sucrose.

5.2.8. *Polyacrylamide gel electrophoresis*

Cell-free products and virus-specific polypeptides in immunoprecipitates were identified by electrophoresis on sodium dodecyl sulfate polyacrylamide slab gels using the discontinuous buffer system as described by Laemmli (259). The slab gel system is superior to gel rods, since up to 20 samples can be run under identical conditions, thus improving the ability to compare complex protein mixtures. Slabs of 15 x 12 x 0.15 cm or 16 x 11 x 0.075 cm (Biorad) gels were made. Routinely, a linear gradient of 7 to 18% polyacrylamide was constructed. The gradient was stabilized against convection currents first by including a 3-20% glycerol gradient and second by including a higher concentration of ammonium persulfate in the 7% gel (0.2%) than in the 18% gel (0.1%) so that the gel polymerized from the top down. Buffer solutions for stacking and resolving gels and the electrophoresis buffer were those described in section 2.2.5. In such a polyacrylamide gradient gel, proteins of the whole range of molecular weights (300,000 down to 8000) are resolved excellently. Shortly before use, a spacer gel (259) containing 14 or 20 slots was applied. Electrophoresis was performed at a constant current of 25 mA for 1.5 mm slabs or at 12.5 mA for 0.75 mm gels, until the bromophenol blue marker reached the bottom of the gel. After electrophoresis, the gel was stained during 15 to 60 min at 50^{o} and destained overnight at 37^{o} (section 2.2.5.). Destained gels were transferred to a piece of Whatman 3 MM filter paper and dried under vacuum as described (321). When indicated, destained gels were dehydrated in dimethyl sulphoxide, soaked in a solution of 22.2% (w/v) 2,5-diphenyl-oxazole (PPO) in dimethylsulphoxide, dried and exposed to Royal X-omatic film at -70^{o} (322).

5.3. ASSAY CONDITIONS FOR CELL-FREE PROTEIN SYNTHESIS IN SYSTEMS SUPPLEMENTED WITH POLYRIBOSOMES

5.3.1. *Effect of ionic conditions*

Studies on the translation of mammalian mRNA in homologous and heterologous cell-free systems indicated that the optimal conditions for the synthesis of specific polypeptides are rather critical (320,323-327). The optimum concentrations of K^+ and Mg^{2+} ions are different for almost each cell-free extract and each mRNA (328). Differences were established between the optimal concentrations of K^+ ions for the translation of globin and Encephalomyocarditis virus RNA in the ascites system (329). Therefore, we

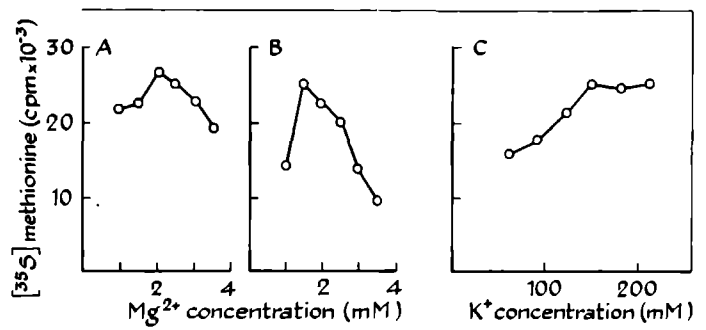


Fig. 26. Effect of magnesium and potassium concentration on the incorporation of $[^{35}S]$methionine in cell-free systems supplemented with polyribosomes. Polyribosomes from JLS-V9 cells (section 5.2.2.) were incubated in the presence of S-100 from rabbit reticulocytes as described (5.2.5.1.), except that the Mg^{2+} dependence of the incorporation was measured at a K^+ ion concentration of 150 mM and the K^+ dependence was determined at a Mg^{2+} ion concentration of 2 mM. Similar ionic conditions were chosen for cell-free incubation of the reticulocyte lysate (section 5.2.5.3.). After incubation 1 µl aliquots were withdrawn and trichloroacetic acid precipitable radioactivity was determined (section 2.2.4.). (A) Mg^{2+} dependency of the amino acid incorporation in a cell-free system supplemented with polyribosomes; (B) Mg^{2+} dependency of the incorporation of amino acids in a reticulocyte lysate; (C) K^+ dependency of the amino acid incorporation in a cell-free system supplemented with polyribosomes.

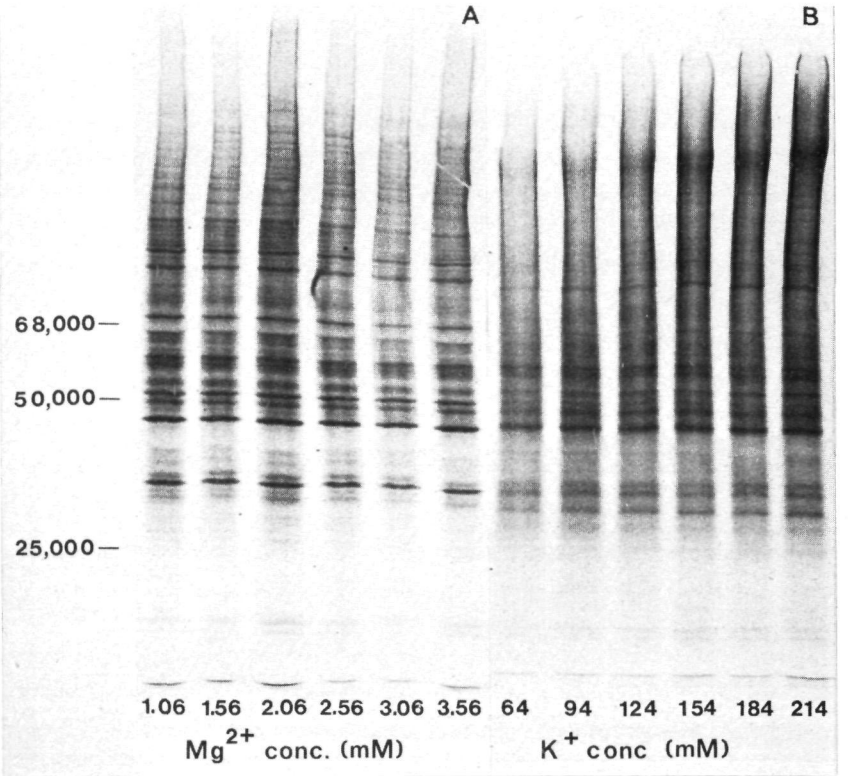


Fig. 27. Autoradiogram of a polyacrylamide slab gel analysis of polypeptides synthesized in cell-free systems supplemented with polyribosomes: effect of the Mg^{2+} and K^{+} ion concentrations. Conditions for cell-free incubation of polyribosomes in the presence of reticulocyte S-100 were as described in the legend to Fig. 26. (A) Mg^{2+} ion dependence; (B) K^{+} ion dependence.

determined the optimum of Mg^{2+} and K^{+} ions for cell-free systems supplemented with polyribosomes, by measuring the incorporation of [^{35}S]methionine into acid precipitable radioactivity. In addition, we analyzed the products synthesized in the subcellular system at different ionic conditions by polyacrylamide gel electrophoresis. Finally, to check the possibility that the synthesis of viral polypeptides would depend in a different way on the ionic conditions, the same analysis was performed after

immunoprecipitation with anti-R-MuLV serum of the newly formed polypeptides.

The Mg^{2+} concentration optimum for the incorporation of $[^{35}S]$methionine in a cell-free system supplemented with reticulocyte S-100 and polyribosomes, isolated by the detergent method, was approximately 2 mM (Fig. 26A). As a rule, this Mg^{2+} curve showed a less sharp optimum than that observed for the synthesis of globin in the reticulocyte lysate (Fig. 26B). The effect of the K^+ ion concentration on the cell-free protein synthesis is shown in Fig. 26C. The amount of incorporated $[^{35}S]$methionine increased up to 150 mM potassium acetate and levels off at higher potassium concentrations. In this context, it has to be noted that the use of potassium acetate instead of KCl eliminates the possibility of a Mg^{2+} shift to lower concentrations due to a weak association of Cl^- with Mg^{2+} (330).

Additional information about the ionic conditions for cell-free protein synthesis is obtained when the newly synthesized polypeptides are analyzed on polyacrylamide gels in the presence of sodium dodecyl sulfate. The autoradiogram of the polypeptides synthesized at different Mg^{2+} ion concentrations and analyzed on a slab gel is shown in Fig. 27A. Almost identical patterns of labeled bands were visible at Mg^{2+} ion concentrations between 1 and 3.5 mM. When the concentration of magnesium was raised to 5 mM or even higher, especially the synthesis of polypeptides in the 100.000 to 200.000 molecular weight region was reduced (not shown). Likewise, analysis was performed on the products synthesized at different K^+ ion concentrations (Fig. 27B). At potassium concentrations between 120 and 210 mM the same set of polypeptides was synthesized in comparable amounts. However, the synthesis of high molecular weight polypeptides was reduced, when the potassium concentration was lowered to 90 and 60 mM.

For the identification of virus-specific polypeptides indirect immunoprecipitation was performed on the *in vitro* reaction mixtures, using antiserum against purified R-MuLV. The gel analysis of the immunoprecipitates is shown in Fig. 28. Several bands were visible on the autoradiogram in the molecular weight range of 40,000 to 70,000. However, no critical dependence on a particular magnesium (Fig. 28,1-6) or potassium concentration (Fig. 28,7-12) was observed. It is clearly visible that the amount of label in specific polypeptides followed the magnesium and potassium curves depicted in Fig. 26A and C.

The optimal salt concentrations for the synthesis of polypeptides in

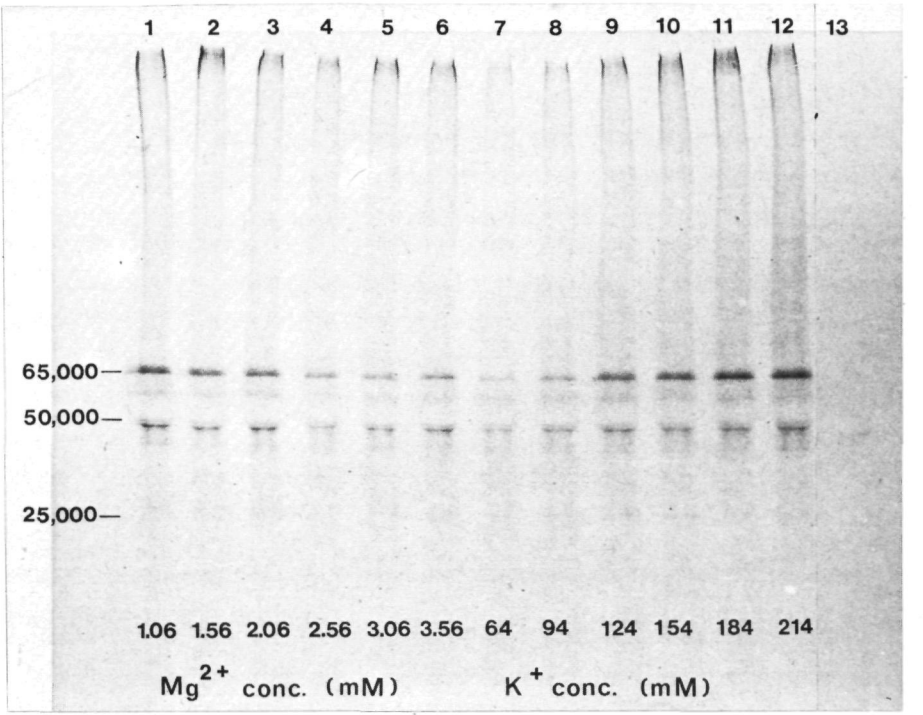


Fig. 28. Polyacrylamide slab gel analysis of the R-MuLV-specific polypeptides synthesized in cell-free systems supplemented with polyribosomes: effect of the Mg^{2+} and K^{+} ion concentration. Polypeptides synthesized in the cell-free systems supplemented with polyribosomes form JLS-V9 cells and S-100 form reticulocytes (legend to Fig. 26) were subjected to immunoprecipitation with a rabbit antiserum to R-MuLV by the indirect precipitation method (gel columns 1-12). Control precipitation with rabbit anti-BSA serum on an equal amount of the *in vitro* extract (gel column 13).

