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Ph. D.

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ANALYSIS OF POLYRIBOSOMES AND INTRACELLULAR RNA FROM FELINE LEUKEMIA VIRUS INFECTED CELLS

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presented by

Anthony Joseph Conley

has been accepted towards fulfillment
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Public Health

Leland F. Velicer
Major professor

Date April 29, 1977

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ABSTRACT

ANALYSIS OF POLYRIBOSOMES AND INTRACELLULAR RNA FROM FELINE LEUKEMIA VIRUS INFECTED CELLS

By

Anthony Joseph Conley

The chronically infected thymus tumor cell line, F-422, produces the Rickard strain of feline leukemia virus (FeLV-R). This oncornavirus contains 50 to 60S genomic RNA, which can be denatured to yield subunits which sediment at 28S, molecular weight of 1.8 X 10^6.

The intracellular virus-specific RNA from this cell line was analyzed by using *in vitro* synthesized complementary FeLV-R DNA. By hybridization kinetics analysis cytoplasmic, polyribosomal, and nuclear ribonucleoprotein (nRNP) particle RNAs were found to be 2.09%, 2.63%, and 1.95% virus-specific, respectively.

Size classes within subcellular fractions were determined by velocity sedimentation in the presence of 99% dimethylsulfoxide (DMSO) and hydridization of the fractionated RNA to the FeLV-R DNA. The nuclear and cytoplasmic extract fractions contained a 28 to 30S size class, which corresponds to the size of the genomic subunit. Both fractions also contained 36S, 23S, and 12 to 18S RNA species. Virus-specific 36S, 23S, and 18S species were present in both the total and the poly(A)+ polyribosomal fraction. The nRNP particle fraction

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contained 36S, 28S, 23S, and 18S virus-specific RNA. The four size classes were also present in the nRNP particle poly(A)+ RNA. The presence in the total nuclear and the nRNP particle fractions of smaller than genomic subunit size RNA, which corresponds to sizes observed in the cytoplasm and polyribosomes, suggests nuclear processing of FeLV-R RNA in these chronically infected cells.

To further understand the mode of oncornavirus RNA translation, the polyribosomes from F-422 cells were examined by using both immunological and nucleic acid hybridization techniques. Virus-specific nascent proteins were detected by binding ^{125}I-labeled anti-FeLV IgG to polyribosomes. Normal rabbit serum (NRS) IgG bound at a level of 0.02% throughout the polyribosome region of the gradient. In contrast anti-FeLV IgG bound to rapidly sedimenting polyribosomes at a level of 0.25 to 0.40%. The peak binding was at 400S polyribosomes. Two preparations of anti-FeLV p30 had little or no binding. The binding of anti-FeLV IgG was further studied to determine its specificity for nascent virus-specific protein. NRS IgG did not compete with anti-FeLV IgG for the polyribosomal binding sites. Total viral protein and p30 absorbed specific antibody from the IgG preparation and the binding of the absorbed IgG was reduced in relation to the amount of protein used. The binding of anti-FeLV IgG to puromycin treated polyribosomes was reduced by the same proportion that nascent proteins were released.

The FeLV-R DNA probe hybridized to two polyribosomal regions, approximately 400 to 450S and 250S, and to a slower sedimenting region, approximatel

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approximately 80S, within the polyribosomal gradients. After EDTA treatment, the DNA still hybridized to RNA from slower sedimenting regions, less than 80S, but not to the two polyribosome regions. The size classes of virus-specific RNA within these regions was determined as described above. The 400 to 450S polyribosomes contained three major peaks at 33S, 22S, and 18S; whereas, the 250S polyribosomes contained only 34S and 18S RNA. RNA from the approximately 80S regions obtained with and without EDTA treatment contained 28S RNA in addition to the 34S, 22S, and 18S virus-specific RNA. The absence of 28S virus-specific RNA in polyribosome regions suggests that FeLV-R subunit RNA is not virus-specific mRNA in infected cells. The presence of 34S, 22S, and 18S RNA within polyribosome regions which also contain nascent virus-specific proteins, suggests that there are three species of FeLV-R-specific mRNA in the F-422 feline thymus tumor cell line.

ANALYSIS OF POLYRIBOSOMES AND INTRACELLULAR RNA FROM FELINE LEUKEMIA VIRUS INFECTED CELLS

By

Anthony Joseph Conley

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1977

DEDICATION

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I acknowledge financial support from the National Institutes of Health and especially from Jane C. Conley. I would also like to express my appreciation to the Department of Microbiology and Public Health for travel assistance to attend scientific meetings.

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INTRODUCTION

The oncornaviruses occupy an unusual position in the field of virology. This class of viruses can infect and transform cells in culture, can cause neoplasia in vertebrates, and can synthesize a DNA copy of its RNA genome. Consequently, the oncornaviruses have received considerable research attention in recent years.

The feline leukemia viruses comprise the one group of mammalian oncornaviruses known to be horizontally transmitted in an outbred species. Thus, the feline leukemia virus systems are excellent model systems for the study of possible virus-induced neoplasia in humans.

An interesting immunological phenomenon is seen in the feline leukemia virus system. Cats which have had an apparent infection but which do not have feline leukemia virus-induced neoplasia, have antibody to a transformed cell surface antigen. The nature of this antigen, feline oncornavirus membrane antigen, is not known. Thus, the study of the origin and molecular nature of feline leukemia virus components or gene products can aid in our understanding of the mechanisms and events which occur in neoplastic diseases.

The presence of an RNA-dependent DNA polymerase is probably the major unique characteristic of the molecular biology of oncornaviruses. The viral polymerase directs the synthesis of a double stranded DNA copy of the viral RNA genome which can be integrated in the host cell genome. Thu

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genome. Thus, there are a number of questions about replication, production, and control of production of the viral macromolecules.

A particularly interesting problem concerns control of the amounts of the various viral gene products synthesized. These products are apparently synthesized in nonequimolar quantities, and some in the form of precursors; a situation which raises questions about the virus-specific messenger RNA and the translational mode of this RNA.

The overall aim of this research was to study the intracellular feline leukemia virus-specific RNA and to determine some of the translational features of this RNA in the chronically infected F-422 thymocyte cell line. Among the specific aims of this research were to:

(i) determine the amount of feline leukemia virus-specific RNA within subcellular fractions.

(ii) determine the sizes of these intracellular RNA species.

(iii) examine virus-specific protein synthesis on F-422 polyribosomes by using anti-feline leukemia virus serum as an immunological probe for nascent viral protein, and in vitro synthesized feline leukemia virus DNA as a nucleic acid probe for virus-specific RNA.

(iv) determine the major size class or polyribosomes synthesizing viral proteins.

(v) specifically determine the size of the polyribosomal-associated presumptive, virus-specific mRNA.

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LITERATURE REVIEW

The major topic of this review is intracellular oncornavirus-specific RNA and the possible translational mode(s) for oncornavirus-specific mRNA. Since other aspects of oncornavirus structure and replication are important in a development of this central theme, a brief discussion of the important properties of these viruses will also be presented. These reviews include short discussions of oncornavirus RNA, proteins, the viral replication cycle, and organization of the viral genome.

1. Oncornavirus RNA

Viral Genome: The genome of oncornaviruses consists of RNA, which has a sedimentation coefficient of 50 to 70S under native conditions (58,148). This RNA, referred to as genomic high molecular weight RNA, can be dissociated to 30 to 40S subunits and various minor heterogenous smaller species (58,148). Dissociation of the genome has been achieved by the use of heat or denaturing agents, such as dimethylsulfoxide or formamide, which disrupt hydrogen bonding and secondary structure (7,38). Electron microscopic studies have shown that, in the native state, the genome consists of subunit dimers noncovalently joined near the subunit 5'-termini (30,80,81). The 3'-termini are probably free and can be detected by electron microscopic techniques which are specific for polyadenylation (12).

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Viral Genomic Subunit: The genomic subunits from most known oncornaviruses have been analyzed. Four techniques have been used for subunit molecular weight determinations. These are: velocity sedimentation in neutral sucrose gradients after denaturation, velocity sedimentation in denaturing sucrose gradients, electrophoresis in polyacrylamide gels (PAGE) after denaturation, and length measurement in the electron microscope. Notwithstanding the considerable variation in size among the oncornaviruses, the subunit is referred to as the 30 to 40S genomic subunit RNA. Subunit sedimentation coefficients range from 28S for the Rickard strain of feline leukemia virus (FeLV-R), the endogenous feline RD-114 virus, and the Soehner-Dmochowski murine sarcoma virus (MSV-SD) (21,36,37), to 35 to 44S for Rauscher murine leukemia virus (R-MuLV) (7). There are comparative electron microscopic (23,88, 175), sedimentation (22), and electrophoretic (31,35) data available for the molecular weight determination of Rous sarcoma virus (RSV) RNA subunits. These data are in agreement and the RSV subunit has a molecular weight in the range of 2.7 to 3.5 x 10^6.

A special case exists for some avian sarcoma viruses, where two electrophoretically separable subunits have been found (34). The a subunit is approximately 10 to 15% larger than the b subunit (34,35), and is the only subunit present within recloned avian sarcoma viruses (35). The b subunit is electrophoretically identical to the single size subunit present in the avian leukosis viruses (35).

Chemical Characteristics of the Subunit: The oncornavirus subunit RNA has properties which are similar to eukaryotic mRNA.

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Polyadenylation of subunit 3'-termini has been detected for the murine leukemia-sarcoma viruses (70,82,197,111), RSV (78,82,109,169), the avian myeloblastosis virus (AMV) (135), and FeLV (21). Subunit RNA from R-MuLV may also contain poly (C) and poly (G) sequences by virtue of its specific binding to poly (G) and poly (C) agarose columns, respectively (103). However, not all oncornaviruse subunits contain these sequences (55).

The general structure $7^{m}G(5')ppp(5')NmpNp$, referred to as a "cap", is a characteristic of the 5'-terminus of most eukaryotic mRNA (115). In early experiments, free phosphorus was not found at the 5'-termini of RSV subunit RNA (128). Further studies on RNA from long-term harvested virus and virus harvested from cell cultures at extremely short intervals confirmed this observation (78). From this work experiments were performed to demonstrate that the cap structures could be detected in avian oncornavirus RNA (77,48,136) and murine oncornavirus RNA (20,112). However, no cap structures could be detected in FeLV-R RNA (147). Subunit RNA from RSV, MuLV, and FeLV-R contain internally methylated nucleotides (20,136,147). N^6-methyladenosine is the predominant methylated species.

2. Oncornavirus Proteins and their Biosynthesis

The oncornavirus protein components have been the subject of considerable study. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) and agarose gel filtration in 6M guanidine hydrochloride (GuHCl) have been the most widely used analytical techniques (45,59,98). It is now assumed that the leukemia viruses

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contain three major classes of proteins, i.e. (i) the low molecular weight structural proteins, (ii) the envelope glycoproteins, and (iii) the RNA-dependent DNA polymerase. The nondefective avian sarcoma viruses contain these three classes of proteins plus a component which is involved in cell transformation (32). The nature of this additional component or its presence in virions is not known. These assumptions are in agreement with the genetic capacity of the viruses and in some cases have been verified (see section 4). Most of the remaining minor proteins observed in analytical SDS-PAGE of virions are considered to be host cell or serum contaminants (137).

Low molecular weight oncornavirus proteins are designated by their molecular weight in thousands, prefixed by a lower case p, glycoproteins the same, prefixed by the lower case gp (5).

Low Molecular Weight Proteins: The avian oncornaviruses contain four low molecular weight structural proteins—p27, p19, p15, and p12 (45,46,64,19). Their structural positions within virions have also been determined (108,138,139,140). They are synthesized by way of an unstable 76,000 dalton, intracellular precursor (166,167). The precursor can be detected by pulse-label and immunoprecipitation of infected cell proteins (166). The precursor-product relationship has been confirmed by pulse-chase experiments and tryptic peptide analysis of the precursor and the viral proteins.

The murine and feline leukemia viruses contain analogous low molecular weight structural proteins (57,59,98,119,137). Synthesis of these proteins also proceeds by intracellular cleavage of comparable

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size precursors (4,72,100,101,124,158). R-MuLV infected cells may further contain a 200,000 dalton precursor (4,95,96,124). This extremely large precursor was not observed by other investigators in similar experiments with the same virus (116,158).

Oncornavirus Glycoproteins: The glycoprotein components from avian, murine, and feline oncornaviruses have been studied (45,59,68, 92,177,178). Morphologically, the glycoproteins are the virus-specific components of the envelope.

Avian oncornaviruses contain two glycoproteins, gp85 and gp37 (18,45) which are linked by disulfide bonds in the envelope as a complex (83). Pulse-chase labeling experiments combined with immunoprecipitation of intracellular proteins suggest that gp85 is produced from a nonglycosylated 70,000 dalton precursor (60). However, no information is available about the relationship of this precursor to the synthesis of both glycoproteins.

Among the variety of murine oncornavirus strains, glycoproteins designated gp70 or gp69/71 appear to be the major envelope glycoproteins (33,68,90,92,93,102). Other minor envelope components have been reported (69,71,93,118). These have been designated gp45 and p15 (E); an apparently nonglycosylated envelope constituent. The structural position and viral specificity of the two smaller proteins has yet to be confirmed (90). R-MuLV gp69/71 and p15 (E) antigenic determinants and tryptic peptides are present in an unstable 90,000 dalton intracellular protein (4,39,124). The unstable precursor is partially glycosylated (96,124) and may be an intermediate in the cleavage of a

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very high molecular weight precursor--260,000 to 300,000 daltons, which has been detected intracellularly (124).

RNA-Directed DNA Polymerase: The oncornaviruses were first shown to contain an RNA-directed DNA polymerase by Baltimore (8) and Temin and Mizutani (146). Since that time numerous investigations have been directed toward the characterization of this enzyme, its catalytic properties, and involvement in oncornavirus infection and replication. These topics have been amply reviewed (58,91,145,148) and will not be discussed here. However, a brief presentation of some characteristics of these enzymes follows.