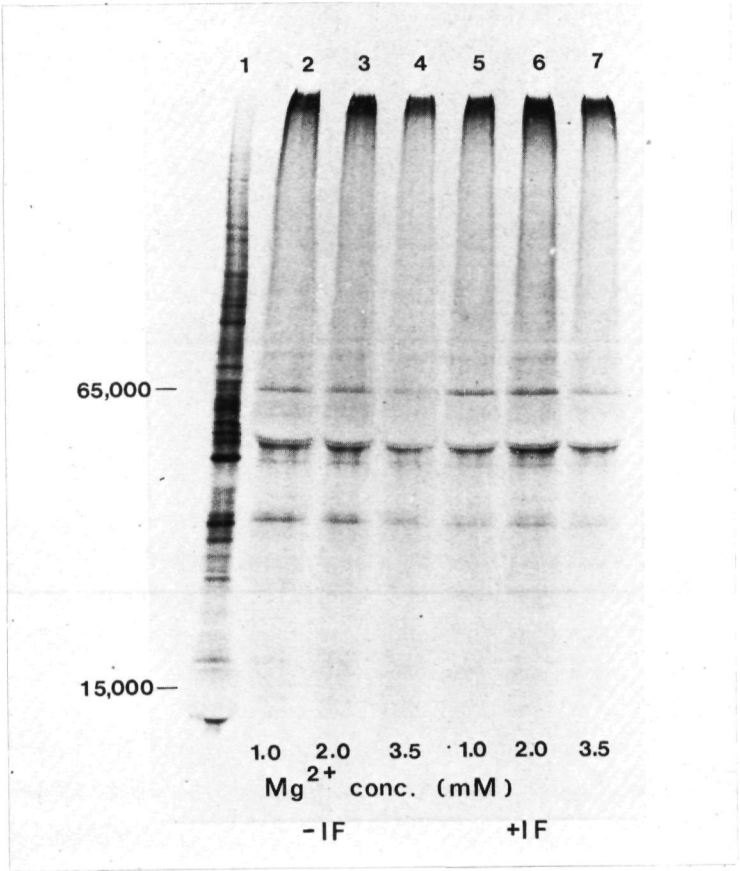


Fig. 29. Effect of crude initiation factors on the synthesis of R-MuLV-specific polypeptides in cell-free systems supplemented with polyribosomes. Polyribosomes isolated from JLS-V9 cells by the detergent method were added to a cell-free system containing S-100 from reticulocytes (section 5.2.5.1.). Incubation was performed at a potassium concentration of 150 mM (1) Polypeptides synthesized by total polyribosomes at 2.0 mM Mg^{2+}; (2-4) Mg^{2+} dependence of the synthesis of R-MuLV-specific polypeptides in the absence of crude initiation factors; (5-7) Mg^{2+} dependence of the synthesis of R-MuLV-specific polypeptides in the presence of crude initiation factors.

cell-free systems supplemented with pH 5 enzymes from reticulocytes or JLS-V9 cells were 2.3 mM for magnesium acetate and 120 - 180 mM for potassium acetate.

Our results indicate that protein synthesis in cell-free systems supplemented with polyribosomes was effective in a rather broad region of magnesium and potassium concentrations. Moreover, there is no dependence on a critical Mg^{2+} or K^{+} ion concentration for the synthesis of a particular virus-specific polypeptide, since at each concentration of ions we tested a similar set of virus-specific polypeptides was synthesized.

5.3.2. *Effect of crude initiation factors*

Abraham *et al.* (331) found no significant difference in the capacity of polyribosomes isolated from cells by the detergent technique or by nitrogen cavitation to incorporate radioactive amino acids. However, these workers showed, by measuring the incorporation of radioactive subunits into polyribosomes, that polyribosomes prepared by the detergent technique were less effective in chain initiation than polyribosomes isolated by nitrogen cavitation. Therefore, we examined the effect of crude initiation factors from rabbit reticulocytes on the synthesis of virus-specific products in a cell-free system supplemented with polyribosomes prepared by the detergent technique. In the presence of initiation factors a slight stimulation (1.5 fold) of amino acid incorporation was observed. Identification of the virus-specific products, synthesized in the presence or absence of initiation factors by immunoprecipitation and gel analysis showed that in both cases the same virus-specific polypeptides were synthesized (Fig. 29). This experiment indicates that the synthesis of virus-specific polypeptides was not essentially improved by the addition of crude reticulocyte initiation factors to cell-free systems supplemented with detergent treated polyribosomes.

5.4. VIRUS-SPECIFIC POLYPEPTIDES SYNTHESIZED BY FREE AND MEMBRANE-BOUND POLYRIBOSOMES

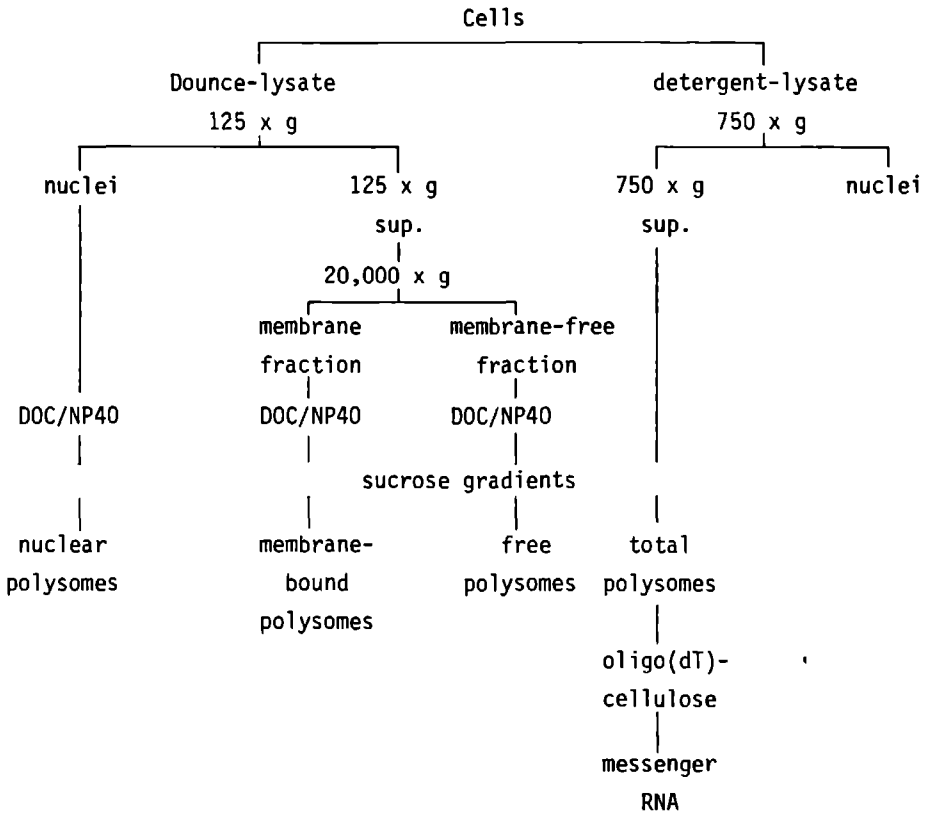
By means of hybridization experiments, it was demonstrated that the relative amount of virus-specific mRNA is 4 to 10 times higher in membrane-bound

than in free polyribosomes (162,163). In accordance with this observation we expected and actually established a similar distribution of newly synthesized virus-specific polypeptides in cell-free systems supplemented with free and membrane-bound polyribosomes. In addition, we identified the virus-specific polypeptides synthesized by both classes of polyribosomes by gel analysis of polypeptides precipitated with rabbit antiserum against R-MuLV.

For the isolation of free and membrane-bound polyribosomes cells were disrupted in hypotonic medium by homogenization in a tight fitting Dounce.

TABLE 4

Fractionation scheme of cells grown in tissue culture



Disruption of JLS-V9 cells grown in monolayer appeared to be difficult since many cells remained intact and large membrane fragments were still attached to nuclei. JLS-V9 cells grown in spinner culture were much better lysed and less cytoplasmic material sedimented with the nuclei. Consequently, polyribosomes present in the 125 x g pellet fraction were also isolated. A complete scheme of the cell fractionation procedure for the isolation of crude cell fractions, free and membrane-bound polyribosomes, total polyribosomes and poly(A)-rich RNA is presented in table 4.

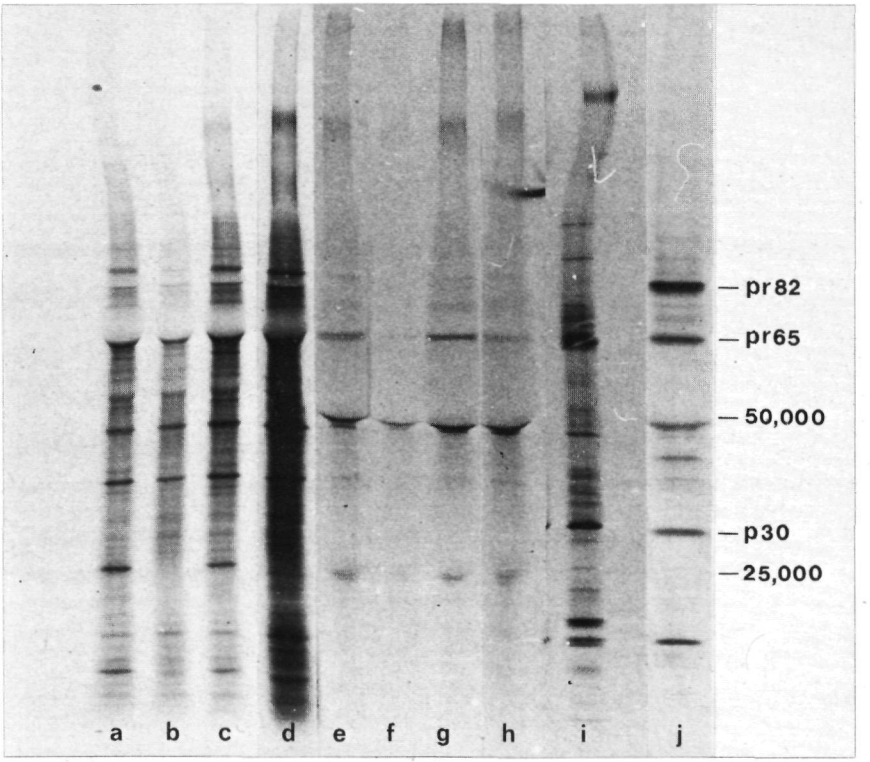


Fig. 30. Polyacrylamide gel autoradiogram of polypeptides synthesized in a cell-free system supplemented with polyribosomes isolated from different cell fractions. Polyribosomes, isolated from cell fractions of JLS-V9 cells grown in suspension culture (section 5.2.2.) were incubated in cell-free systems containing pH 5 enzymes from JLS-V9 cells (section 5.2.5.1.). After incubation, equal amounts of the *in vitro* extract (equivalent to 5 µl of each 75 µl reaction mixture) were prepared for direct analysis by

polyacrylamide gel electrophoresis. For indirect immunoprecipitation with rabbit antiserum to R-MuLV, aliquots containing equal amounts of radioactivity (10^6 cpm) incorporated into protein were withdrawn from the reaction mixtures. Immunoprecipitation on the extracts of cells labeled *in vivo* with [^{35}S]methionine was performed as described in section 5.2.6. (a) Polypeptides synthesized in the cell-free system supplemented with polyribosomes isolated from the 125 x g pellet fraction; (b) with free polyribosomes; (c) with membrane-bound polyribosomes; (d) with total polyribosomes; (e) R-MuLV-specific polypeptides mediated by polyribosomes associated with the nuclear fraction; (f) by free polyribosomes; (g) by membrane-bound polyribosomes; (h) by total polyribosomes; (i) proteins of R-MuLV labeled with [^{14}C]amino acids; (j) R-MuLV-specific polypeptides synthesized *in vivo*.