The avian polymerase consists of two subunits with molecular weights of approximately 65,000 and 105,000 (44,76). The smaller subunit can be generated from the larger by proteolytic cleavage (92), and appears to be the major enzymatically active subunit (56).

Unlike the avian enzyme, the mammalian oncornavirus polymerases consist of one protein with an estimated molecular weight of 70,000 (2,50,113,149). Although the mode of biosynthesis of the RNA-directed DNA polymerase is not known, the avian polymerase is a virus-specific product (164, and section 4).

3. Integration of Oncornavirus DNA--the Provirus

Early observations suggested that productive infection and transformation of cells with oncornaviruses required DNA synthesis immediately after infection (6,142). These observations led to the hypothesis that oncornaviral RNA is copied into DNA, the provirus (144), which can

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be incorporated into the host genome (143). The discovery of the RNA-directed DNA polymerase (8,146) confirmed that genomic RNA can be "reversely transcribed" into DNA. Since then, infectious proviral DNA has been recovered from RSV and Moloney-MuLV infected cells (65,130), and has been demonstrated to be covalently integrated into the host genome (89,162). The DNA intermediate appears to be synthesized in the infected cell cytoplasm (51,161), where it can exist in a variety of forms ranging from linear molecules of 2.5 to 3.0 X 10^6 daltons for RSV and M-MuLV (87,160), to closed circular molecules of 5 to 6 X 10^6 daltons for M-MuLV (51,130). This suggests that double-stranded DNA copies of the viral genomic subunit are the major proviral molecules which are eventually integrated (89,127,159,162). The integrated DNA is considered the transcriptional template for the production of progeny viral RNA and virus-specific mRNA (58,145).

4. Organization of the Oncornavirus Genome

Since the oncornavirus genome consists of subunits, a number of problems had to be resolved before genetic and physical maps could be constructed. The major obstacle concerned the complexity of the genome, i.e., is each subunit identical, a polyploid genome; or is each subunit unique, a haploid genome?

In avian oncornavirus systems, high frequency of recombination was observed for the viral markers-host range, transformation, and RNA-dependent DNA polymerase (32,76,165,176). These observations would suggest reassortment of genomic subunits. However, analysis of the RNA of specific recombinants suggested that this was not the case.

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Nondefective sarcoma-positive recombinants, containing a leukosis virus host range marker, retains the a subunit RNA (11,32). This result could not be obtained from reassortment and indicated that crossing-over is the probable mechanism of recombination. These analyses lend indirect support to polyploidy.

Two direct approaches have been used to relate the size of the oncornaviral genome to the complexity of its RNA. In the first, reassociation kinetics analysis is used, in the second a chemical analysis which determines an apparent complexity (11,14) is used.

An empirical relationship has been established between the rate of annealing of RNA to a complementary probe and the complexity of that RNA. In vast RNA excess, the rate of annealing is inversely proportional to the complexity (15,17). By this method, the complexity of RSV RNA was determined to be 9.3×10^6 daltons (141) and that of M-MuLV, approximately 9.0×10^6 daltons (43). These values are close to the molecular weight determinations of 1×10^7 for the 70S genomic RNA, and are suggestive of unique subunits. Baluda et al. (10) performed the same type of analysis on AMV RNA, employing strict conditions for the purification of RNA standards. Their results indicate a complexity of approximately 3×10^6 daltons, suggesting identical subunits, and are opposite the results obtained in the previous two investigations.

The second, chemical method has consistently yielded a complexity measurement of approximately 3.5×10^6 daltons for RSV RNA (11,14,32). The general approach for this method is the isolation and molar yield determination of a series of unique oligonucleotides from T_1 RNase

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digests of subunit RNA. This data can then be used to calculate the complexity of the RNA (11,14). Since these data support a complexity of approximately 3.5 X 10^6 daltons, a polyploid genome is most likely and is now the accepted complexity. This further implies that oncornaviruses encode approximately 300,000 daltons of virus-specific protein (9).

The construction of genomic physical maps and the identification of RSV gene order has been achieved by using the same technique of T_1 RNase-derived oligonucleotide isolation. Although there are probably slight differences among the oncornaviruses, the general deduced gene order can be considered valid for both avian and mammalian sarcoma-leukemia viruses.

In these experiments, the oligonucleotides were oriented to the 3'-polyadenylated end of the genomic subunits. Maps were constructed from these orientations by comparison of deletion mutant and recombinant RNA oligonucleotides (24,73,170,171,172,173). Wang et al. (170) located sarcoma marker specific oligonucleotides, designated-sarc, near the 3'-termini. They also observed conserved sequences at the 3'-terminus, 5' to the polyadenylation (170). The location of the sarc oligonucleotides and the presence of conserved sequences at the 3'-terminus were confirmed in a similar fashion (24,73). Host range marker oligonucleotides, designated-env, were found in the range of 2800 to 5000 nucleotides from the 3'-terminus, i.e., near the middle of the subunit, in nondefective RNA (171). This analysis employed comparison of nondefective RNA with RNA from defective deletion mutants (171). The same map location for env was determined by correlation of

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recombinant and parental oligonucleotides (73,172). Finally, Wang et al. (173) mapped RNA-dependent DNA polymerase ts markers, designated-pol, between 6000 and 8000 nucleotides from the 3'-terminus. Correlations were also made in these recombinants between the parental protein marker p27, and the origin of oligonucleotides at or near the 5'-terminus (173). The viral structural protein sequences, designated-gag, are probably located at the 5'-terminus. Evidence for gag at the 5'-terminus is also found from *in vitro* protein synthesis experiments using viral RNA. Structural protein precursors are the major products observed (54,79,105,168).

The gene order consistent with all these data and which is considered essentially correct for nondefective avian sarcoma viruses is: 5'-gag-pol-env-sarc-50 conserved N-poly(A)-3' (9,173). Avian leukosis viruses do not have the sarc sequences (170), and a virus-specific sarc marker has not yet been identified for the murine and feline oncornaviruses.

5. Intracellular Oncornavirus-Specific RNA

The RNA-dependent DNA polymerase can be used in an *in vitro* DNA synthesis reaction to prepare DNA copies of the oncornaviral RNA. DNA probes were first used to detect virus-specific RNA in both avian and mammalian oncornavirus infected cells (26,155). The initial experiments suggested that intracellular RNA was of the same polarity as viral RNA and could be best detected using preparations of complementary DNA preselected by hybridization with viral RNA (26). Garapin et al. (49) found that when the reaction was performed in the presence of

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actinomycin D, single stranded DNA was the major product. This DNA was also found to be a relatively uniform copy of the genome (49). The methods involved in complementary DNA preparation are well documented (27,74,110,114). The complementary DNA used for analyzing intracellular RNA usually has been prepared by synthesis in the presence of actinomycin D or preselected by hybridization with viral RNA.

Reassociation kinetics analysis, in RNA excess, with viral RNA as the standard has been generally used to quantitate intracellular virus-specific RNA under steady state conditions. Hybridization saturation experiments have been used for determinations of homology of RNA species from various cell lines to the DNA probes. Recently, techniques have been developed for specific binding of RNA:DNA-poly(dC) hybrids to poly(I) sephadex columns (25) and specific binding of RNA to matrix bound complementary DNA (16). Besides use in quantitation, these techniques allow for the analysis of newly synthesized virus-specific RNA. In the presentation that follows these types of hybridization techniques have been widely used.

<u>Virus-Specific RNA in Infected Avian Cells</u>: Virus-specific RNA has been found in cells infected with avian oncornaviruses. The major characteristics of these RNAs are summarized in Table I. In most studies with the avian oncornaviruses, chicken embryo fibroblast (CEF) cells have been the usual host cell and RSV has been used for infection. In all these studies, the amount of virus-specific RNA has been quantitated relative to the total amount of intracellular RNA. The values for virus-specific RNA range from approximately 0.1% (120) to 2.0% (16).

TABLE I: Virus-Specific RNA in Infected Avian Cells.

Virus	Cell	Subcellular Location	(% Virus-Specific) Quantity	(Sedimentation Coefficient) Size	Note	Reference
RSV-B77	CEF	Cytoplasmic	1%	---	(+) genomic homology	(26)
RSV-Prague C	CEF	Total Cell	.14%	---	---	(120)
		Polyribosomal	---	35S, 10 to 30S	---	
RSV-Schmitt-Rupin	CEF	Total Cell	.3% to .4%	heterogenous 4S to 50S	---	(84)
		Cytoplasmic	.25% to .6%	---	---	
		Nuclear	.1% to .2%	---	---	
RSV-Schmitt-Rupin	CEF	Total Cell	.36%	---	---	(104)
		Cytoplasmic	.36%	---	---	
		Nuclear	.46%	---	---	
RSV-Schmitt-Rupin	CEF	Total Cell	.6% to .9%	---	---	(25)
RSV-B77	DEF	Nuclear	2%	---	poly(A)+	(16)
RSV-Prague C	CEF	Cytoplasmic	.2%	35S + 24S	poly(A)+	
RAV-2	CEF	Total Cell	.7%	35S + 24S	---	(63)

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In general, these values constitute the accepted range for measurement of RNA under steady state conditions.

It has also been possible to measure newly synthesized RNA by labeling and specific selection or by rapid infection and transformation. Parsons et al. (104) measured newly synthesized RNA for virus-specific sequences by labeling, annealing the RNA to a heterologous DNA probe, and selecting virus-specific RNA:DNA hybrids. Nuclear virus-specific RNA was detected within 15 minutes and its level remained stable. Cytoplasmic virus-specific sequences were also present at early time points, however the level continued to increase.

In another experiment, virus-specific intracellular RNA was examined immediately after infection and transformation of cells by RSV (120). The amount of RNA at various times postinfection was determined by hybridization kinetics analyses. Within 12 hours postinfection, virus-specific sequences were detected intranuclearly and shortly thereafter reached a stable level of approximately 0.03%. In contrast, cytoplasmic virus-specific RNA levels continued to increase.

Recently, the pulse labeling and selection techniques have been applied to an examination of the size of newly synthesized virus-specific nuclear RNA (16). An RNA species, approximately 15% larger than genomic subunits was detected. This species was not detected in longer labeling periods, suggesting that the viral RNA species are synthesized as a precursor which must undergo intranuclear processing (16).

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The sizes of intracellular avian oncornavirus-specific RNA have been examined (16,63,88,120). When analyzed under native conditions, whole cell virus-specific RNA is quite heterogenous (88). Denatured RNA (16,63) or specific subcellular fraction RNA (120), were found to consist of more discrete species. Specifically, 36S and 24S size classes were found (16,63). In another study, polyribosomal associated virus-specific RNA was found to consist of 35S and 10 to 30S species (120).

Detection of Intracellular Avian Oncornavirus-Specific RNA with Probes of Specific Sequence: Stehelin et al. (132,133) devised methods for preparation of specific RSV gene sequence DNA probes. Briefly, DNA synthesized from a nondefective RSV template was hybridized to transformation defective RNA. The nonhybridizing DNA sequences, representative of the sarc gene, designated DNA_{sarc}, were separated from the hybrids. In an analogous manner env gene DNA sequences, designated DNA_{env}, were prepared (62).

DNA_{sarc} was found to hybridize to 24S RNA present in the cytoplasm and on polyribosomes of Schmitt-Rupin-RSV transformed hamster cells (16,28). Since the 24S RNA is also present in SR-RSV infected virus-producing and transformed CEF cells, the DNA_{sarc} hybridization suggests that the RSV sarc gene is transcribed (or processed) into both 36S and 24S intracellular virus-specific RNA (28).

DNA_{env} as well as representative DNA, hybridized to both 36S and 24S intracellular RNA (63). Either the 24S RNA is a heterogenous population of transcripts, or both sarc and env sequences are present within one transcript.

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Viral RNA has been used as a specific hybridization probe for the detection of intracellular virus-specific RNA. Stavnezer et al. (131) reported the presence of negative strand sequences, i.e., RNA complementary to viral RNA. These sequences are homologous to approximately 40% of the viral RNA. They exist in nuclei and in the cytoplasm as part of RNA duplex structures at relative concentrations of 0.005% and 0.0005%, respectively. Their function is not known. They may however, be involved in virus-specific mRNA processing (131).

Virus-Specific RNA in Infected Mammalian Cells: The major characteristics of virus-specific RNA in infected mammalian cells are summarized in Table II. M-MuLV and R-MuLV infected cells have been widely used for these analyses. The characteristics of intracellular virus-specific RNA which have been studied and determined include subcellular location, quantity of RNA, and sedimentation values.

Green and colleagues (126,150,153,155,163) examined M-MuLV specific RNA in infected cells. Virus-specific sequences were present in total cellular RNA as two distinct sizes with sedimentation coefficients of 35S and 20S (155). These sequences comprised approximately 0.7% of the total RNA and approximately 0.6% of polyribosomal-associated RNA (163). Both species appear to contain 3'-terminal polyadenylation (150), and these sizes were found to be unevenly distributed on cellular polyribosomes. Free polyribosomes only contained the 35S RNA whereas the membrane-bound polyribosomes contained both the 35S and 20S (126). The 350S sedimenting polyribosomes of both classes contained the majority of virus-specific sequences in the 78A1 M-MuLV infected cells (163).

TABLE II: **Virus-Specific RNA** *in* **Infected Murine Cells.**

Virus	Cell Line	Subcellular	(% Virus-Specific)	(Sedimentation Coefficient)

TABLE II: Virus-Specific RNA in Infected Murine Cells.

Virus	Cell Line	Subcellular Location	(% Virus-Specific) Quantity	(Sedimentation Coefficient) Size	Note	Reference
MuLV-Moloney	78A1	Cytoplasmic	---	35S + 20S	---	(155)
MuLV-Moloney	78A1	Polyribosomes	.58%	---	Major Virus-Specific Polysomes at 350S	(163)
		Total Cell	.68%	---		
MuLV-Moloney	78A1	Cytoplasmic Poly(A)+	---	35S + 20S	---	(150)
MuLV-Moloney	78A1	Free Polyribosomes	---	35S	---	(126)
		Membrane-Bound Polyribosomes	---	35S + 20S	---	
MuLV-Moloney	78A1	Total Cell	---	35S + 20S	---	(153)
MuLV-Moloney	Balb/3T3	Total Cell	---	35S + 20S	---	
MuLV-Moloney	NIH/3T3	Total Cell	---	35S + 20S	---	
MuLV-Moloney	JLS-V11	Cytoplasmic	.25%	(70S), 35S, 20 to 30S	---	(40)
MuLV-Moloney	SCRF-60A	Cytoplasmic	.9%	(70S), 35S	---	
		Polyribosomes	---	35S + 20S	Major Virus-Specific Polysomes at 150-200S	
MuLV-Moloney	NIH/3T3 (cloned)	Cytoplasmic	.14%	---	---	(42)
		Polyribosomes	.03%	---	Major Virus-Specific Polysomes at 300S	
MuLV-Moloney	NRK	Cytoplasmic	1.3%	---	---	(61)
		Nuclear	.7%	35S + 25S	Virus-Specific RNA >35S Detected	
MuLV-Rauscher	JLS-V5	Cytoplasmic	.1% to .4%	38S	No Hybridization	(174)
MuLV-Rauscher	JLS-V9	Membrane-Bound Polyribosomes	.3%	36S, 21S, 14S	36S RNA only on >230S Polysomes	(52)
		Free Polyribosomes	.05%	36S	---	
MuLV-Rauscher	JLS-V9	Total Polyribosomes	---	36S, 21S, 14S	---	(54)
		Polyribosomal Poly(A)+	---	36S + 21S	14S Minimal	

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Fan and Baltimore (40) also studied the intracellular M-MuLV-specific RNA. The viral sequences were 0.25% of the cytoplasmic RNA in JLS-V11 cells and 0.9% in SCRF-60A cells. High molecular weight RNA of approximately 70S was found associated with cytoplasmic membrane material and could be removed from RNA preparations by mild trypsin pretreatment of the cells. The polyribosomes were shown to contain the 35S and 20S size virus-specific RNA. Polyribosomes sedimenting in the 150 to 200S range contained the majority of virus-specific sequences. However, later examination of M-MuLV infected JLS-V11 cell polyribosomes, showed that the greater than 300S polyribosomes contained the majority of virus-specific sequences (42).

Three virus-specific classes of RNA were found in R-MuLV infected cell polyribosomes (52,54). The quantity and distribution of sizes differed between free and membrane-bound polyribosomes. The free polyribosomes contained 0.05% virus-specific sequences and three size classes--36S, 21S, and 14S. Relatively more 36S RNA was found in membrane-bound polyribosomes of fast sedimenting classes than in slower sedimenting classes. The 36S and 21S virus-specific RNA sizes were also shown to contain polyadenylate sequences (54).

Virus-specific M-MuLV sequences were examined in infected NRK cell nuclei (61). These sequences comprised 0.7% of nuclear RNA and appeared heterogenous when analyzed under native conditions. After denaturation, most of the virus-specific RNA had a sedimentation coefficient of 35S and approximately 25S. However, a small amount of virus-specific RNA sedimenting slightly faster than the 35S RNA was still present when analyzed under denaturing conditions.

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Endogenous Oncornavirus-Specific RNA in Mammalian Cells: Type-C viruses, morphologically similar to the leukemia and sarcoma viruses, have been isolated from a variety of vertebrate species. These viruses, referred to as endogenous RNA viruses, apparently exist as stable genetic constituents of the host cell and can sometimes be induced to express oncornavirus proteins and complete virions (1). The biology and regulation of the endogenous viruses will not be discussed here. However, a description of intracellular virus-specific RNA of some of the endogenous viruses is presented.

Fan and Besmer (41) examined the endogenous virus-specific RNA in the JLS-V9 uninfected murine cells. These cells contained two species of RNA with sedimentation coefficients of 38S and 27S which hybridized to a DNA probe prepared from iododeoxyuridine-induced endogenous virus. Based on the criteria of EDTA release from polyribosomes, virus-specific mRNA could not be detected under the conditions of analysis. In later experiments involving a more complete fractionation of the cytoplasm and using EDTA release from polyribosomes as the criteria, endogenous virus-specific mRNA was detected in these JLS-V9 cells (42). The mRNA had sedimentation coefficients of 38S and 27S. The total cytoplasmic RNA was found to be 0.22% virus-specific, and the mRNA approximately 5% of this value. Thus, approximately 0.01% of the cytoplasmic RNA was virus-specific mRNA. Although viral sequences can be detected, these cells remain negative for virus production and viral gene products (42).

The other mammalian endogenous virus which has been studied is the feline RD-114 virus. Okabe et al. (99) studied RD-114 virus-specific RNA in the RD-114 human cells and a number of cat cells.

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Virus producing RD-114 cells contained approximately 0.15% virus-specific sequences, which were heterogenous in size. Various cat cell lines and normal cat tissues contained significant amounts of RD-114 RNA, which however were less than virus producing cells. The normal cat cell RD-114-specific RNA was found in two distinct size classes of 35S and 18S (99).

Mammalian Sarcoma Virus-Specific Intracellular RNA: Mammalian sarcoma viruses can cause tumors in animals and transform cells in culture. In this respect, they are similar to avian sarcoma viruses. In contrast to the avian system, mammalian sarcoma viruses are defective for replication, requiring a "helper" leukemia virus for envelope proteins (148) and probably also for RNA-dependent DNA polymerase (134). These viruses are also rarely isolated. Two of the major murine sarcoma viruses studied, Kirsten-murine sarcoma virus (Ki-MSV) and Harvey-MSV (H-MSV) were isolated after passage of Ki-MuLV and M-MuLV in rats, respectively. In consideration of their unique position among oncornaviruses, the characteristics of the intracellular virus-specific RNA of mammalian sarcoma viruses are presented separately.

In early experiments, a major difference between virus-specific intracellular RNA of virus-producing and non-producing, transformed cells was noticed by Tsuchida et al. (155). A cell line, designated HT-1 (nonproducing, but transformed by H-MSV) was found to contain a single 35S size species of virus-specific RNA. The detection was performed using DNA probe prepared with M-MuLV. The intracellular HT-1 RNA had only 30 to 40% homology to the M-MuLV DNA probe. Further experiments were performed in which the HT-1 intracellular RNA was shown to

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shown to be 33S and have approximately 50% homology to the M-MuLV DNA probe (153).

An analogous homology between Ki-MuLV DNA probe and RNA from cells transformed by Ki-MSV was observed by Benveniste et al. (13). In these studies, RNA from a number of normal, Ki-MSV transformed, spontaneous or DNA tumor virus transformed, and virus-producing transformed cells was annealed with Ki-MuLV DNA probe. RNA from either rat or mouse virus-producing cell lines had high homology, approximately 68%, with the DNA probe. RNA from either rat or mouse transformed, non-producing cells had only approximately 40% homology to the DNA probe. Normal rat and mouse cell RNA, and the non-oncornavirus transformed cell RNA, had little homology to Ki-MuLV DNA probe. Slightly more than 5% DNA hybridization was detected with some of the normal cell RNA. These results suggested that non-producing, Ki-MSV transformed cells contain some but not all of the sequences present in viral RNA, and that there is a possible partial gene expression (12).

Another question which pertains to a determination of which sequences of the mammalian sarcoma viruses are expressed in transformed cells is; what other homologies are contained within the sarcoma RNA other than the homologous leukemia virus sequences. This problem has been partially clarified in a number of hybridization experiments (122, 123).

RNA from either rat or mouse Ki-MSV transformed cells hybridized approximately 15% of rat endogenous leukemia virus (RaLV) DNA probe (123). This suggested an additional homology with genomic information

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of RaLV. RaLV DNA probe did not hybridize to Ki-MuLV infected cell RNA, M-MSV transformed cell RNA, or normal mouse cell RNA. The same relationship of partial homology with RaLV DNA probe was found to exist for H-MSV (122,156).

Tsuchida et al. (156) extended the analysis of intracellular sarcoma-specific RNA. In these experiments a "sarcoma virus-specific" DNA probe, designated 58-2TS DNA, was prepared by annealing and separating helper-specific sequences from DNA made by viruses from M-MuLV rescued Ki-MSV transformed cells. RNA from non-producer, transformed cells and transformed, induced cells had high, approximately 80%, homology with 58-2TS DNA probe. The intracellular RNA detected with 58-2TS probe consisted of a 30S RNA size species. R-MuLV DNA probe did not hybridize to the 30S RNA.

Further hybridization analyses with the 58-2TS DNA probe demonstrated the presence of the 30S RNA in normal rat cells (151). In agreement with the studies on the levels of rat endogenous sequences in Ki-MSV transformed mouse and rat cells (122,123), it was shown that the hybridizing sequences specific for mouse (Ki-MuLV) and rat (RaLV) in Ki-MSV sequences, are both present within the same 30S intracellular virus-specific RNA.

It was hypothesized that the initial passages of Ki-MuLV or M-MuLV *in vivo*, in rats, was accompanied by a recombinational event between the MuLV and rat endogenous sequences to generate the sarcomagenic but yet replication-defective Ki-MSV and H-MSV, respectively (122,123). Since these sequences reside on a single intracellular RNA

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molecule, unique to the initial rat cell and the resultant MSV transformed cells, the recombinational hypothesis is probably correct.

To verify the hypothesis, and to examine whether the 30S RNA of H-MSV comes from a stable genetic element, Tsuchida et al. (152) studied the level and size distribution of intracellular RNA sequences related to hamster, mouse, and rat oncornaviruses in the B-34 transformed hamster cell line. The B-34 cell line was derived from a H-MSV induced mouse tumor passed in hamsters. B-34 cellular RNA was found to contain the rat derived sequences, the mouse derived sequences, and additional sequences homologous to the endogenous hamster leukemia virus (G-HaLV). All sequences were detected by hybridization with DNA probes of strict viral specificity. As with the H-MSV sequences in murine and rat cells, the RaLV DNA probe, the Ki-MSV and H-MSV DNA probes detected a 30S intracellular virus-specific RNA; whereas the G-HaLV DNA probe detected a 35S RNA and had no homology to the 30S RNA species.

A hamster-mouse hybrid cell line which produces an excess of H-MSV over R-MuLV was examined for intracellular H-MSV-specific sequences (154). Again the intracellular RNA detected was a 30S size species. Finally, intracellular MSV-specific RNA has been detected in a number of experiments, in which sarcoma-specific DNA probes have been used (47,121).

The intracellular RNA of rat tumor cells has also been shown to contain the sarcoma-specific sequence (3). It appears that the sequences represented in the sarcoma-specific RNA are not only involved in *in vitro* transformation, but also in the specific neoplasias of susceptible species.

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6. Translation of Oncornavirus RNA

An understanding of the translational mode of oncornavirus mRNA has yet to be achieved. Discrete message production is not inherent based on a fragmented genome as is the case for reovirus or myxovirus RNA. Although post-translational cleavage mechanisms are operative in the production of oncornavirus low molecular weight structural proteins, there probably is not an entire dependence on cleavage of a 'polyprotein' for the generation of all viral proteins as is the case for pincornavirus RNA. Since both progeny virion and virus-specific mRNA arise by transcription of the integreated DNA genomic copy, the effects of control on viral transcription and intranuclear processing are probably just as important as the polyribosomal functioning of virus-specific RNA.

This section will serve two functions: (1) to present what information is known about possible control of virus-specific transcripts which could serve as viral mRNA, (2) to review the information on the polyribosomal-associated virus-specific RNA and the criteria for its consideration as viral mRNA.

Transcriptional Control and Intranuclear Processing: Humphries and Temin (67) studied the initiation of transcription in the initial times after infection of cells with RSV. Virus-specific RNA was not transcribed in stationary cells and cell division inhibitors; such as, colchicine, would prevent the initiation of transcription. The effect was only on the first cell division, i.e., colchicine added to infected cells after the first division would not prevent the transcription of virus-specific RNA. Further studies were undertaken to examine the

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rate of synthesis of virus-specific RNA throughout the cell cycle (66). There was never more than a two-fold variation in the amount of virus-specific RNA synthesized, leading to the conclusion that after the initial cell division, the rate of virus-specific RNA synthesis is independent of the cell cycle (66).

Since the production of virus-specific RNA is probably controlled by cellular mechanisms, experiments were also performed to gain insights into the enzyme system involved in viral RNA synthesis. α-amanitin, an inhibitor of DNA-dependent RNA polymerase-form II, was found to inhibit the synthesis of viral RNA within 2 hours after addition (29). Under these conditions, very little inhibition of ribosomal RNA synthesis was observed. Since the transcription of viral RNA has a sensitivity to α-amanitin which is similar to that of cell mRNA, these experiments further suggest that virus-specific RNA synthesis is dependent on cellular mechanisms.

Actinomycin D has also been used to perturb intracellular virus-specific RNA synthesis. Levin et al. (85,86) have found that de novo synthesis of M-MuLV proteins occurs in the presence of actinomycin D. The functional half-life of viral mRNA measured in this manner was 6 to 8 hours. Under the same conditions, progeny virion RNA had a half-life of 1.5 to 2 hour. These experiments indicate a functional separation of viral transcripts; progeny virion RNA turns over rapidly; whereas, viral mRNAs are much more stable under these conditions. Since virion RNA and intracellular virus-specific RNA are of the same polarity, the differences in half-life would not be expected if

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transcripts served both message and progeny functions in an equilibrated intracellular pool.

Hayward and Hanafusa (62) examined both endogenous and exogenous intracellular virus-specific RNA levels in cells containing endogenous viral sequences which were infected with the Bryan strain of RSV (BH-RSV, defective in env sequences). In these experiments, the intracellular levels of endogenous virus-specific RNA were not affected by infection with BH-RSV. Likewise, the intracellular BH-RSV-specific RNA levels were not affected by the presence or absence of endogenous sequences. It was concluded that the presence or absence of endogenous sequences is not involved in the control of transcription of exogenous virus-specific RNA.