Polyribosomes isolated from different cell fractions of JLS-V9 cells grown in spinner culture were allowed to translate their mRNAs in a cell-free system supplemented with an optimal concentration of pH 5 enzymes. The autoradiogram of the polyacrylamide gel displaying the polypeptides synthesized *in vitro* is shown in Fig. 30. A large number of labeled bands in the molecular weight range of 8000 up to 150,000 can be seen. The most striking point, however, is the high degree of similarity among the polypeptide patterns visualized on the autoradiogram. The different classes of polyribosomes programmed the synthesis of almost the same set of polypeptides, with the exception of one polypeptide band (molecular weight 25,000) which seems to be made exclusively on polyribosomes isolated from membrane-rich fractions (Fig. 30a and c). This polypeptide is present in a much less amount in the cell-free products of the total polyribosomes (Fig. 30d).

The virus-specific polypeptides synthesized *in vitro* can be distinguished from labeled host polypeptides by immunoprecipitation with rabbit antiserum against R-MuLV. Therefore, specific immunoprecipitation was performed on equal amounts of [^{35}S]methionine incorporated into polypeptides from each reaction mixture, by the indirect immunoprecipitation technique, and the precipitates were analyzed on a polyacrylamide slab gel (Fig. 30e,f,g and h). Radioactive material which migrates in front of the heavy chain (50,000 M_r) of the immunoglobulins was always visible on the film as a curved band, due to overloading of the gel by almost 75 µg of the heavy chain. Since a labeled band, although less pronounced, was also visible in this region in the gel analysis

of control immunoprecipitations (Fig. 28,14), this indicates that at least part of the polypeptides in front of the heavy chain were aspecifically precipitated. In some analysis a curved band was also visible in front of the light chain (23,500 M_r) of the immunoglobulins. Besides these more or less artificial bands, one major polypeptide with a molecular weight of 65,000 is clearly visible. This polypeptide was synthesized *in vitro* by all polyribosomal fractions. However, comparison of Fig. 30f and Fig. 30g clearly shows that the synthesis of virus-specific polypeptides was significantly higher in systems supplemented with membrane-bound polyribosomes than in those with free polyribosomes. In addition to the 65,000 daltons polypeptide, two rather faint bands were recognized with molecular weights of 74,000 and 82,000. These polypeptides had apparent molecular weights corresponding to the virus-specific precursor polypeptides of 65,000, 74,000 and 82,000 M_r, synthesized *in vivo* (Fig. 30j). Thus, these initial observations suggest that a similar set of virus-specific polypeptides was synthesized *in vitro* as *in vivo*. No evidence, however, was obtained for proteolytic cleavage of the polypeptide chains made *in vitro* to yield the immunoprecipitable mature virion proteins p30 and p15 (compare Fig. 30g and Fig. 30i). It seems possible, therefore, that either appropriate proteolytic enzymes were absent in the pH 5 enzyme fraction or incubation was performed at a temperature not optimal for processing of precursor polypeptides. One of the larger virion proteins comigrated with the virus-specific polypeptide of 65,000 M_r synthesized *in vitro*, suggesting the presence of one of the precursor polypeptides in mature virus (Fig. 30g and i). This idea is strengthened by studies of van Zaane (personal communication) showing the precipitation of the virion 65,000 M_r protein with monospecific antiserum against p30.

In another set of experiments, we studied the synthesis of virus-specific polypeptides in cell-free systems supplemented with polyribosomes isolated from monolayer cells and pH 5 enzymes from these cells or reticulocyte S-100. In addition, in view of the observation of van Zaane *et al.* (255) that the arginine analog canavanine prevented formation of the 65,000 M_r polypeptide *in vivo*, we examined the effect of canavanine, *p*-fluorophenylalanine and azetidine-2-carboxylic acid on the synthesis of virus-specific polypeptides *in vitro*.

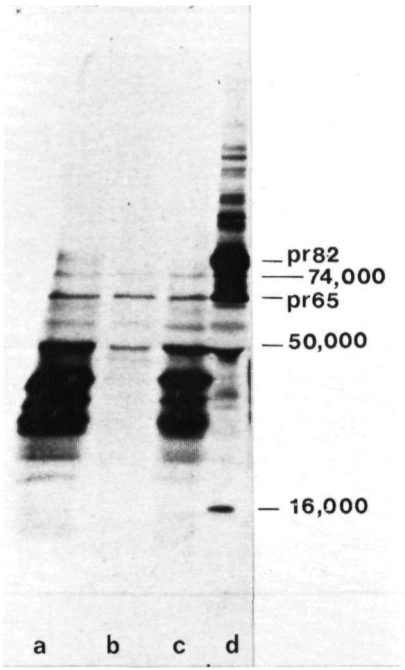


Fig. 31. Polyacrylamide slab gel analysis of R-MuLV-specific polypeptides synthesized in cell-free systems supplemented with polyribosomes from monolayer cells and pH 5 enzymes. Polyribosomes from JLS-V9 cells grown in monolayer (section 5.2.2.) were incubated in cell-free systems containing pH 5 enzymes from JLS-V9 cells (section 5.2.5.1.). Immunoprecipitation was performed on equal samples of radioactivity incorporated into protein with antiserum to R-MuLV, by the direct immunoprecipitation method. Prior to autoradiography the gel was treated with PPO. (a) R-MuLV-specific immunoprecipitate of polypeptides synthesized in the reaction mixture supplemented with nuclear polyribosomes; (b) with free polyribosomes; (c) with membrane-bound polyribosomes; (d) Specific immunoprecipitate of polypeptides synthesized *in vivo*.

In this case, immunoprecipitation on newly synthesized radioactive products was performed by the direct immunoprecipitation technique with antiserum against R-MuLV. Fig. 31a,b and c show the virus-specific products synthesized by polyribosomes isolated from monolayer cells in a cell-free system supplemented with pH 5 enzymes. The 65,000 M_r polypeptide was present as a minor product in incubations supplemented with polyribosomes associated with the 125 x g pellet (Fig. 31a) or membrane-bound polyribosomes (Fig. 31c), whereas several major bands were visible in the low molecular weight range of 20,000 - 50,000 M_r. Remarkably, these low molecular weight polypeptides were only minor products of cell-free systems supplemented with free polyribosomes (Fig. 31b) or membrane associated polyribosomes from cells grown in suspension culture (Fig. 30e and g). Since low molecular weight polypeptides were almost absent in the cell-free products mediated by free polyribosomes, the membrane associated polyribosomes isolated from monolayer cells were presumably

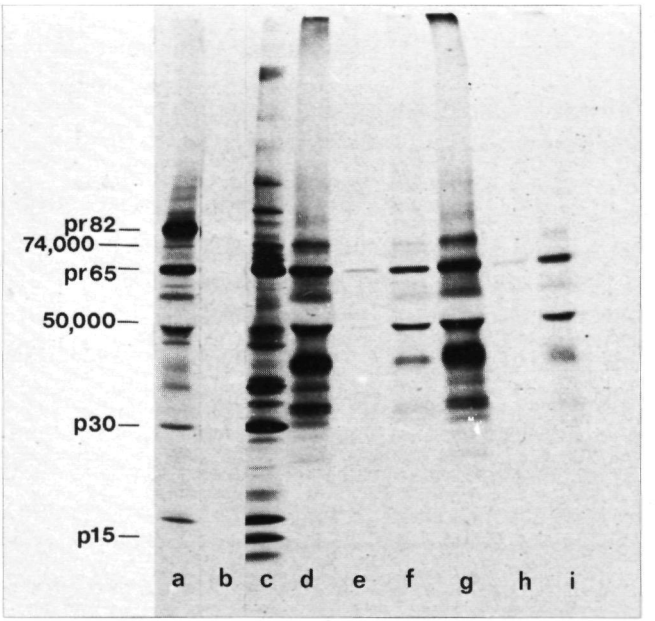


Fig. 32. Autoradiogram of the polyacrylamide gel analysis of R-MuLV-specific polypeptides synthesized in cell-free systems supplemented with polyribosomes from monolayer cells and S-100 from reticulocytes. Isolation of polyribosomes and immunoprecipitation of the newly synthesized polypeptides was performed as described in the legend to Fig. 31. Purified polyribosomes were incubated in the presence of S-100 from reticulocytes. Prior to autoradiography the gel was treated with PPO. (a) R-MuLV-specific polypeptides synthesized in JLS-V9 cells labeled with [^{35}S] methionine for 60 min; (b) aspecific immunoprecipitation of *in vivo* labeled cell extract; (c) R-MuLV labeled with [^{14}C] amino acids; (d) R-MuLV-specific polypeptides synthesized in the cell-free system supplemented with nuclear polyribosomes; (e) with free polyribosomes; (f) with membrane-bound polyribosomes; (g), (h) and (i) same as (d), (e) and (f) except that incubation was performed in the presence of 200 μM of amino acid analogs (*p*-fluorophenylalanine, canavanine and azetidine-2-carboxylic acid).

contaminated with RNAase or proteases causing premature termination or proteolytic cleavage of newly synthesized polypeptides. Anyway, it is obvious that both free and membrane-bound polyribosomes gave rise to the synthesis of virus-specific polypeptides with molecular weights of 55,000, 65,000 and 74,000. From the latter products, the 55,000 and 74,000 M_r polypeptides were always present as minor bands in the protein pattern of cells labeled *in vivo* (Fig. 31d). Only in the gel analysis of the virus-specific *in vitro* products of membrane associated polyribosomes (Fig. 31a and c) a faint band is visible comigrating with the viral glycoprotein precursor of 82,000 M_r (Fig. 31d).

A different result was obtained when polyribosomes isolated from monolayer cells were allowed to translate their mRNAs in the presence of reticulocyte S-100 (Fig. 32). Only one low molecular weight polypeptide of approximately 45,000 M_r was synthesized in minor amounts by membrane-bound polyribosomes (Fig. 32f), whereas several low molecular weight bands were still present in the reaction mixtures supplemented with polyribosomes associated with the 125 x g pellet fraction (Fig. 32d). Obviously, all polyribosomal fractions mediate the synthesis of a major polypeptide of 65,000 M_r (Fig. 32d,e and f). In the same molecular weight range two minor bands were seen with molecular weights of 55,000 and 74,000, when polyribosomes isolated from membrane-rich cell fractions were incubated *in vitro* (Fig. 32d and f). Although evidence for the synthesis of these polypeptides by free polyribosomes was obtained in the preceding experiment (Fig. 31b), they were not detected among the specific polypeptides shown in Fig. 32e. However, we were able to demonstrate that the 55,000 and 74,000 M_r polypeptides were precipitated from the cell-free products mediated by the same batch of polyribosomes by indirect immunoprecipitation (not shown).

Addition of amino acid analogs (*p*-fluorophenylalanine, canavanine and azetidine-2-carboxylic acid) to the cell-free system supplemented with polyribosomes did not result in the synthesis of higher molecular weight polypeptides. In addition, in contrast to the results obtained from *in vivo* studies (255) the synthesis of the 65,000 M_r precursor polypeptide was not prevented *in vitro* (Fig. 32g,h and i). One reason for this result is possibly the essentially different experimental approach: while in the *in vitro* studies mainly nascent polypeptide chains were completed, in the *in vivo* studies amino acid analogs were added prior to the radioactive amino acids.