Processing of transcripts in eukaryotic cells includes intranuclear cleavage of presumptive mRNA precursor molecules, adenylation of 3'-termini, methylation of internal nucleotides, and modification of 5'-termini (106). The extent to which intracellular oncornavirus RNA is subjected to these events can be inferred from some studies on virion RNA molecules. Further information has also been recently obtained on processed intracellular RNA.

As mentioned in section (1), it is known that oncornavirus RNA is internally methylated, contains modified 5'-termini, and 3'-polyadenylation. For certain intracellular virus-specific RNAs, polyadenylated 3'-termini are presumed to be present (28,54,150). However, the low levels of intracellular virus-specific RNA have, to the present precluded an analysis of these sequences for methylation and modified 5'-termini.

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Intranuclear cleavage processing of larger than genomic subunit RNA has been shown to occur for RSV (16). Further, the intranuclear presence of specific sizes of smaller than genomic subunit size RNA, also suggests nuclear events which could lead to the generation of separate viral gene mRNA molecules (16,61).

Polyribosomal Virus-Specific RNA and Cell Free Translation: Virus-specific RNA has been detected on infected cell polyribosomes (see Section 5--Tables I and II). In general, the criteria for considering these RNAs as viral mRNA has been a change in sedimentation after EDTA treatment of polyribosome preparations (40,42,28,120,163). In certain cases, this procedure has been useful in the detection of low levels of presumptive mRNAs which were obscured by other virus-specific sequences in cytoplasmic EDTA-resistant structures of comparable sedimentation coefficients (42).

The size of the major polyribosomal virus-specific RNA has usually been the same as the genomic subunit (40,52,120). It has recently been demonstrated that the genomic subunit RNA, derived from the initial infecting virus genome, can be detected on polyribosomes (117). The genomic subunit itself probably functions as mRNA *in vivo* (9).

The smaller than genomic subunit size viral sequences present on polyribosomes would serve an important function. Viral mRNA of these sizes would permit the separate or independent biosynthesis of specific viral products (16). This is important because viral products are not synthesized in equimolar quantities.

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Other than transcription or nuclear cleavage processing, post-translational recycling of subunit molecules has been postulated as an alternative method for the generation of smaller than genomic subunit size viral mRNA (94). Mueller-Lantzsch et al. (94) have reported that 35S virus-specific RNA is the only size species obtained from polyribosomes which were specifically purified by anti-p30 serum precipitation of polyribosomes containing p30 nascent chains. They postulated that after the translation of structural protein sequences-*gag*; the 35S RNA is released, *gag* sequences are removed enzymatically, and the next gene, *pol* in the resultant mRNA would be translated. This would continue until all viral genes are translated. An interesting observation, which partially supports this hypothesis, was made by Shanmugam (125). Microsomal fractions were found to contain nucleolytic activity. M-MuLV 35S RNA added to these fractions would be cleaved to molecules of approximately 29 to 31S and 24 to 25S. However, the sizes of these fragments does not correspond to the *in vivo* sizes of virus-specific RNA.

In vitro translation of oncornavirus RNA has also been used as an approach to understand the translational mode of viral mRNA. Early work using viral RNA in *E. coli* lysates or reticulocyte lysates was equivocal (53,129,157). Better results have been obtained in translation of the RNA in lysates of other mammalian cells.

In mouse ascites Krebs II lysates, RSV 30 to 40S RNA directed the synthesis of a 75,000 to 80,000 dalton protein (168). This protein was specifically precipitated with antiviral serum, and tryptic peptide analysis demonstrated similarities to the low molecular weight viral

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structural proteins. Similar results were obtained with transformation defective RSV RNA (105). It appears that viral subunit RNA directed the synthesis of the precursor to the low molecular weight viral proteins, i.e., the *gag* gene was translated. Since *gag* is located at the 5'-end (173), this result would be expected if translation proceeded through the first gene, and then terminated.

Larger than low molecular weight structural protein precursor size products (140,000 to 185,000 daltons) have been obtained in cell free translation of M-MuLV (79) and R-MuLV (97) RNA. However, Salden et al. (116) only found precursor size (50,000 to 72,000 daltons) products in response to R-MuLV RNA.

The intracellular virus-specific RNAs were used in later cell free reactions and again were found to only direct the synthesis of precursor size products (54). In these experiments 65,000 to 72,000 dalton products were obtained using the intracellular 35S RNA, a 70,000 dalton product using 20 to 22S RNA, and heterogenous smaller products using the 14S RNA. Not enough material was synthesized in these reactions to perform tryptic peptide analysis.

It appears then, that the avian oncornavirus RNA can only direct the synthesis of *gag* gene products *in vitro*; whereas, murine oncornavirus RNAs may sometimes direct the synthesis of very large proteins. It has been postulated that the largest precursors are the primary translational product and that very rapid cleavage, either *in vitro* or *in vivo*, accounts for their absence (4). The resolution of these problems must await further study on the *in vivo* and *in vitro* translation of viral and intracellular virus-specific RNA molecules.

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ARTICLE I

DETECTION AND ANALYSIS OF INTRACELLULAR FELINE LEUKEMIA VIRUS-SPECIFIC RNA

Anthony J. Conley and Leland F. Velicer

J. Virol. (submitted) 1977

[1] Article No.

DETECTION AND ANALYSIS OF INTRACELLULAR FELINE LEUKEMIA VIRUS-SPECIFIC RNA[1]

Anthony J. Conley and Leland F. Velicer

Department of Microbiology and Public Health
Michigan State University
East Lansing, Michigan 48824

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[1] Article No. 7969 from the Michigan Agricultural Experiment Station

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ABSTRACT

Intracellular virus-specific RNA from a chronically infected feline thymus tumor cell line, F-422, was analyzed by using *in vitro* synthesized feline leukemia virus (Richard strain) (FeLV-R) DNA probe. By hybridization kinetics analysis cytoplasmic, polyribosomal, and nuclear ribonucleoprotein (nRNP) particle RNAs were found to be 2.09%, 2.63%, and 1.95% virus-specific, respectively. Size classes within subcellular fractions were determined by sucrose gradient centrifugation in the presence of dimethylsulfoxide (DMSO) followed by hybridization. Nuclear and cytoplasmic fractions contained a 28S to 30S size class, which corresponds to the size of the genomic subunit. Both fractions also contained 36S, 23S, and 12S to 18S RNA species. Virus-specific 36S, 23S, and 18S species were present in both the total and poly(A)+ polyribosomal fraction. The nRNP particle fraction contained 36S, 28S, 22S, and 18S virus-specific RNA. These four size classes were also present in the nRNP particle poly(A)+ RNA. The presence in the nuclear and nRNP particle fractions of smaller than genomic subunit size RNA, which corresponds to sizes observed in the cytoplasm and polyribosomes, suggests nuclear processing of FeLV-R RNA in these chronically infected cells.

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INTRODUCTION

Oncornavirus high molecular weight 60 to 70S genomic RNA can be dissociated to 30 to 40S subunits and various minor heterogenous smaller species (49). The genomic RNA of the Richard strain of feline leukemia virus (FeLV-R) differs from exogenous avian, murine, and other feline oncornavirus isolates in that it sediments in aqueous sucrose gradients as a 50 to 60S species (6,9). The FeLV-R high molecular weight genomic RNA can be denatured to yield subunits which co-sediment with 28S (1.8×10^6 molecular weight) ribosomal RNA in 99% dimethylsulfoxide (DMSO) sucrose gradients, and migrate with an estimated molecular weight of 3.2×10^6 in composite acrylamide-agarose gels (6). These subunits are further unique in that they do not have a modified 5' terminal-'cap' (48) as reported for avian (13,25,46) and murine (5,39) oncornavirus RNA subunits.

Genomic subunits are considered to be the main transcriptional product of the integrated provirus (19,20). Using complementary viral DNA synthesized in vitro, and the techniques of nucleic acid hybridization, oncornavirus specific RNA can be detected in avian (7,28,36,43), murine (11,15,21,42,45,50,51,52), and feline RD-114 (33) oncornavirus infected cells. Sucrose gradient sedimentation or electrophoretic analysis of intracellular RNA followed by hybridization of the fractionated RNA shows the presence of some virus-specific RNA sequences comparable

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comparable in size to both the viral genome (11,28), and to the subunit (11,15,23,28,43,45,50,51). In other analyses smaller than subunit size RNA is found (11,15,16,23,28,33,43,45,50).

From a consideration of the many unique properties of FeLV-R genomic subunit RNA, it became important to examine the intracellular virus-specific RNA in FeLV-R producing cells. We were especially interested to determine whether smaller than genomic subunit size RNA species would be present and if they were of comparable size to those found in murine and avian oncornavirus infected cells. In this study, FeLV-R specific RNA in a chronically infected thymus tumor cell line (F-422) was analyzed to, (i) locate and quantitate virus-specific RNA within the subcellular fractions of infected cells, (ii) identify the size classes of virus-specific RNA, and (iii) examine cellular polyribosomes and nRNP particles for the presence of virus-specific RNA.

Most of this work was submitted by A. J. Conley in partial fulfillment of the requirements for the Ph.D. degree, Michigan State University, East Lansing, 1977.

This paper was presented in part at the 77th Annual Meeting of the American Society for Microbiology, 8 May - 13 May, 1977, New Orleans, Louisiana.

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MATERIALS AND METHODS

Cells and Virus: The chronically infected feline thymus tumor cell line, F-422, which produces the Rickard strain of FeLV (FeLV-R), was used as the source of virus and cells. This cell line, grown in suspension culture, was propagated as previously described (18). Uninfected feline embryo lung fibroblasts (FLF-3) were obtained from W. D. Hardy, Jr. (Memorial-Sloan Kettering, New York) through Allan Haberman (Michigan State University). These cells were propagated as monolayer roller bottle cultures in medium consisting of 40% McCoys 5A-60% Leibowitz L15 with 15% fetal calf serum.

Isotopic Labeling: Viral or cellular RNA labeled with ^{3}H-uridine was obtained by incubating cells in fresh medium containing 5.0 μCi ^{3}H-uridine/ml (New England Nuclear Corp., 40-50 Ci/mmol) at a cell density of 2 X 10^{6}/ml for 4 h or less.

Viral RNA labeled with ^{32}P was obtained by incubating cells in Swim's phosphate free medium for one hour followed by resuspension in fresh Swim's phosphate free medium containing 0.1 mCi ^{32}P/ml (Amersham/Searle, 26 Ci/mg of P, as orthophosphate, in dilute HCl), 0.5 μg/ml amphotericin B (P/L Biochemicals) and 1% DMSO (53). These cells were incubated for 4 hours followed by collection of the culture medium and resuspension of the cells in fresh Swim's phosphate free medium for two additional

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additional hours. Both collections of culture medium were pooled and clarified at 10,000 rpm for 10 minutes in the Sorvall GSA rotor.

Preparation of Virus: All culture supernatants used were from cells grown in fresh medium for four hours or less. Virus was prepared from this material by either of two methods. In the first, clarified medium was layered over 5 ml of 20% glycerol (vol/vol) in 0.06 M NaCl, 0.05 M Tris HCl (pH 8.5), for rapid collection of virus used in the synthesis of DNA; or layered over 5 ml of 20% glycerol (vol/vol) in 0.1 M NaCl, 0.1 M Tris HCl (pH 7.5), 1 mM EDTA, for rapid collection of unlabeled virus for RNA purification, and centrifuged at 27,000 rpm in the SW27 rotor (Beckman) for 1.5 hr. In the second method FeLV was prepared from culture medium by a discontinuous sucrose gradient centrifugation as described (34).

Cell Fractionation Techniques: Various subcellular fractions were prepared from F-422 cells in the following manner. Cells were harvested by low speed centrifugation, washed with phosphate buffered saline, and repelleted. Cells were allowed to swell in hypotonic buffer (RSB) 0.01 M Tris (pH 7.4), 0.01 M NaCl, 1.5 mM $MgCl_2$ at 4°C for 10-15 minutes. Disruption was performed with a dounce homogenizer and a tight fitting pestle using 15 to 20 strokes.

A cytoplasmic extract was prepared by pelleting nuclei at 950 X g for 5 minutes. The supernatant was removed and the nuclei were resuspended in RSB containing 0.1% Nonidet P-40 (NP-40), and repelleted. The second supernatant was removed, combined with the first, and designated cytoplasmic extract.

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Total polyribosomes were prepared from the cytoplasmic extract containing 50 μg/ml sodium heparin, 0.2% sodium deoxycholate, and 0.2% NP-40. This suspension was centrifuged at 27,000 X g for 5 minutes, and the supernatant was removed, applied to a column (18 X 1.5 cm) of Sepharose 2B previously equilibrated in RSB, and chromatographed (10). The excluded material, containing the polyribosome fraction almost devoid of ribosomal subunit and ribosomal monomer material, was designated total cellular polyribosomes.

Pelleted crude nuclei, obtained as described above, were washed twice in RSB containing 0.2% NP-40, 0.1% sodium deoxycholate by resuspension and centrifugation at 950 X 0 for 5 minutes and twice in RSB by the same procedure.

Nuclear ribonucleoprotein (nRNP) particle fractions were prepared from washed nuclei by either the methods described by Bhorjee and Pederson (2), and Kish and Pederson (26) (designated Method I), or by the method described by Quinlan et al. (38) (designated Method II). In Method I, washed nuclei were resuspended at approximately 4 X 10^7/ml in RSB and sonicated by using a MSE sonicator for 40 total seconds in 10 second intervals. The suspension was made 50 μg/ml sodium heparin between the third and fourth intervals. The sonicate was layered on 30% (w/v) sucrose in 0.01 M NaCl, 2.5 mM Tris HCl (ph 7.2) and centrifuged at 4500 X g for 5 min in the SW27 rotor. The material remaining above the interface was removed and layered on a discontinuous sucrose gradient consisting of 8 ml 10% (w/v) sucrose, 2 ml 45% (w/v) sucrose and 15 ml of 60% (w/v) sucrose, all in RSB. The gradient was centrifuged

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for 90 min at 26,000 rpm in the SW 27 rotor (26). The nuclear ribonucleoprotein band, spanning the 45% sucrose layer was removed, diluted three-fold with RSB, and pelleted in the SW50.1 rotor (Beckman), 1 h at 50,000 rpm.

In Method II nuclei were washed once again in 0.1 M NaCl, 10 mM Tris HCl (pH 9.0), 1 mM $MgCl_2$, pelleted, as described for nuclei above, and resuspended in the same buffer. This suspension was incubated with constant stirring for 3 to 4 hours at 4°C. After incubation the nuclei were again pelleted and the extract collected (38). The nRNP particles were prepared from this extract by the discontinuous gradient centrifugation procedure described above.

RNA Extraction: RNA was extracted from pelleted material other than nuclei, by the TNE-9 SDS-phenol procedure as previously described (6). RNA extraction from supernatant materials was performed by the same method, except that the supernatants were first made 0.1 M NaCl, 0.1 M Tris (ph 9.0), 1 mM EDTA and 1% SDS. RNA was extracted from nuclear pellets by the hot SDS-phenol method (17).

Polyacrylamide Gel Electrophoresis: Electrophoresis of nucleic acids in 2% polyacrylamide-0.5% agarose composite gels was performed as previously described (6).

Velocity Sedimentation Analysis of RNA: Neutral sucrose gradient analysis of viral RNA and velocity sedimentation on 5% to 20% (w/v) sucrose gradients in the presence of 10 mM LiCl, 1 mM EDTA, 99% DMSO was performed as described (6). RNA from DMSO gradient fractions used in hybridization experiments was collected by ethanol precipitation in the presence of 0.2 M NaCl and 50 μg/ml carrier yeast RNA.

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Preparation of FeLV DNA: The endogenous RNA-directed DNA polymerase reaction was used to prepare radioactive DNA complementary to FeLV RNA. A modification of the reaction mixture described by Rothenberg and Baltimore (40) was used. It contained 50 μg/ml actinomycin D, 50 mM Tris HCl (pH 8.3), 60 mM NaCl, 0.013% NP-40, 5 mM each dCTP, dATP, dGTP, 0.1 mM TTP, generally 115 μCi/ml ^{3}H-TTP (I.C.N. Pharmaceuticals, 40 Ci/mmole) and virus at 1.5 to 3.0 mg protein/ml. The mixture was incubated at 37°C for 12 to 16 hours followed by making the sample 0.2 M sodium acetate, 20 mM Tris HCl (pH 8.3), 50 mM EDTA, and 0.5% SDS. The DNA product was extracted and purified according to the method of Rothenberg and Baltimore (40), chromatographed on Sephadex G-50, incubated for 4 hours at 37°C in 0.4 M NaOH, neutralized, and precipitated in 67% ethanol at -20°C.

RNA-DNA Hybridization: Cellular or viral RNA was incubated with ^{3}H-FeLV DNA probe (generally 1000-1500 cpm) in a hybridization mixture consisting of (final concentrations) 0.01 M Tris HCl (pH 7.2), 0.4 M NaCl, 0.05% SDS, 0.25 mM EDTA, 0.075 mg/ml calf thymus DNA, and 0.15 mg/ml yeast RNA at 66°C as described (44). The reactions were assayed for DNA hybrid formation by the use of a crude preparation of S1 nuclease (31). The crude nuclease was prepared by extraction of 8 gm of α-amylase (Sigma) into 100 ml of nuclease buffer, 25 mM potassium acetate (pH 4.5), 0.1 mM $ZnSO_4$, 0.1 M NaCl (47), followed by clarification of the extract. The extract was made 50% glycerol and stored at -20°C. Two ml of nuclease buffer containing 0.0125 ml crude nuclease preparation per ml was added to each reaction, incubated at 45°C for

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30 min, precipitated with trichloroacetic acid (TCA), collected on 0.45 nm filter discs (Millipore Corp.) and assayed for radioactivity as previously described (18).

Viral RNA labeled with ^{32}P was hybridized to varying concentrations of the single stranded ^{3}H-labeled DNA in the above reaction mixture for 48 hours at 66°C. After annealing samples were digested with pancreatic RNase A, 50 μg/ml in 0.3 M NaCl for 30 min at 37°C (14). Samples were TCA precipitated, collected on filter discs and assayed for RNase resistant ^{32}P.

Oligo dT Cellulose Chromatography: Oligo (dT) cellulose chromatography was performed using oligo (dT) cellulose (Collaborative Research, T3) by a modification of the procedure described (37). Poly A(+) RNA was bound in 0.5 M KCl, 20 mM Tris (pH 7.5) and eluted in 20 mM Tris (pH 7.5).

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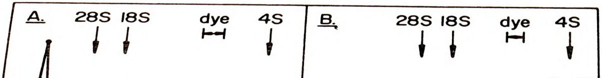
RESULTS

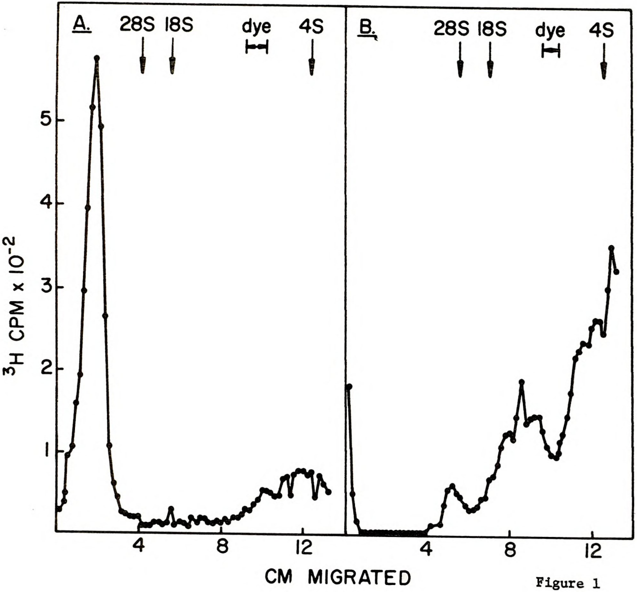
Characteristics of FeLV DNA Probe: DNA synthesized in the endogenous viral RNA directed DNA polymerase reaction in the presence of actinomycin D has been reported to consist almost entirely of single stranded species (14). The FeLV DNA, synthesized in the presence of 50 μg/ml actinomycin D, was also found to be single stranded, based on its being rendered greater than 98% TCA soluble after treatment with S1 nuclease (47).

The average size of the DNA transcripts was determined by electrophoresis of both native and alkali treated products on 2% polyacrylamide-0.5% agarose composite gels. As seen in Figure 1A, the native product contains a very high proportion of slow migrating material. This material represented DNA product complexed with viral genomic RNA, which after alkali treatment to destroy the RNA, migrated faster as heterogenous smaller species (Figure 1B). A small amount of the product migrated with an estimated molecular weight of 2.0 X 10^6 (an equivalent sedimentation value of approximately 30S). There were present a number of species migrating in the molecular weight range of 1.7 to 4.5 X 10^5 9 to 15S). However, the majority of the DNA migrated with estimated molecular weights of less than 0.8 x 10^5 (less than 6S).

To assess the extent and uniformity of the transcription, the ^{3}H-DNA product was hybridized to ^{32}P-viral RNA at various DNA/RNA ratios

Figure 1: Polyacrylamide gel electrophoresis of the product of the FeLV endogenous RNA directed DNA polymerase reaction. Electrophoresis of the nucleic acids extracted and purified from the reaction mixture described in the text was performed in 2.0% polyacrylamide-0.5% agarose composite gels as described (6). (A) Native ^{3}H-DNA product; (B) ^{3}H-DNA product, alkali treated as described in the text. Dye indicates position of bromophenol blue. Cellular 28S, 18S, and 4S RNA were run in parallel.

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and the hybrids formed were assayed for RNase resistance (14). As seen in Figure 2, the DNA product protected 32.5% of the viral genome at a DNA/RNA ratio of one. At a ratio of 17.5/1.77% of the RNA was resistant to RNase digestion. This was the greatest ratio tested due to limitations on the amount of DNA product which could be prepared. The incomplete protection indicated that the entire genome was not transcribed. Since the maximum protection observed was at a DNA/RNA ratio of 17.5/1, the data further indicates non-uniform transcription.

Amount of Intracellular Virus-Specific RNA: Experiments were performed to determine the rates of hybridization of various intracellular RNA fractions with the FeLV DNA. RNA-DNA hybridization in RNA excess can be performed to determine the value of $C_rt(\frac{1}{2})$; the product of the concentration of RNA and the time at half saturation (3). The relative concentration of virus-specific RNA in those fractions tested, can be determined by comparison of the $C_rt(\frac{1}{2})$ obtained for the RNA from each fraction with that $C_rt(\frac{1}{2})$ obtained for the viral RNA (11,43). These experiments were performed with either varied time or concentration, with essentially identical results obtained by both methods. The results of these experiments are seen in Figure 3. The amount of viral DNA hybridized is plotted as a function of $\mathrm{Log}[10^4 C_rt]$. By comparison to the viral $C_rt(\frac{1}{2})$ for 28S FeLV subunit RNA, unfractionated F-422 cytoplasmic RNA was found to contain 2.09% virus-specific RNA. Total polyribosomal RNA contained 2.63% virus-specific RNA, and nRNP particle RNA, 1.95% virus-specific RNA. Under these conditions, cytoplasmic RNA from uninfected feline embryo lung fibroblasts (FLF-3) did not hybridize to

Figure 2. Hybridization of ^{32}P-labeled FeLV 28S subunit RNA with ^{3}H-labeled FeLV DNA prepared as described for Figure 1B. The specific activity of the RNA was 158 cpm/pmol nucleotide, and that of the DNA was 218 cpm/pmol nucleotide. After incubation, samples were digested with pancreatic RNase A as described (14). The percent of ^{32}P-FeLV 28S RNA resistant to digestion is plotted as a function of the DNA/RNA ratio. Values are corrected for an RNase resistant background of 6%.

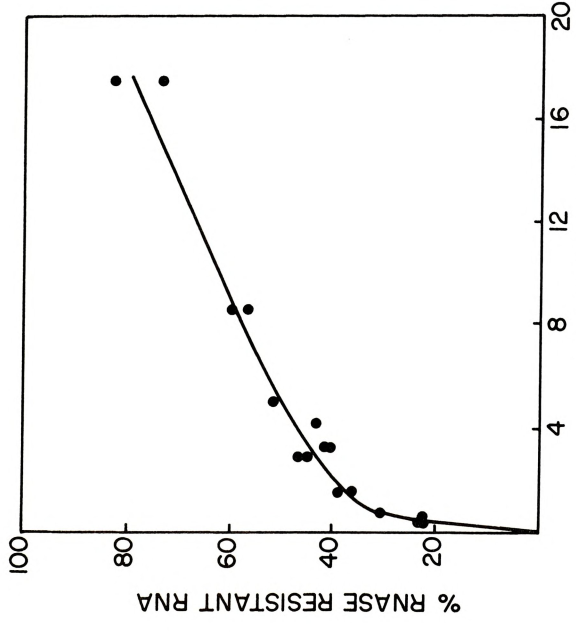
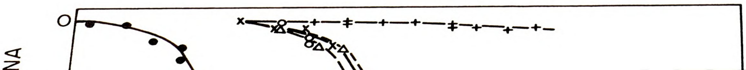
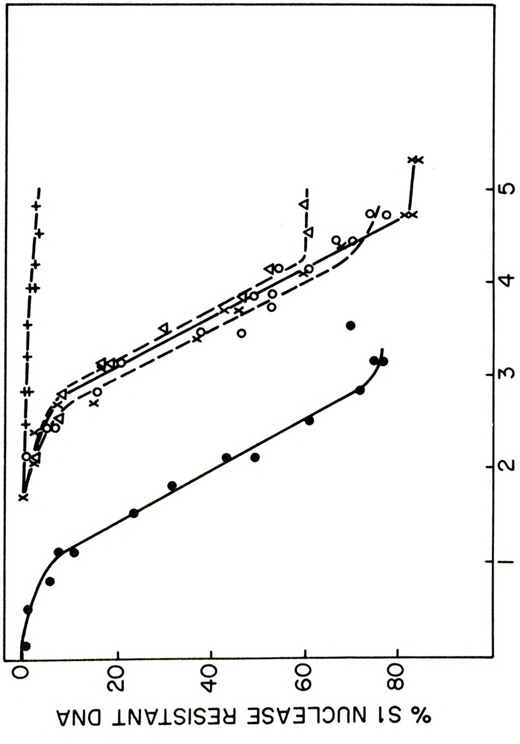


Figure 2

Figure 3. Relative concentrations of virus-specific RNA in subcellular fractions from F-422 cells. The relative concentrations were determined by hybridization of RNA from the various subcellular fractions and FeLV 28S subunit RNA at varying concentrations with ^{3}H-FeLV DNA (1400 cpm per reaction mixture) in the reaction mixture described in the text. Hybrid formation was detected by S1 nuclease digestion as described in the text. Symbols: (●——●) FeLV 28S RNA, (O——O) total F-422 polyribosomal RNA, (X——X) F-422 cytoplasmic RNA, (Δ——Δ) F-422 nuclear RNP particle RNA, (+——+) FLF-3 cytoplasmic RNA.