The above experiments indicate that both free and membrane-bound polyribosomes gave rise to the synthesis of a virus-specific polypeptide, which is presumably identical to the virus-specific precursor polypeptide of 65,000 M_r. Our finding that only occasionally a minor polypeptide band of 82,000 M_r was observed in the gel analysis of membrane-bound polyribosomes, suggests that we had difficulties with the detection or the accurate synthesis of the viral glycoprotein precursor. Variable amounts were observed of virus-specific polypeptides of 55,000 and 74,000 M_r, which comigrated with two minor polypeptides synthesized *in vivo*. Finally, in all experiments the relative content of virus-specific polypeptides mediated by membrane-bound polyribosomes was much higher than in systems supplemented with free polyribosomes.

5.5. VIRUS-SPECIFIC POLYPEPTIDES SYNTHESIZED IN FRACTIONATED CELL EXTRACTS

Monolayer cells were lysed in hypotonic buffer and nuclei were removed by low speed centrifugation (Table 4). The supernatant was fractionated by centrifugation at 20,000 x g into a pellet (membrane fraction) and supernatant fluid (membrane-free fraction). These crude fractions were tested for their protein synthesizing activity in the presence or absence of crude initiation factors from reticulocytes.

Fig. 33a-j shows the patterns of labeled polypeptides made *in vitro* in the crude fractionated cell extracts. For comparison the pattern of newly synthesized polypeptides of cells labeled *in vivo* is included (Fig. 33k). Recognition of distinct polypeptide bands in the gel analysis of products made in the resuspended cytoplasmic pellet (Fig. 33c,d,e and h,i,j) is difficult due to the high background in these gels: This background radioactivity may be caused by inaccurately synthesized polypeptide chains or the poorer resolution in this gel system of membrane associated proteins (332). In contrast, sharp separations were observed of those polypeptides that were made in the supernatant fractions (Fig. 33b and g) and to a lesser extent in the 125 x g supernatant (Fig. 33a and f). The most obvious feature of this gel analysis is the high degree of similarity in polypeptide patterns, independent of the fraction used for cell-free synthesis. A closer observation of the autoradiogram reveals that addition of initiation factors, S-100 or pH 5 enzymes, resulted in a slight stimulation of the

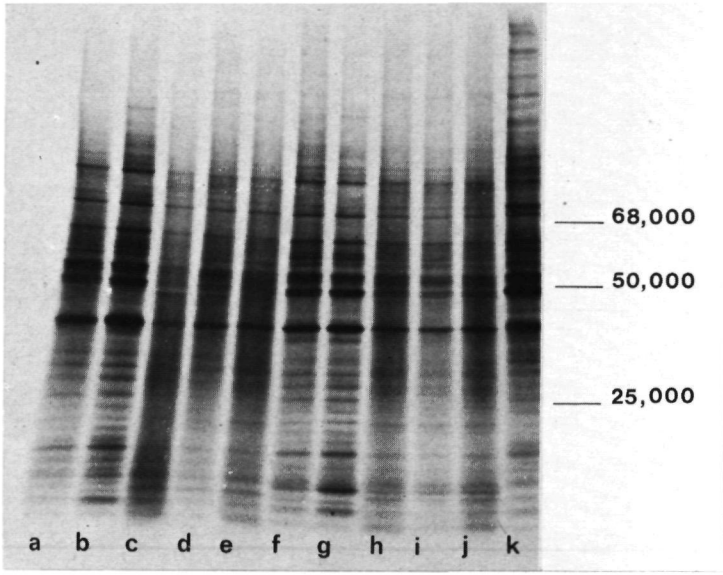


Fig. 33. Autoradiogram of the polypeptides synthesized in crude fractionated cell extracts from JLS-V9 cells electrophoresed on a polyacrylamide slab gel. The procedures for the preparation of subcellular fractions from JLS-V9 cells and for incubation *in vitro* were as described (sections 5.2.3. and 5.2.5.2.). Prior to autoradiographic analysis the slab gel was treated with PPO. Polypeptides synthesized in a cell-free system supplemented with (a) the 125 x g supernatant fraction; (b) the 20,000 x g supernatant fraction; (c) the resuspended 20,000 x g pellet fraction; (d) the resuspended 20,000 x g pellet fraction and S-100 from JLS-V9 cells; (e) the resuspended 20,000 x g pellet fraction and pH 5 enzymes from JLS-V9 cells; (f) to (j) cell-free incubations specified in (a) to (e) repeated in the presence of crude initiation factors from reticulocyte ribosomes; (k) polypeptides synthesized in JLS-V9 cells labeled with $[^{35}S]$ methionine for 60 min.

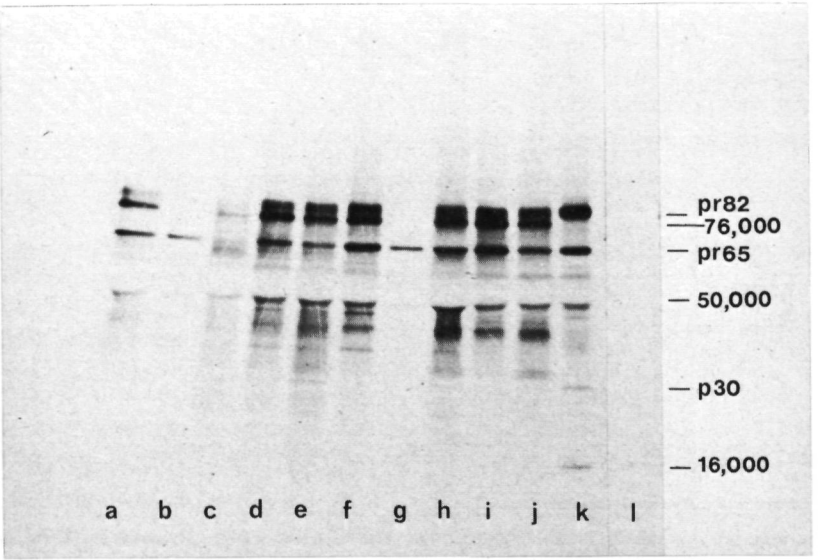


Fig. 34. Polyacrylamide gel electrophoresis of R-MuLV-specific polypeptides synthesized in cell-free systems supplemented with subcellular fractions from JLS-V9 cells. Virus-specific polypeptides were precipitated from the *in vitro* reaction mixtures (see legend to Fig. 33) with rabbit antiserum to R-MuLV (direct method). In this experiment, from each reaction mixture equal amounts of radioactivity incorporated into protein were used for specific and aspecific immunoprecipitation. Prior to autoradiographic analysis the gel was treated with PPO. R-MuLV-specific polypeptides synthesized in a cell-free system supplemented with (a) the 125 x g supernatant; (b) the 20,000 x g supernatant; (c) the resuspended 20,000 x g pellet; (d) the resusended 20,000 x g pellet and S-100 from JLS-V9 cells; (e) the resuspended 20,000 x g pellet and pH 5 enzymes from JLS-V9 cells; (f) to (j) cell-free incubations specified in (a) to (e) repeated in the presence of crude initiation factors; (k) R-MuLV-specific polypeptides synthesized *in vivo*; (l) aspecific immunoprecipitation of polypeptides synthesized *in vitro* in the 125 x g supernatant fraction.

synthesis of several polypeptides in the 20,000 x g pellet fraction (compare Fig. 33c with Fig. 33d,e and h). Finally, with the exception of a number of high molecular weight polypeptides, similar polypeptides were synthesized *in vivo* and in the *in vitro* system (Fig. 33f and k).

Virus-specific polypeptides synthesized in the crude extracts were precipitated by the direct immunoprecipitation method, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and located by scintillation autoradiography of dried slab gels (Fig. 34). In the presence of crude initiation factors predominantly three virus-specific polypeptides were synthesized in the membrane-rich 125 x g supernatant (Fig. 34f) and the resuspended 20,000 x g pellet fraction (Fig. 34h,i and j) with molecular weights of 65,000, 76,000 and 82,000. Among the virus-specific polypeptides synthesized *in vitro* one polypeptide was present, designated 76,000 M_r, which is not detected in the gel analysis of virus-specific polypeptides made *in vivo* (compare Fig. 34h,i and j with Fig. 34k). Incubation of the resuspended 20,000 x g pellet in the presence (Fig. 34i and j) or absence (Fig. 34h) of S-100 or pH 5 enzymes from JLS-V9 cells resulted in the same set of polypeptides.

When the crude initiation factors were omitted, especially the virus-specific polypeptide synthesis in the cytoplasmic pellet fraction was reduced (Fig. 34c). However, addition of S-100 or pH 5 enzymes to this fraction restored the specific polypeptide synthesis completely (Fig. 34d and e). This was apparently due to a deficiency of factors required for protein synthesis in the resuspended 20,000 x g pellet fraction. Among the virus-specific polypeptides synthesized in membrane-rich fractions a number of minor bands were visible with molecular weights of 55,000 and 20,000 - 50,000, similar to those observed in previous experiments employing membrane-bound polyribosomes.

A different result was obtained when the membrane-free 20,000 x g supernatant was incubated *in vitro*. Gel analysis of the immunoprecipitate shows that a polypeptide of 65,000 M_r is the only virus-specific product synthesized (Fig. 34b and g). After direct immunoprecipitation the relative content of labeled polypeptides was 3 to 5 times less in the immunoprecipitate of the 20,000 x g supernatant than in that of the 20,000 x g pellet. This observation is consistent with a previous result obtained with free and membrane-bound polyribosomes. In this context, it should be noted that

the proportion of input radioactivity in the immunoprecipitate is only a rough reflection of the exact percentage of virus-specific polypeptides newly synthesized *in vitro*. From these results, we may conclude that *in vitro* incubation of all crude cytoplasmic fractions gave rise to the synthesis of the virus-specific precursor polypeptide of 65,000 M_r. On the other hand the synthesis of the viral glycoprotein precursor (82,000 M_r) could only be detected by specific immunoprecipitation when membrane-rich fractions were incubated *in vitro*.

5.6. TRANSLATION OF VIRUS-SPECIFIC mRNA PURIFIED ON OLIGO(dT)-CELLULOSE

In previous studies the presence was reported of virus-specific RNA in free and membrane-bound polyribosomes from cells infected with Mo-SV(Mo-MuLV) or R-MuLV (162,163). Virus-specific 35S RNA appeared to be the predominant RNA species present in free polyribosomes. On the other hand, membrane-bound polyribosomes also contained smaller RNA molecules with sedimentation coefficients of about 14S and 20-22S (162,163). However, these studies did not yet demonstrate that these virus-specific RNA species direct the synthesis of specific polypeptides. Therefore, evidence that these RNA species would be translated in certain virus-specific polypeptides could give valuable information about the interrelationship between the smaller RNAs and the 35S RNA.

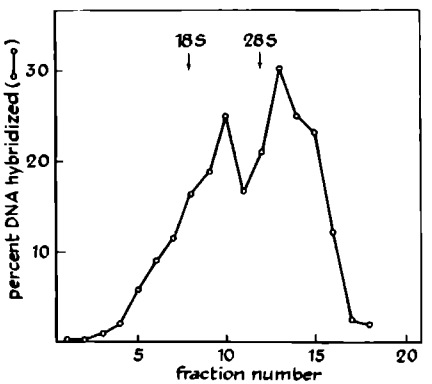


Fig. 35. Sedimentation analysis of poly(A)-containing RNA isolated from polyribosomes of R-MuLV infected cells in sucrose gradients containing 50% formamide. Experimental details were described in the legend to Fig. 25.

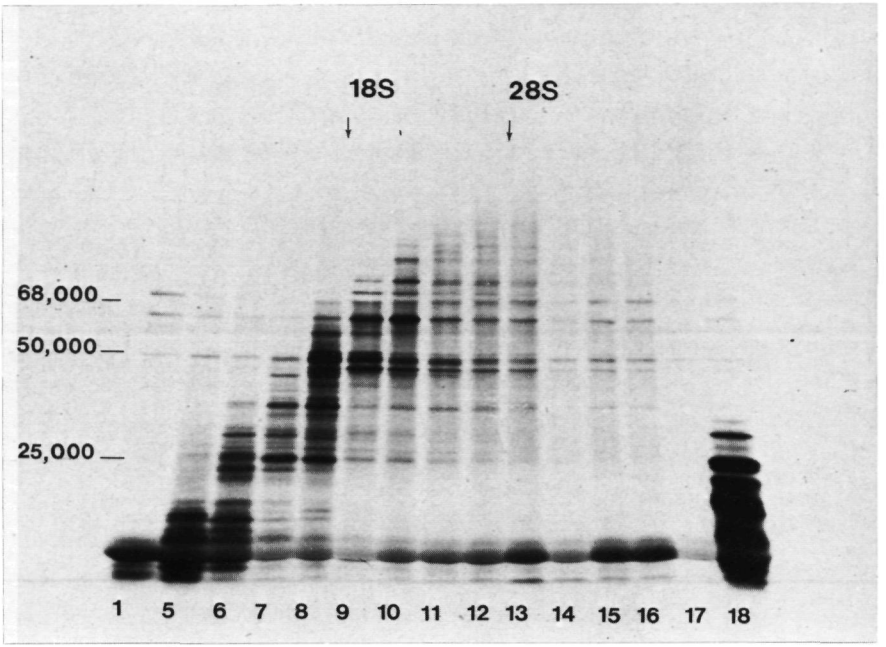


Fig. 36. Electrophoresis on a 7-18% polyacrylamide slab gel of polypeptides newly synthesized *in vitro* under the direction of mRNA fractionated according to size. mRNA from fractions of the gradient shown in Fig. 35 was translated in a lysate from rabbit reticulocytes (section 5.2.5.3.). Equal amounts of the *in vitro* incubation mixtures (equivalent to 1 µl of a 25 µl reaction mixture)were prepared for gel electrophoresis by addition of sample buffer (section 5.2.8.) containing 6 M urea, heated at 95^{o} for 2 min and applied to the sample wells. Prior to autoradiographic analysis the gel was treated with PPO. Polypeptides synthesized in the reticulocyte lysate without exogenous mRNA (1). Columns 5 to 17 refer to the fractions of the gradient shown in Fig. 35. Polypeptides synthesized in the reticulocyte lysate under the direction of 1 µg 10S lens mRNA (18).

In chapter 4, we described the isolation of poly(A)-rich virus-specific RNA by oligo(dT)-cellulose chromatography. In order to prevent degradation of RNA by multiple step isolation procedures, polyribosomes were dissociated with sodium dodecyl sulfate and directly applied to the column. The poly(A)-rich material recovered from the column had a E_{260}/E_{280} ratio of 2.1 to 2.2,

indicating that a pure RNA preparation was obtained. Because of aggregation of this poly(A)-rich RNA, it appeared necessary to use denaturing agents during centrifugation (see chapter 4). Fig. 35 shows the profile of poly(A)-rich RNA hybridizing with the labeled R-MuLV virus DNA probe after gradient centrifugation in the presence of 50% formamide. From the absence of hybridizable material at the bottom of the gradient, it is obvious that no aggregation of virus-specific mRNA had occurred.

Messenger RNA present in individual fractions of this gradient was translated in a reticulocyte cell-free system and the newly synthesized polypeptides were analyzed on a polyacrylamide slab gel. The analysis confirms that there was no aggregation of the bulk of the mRNA in the gradient as there was a clear relationship between the molecular weights of the polypeptides which were synthesized and the sedimentation value of the corresponding messenger fraction (Fig. 36 1,5-17). A large number of polypeptides were made, ranging in molecular weight from 10,000 up to 150,000. After longer exposure times of the dried slab gel even higher molecular weight polypeptides were visualized and a gradual increase of the molecular weights of newly synthesized polypeptides was clearly visible up to fraction 14 (30 - 35S region). For comparison, the translation products of an optimal concentration of 10S calf lens mRNA is shown (Fig. 36,18). It is evident that this mRNA preparation was translated more efficiently than most of our poly(A)-containing RNA fractions. This is not surprising, since the 10S calf lens mRNA preparation is rather homogeneous. The poly(A)-containing mRNA fractions from infected cells, on the other hand, are essentially heterogeneous. Apparently, certain RNA molecules interfere with the translation of others since it follows from the data presented below that optimal synthesis of certain polypeptides was critically dependent on the concentration of poly(A)-rich RNA added to the lysate. Because it was not feasible to determine the optimal concentration of mRNA present in each fraction of the gradient for the synthesis of each virus-specific product, mRNA from each fraction was translated at three arbitrary concentrations.

Fig. 37 and 38 show the polypeptides precipitated from each reaction mixture by the indirect immunoprecipitation method, at the two highest concentrations of mRNA tested. As a reference, the analysis of virus-specific polypeptides synthesized *in vivo* was included (Fig. 37,18 and Fig. 38,18). In a control experiment, immunoprecipitation was carried out on polypeptides

made in the endogenous reticulocyte lysate (Fig. 37,19). One polypeptide with a molecular weight of about 32,000 was precipitated aspecifically by the R-MuLV antiserum; it was present in variable amounts in all analyses.

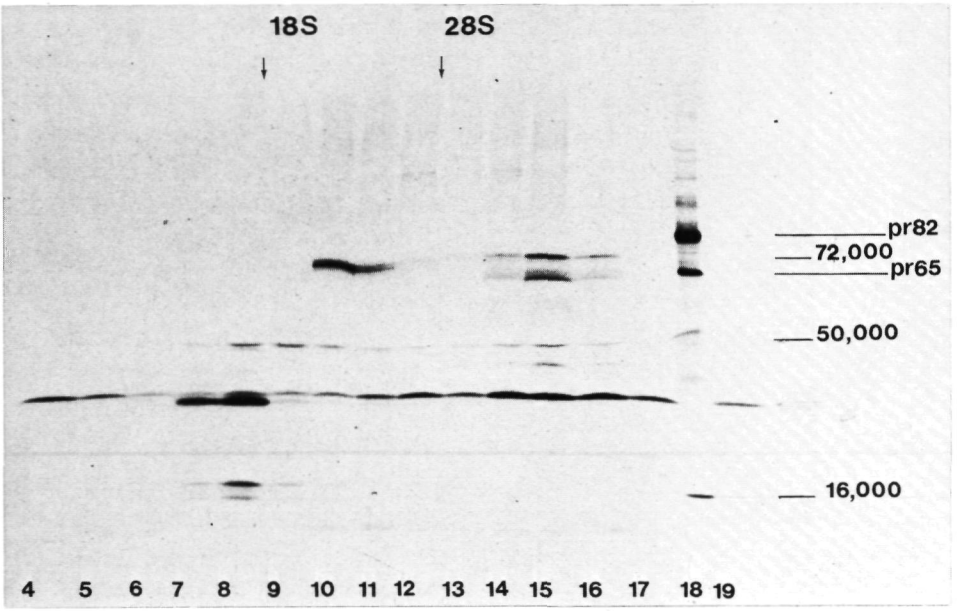


Fig. 37. Autoradiographic analysis of the R-MuLV-specific polypeptides synthesized *in vitro* under the direction of mRNA fractionated according to size. RNA from each fraction of the gradient shown in Fig. 35 was dissolved in 50 µl of distilled water and stored at -20°. The reticulocyte cell-free system was programmed with 3 µl of RNA from each fraction (section 5.2.5.3.). After incubation, the products synthesized in each 25 µl reaction mixture were subjected to indirect immunoprecipitation with rabbit antiserum to R-MuLV. As a control, immunoprecipitation was performed on the products synthesized in the lysate without exogenous RNA. Prior to autoradiographic analysis the gel was treated with PPO. Columns 4 to 17 refer to the fraction of the gradient shown in Fig. 35. (18) R-MuLV-specific polypeptides synthesized *in vivo*. (19) Aspecific immunoprecipitation of polypeptides synthesized in the lysate in absence of exogenous RNA.

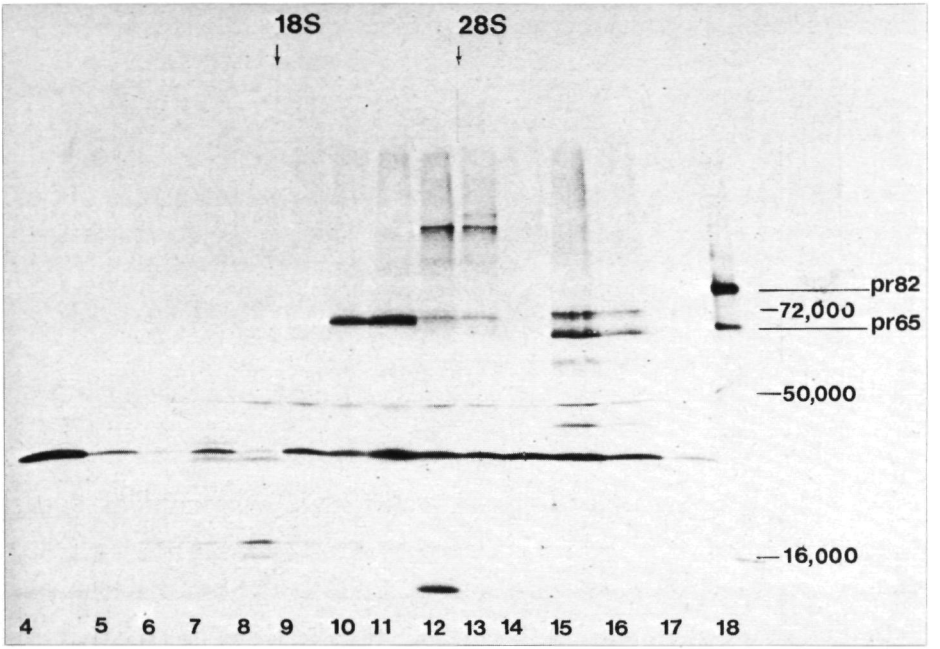


Fig. 38. Autoradiographic analysis of the R-MuLV-specific polypeptides synthesized *in vitro* under the direction of mRNA fractionated according to size. Conditions for cell-free protein synthesis, immunoprecipitation and autoradiographic analysis were as described in the legend to Fig. 37, except that 7 µl of RNA from each fraction was added to the lysate. Columns 4 to 17 refer to the fractions of the gradient shown in Fig. 35. (18) R-MuLV-specific polypeptides synthesized *in vivo*.

mRNA present in the 35S region directed predominantly the synthesis of two major specific polypeptides with molecular weights of 65,000 and 72,000 (Fig. 37,14,15 and 16 and Fig. 38,14,15 and 16) of which the first comigrated with the 65,000 M_r band made *in vivo* (Fig. 39a,b and c). At the highest concentration of mRNA two additional minor polypeptides were visible with molecular weights of 55,000 and approximately 45,000 (Fig. 38,15). No evidence was obtained for the synthesis of the glycoprotein precursor (82,000 M_r) under the direction of the 35S mRNA. The specificity of the polypeptides directed by the 35S mRNA is strongly supported by recent studies of Salden *et al.* (157) on the translation of R-MuLV RNA. These investigators were able to demonstrate the synthesis of two major polypeptides with