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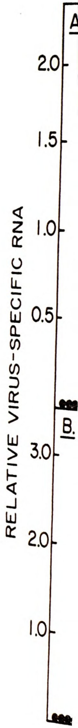
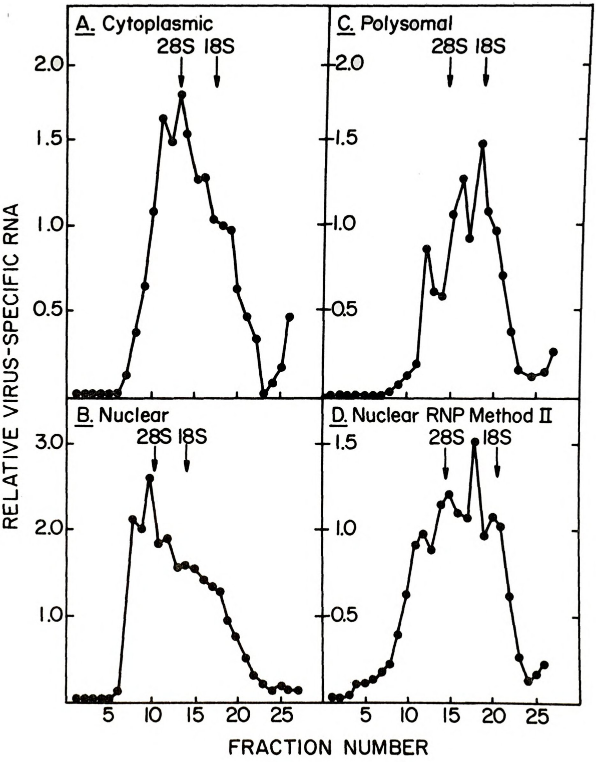
the FeLV DNA probe. Maximum hybridization for the viral, cytoplasmic, and polyribosomal RNA ranges from 76 to 84%. The nRNP particle RNA had a maximum at slightly more than 60%. This may indicate that not all the sequences present in the other fractions were present in nRNP particle RNA.

Size of Virus-Specific RNA in F-422 Cytoplasmic Extracts: RNA was extracted directly from F-422 cytoplasmic extracts as described in Materials and Methods. After sedimentation in a 5% to 20% (w/v) sucrose gradient, containing 99% DMSO, the RNA was precipitated from fractions and analyzed for virus-specific RNA using ^{3}H-FeLV DNA probe. As seen in Figure 4A, the major cytoplasmic species present sedimented with a value of 28S (molecular weight estimate of 1.8×10^6). There was also present faster sedimenting material with a value of 36S (molecular weight estimate of 2.8×10^6). In an earlier analysis using only a parallel marker gradient, this RNA was present as a more completely resolved peak sedimenting at approximately 36S (data not shown). Minor amounts of material sedimenting at approximately 23S and less than 18S were also present. Due to the denaturing conditions of the analysis, cytoplasmic RNA of genomic size (50-60S), would not be detected. The RNA detected would include genomic subunits from virion precursor particles present in the cytoplasm as well as non-virion virus-specific RNA.

Size of Total Nuclear Virus-Specific RNA: The results of nuclear RNA size analysis are shown in Figure 4B. Peaks with values of 30S (mol wt $\sim 2 \times 10^6$), 39S (mol wt $\sim 3.5 \times 10^6$) and 24S (mol wt $\sim 1.2 \times 10^6$)

Figure 4. Sizes of intracellular virus-specific RNA, as determined by analysis under denaturing conditions. RNA extracted from the various subcellular fractions were analyzed on sucrose gradients containing 99% DMSO by the method previously described (5). Parallel ribosomal RNA gradients were run with each analysis. Fractions (A), (C), and (D) also contained internal ribosomal RNA markers. Arrows indicate internal marker position in panels (A), (C), (D), and parallel marker in panel (B). After centrifugation, gradients were fractionated, processed, and assayed for virus-specific RNA as described in the text. The amount of RNA was plotted as relative virus-specific RNA, using the relationship described by Fan and Baltimore (11), where 50% hybridization has a relative value of one. (a) 8.1 μg total cytoplasmic RNA. (B) 14.5 μg total nuclear RNA. (C) 4.3 μg polyribosomal RNA. (D) 9.1 μg nuclear RNP particle RNA.

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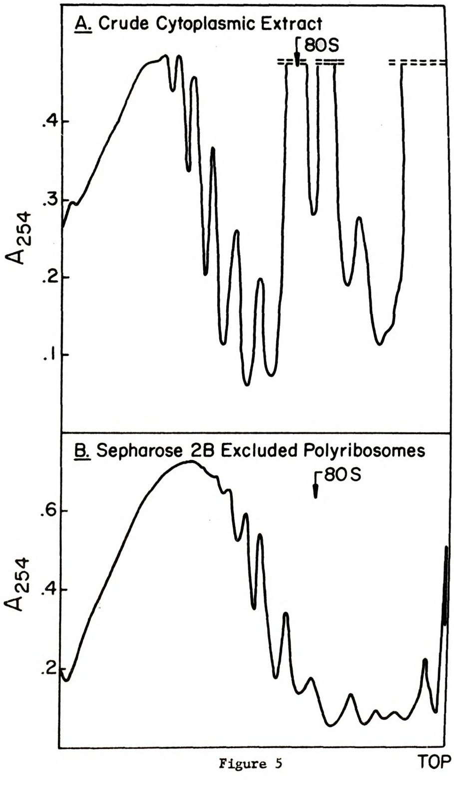
Virus-Specific RNA from Cellular Polyribosomes: Polyribosomes from F-422 cells were purified by the Sepharose 2B exclusion method as described (10). A 20% to 45% (w/v) sucrose gradient analysis of polyribosomes isolated by this procedure compared to an analysis of a total cytoplasmic extract is shown in Figure 5. The Sepharose 2B excluded material consisted entirely of polyribosomes, almost devoid of ribosomal monomers and slower sedimenting material.

This polyribosome fraction was extracted and analyzed for virus-specific RNA as described for the cytoplasmic extract RNA. The analysis is seen in Figure 4C. There were three distinct peaks of hybridizing material with values of 36S, 23S, and 18S; molecular weight estimates of 2.8 X 10^6, 1.1 X 10^6, and 0.7 X 10^6, respectively. The two slower sedimenting peaks present here, are more discernable than in the gradient of the cytoplasmic extract RNA. The same three species were obtained when Sepharose 2B excluded material was pelleted through 2.0 M sucrose before extraction and DMSO gradient analysis (data not shown). It is notable that there was no peak of hybridizing material at a value of 28S in this polyribosomal RNA.

Figure 5. Sucrose gradient sedimentation analysis of Sepharose 2B excluded polyribosomes and unfractionated total cytoplasmic extract. Total polyribosomes were prepared as described in the text. Analysis was performed on 4.8 ml 20% to 45% (w/v) linear sucrose gradients in the SW50.1 rotor, 40,000 rpm for 1.25 hr. (A) Sepharose 2B excluded polyribosomes. (B) Unfractionated cytoplasmic extract.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Virus-Specific RNA from Intranuclear Ribonucleoprotein Particles: Many aspects of the post-transcriptional processing and maturation of presumptive mRNA in eukaryotic cells remain to be elucidated. One present hypothesis is that some aspects of processing and transport of mRNA in eukaryotic cells involves an association of those RNA species with protein in complexes designated ribonucleoprotein particles (1,27, 54). The mRNA transcripts of the integrated oncornavirus DNA may be processed and transported by the same mechanisms as cellular mRNA. An analysis of nRNP particles for virus-specific RNA, could characterize virus-specific RNA processed in this manner and give further insight into possible viral mRNA species which arise by transcription and nuclear processing.

The hybridization pattern of RNA extracted from nRNP particles isolated by the method of Quinlan et al. (38) (Method II) is shown in Figure 4D. This fraction contained four discernable virus-specific RNA species with values of 34S, 28S, 22S, and 18S; molecular weight estimates of 2.6 X 10^6, 1.8 X 10^6, 1.05 X 10^6, and 0.7 X 10^6, respectively. The two slower sedimenting peaks were more discernable than in the total nuclear fraction. Since the two slower sedimenting species were present in high proportions in nRNP particle RNA and polyribosome RNA fractions, pulse-labeled total RNA, from these two fractions was prepared in order to compare the mean size of total RNA in these two fractions. Comparable sizes of total RNA from the two fractions may indicate a similar relationship between the virus-specific RNAs of these two fractions. The DMSO gradient analyses of these fractions is shown in Figure 6. The nRNP particle RNA, labeled for ten minutes with

Figure 6. DMSO sucrose gradient analysis of rapidly labeled RNA from polyribosomes and nuclear RNP particles. Polyribosomes labeled for twenty minutes with ^{3}H-uridine and nRNP particles labeled for ten minutes were prepared and RNA extracted as described in the text. Analyses were performed as described in Figure 4 in parallel gradients with a parallel marker gradient. Symbols: (●——●) RNA from polyribosomes labeled for 20 minutes with ^{3}H-uridine. (●---●) RNA from nRNP particles labeled for 10 minutes with ^{3}H-uridine.

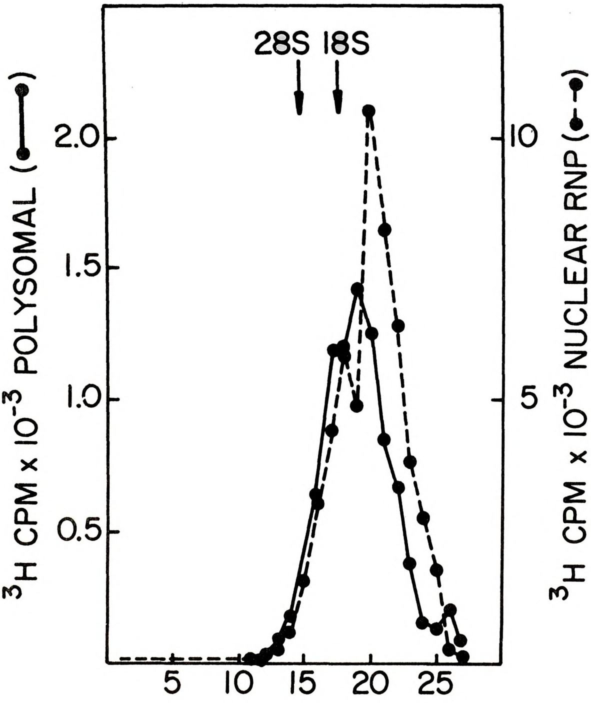
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Figure 6

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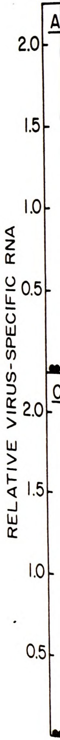
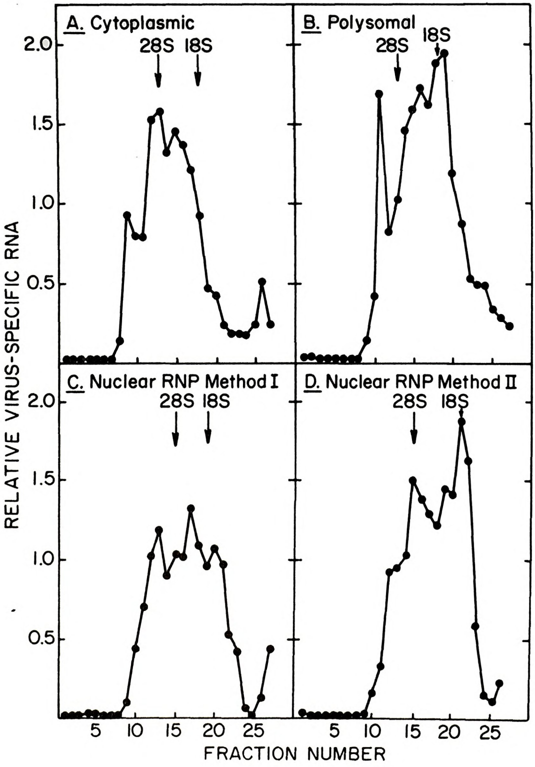
^{3}H-uridine, had a mean sedimentation value of approximately 12S, while the mean value for polyribosomal RNA labeled for twenty minutes with ^{3}H-uridine was approximately 14S. Both preparations contained a significant amount of RNA which sedimented faster than the mean value. This analysis shows that the sizes of rapidly labeled total RNA in nRNP particles and polyribosomes were similar.

Presumptive Poly(A) Containing Intracellular RNA: To further characterize the virus-specific RNA species detected, the cytoplasmic, polyribosomal, and nRNP particle RNAs were subjected to oligo(dT) cellulose chromatography before DMSO gradient analysis. The results of these analyses are shown in Figure 7. Cytoplasmic poly(A) containing virus-specific RNA (Figure 7A) contained three RNA species with approximate sedimentation values of 33 to 38S, 28 to 31S, and 22 to 24S (estimated molecular weights of 2.5 to 3.2 X 10^6, 1.8 to 2.2 X 10^6, and 1.0 to 1.2 X 10^6, respectively). The 33 to 38S and 28 to 31S species were present in the total cytoplasmic RNA fraction. However, oligo(dT) cellulose chromatography increases the relative proportion of the 22 to 24S species when compared to the total extract. Since only approximately 66% of FeLV-R 28S RNA contains poly(A) (6), this relative increase would be expected if the oligo(dT)-cellulose chromatography procedure selected only the poly(A)+ intracellular 28S RNA.

Polyribosomal virus-specific RNA contained three predominant species of poly(A) containing RNA (Figure 7B). These species sedimented with values of 35S, 22 to 24S, 16 to 18S, and were similar to the total polyribosomal virus-specific RNA.

Figure 7. Sizes of intracellular virus-specific poly(A) + RNA. Poly(A) containing RNA was prepared as described in the text. Analysis was performed as described in Figure 4. Arrows indicate internal marker position in all four panels. (A) 22 μg cytoplasmic poly(A) + RNA. (B) 17 μg polyribosomal poly(A) + RNA. (C) 3.2 μg nRNP particle poly(A) + RNA prepared by Method I as described in the text. (D) 4.5 μg nRNP particle poly(A) + RNA prepared by Method II as described in the text.