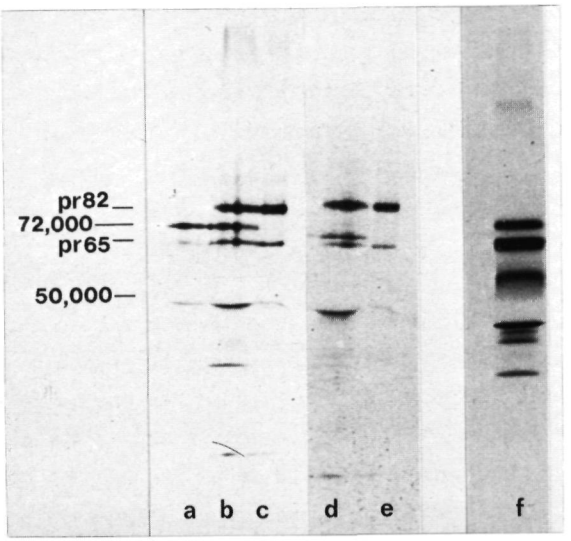


Fig. 39. Comparison of *in vitro* and *in vivo* synthesized virus-specific polypeptides by electrophoresis on polyacrylamide gels. (a) Immunoprecipitate of polypeptides synthesized *in vitro* by mRNA from fraction 15 (Fig. 35); (b) co-electrophoresis of the immunoprecipitates of polypeptides synthesized *in vivo* and those synthesized *in vitro* under the direction of mRNA from fraction 15; (c) and (e) immunoprecipitates of polypeptides synthesized *in vivo*; (d) co-electrophoresis of the immunoprecipitates of polypeptides synthesized *in vivo* and those synthesized *in vitro* under the direction of mRNA from fraction 10 (Fig. 35); (f) immunoprecipitate of polypeptides synthesized in the reticulocyte lysate programmed with 1 µg of denatured R-MuLV RNA.

molecular weights of 65,000 and 72,000 in cell-free systems supplemented with native or denatured R-MuLV RNA (Fig. 39f). Minor virus-specific products were a 55,000 M_r polypeptide and some polypeptides in the 30,000-50,000 M_r region. This pattern of virus-specific products was strikingly similar to that observed after cell-free translation of 35S mRNA (compare Fig. 38,15 with Fig. 39f).

Virus-specific RNA in the 20-22S region gave rise to the synthesis of a 70,000 M_r polypeptide (Fig. 37,10 and 11 and Fig. 38,10 and 11), which was not detected among the virus-specific polypeptides synthesized *in vivo* (Fig. 39d and e). Upon close observation of the gel, it was seen that this

band was actually a double band. At the highest RNA concentration (Fig. 38) the slowest migrating component of both bands was also present in fraction 12 and 13. Moreover, the total picture gives the impression that looking from the lower to the higher fractions the 70,000 M_r band in fraction 10 undergoes gradually a transition to the 72,000 M_r band in fraction 15. The significance, however, of this suggestive observation is still unknown.

Messenger RNA in the 14S region directed the synthesis of low molecular weight polypeptides, one polypeptide band of about 30,000 M_r and two bands in the 15,000 M_r region were visible (Fig. 37,7 and 8 and Fig. 38,7 and 8). Comparison of these figures clearly shows that the synthesis of the 30,000 M_r band was strongly inhibited at the highest RNA concentration. Moreover, especially at the highest RNA concentration we observed the synthesis of high molecular weight polypeptides (100,000 - 200,000 M_r) directed by mRNA of approximately 28S (Fig. 38,13). More experimental evidence is required to explain the significance of this latter observation.

Finally, it is worth noticing the percentage of newly synthesized polypeptides which could be precipitated with antiserum against R-MuLV. At most about 0.2% of the input radioactivity was precipitated from incubation mixtures supplemented with the different mRNA fractions and, thus, clearly shows the high sensitivity of the method we employed to detect virus-specific polypeptides. On the other hand, it is quite obvious that the slightest contamination of the immunoprecipitate, caused by coprecipitation or insufficient washing, may give rise to erroneous results.

5.7. DISCUSSION

The biosynthesis of RNA tumor virus-specific proteins *in vivo* and *in vitro* can be examined despite a vast background of cell proteins by employing antisera prepared against purified virions. Using this technique, two major high molecular weight polypeptides were detected in cells infected with Rauscher leukemia virus. The mature viral proteins p30 and p15 are formed by proteolytic cleavage of a precursor polypeptide of 65,000 molecular weight (designated pr65). This was established by employing monospecific antisera to the purified viral proteins p30 and p15, and by pulse-chase experiments (van Zaane, personal communication, 257). It is likely, however, that the mature virion proteins p12 and p10 are also derived from pr65.

Similarly, a high molecular weight polypeptide of 82,000 - 85,000 M_r (designated pr82) was detected with monospecific antiserum to gp69/71 (van Zaane, personal communication, 257). In further experiments, it was established that pr82 could be labeled with [^{3}H]glucosamine. Moreover, in addition to the viral glycoprotein gp69/71, the viral protein p15(E), which is most likely a constituent of the viral envelope, was also demonstrated to arise by proteolytic cleavage of the intracellular precursor polypeptide pr82 (van Zaane, personal communication).

TABLE 5

R-MuLV-specific polypeptides* synthesized *in vitro*

fraction used for translation	major products (molecular weight x 10^{-3})	minor products (molecular weight x 10^{-3})
125 x g sup	65, 76, 82	30-50, 55
20,000 g pellet	65, 76, 82	30-50, 55
20,000 x g sup	65	
free polyribosomes	65	55, 74
membrane-bound polyribosomes	45, 65	30-50, 55, 74
14S mRNA	10, 15, 30	
20-22S mRNA	70	
35S mRNA	65, 72	30-50, 55
35S viral RNA	65, 72	30-50, 55

* Polypeptides synthesized *in vivo*, major: 82,000 M_r and 65,000 M_r, minor: 74,000 M_r, 70,000 M_r, 28,000 M_r and 16,000 M_r.

In this chapter, we described the synthesis *in vitro* of R-MuLV-specific polypeptides in crude cell extracts, mediated by free and membrane-bound

polyribosomes or directed by mRNA fractionated according to size (compare table 5). All cytoplasmic fractions synthesized a polypeptide with a molecular weight of 65,000, presumably identical with the intracellular precursor polypeptide pr65. However, only the membrane-rich fractions synthesized a polypeptide of 82,000 M_r, probably identical to the intracellular precursor polypeptide pr82, and a polypeptide of 76,000 M_r. Although other explanations are possible, the absence of this latter polypeptide in the cell-free products of the cytoplasmic supernatant suggests that the 76,000 M_r polypeptide was either not completely glycosylated or represented one of the first intermediates in the processing of pr82 from which the viral glycoproteins gp69/71 and the envelope protein p15(E) are derived.

Among several immunoprecipitable polypeptides the 65,000 M_r polypeptide was detectable after cell-free incubation of all polyribosomal fractions. Whereas the glycosylated 82,000 M_r polypeptide was easily recognized as one of the major products synthesized by polyribosomes still attached to membranes, only occasionally a minor labeled band was visible in the cell-free products of polyribosomes released from membranes and never in those of free polyribosomes. Besides, we were not able to detect the 82,000 M_r polypeptide in lysates supplemented with the different mRNA fractions. The simplest explanation for these observations is that although the protein moiety was synthesized in cell-free systems supplemented with the cytoplasmic supernatant, purified polyribosomes or mRNA, this polypeptide was not precipitated by the antiserum, since the protein moiety is less antigenic than the carbohydrate moiety. However, Halpern *et al.* (317) reported that the incompletely glycosylated precursor (70,000 M_r) of the AMV viral glycoprotein (85,000 M_r) was precipitated by a monospecific antiserum against purified gp85, suggesting that at least in that case part of the antigenic determinants are located in the protein moiety. On the other hand, it could be speculated that association with membranes is a prerequisite for the correct synthesis of the glycoprotein. In this context, it is interesting to notice that other groups have also had difficulty in demonstrating the synthesis of viral glycoproteins *in vitro* (333-336).

That membranes are involved in the synthesis of virus-specific polypeptides is evident from the more general observations that they were synthesized in relatively high amounts in membrane-rich fractions or by polyribosomes isolated from these fractions. The earlier finding of our group

(163) and others (162) that the relative content of virus-specific RNA, as measured by hybridization, was 4 - 10 times less on free polyribosomes than on membrane-bound polyribosomes is consistent with these results. Analysis of the cell-free products directed by the R-MuLV-specific mRNA fractionated according to size, shows that at least the 35S mRNA and the 20-22S mRNA fractions directed the synthesis of virus-specific products. Employing anti-R-MuLV serum the same set of polypeptides (65,000 and 72,000 M_r) was detected after translation of 35S mRNA and native or denatured viral RNA. Moreover, in recent experiments evidence was obtained that both polypeptides were also precipitated form the cell-free products directed by denatured viral RNA, using a monospecific antiserum against purified p30 (Salden, personal communication). Together with the finding that a 72,000 M_r polypeptide was synthesized instead of pr65 in canavanine treated cells (van Zaane, personal communication), this implies that the 72,000 M_r polypeptide shares common amino acid sequences with the precursor polypeptide pr65. We never observed the synthesis of the 72,000 M_r polypeptide by purified polyribosomes. On the other hand, we also never observed the synthesis of a 74,000 M_r polypeptide under direction of viral RNA or 35S mRNA, while this polypeptide was actually a minor product synthesized by purified polyribosomes. In addition, preliminary evidence indicates that a monospecific antiserum against p30 precipitated the 65,000 and 74,000 M_r polypeptides from lysates of cells labeled *in vivo* and from the cell-free products made by purified polyribosomes (van Zaane and Gielkens, unpublished observations). If the difference in molecular weight of the 72,000 and 74,000 polypeptides is real, we may speculate that pr65 is derived from a higher molecular weight polypeptide of at least 74,000.

RNA sedimenting in the 20-22S region directed the synthesis of a polypeptide of 70,000 M_r, which was not detected among the cell-free products directed by the viral RNA and 35S mRNA. Although a definite proof is still lacking, it is attractive to speculate that the 70,000 M_r polypeptide shares amino acid sequences with the 72,000 and 65,000 M_r polypeptides. More experimental evidence is also needed to support the virus-specificity of the polypeptides synthesized by mRNA in the 14S region. Since 14S RNA, presumably, has a coding capacity of about 40,000 daltons of protein and a number of low molecular weight polypeptides are synthesized under its direction, it follows that this RNA fraction consists of different virus-specific RNA species or

that the polypeptides are derived from a common precursor. This question should also be settled by further experiments.

In the *in vitro* reaction mixtures several immunoprecipitable polypeptides were detected with molecular weights of 30,000 - 55,000 dependent on the RNA containing fraction added to the cell-free system. Variable amounts of these polypeptides were made. This group of polypeptides may result from premature termination or cleavage of high molecular weight polypeptides due to proteases. Striking is that most of these polypeptides are also visible as faint bands in the lysates of cells labeled *in vivo* (compare Fig. 32a with Fig. 32d and f; Table 5). It is conceivable that the high proportion of these polypeptides in the cell-free products of purified membrane associated polyribosomes is caused by contamination of these polyribosomes with RNAase and proteases released from the membranes by the detergent treatment.

Several pathways are feasible for the synthesis of mature virion proteins,
1. The formation of mature viral proteins by the device of posttranslational cleavage of a single large precursor polypeptide, encoded by the viral RNA, which functions as messenger RNA in the infected cells.
2. The population of messengers required for the synthesis of viral proteins is heterogeneous: some are smaller and others are identical in size to the viral genome; some of the viral proteins are formed by cleavage of precursor polypeptides.
3. Mature virion proteins are synthesized by small monocistronic messengers.

The first possibility, posttranslational cleavage of a single large polypeptide to bypass the apparent inability of mammalian cells to initiate and terminate translation at internal points of the mRNA (337) is known to occur in cells infected with picornaviruses (337-339). The second possibility seems to be, at least partially, met in cells infected with Sindbis or Semliki Forest virus (Togaviruses). In cells infected with these viruses the viral 42S RNA was found in association with polyribosomes. However, the most prominent virus-specific mRNA associated with polyribosomes was smaller in size (26S) than the viral 42S RNA genome (340-343) and contained sequences identical to a part of the viral genome (344). Both mRNAs stimulated the synthesis *in vitro* of core and envelope proteins of the virus (335,336,345,346). However, no translation products of the nonstructural part of the 42S RNA genome have been identified. On account of these observations, Smith *et al.* (335)

suggested that the 26S mRNA is an amplified form of the genes coding for the structural proteins, while translation of the non-amplified portion of the 42S RNA provides the other virus coded proteins, that are required in only small amounts. The fact that 26S mRNA was almost 20 times more active in cell-free systems than the 42S mRNA substantiates this idea (347). Finally, pathway 3 is most likely the mechanism which is operative for Rhabdoviruses which contain a RNA genome of negative polarity. Several messengers with sedimentation values of 12S to 18S have been identified in vesicular stomatitis virus infected cells (348,349).

If the entire genome of RNA tumor viruses would be translated into a single polyprotein of about 300,000 M_r which is cleaved by proteolytic enzymes to form the functional virus proteins, only one virus-specific messenger RNA species should be associated with polyribosomes. However, apart from the 35S mRNA which is identical in size to the RNA subunits of these viruses, lower molecular weight mRNAs of 20-22S and about 14S have been found (160-163). Furthermore, we provided evidence that apart from the 35S RNA also the RNA species of smaller size served as messenger and directed the synthesis of virus-specific polypeptides *in vitro*. In these studies and those on the identification of intracellular virus-specific polypeptides no evidence was obtained for the synthesis of a giant polypeptide of about 300,000 M_r. In fact, the precursor for the mature virus capsid proteins and the viral envelope proteins had molecular weights smaller than 85,000 (253-257). Thus, these observations do not support the possibility that the entire genome of RNA tumor viruses is translated into a single polypeptide. It should be noted, of course, that higher molecular weight precursor polypeptides would escape detection if they would lack the proper antigenic determinants. Finally, if the entire genome is translated into a single polypeptide, differential expression of virus genes (29,350) would be difficult to explain, since such a mechanism predicts that the viral proteins should always be synthesized in equal molar amounts. Therefore, although these data are not yet complete enough to allow final conclusions on the functions of the intracellular viral mRNAs of different size classes, they do favor model 2 in our opinion. This view is based, largely, on the observation that in addition to the viral 35S mRNA smaller messengers also code for virus-specific proteins in our cell-free system.

Evidence for the etiological role of certain RNA-containing viruses in the development of tumors in some animals has been provided by many investigators. The viral theory of cancer has led to an enormous research on the biochemistry and the mode of replication of RNA tumor viruses.

A short review on some of the main topics of the molecular biology of these particles is presented in Chapter 1. The most essential characteristic of RNA tumor viruses is their mode of replication via a double-stranded DNA intermediate, which becomes integrated as a provirus in the cellular DNA of the host. A second striking feature of these viruses is their widespread occurrence, suggesting that these particles may be involved in the development of cancer in human as well.

The Rauscher leukemia virus, a murine-type RNA tumor virus, causes an erythroid leukemia in mice. We have used tissue culture cells infected with this virus as a model system to study the biosynthesis of virus proteins. When these investigations were started details on this phase of the 'life-cycle' of RNA tumor viruses were still lacking.

In the experimental part of Chapter 1, we described the separation of the proteins of purified Rauscher leukemia virus particles by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. By this method virion proteins are separated according to the molecular weights of their polypeptide chains. The autoradiograph of the gels containing a linear gradient of acrylamide, clearly shows that the virion contains in addition to the major structural proteins a large number of minor proteins, which have not yet been characterized. Apparently, the minor bands do not represent contamination since they were present in virions isolated from different cell lines as well as from plasma of leukemic mice. Furthermore, we obtained evidence for heterogeneity in virion protein p10, which was found to consist of two or three distinct protein components.

In order to establish whether the viral RNA genome itself acts as messenger for the synthesis of viral proteins, purified RNA from Rauscher leukemia virus, avian myeloblastosis virus and mammary tumor virus was translated in a cell-free system from bacteria (Chapter 2). We could show that addition of the individual viral RNAs from these three unrelated RNA tumor viruses resulted in the synthesis of a specific set of polypeptides. Thus, the viral

RNA has messenger properties and most likely contains the sequences of the virus-specific messenger RNA in infected cells.

Convincing evidence for the presence in infected cells of virus-specific messenger RNA, containing sequences identical to the viral genome, was provided in Chapter 3. By hybridization with radioactive DNA complementary to the viral RNA, we were able to detect virus-specific RNA sequences in free and membrane-bound polyribosomes of cells infected with the Rauscher leukemia virus. Determination of the relative content of virus-specific RNA sequences in free and membrane-bound polyribosomes indicated that virus-specific proteins are preferentially synthesized in association with membranes. Size analysis of the virus-specific RNA associated with polyribosomes by density gradient centrifugation revealed the presence of three size classes of virus-specific messenger RNA. The most prominent class of virus-specific messenger RNA has a sedimentation value of approximately 35S and is identical in size and polarity to the virion RNA subunits produced by thermal or solvent dissociation of the 60-70S viral RNA genome. In addition, in the 20-22S and 14S region of the gradient two minor classes of virus-specific RNA were found, suggesting that virus-specific RNA components smaller in size than the viral RNA subunit of 35S may serve as messengers for the synthesis of viral proteins. After separation of the cellular ribosomes in free and membrane-bound polyribosomes a striking difference in the size distribution of virus-specific RNA of both types of polyribosomes was observed. Whereas one major virus-specific RNA species (35S) was associated with free polyribosomes, three major size classes (35S, 20-22S and 14S) were found in membrane-bound polyribosomes.

In Chapter 4 we described the effect of heat and formamide dissociation on the size distribution of virus-specific RNA. Dissociation of intermolecular and intramolecular hydrogen bonds resulted in a drastic change of the size distribution of virus-specific RNA. Upon exposure to high temperatures or to organic solvents virus-specific RNA accumulated in the 14S and 20-22S region at the expense of the 35S RNA indicating the presence of noncovalent aggregates of virus-specific RNA in the latter part of the gradient. This shift in the size distribution was also observed when cytoplasmic RNA of cells transformed by a mouse sarcoma virus was analyzed.

In addition, we provided evidence for the presence of poly(A)-rich tracks, presumably located at the 3'terminal end, in virus-specific mRNA isolated

from polyribosomes. Analysis of this RNA in sucrose gradients containing formamide indicated the presence of two prominent classes of virus-specific RNA with sedimentation values of 35S and 20-22S.

Although these observations provided very suggestive evidence for the messenger properties of the various virus-specific RNA species, definite proof could only be provided by demonstrating the synthesis of virus-specific polypeptides directed by these RNA fractions. Since the virus-specific polypeptides constitute a few percent only of the total protein content of infected cells, they should be distinghuished from the vast background of cellular proteins by immunoprecipitation with specific antisera directed against purified virus. Thus, we could detect the virus-specific polypeptides synthesized in cell-free systems supplemented with polyribosomes, fractionated cell extracts or fractionated mRNA (Chapter 5). Purified free and membrane-bound polyribosomes mediated the synthesis *in vitro* of a polypeptide with a molecular weight of 65,000, which is immunologically and electrophoretically identical to the intracellular precursor polypeptide (pr65) of several mature structural virus proteins. In contrast, cell-free incubation of polyribosomes still attached to membranes resulted in the synthesis of an additional polypeptide (82,000 M_r) which appears to be identical to the intracellular precursor polypeptide (pr82) of two viral proteins located in the virion envelope. This precursor polypeptide (82,000 M_r), however, was not detected among the cell-free products synthesized in a cell fraction containing free polyribosomes only. In addition, virus-specific polypeptides appeared to be preferentially synthesized on membrane-bound polyribosomes. This observation was consistent with our results from hybridization experiments.

Finally, by translation of mRNA fractionated in sucrose gradients containing formamide in a mammalian cell-free system, we were able to verify the messenger properties of the virus-specific RNA associated with polyribosomes. Messenger RNA sedimenting in the 35S region directed the synthesis *in vitro* of two polypeptides of 65,000 and 72,000 molecular weight. The former polypeptide is identical in size to the intracellular precursor polypeptide (pr65) of several low molecular weight virus proteins. No evidence, however, was obtained for the synthesis of a polypeptide corresponding in size to the virus-specific glycosylated precursor of 82,000 molecular weight (pr82). One polypeptide of 70,000 molecular weight was detected among the cell-free

products directed by mRNA sedimenting in the 20-22S region. However, this virus-specific polypeptide was not found among the virus-specific polypeptides synthesized in infected cells. The synthesis of some low molecular weight virus-specific polypeptides of approximately 30,000 and 15,000 molecular weight was observed, when the cell-free system was programmed with mRNA from the 14S region.

These findings further substantiate our idea that in addition to the virus-specific 35S mRNA smaller virus-specific RNA species may serve as messengers for the synthesis of virus proteins in cells infected with Rauscher leukemia virus.

Het bestaan van een causaal verband tussen infecties met een aantal RNA-bevattende virussen en het ontstaan van tumoren bij de besmette dieren is door een groot aantal onderzoekers overtuigend bewezen. De theorie, dat virussen betrokken zijn bij de ontwikkeling van kanker heeft er dan ook toe geleid dat vele moleculair biologen hun aandacht gericht hebben op de biochemische eigenschappen en het replicatie-mechanisme van RNA tumorvirussen.

In hoofdstuk 1 worden de belangrijkste moleculair biologische aspecten van deze virusdeeltjes besproken. De belangrijkste ontdekking, die het onderzoek aan deze virussen heeft opgeleverd, is ongetwijfeld de replicatie van de genetische informatie van deze virussen via een dubbel-strengig DNA intermediair, dat als provirus geïntegreerd wordt in het cellulaire DNA van de gastheer. Opvallend is ook het voorkomen van RNA tumorvirussen in een grote verscheidenheid van diersoorten, hetgeen suggereert dat deze virussen eveneens betrokken kunnen zijn bij de ontwikkeling van kanker bij de mens.

Het Rauscher leukemie-virus, dat behoort tot de groep van RNA tumorvirussen, veroorzaakt leukemie in een aantal muizenstammen. Als een modelsysteem voor de bestudering van de biosynthese van Rauscher leukemie-virus-eiwitten kozen wij weefselkweek-cellen die geïnfecteerd waren met dit virus. Aan deze phase uit de 'levens-cyclus' van RNA tumorvirussen, was vrijwel geen onderzoek verricht toen wij dit onderzoek aanvingen.

In het experimentele gedeelte van hoofdstuk 1 beschrijven wij de karakterisering van de virale eiwitten van gezuiverd Rauscher leukemie virus met behulp van polyacrylamide-gelelectroforese in aanwezigheid van natrium dodecylsulfaat. Deze techniek maakt het mogelijk de virale eiwitcomponenten te scheiden op basis van het molecuulgewicht van hun polypeptideketens. De resultaten van de gelanalyse tonen aan dat het virus naast een aantal hoofdcomponenten ook een groot aantal eiwitten bevat die slechts in geringe hoeveelheden vertegenwoordigd zijn. Dit zijn kennelijk geen verontreinigingen, want een aantal van deze laatste eiwitcomponenten bleken aanwezig te zijn zowel in virusdeeltjes die geproduceerd werden in verschillende cellijnen, als ook in gezuiverd virus uit het plasma van leukemische muizen. Verder vonden wij aanwijzingen dat een van de virale eiwitten, namelijk p10, bestaat uit twee of drie eiwitcomponenten, die slechts zeer geringe verschillen in molecuulgewicht vertoonden.