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Poly(A) containing RNA from nRNP particles isolated by both methods was analyzed. Virus-specific poly(A) containing RNA from nRNP particles isolated by the methods of Bhorjee and Pederson (2) and Kish and Pederson (26) (Method I), is shown in Figure 7C. The RNA present had sedimentation values of 35S, 28S, 23S, and 17S. The poly(A) + RNA from particles isolated by the method of Quinlan et al. (38) Method II (Figure 7D), containee major species with sedimentation values of 28S and 18S. In this fraction a 33 to 35S shoulder and a small 22S peak were also present. The molecular weight estimates for the species isolated by both methods were similar and were estimated at 2.5 to 2.8 X 10^6 for the 33 to 35S, 1.8 X 10^6 for the 28S, 1.0 to 1.1 X 10^6 for the 22 to 23S, and 0.6 to 0.7 X 10^6 for the 17 to 18S virus-specific RNA.

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DISCUSSION

In this study we have used complementary single stranded viral DNA to study intracellular FeLV specific RNA. The major qualifications of this approach concern the uniformity and completeness of the transcript. The combination of analyses shown in Figure 1 and Figure 2 describe the FeLV DNA probe and indicate the level of transcription. The majority of the native DNA transcript migrates as material remaining bound to the high molecular weight RNA template. This result has been noted by others and is assumed to represent a faithful transcript of the template (22). As seen in Figure 1B, most of the denatured product migrates as small molecules. The small amount of 2.0 X 10^6 dalton material present is still approximately 1.2 X 10^6 daltons smaller than 28S FeLV-R genomic subunit RNA, which migrates with an estimated molecular weight of 3.2 X 10^6 in 2% polyacrylamide-0.5% agarose gels (6).

The template protection experiment described in Figure 2 allows us to make an evaluation of the quality of the FeLV-R DNA probe. This analysis and indirectly the electrophoretic analysis suggests that not all FeLV-R RNA sequences are present in the DNA probe. DNA probes of this quality can be used in analyzing intracellular RNA (11), but it should be emphasized that certain intracellular virus-specific RNA sequences may not be detected.

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Recently, larger and more uniform transcripts of oncornavirus RNA have been obtained (8,24,40). Even though the conditions of synthesis used here are similar to those described by Rothenberg and Baltimore (40), high uniformity and complete transcription was not obtained. Since this work was completed, published results suggest that more uniform transcription occurs under conditions of restricted magnesium ion (41).

The value of approximately 2% FeLV-R-specific RNA in unfractionated F-422 cytoplasmic extracts was higher than the values reported for Moloney murine leukemia virus (M-MuLV) infected cells, 0.69% (52), 0.3 to 0.9% (11), 22% (12), 1.0 to 1.3% (23), or Rous sarcoma virus infected cells, 0.27% (43), 0.25 to 0.6% (28) and 0.2 to 2.0% (4). The same was true for the value of 2.63% FeLV-R-specific RNA from total F-422 polyribosomes. The virus-specific polyribosomal RNA levels reported for Rauscher murine leukemia virus (R-MuLV) (15) and M-MuLV (12.52) range from approximately 0.05% to 0.58%. The F-422 line is one of the higher oncornavirus producing cell lines and the higher levels of FeLV-R-specific RNA may be related to the apparently high viral synthetic rate (18). Although the amount of virus-specific RNA in the nRNP particle fraction was found to be less than the other fractions, this lower value would be expected if nRNP particle RNA contained a transient subpopulation of viral RNA molecules, which could be undergoing processing for function as mRNA.

The consistent appearance of very reproducible sized species, within narrow ranges, allows us to use the average RNA size designations

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36S, 28S, 23S and 18S to identify the intracellular FeLV-R-specific RNA in subsequent discussions.

The size of the major species of virus-specific RNA in total nuclear (Figure 4B) and unfractionated cytoplasmic extracts (Figure 4A) is comparable in size to the FeLV-R genomic subunit RNA. This relationship is similar to intracellular R-MuLV, M-MuLV, and RSV RNA (11,23,50, 51). However, both the nuclear and cytoplasmic fractions contain FeLV-R-specific RNA which sediments faster than the genomic subunit. This phenomenon has been reported for nuclear virus-specific RNA from M-MuLV infected cells (23) and RSV infected cells (4). In these cases the largest nuclear species is not more than 15% larger than the 35S genomic subunit. The largest intranuclear RSV-specific RNA is considered a precursor to the genomic subunit since it is not detected in longer labeling periods and steady state RNA (4). Although the resolution in our experiments does not permit an exact size determination of the 36S species found in all subcellular fractions, its estimated molecular weight is approximately 50 to 70% larger than that of the genomic subunit.

When cytoplasmic virus-specific RNA is analyzed under non-denaturing conditions, larger than genomic subunit species have been found (11,28). These species are considered cytoplasmic genomic RNA precursors. The presence of smaller than genomic subunit size RNA in unfractionated cytoplasm has been reported in M-MuLV infected cells (11,50,51), RSV infected cells (28,43), and RD-114 cells (33). Although the sizes of these smaller molecules vary among the virus

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systems studied, their presence appears to be a general phenomenon. The unfractionated F-422 cytoplasm (Figure 4A) contained a minimal amount of the smaller FeLV-R-specific species. However, when poly(A) containing RNA from this fraction is analyzed (Figure 7A), a 23S species is resolved, probably due to its relative enrichment in the fraction by oligo(dT) cellulose chromatography.

Polyribosomal RNA from F-422 cells contains three (36S, 23S, and 18S) distinct species (Figure 4C). It is important to note that 28S RNA was not detected in this fraction suggesting that the FeLV-R genomic subunit may not be present on polyribosomes. Three species are present in the polyribosomal poly(A) containing virus-specific RNA (Figure 7B), with approximately the same sedimentation values as total polyribosomal virus-specific RNA (Figure 4C) and probably represent the same molecules. The detection of three distinct FeLV-R-specific RNA species on F-422 polysomes is in part similar to the findings of Gielkens et al. (15) for R-MuLV polyribosomal RNA. Their analysis showed the presence of 36S, 21S and 14S virus-specific RNA from infected cell polyribosomes, with the species being unequally distributed on polyribosomes. Free polyribosomes contained mostly 36S RNA, slower sedimenting membrane-bound polysomes contained the smaller 21S and 14S RNA, while the faster sedimenting membrane bound polysomes were enriched for the 36S RNA (15). These same species were also found by analysis on denaturing, 85% formamide, gradients (16). They further showed that 21S and 36S RNA were the major poly(A) containing intracellular species (16). The 36S R-MuLV polyribosomal RNA is the same size as R-MuLV

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subunit RNA; whereas, the FeLV-R 36S polyribosomal species is not the same size as the FeLV-R subunit RNA.

Two methods were used to obtain nRNP particles. From control mixing experiments we found that purified viral RNA is cleaved during the isolation of nRNP particles by the method of Bhorjee and Pederson (2) and Kish and Pederson (26), Method I. Cleavage was not observed during Method II (38) isolation or any of the other isolation procedures. Consequently, the integrity of nRNP particle RNA isolated by Method I, is suspect (Figure 7C).

The presence of virus-specific RNA in nRNP particles is important from several aspects. Primary among these is that the transport and processing of the various virus-specific RNA size species could be performed by an apparently normal cellular mechanism. We presently have no evidence to eliminate the possibility that the smaller species arise by transcription separate from that of the larger species since our analysis was under steady state conditions. However, their presence does indicate a nuclear site of generation and a post-translational cleavate-recycling model as proposed for M-MuLV RNA (32), need not be evoked to account for the presence of all the smaller than subunit size polyribosomal virus-specific molecules. If the smaller species did arise by cleavage during translation, their presence in the nucleus under steady state conditions would require return transport to the nucleus.

A steady state level of RNA smaller than genomic subunit size could reflect a transcriptional or nuclear post-transcriptional control

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mechanism for the production of non-equimolar amounts of oncornaviral gene products. These smaller RNA species, in nRNP particles and polyribosomes, may further indicate a physical basis for the possibility of two separate pools of oncornaviral RNA transcripts. Levin and colleagues (29,30) have found that de novo synthesis of M-MuLV viral protein occurs in the presence of actinomycin D, with the expected absence of new progeny virion RNA. Functionally, the half life of M-MuLV messenger RNA ($T\frac{1}{2}$ 1.5-2 hr) (30). It could be possible that the smaller virus-specific RNA species represent the functionally stable virus-specific mRNA. The data presented here shows that some possible species of viral precursor mRNA may exist in the nuclei as smaller than subunit size RNA, and nuclear fractionation greatly increases the ability to find these species.

The analyses presented here and other preliminary data concerning FeLV-R in F-422 cells, lead to some interesting observations about FeLV-R RNA metabolism in F-422 cells. Preliminary observations in this laboratory indicate that FeLV-R from F-422 cells is 2.5 logs less infectious than virus from the original FeLV-R isolate passed in feline fibroblasts, based on endpoint dilution assays of supernatant DNA polymerase activity on FLF-3 cells (Haberman and Velicer, unpublished results). Since the genomic subunit size RNA is not the largest species found intracellularly, the majority of progeny virions produced by F-422 cells may be defective in the RNA molecules incorporated into virions. Further, we have preliminary evidence that FeLV-R 50-60S RNA preparations contain a minor subunit of approximately 36S as well as

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the major 28S subunit (Conley and Velicer, unpublished results). The approximately 36S molecule may simply contain host cell sequences generated by transcriptional readthrough, and the extra sequences are cleaved from most virus-specific molecules as is the case for the larger precursor of the 35S RSV RNA (4). However, we do not yet know which size molecule contains the entire genomic capacity of FeLV-R.

If the sequences present in 36S RNA but not in 28S RNA are virus-specific, some interesting processing schemes could be devised. The combined estimated molecular weights of the 18S RNA found on polyribosomes and in nRNP particles, and the 28S genomic subunit RNA, is 2.5×10^6. This corresponds to molecules of approximate sedimentation value of 34S which is of similar size to the largest species observed in all the subcellular fractions. It is tempting to speculate that the smallest polyribosomal species (18S) is a nuclear cleavage product of 34-36S RNA, with most of the remaining RNA being eventually incorporated into virions as a 28S species. The minimal amount of RNA needed for FeLV-R structural protein precursor Pp70 (35) is 6.3×10^5 (a 17.5S molecule). The 18S species is compatible to that needed for separate biosynthesis of structural protein precursor. In this model the 36S RNA could still function as structural protein precursor mRNA as is true of M-MuLV 36S RNA (32). However, this speculation would need to be proven by cell-free translation of individual intracellular species and by hybridization-competition experiments with the various intracellular RNA species.

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ARTICLE II

ANALYSIS OF POLYRIBOSOMES FROM CELLS INFECTED WITH FELINE LEUKEMIA VIRUS

Anthony J. Conley and Leland F. Velicer

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ANALYSIS OF POLYRIBOSOMES FROM CELLS INFECTED WITH FELINE LEUKEMIA VIRUS

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ABSTRACT

Polyribosomes from a chronically infected feline thymus tumor cell line, F-422, were analyzed by using antisera specific for feline leukemia virus (Rickard strain) (FeLV-R) proteins and _in vitro_ synthesized FeLV-R DNA probe. Virus-specific nascent proteins were detected by binding ^{125}I-anti-FeLV IgG to polyribosomes. Normal rabbit serum (NRS) IgG bound at a level of 0.02% throughout the polyribosome region of the gradient. Anti-FeLV IgG bound to rapidly sedimenting polyribosomes (peak binding at 400S) at a level of 0.25 to 0.40%. This binding was further studied to determine its specificity for nascent virus-specific protein. NRS IgG did not compete with anti-FeLV IgG for the polyribosomal binding sites. Total viral protein and p30 absorbed specific antibody from the IgG preparation and the binding of the absorbed IgG was reduced in relation to the amount of protein used. The binding of anti-FeLV IgG to puromycin treated polyribosomes was reduced by the same proportion that nascent proteins were released.

The FeLV-R DNA probe hybridized to two polyribosomal regions (approximately 400 to 450S and 250S) and to a slower sedimenting region (approximately 80S) within the polyribosomal gradients. The DNA still hybridized to RNA in slower sedimenting regions (<80S) but not in the two polyribosome regions after EDTA treatment. The size classes of virus-specific RNA within these regions were determined by velocity sedimentation in the presence of 99% dimethylsulfoxide (DMSO). The 400 to 450S polyribosomes contained three major peaks at 33S, 22S, and 17S;

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whereas, the 250S polyribosomes contained only 34S and 18S RNA. RNA from the approximately 80S regions obtained with and without EDTA treatment contained 28S in addition to the 34S, 22S, and 18S virus-specific RNA. The presence of 34S, 22S, and 18S RNA within polyribosome regions which also contain nascent virus-specific proteins suggests that there are three species of FeLV-R-specific mRNA in the F-422 feline thymus tumor cell line.

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INTRODUCTION

The synthesis of oncornavirus proteins has been extensively examined in recent years. By using immunological techniques, high molecular weight intracellular precursors have been detected for the structural proteins of avian myeloblastosis virus (40,41) and various murine oncornaviruses (1,19,33,38). Further, the immunological detection of extremely large intracellular virus-specific proteins of 200,000 to 300,000 daltons (1,38) suggests the possibility that all the oncornavirus proteins may be synthesized as a single precursor. The structural proteins of the Rickard strain of feline leukemia virus (FeLV-R) are generated by cleavage of an intracellular 70,000 dalton precursor (26). These observations raise further questions concerning the size and number of possible virus-specific mRNA(s) in oncornavirus infected cells.

In most oncornavirus infected cells a 30 to 40S RNA, which corresponds in size and polarity to the viral subunit, is the major intracellular virus-specific species (12,32,37). It has been suggested that the intracellular viral genomic subunits function as viral mRNA (12). Since species of subunit size are present on polyribosomes (2,8,12,13,32, 35), and since RNA from these various oncornaviruses directs the synthesis of virus-specific products <u>in vitro</u> (16,20,24,29,30,42), this hypothesis is probably correct. However, the presence in polyribosomes of smaller than subunit size virus-specific RNA (2,8,15,16,35), and the ability of these smaller species to direct the synthesis of virus-s

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virus-specific products in vitro (16) also suggests an mRNA function.