Teneinde vast te stellen of het virale RNA genoom van RNA tumorvirussen als boodschapper kan fungeren, werd het virale RNA van Rauscher leukemie-virus, kippen leukose-virus en mamma tumor-virus toegevoegd aan een cel-vrij systeem verkregen uit bacteriën. Vertaling van het gezuiverde RNA van deze drie niet immunologisch verwante virussen in het bacteriele systeem leidde tot de synthese van polypeptiden, die na gelanalyse specifiek bleken voor het toegevoegde virale RNA. Hiermede was bewezen dat het virale RNA als boodschapper kan fungeren en daarom zeer waarschijnlijk dezelfde base-sequenties bevat als de virus-specifieke boodschapper in de geïnfecteerde cel.

Het definitieve bewijs voor de aanwezigheid in geïnfecteerde cellen van virus-specifieke boodschappers, die dezelfde sequenties bevatten als het virale RNA genoom, werd verkregen door middel van hybridisatie-experimenten (hoofdstuk 3). Met radioactief DNA, dat de complementaire base-sequenties van het virale RNA bevat, waren wij aldus in staat virus-specifieke RNA sequenties aan te tonen in vrije en membraan-gebonden polyribosomen, verkregen uit cellen geïnfecteerd met het Rauscher leukemie-virus. Bovendien verkregen wij met behulp van deze hybridisatie-experimenten duidelijke aanwijzingen dat de biosynthese van virale eiwitten preferentiëel in associatie met membranen plaats vindt.

Na fractionering van het polyribosomale RNA in glycerol-gradienten werd met behulp van hybridisatie de verdeling van het virus-specifieke RNA in de gradient vastgesteld. Deze experimenten lieten zien dat er in de geïnfecteerde cel drie grootte-klassen van virale boodschappers aanwezig zijn. De belangrijkste virale boodschapper heeft een sedimentatiewaarde van ongeveer 35S, en is wat lengte en polariteit betreft identiek aan de virale RNA subunits die ontstaan na verbreken van de intermoleculaire waterstofbruggen in het 60-70S RNA van het virus. Daarnaast vonden wij virus-specifieke RNA componenten met sedimentatiewaarden van ongeveer 20-22S en 14S. Deze waarneming suggereert dat deze laatste RNA fracties evenals het 35S RNA fungeren als boodschapper voor de synthese van virale eiwitten.

Na scheiding van de cellulaire ribosomen in vrije en membraan-gebonden polyribosomen, werd een opvallend verschil waargenomen in de grootte-verdeling van de virus-specifieke RNAs uit beide typen polyribosomen. De virus-specifieke RNA populatie geassocieerd met vrije polyribosomen bestond voornamelijk uit 35S RNA. Daarentegen waren drie virus-specifieke RNA componenten duidelijk te onderscheiden na sedimentatie in glycerol-gradienten van het

RNA dat geassocieerd is met membraan-gebonden polyribosomen.

In hoofdstuk 4 beschreven wij de gevolgen die dissociatie van intermoleculaire en intramoleculaire waterstofbruggen heeft op de verdeling van de virus-specifieke RNA moleculen in gradienten. Dissociatie van waterstofbruggen werd bewerkstelligd door het virus-specifieke RNA te incuberen bij hoge temperatuur of in aanwezigheid van formamide. Na analyse van het behandelde RNA in glycerol-gradienten bleek een duidelijke verandering in de verdeling van virus-specifieke RNA moleculen te hebben plaatsgevonden. De virus-specifieke RNA-populatie in het 14S en 20-22S gebied van de gradient, was duidelijk toegenomen ten koste van het 35S RNA. Deze bevinding wijst op de aanwezigheid van niet covalent gebonden aggregaten van virus-specifiek RNA, die onder niet-denaturerende condities sedimenteren in het 35S gebied van de gradient.

Tevens toonden wij aan dat het virus-specifieke RNA in polyribosomen poly(A)-rijke base-sequenties bevat, die zeer waarschijnlijk covalent gebonden zijn aan het 3'einde van de virale boodschappers. Het poly(A)-bevattende RNA bleek na analyse in suiker-gradienten te bestaan uit twee grootte-klassen met sedimentatiewaarden van 35S en 20-22S.

Hoewel deze waarnemingen duidelijk wijzen op de mogelijkheid dat de verschillende virus-specifieke RNA moleculen een boodschapper functie vervullen, kan de definitieve bewijsvoering voor een dergelijke functie slechts geleverd worden door translatie-experimenten. Aangezien de virus-specifieke eiwitten echter slechts 1-3 procent uitmaken van de totale eiwitpopulatie in geïnfecteerde cellen, is het alleen mogelijk de virale eiwitten van de overmaat cellulaire eiwitten te onderscheiden door middel van immunoprecipitatie met specifieke antilichamen gericht tegen gezuiverd virus. Met deze techniek bleek het mogelijk de virus-specifieke eiwitten te identificeren, die nieuw gesynthetiseerd werden in cel-vrije systemen waaraan polyribosomen, gefractioneerde celextracten of gefractioneerd boodschapper RNA werd toegevoegd (hoofdstuk 5).

Toevoeging van vrije en membraan-gebonden polyribosomen aan een cel-vrij systeem leidde tot de synthese van voornamelijk een polypeptide met een molecuulgewicht van 65.000. Dit nieuw gesynthetiseerde eiwit bleek electroforetisch en immunologisch identiek aan het intracellulaire precursor-eiwit (pr65) waaruit een aantal laag moleculaire viruseiwitten ontstaan door proteolytische afsplitsing. Wanneer echter aan het cel-vrije

incubatiemengsel polyribosomen werden toegevoegd die niet vrijgemaakt waren van de membranen, werd de synthese waargenomen van een additioneel eiwit met een molecuulgewicht van 82.000. Dit cel-vrije produkt is waarschijnlijk identiek aan de intracellulaire precursor (pr82) van twee eiwitten die in de buitenmembraan van het virus gelokaliseerd zijn. Het 82.000 eiwit kon niet aangetoond worden na toevoeging van een cel-fractie die alleen vrije polyribosomen bevat. In deze experimenten werd bovendien aangetoond dat de virale eiwitsynthese preferentiëel in de membraan-gebonden polyribosomale fractie plaatsvindt. Deze laatste waarneming bevestigt onze resultaten verkregen door middel van hybridisatie proeven.

Tenslotte waren wij in staat de boodschapperfunctie van in gradienten gefractioneerd virus-specifiek RNA te bevestigen door vertaling in een celextract van konijnen-reticulocyten. Toevoeging van 35S boodschapper RNA leidde tot de synthese van twee eiwitten met molecuulgewichten van 65.000 en 72.000. Het 65.000 eiwit is waarschijnlijk identiek aan de intracellulaire precursor pr65 van een aantal laag moleculaire viruseiwitten. Geen aanwijzingen werden verkregen, dat deze RNA fractie in staat is de synthese te bewerkstelligen van het precursoreiwit met een molecuulgewicht van 82.000 (pr82), dat in de geïnfecteerde cel aangemaakt wordt. De 20-22S boodschapperfractie gaf aanleiding tot de synthese van een virus-specifiek eiwit met een molecuulgewicht van 70.000. Er zijn geen duidelijke aanwijzingen dat een virus-specifiek produkt van deze grootte in gemakkelijk te detecteren hoeveelheden in de geïnfecteerde cel wordt gemaakt. De synthese van enkele laag moleculaire virus-specifieke eiwitten met een molecuulgewicht van ongeveer 30.000 en 15.000 werd waargenomen na toevoeging van de 14S boodschapperfractie. Opgrond hiervan poneren wij, dat naast het virus-specifieke 35S boodschapper RNA ook kleinere virus-specifieke boodschappers betrokken zijn bij de synthese van virale eiwitcomponenten in cellen geïnfecteerd met het Rauscher leukemie-virus.

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CURRICULUM VITAE

A.L.J. Gielkens werd geboren op 26 april 1944 in Oirsbeek. Na het behalen van het diploma gymnasium β in 1963 aan het Bernardinuscollege te Heerlen, studeerde hij Scheikunde aan de Katholieke Universiteit te Nijmegen. Het doktoraalexamen met als hoofdvak biochemie, werd afgelegd in november 1969. Vanaf december 1969 was hij als Fellow van het Koningin Wilhelmina Fonds verbonden aan het laboratorium voor Biochemie van de Katholieke Universiteit te Nijmegen. Sinds 1 juni 1975 is hij als wetenschappelijk medewerker verbonden aan het Centraal Diergeneeskundig Instituut, afdeling Virologie, te Lelystad.

STELLINGEN

I

De volgorde van de genetische elementen, env, gag, onc en pol, binnen het RNA-genoom van aviaire sarcomavirussen is: 5'-gag-pol-env-onc-3'; voor aviaire leukosevirussen: 5'-gag-pol-env-3'.

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II

Een werkhypothese voor verder onderzoek aan de gen-expressie van RNA-tumorvirussen zou kunnen luiden: 'Op de virale mRNA's van verschillende grootteklassen is alleen het 5'-terminale cistron voor vertaling beschikbaar.' Deze hypothese zou inhouden dat het gag-gen ligt aan de 5'-zijde van het 35S RNA, een implicatie, die in goede overeenstemming is met de tot dusver beschikbare gegevens over de gen-volgorde bij deze virussen.

Referenties bij stelling I en dit proefschrift.

III

Het ontbreken binnen het RNA-genoom van aviaire leukosevirussen van het gen dat verantwoordelijk is voor transformatie van fibroblasten in vitro en het gebrek aan ruimte voor andere genen dan gag, pol en env, doet vermoeden dat leukose bij kippen het gevolg is van een virus geïnduceerde expressie van oncogene informatie die bij de gastheer aanwezig is.

IV

Een aantal van de eiwitcomponenten die in geringe hoeveelheden aanwezig zijn in RNA-tumorvirusdeeltjes, zijn waarschijnlijk identiek aan de proteolytische splitsingsproducten van de gag-precursor.

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mRNA's coderend voor eiwitten die niet door de cel worden uitgescheiden zijn wellicht op andere wijze aan het ruwe endoplasmatische reticulum gebonden als die welke informatie bevatten voor eiwitten die wel voor export bestemd zijn.

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VII

Berekeningen van het aantal moleculen interferon per cel nodig om de virusproduktie van die cel te remmen, berusten, voor zover zij op het werk van Ng en Vilcek teruggaan, zowel op een verkeerde toepassing van de verdelingswet van Poisson als op een storende rekenfout.

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VIII

In hun ijver prostaglandine E-receptoren in rattelevermembranen aan te tonen, hebben Smigel en Fleischer zich ten onrechte niet verdiept in de fysiologische relatie tussen receptor en ligand.

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IX

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X

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XI

De grote toepassing die vaccinatie met avirulente virusstammen heeft gevonden bij de preventie van de ziekte van Marek, een door een herpesvirus geïnduceerde lymfoproliferatieve ziekte bij kippen, vraagt om meer onderzoek naar de mogelijkheden van vaccinatie ter voorkoming van kanker bij andere species.

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XII

De betrouwbaarheid van voorspellingen van influenza epidemieën is vergelijkbaar met de betrouwbaarheid van de weervoorspellingen in de Enkhuizer almanak.

A.L.J. Gielkens
6 mei 1976.