FeLV-R genomic subunits sediment as 28S RNA in sucrose gradients containing 99% dimethylsulfoxide (DMSO) (5,9). Although a virus-specific species of this size is present intranuclearly and intracytoplasmicly, a 28S virus-specific species could not be detected in total polyribosomes from FeLV-R infected cells (6). These polyribosomes were shown to contain an approximately 34S virus-specific species in addition to 23S and 18S FeLV-R specific RNA.

To further understand the mode of oncornavirus RNA translation, the cellular polyribosomes from FeLV-R infected cells were examined by using both immunological and nucleic acid hybridization techniques. These polyribosomes were analyzed to (i) determine whether nascent virus-specific determinants could be detected by using antibody preparations specific for FeLV-R proteins, (ii) detect the size of polyribosomes containing virus-specific RNA by using an in vitro synthesized DNA probe, (iii) correlate the detection of virus-specific nascent protein and RNA, and (iv) determine the size classes of virus-specific RNA present within the major size class of polyribosomes containing virus-specific RNA and nascent proteins.

Most of this work was submitted by A. J. Conley in partial fulfillment of the requirements for the Ph.D. degree, Michigan State University, East Lansing, 1977.

This paper was presented in part at the Cold Spring Harbor meeting on RNA Tumor Viruses, May 25-29, 1977, Cold Spring Harbor, New York.

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MATERIALS AND METHODS

Cells and Virus: The chronically infected feline thymys tumor cell line (F-422) which produces the Rickard strain of feline leukemia virus (FeLV-R), was used as the source of cells. This line, grown in suspension culture, was propagated as previously described (14).

Isotopic Labeling: Cells were labeled with ^{14}C-uridine by incubation in fresh medium containing 0.5 μCi ^{14}C-uridine/ml (New England Nuclear Corp., 53 mCi/mmole) at a cell density of 2 X 10^6/ml for 4 hr. Cells labeled for one minute with ^{3}H-amino acids were obtained by incubation in amino acid deficient medium containing 25 μCi ^{3}H-amino acid mixture/ml (New England Nuclear Corp.) at a cell density of 50 X 10^6/ml. After one minute at 37°C the pulse was terminated by addition of the cells to partially frozen medium. ^{14}C-amino acid labeled virus was prepared as described (14).

Cell Fractionation: Polyribosomes were prepared from harvested F-422 cells by the method described (6). Briefly, washed cells were disrupted by dounce homogenization after swelling in hypotonic buffer (RSB), 0.01 M Tris HCl (pH 7.4), 0.01 M NaCl, 1.5 mM $MgCl_2$. A cytoplasmic extract was prepared and solubilized with (final concentrations) 0.2% sodium deoxycholate (w/v) and 0.2% Nonidet P-40) (w/v). Sodium heparin was added to 50 μg/ml and the extract was centrifuged at 27,000 X g for 5 min in the SS34 rotor (Sorvall). The supernatant was chromatographed on a column of Sepharose 2B (10). The excluded fractions containing polyribosomes were pooled, made 50 μg/ml sodium heparin and used immediately in experiments.

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Preparation of Antisera: Rabbit anti-FeLV (Tween 80-ether disrupted), rabbit anti-FeLV p30 (prepared with guanidine HCl-agarose chromatography purified p30, designated-I), and normal rabbit serum (NRS) were prepared as described (14). Goat anti-FeLV p30 (designated-II) was obtained from F. de Noronha (Cornell University, Ithaca, New York). All sera were subjected to two successive precipitations with 40% saturated ammonium sulfate followed by extensive dialysis against phosphate buffered saline (PBS). These proteins were chromatographed on a column of Sephadex G-200 equilibrated with PBS. The gamma globulin (IgG) fractions were pooled and concentrated by diafiltration.

Radioiodination of Proteins: The iodine monochloride method of Helmkamp et al. (18) was used for ^{125}I labeling of IgG. The procedure was modified for use in PBS and was performed at a level of 50 μCi ^{125}I (New England Nuclear Corp., carrier free-17 Ci/mg) per iodination reaction. After iodination the proteins were extensively dialyzed against several changes of PBS. Specific activities ranged from 1.5 to 2.4 X 10^4 cpm/μg protein.

Determination of Protein Concentrations: The method of Lowry et al. (21), was used to determine protein concentrations. Bovine serum albumin was used as the standard.

Binding of ^{125}I-IgG Preparations to Polyribosomes: Binding of the labeled IgGs was performed by direct addition of the preparations to purified polyribosomes (generally 5 to 8 μg ^{125}I-IgG per A260 unit of polyribosomes). For direct binding experiments the mixtures were incubated at 4^oC for 45 min. A NRS ^{125}I-IgG:polyribosome and an

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unlabeled NRS IgG:polyribosome mixture (control for degradation of polyribosomes under the conditions of incubation and analysis) were run with each analysis.

Gradient analyses of IgG:polyribosome mixtures were performed on 20% to 45% (w/v) sucrose gradients in RSB. Gradients were centrifuged at 40,000 rpm for 1.25 hr at $4^{o}C$ in the SW 50.1 rotor (Beckman). Equal fractions were collected and counted directly in an autogamma spectrometer (Packard).

Puromycin Treatment of Polyribosomes: The method described by Blobel and Sabatini (3) was modified to use for partial release of nascent proteins without polyribosomal disaggregation. Polyribosomes from cells labeled for one min with ^{3}H-amino acids, were incubated with (final concentrations) 10 m$\underline{M}$ puromycin and 50 m$\underline{M}$ KCl-total monovalent cation concentration 60 m$\underline{M}$, at $0^{o}C$ for 45 min. For the binding of ^{125}I-IgG under these conditions, unlabeled polyribosomes were incubated for 15 min in the mixture above, followed by addition of ^{125}I-IgG and incubation for the additional 30 min. The mixture which did not contain puromycin was incubated only with KCl for the first 15 min.

Absorption of ^{125}I-anti FeLV IgG with Viral Proteins: Varying amounts of soluble FeLV proteins (0.4 to 32 μg) or FeLV p30 (1.0 to 50 μg) were incubated with a constant amount of ^{125}I-anti FeLV IgG at $37^{o}C$ for 30 min. The mixtures were centrifuged at 5,000 rpm in the SS34 rotor (Sorvall) for 15 min. A constant amount of supernatant from each mixture was used in the binding experiments with polyribosomes.

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Preparation of Intracellular RNA: RNA from polyribosomal gradient fractions was collected by ethanol precipitation in the presence of 0.2 M NaCl and 50 μg/ml carrier yeast RNA. RNA was extracted from total polyribosomes and from pooled polyribosomal gradient fractions by the TNE-9 (0.1 M NaCl, 1 mM EDTA, 0.1 M Tris HCl, pH 9.0) SDS-phenol procedure as described (5).

FeLV DNA and DNA-RNA Hybridization: Single stranded FeLV ^{3}H-DNA, synthesized in the endogenous RNA directed DNA polymerase reaction was prepared as described (6). Hybridization of this DNA probe to RNA from gradient fractions was performed as described (6). Briefly, the hybridization conditions were 66°C in 0.01 Tris HCl (pH 7.2), 0.4 M NaCl, 0.05% SDS, 0.025 mM EDTA, 0.075 mg/ml calf thymus DNA, 0.15 mg/ml yeast RNA, sample RNA, and FeLV ^{3}H-DNA (generally 1000-1500 cpm). Hybrid formation was determined by digestion with S1 nuclease.

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RESULTS

<u>Binding of ^{125}I-IgG to Purified Polyribosomes</u>: Initial experiments were performed to determine if IgG preparations specific for viral proteins would bind to nascent virus-specific proteins. The results of these experiments are seen in Figure 1. Binding of rabbit anti-FeLV p30 IgG (prepared with p30 purified by guanidine HCl-agarose chromatography, designated anti-p30 (I) and NRS IgG are seen in Figure 1A. Also included in Figure 1A is an absorbance profile of the polyribosomes. The NRS IgG binding was at a level of approximately 0.02 to 0.03% throughout the polyribosome region of the gradient. The anti-p30 (I) bound only slightly higher, with values of 0.03 to 0.04%. In comparison, anti-p30 (II), Figure 1B, bound to polyribosomes with higher values of approximately 0.1 to 0.15%. In contrast, anti-FeLV IgG bound at a level of 0.25 to 0.32% in the polyribosome region. The binding was highest in the regions containing the fastest sedimenting polyribosomes. Peak binding in this gradient (Figure 1C) was equivalent to polyribosomes of sedimentation coefficient of approximately 400S, as calculated by the method of McEwen (22). Since this level was significantly greater than the levels obtained with the other IgGs, the anti-FeLV IgG was used in further experiments.

The specificity of binding was further examined by preincubation of polyribosomes with an excess of unlabeled NRS IgG, followed by incubation with ^{125}I-anti FeLV. The results are seen in Figure 2. The binding of anti-FeLV to polyribosomes preincubated with NRS IgG was

Figure 1. Sedimentation analysis of ^{125}I-IgG:polyribosome mixtures. F-422 polyribosomes purified by sepharose 2B chromatography were incubated with the ^{125}I-IgG preparation indicated in each panel and analyzed as described in the text. All analyses were performed in parallel gradients. Each fraction of the gradient was plotted as the percent of the total ^{125}I-IgG. (A) (●——●) rabbit anti-p30 I, (O---O) normal rabbit serum (NRS) IgG, (———) A_{254} total polyribosomes; (B) (●——●) goat anti-p30 II, (O---O) NRS; (C) (●——●) rabbit anti-FeLV, (O---O) NRS.

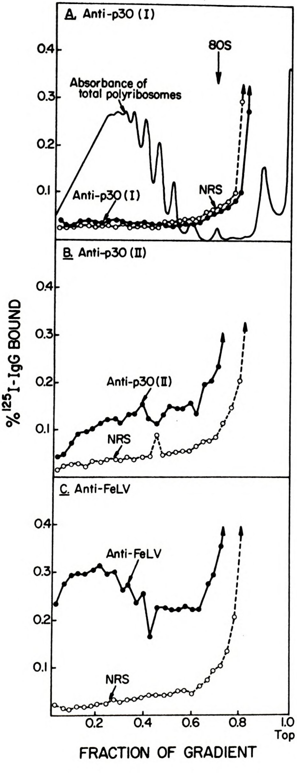
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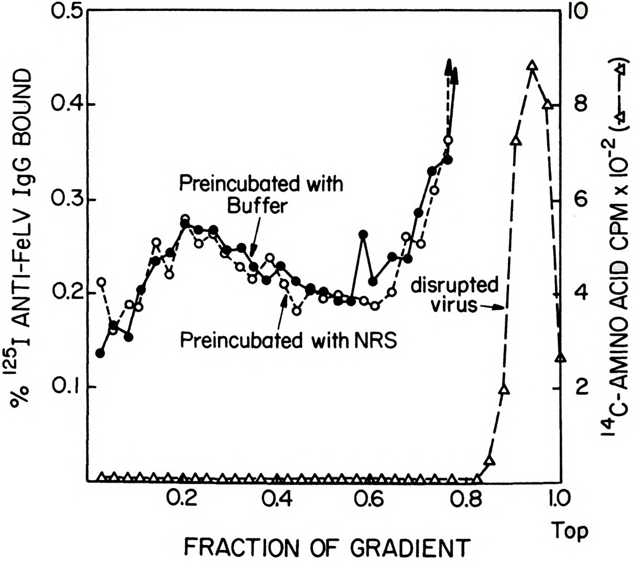
Figure 2. Sedimentation analysis of ^{125}I-anti-FeLV:polyribosome mixture preincubated with an excess of unlabeled NRS and sedimentation analysis of disrupted ^{14}C-amino acid labeled FeLV:polyribosome mixture. Purified polyribosomes were incubated for 15 min with an excess of unlabeled NRS (approximately 200 μg protein) followed by incubation with ^{125}I-anti-FeLV as described in the text. A parallel mixture was incubated with an equal volume of buffer followed by incubation with the specific IgG. The analyses were performed as described in Figure 1 (O---O) mixture preincubated with NRS, (●——●) mixture preincubated with buffer. ^{14}C-amino acid labeled FeLV proteins were incubated with polyribosomes as described in the text. The analysis was performed as described above. After fractionation, samples were assayed for TCA insoluble radioactivity as previously described (14). (Δ---Δ) ^{14}C-amino acid labeled FeLV protein.

activity as previously described (14). (Δ---Δ) ^{14}C-amino acid labeled FeLV protein.



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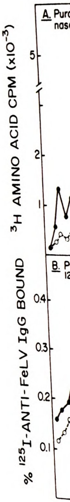
Nature of the Polyribosomal Sites: A mixing experiment was performed to determine if soluble viral proteins could nonspecifically adsorb to polyribosomes and cause artificial binding of ^{125}I-IgG which would not be due to antibody reaction with nascent virus-specific protein. FeLV, which was labeled with ^{14}C-amino acids, was freeze-thaw disrupted and 0.02% NP-40 solubilized. This preparation was incubated with polyribosomes and analyzed as described. As seen in Figure 2, there is no radioactivity associated with the polyribosome region of the gradient. The labeled viral proteins were found only within the first 0.2 fraction of the gradient. Further, unlabeled viral protein added to the cytoplasmic extract during polyribosome purification did not increase the binding of ^{125}I-anti FeLV IgG compared to control polyribosomes (data not shown).

To further define the nature of the polyribosomal binding sites, experiments were performed to determine the effect of puromycin release of nascent protein on ^{125}I-anti-FeLV IgG binding. The conditions of release were established as described in Materials and Methods. Disaggregation of the ribosomal-mRNA complexes would be reflected by an increase in absorbance in the slower sedimenting part of the gradient due to release of ribosomal subunits from polyribosomes (3). As seen in Figure 3D there is very little disaggregation of puromycin treated polyribosomes. The amount of slower sedimenting material present is only slightly more than in control polyribosomes (Figure 3C).

Figure 3. The partial release of nascent protein and partial reduction of anti-FeLV binding by puromycin treatment. (A) Polyribosomes from cells labeled for one min with ^{3}H-amino acids were prepared and samples treated with or without puromycin as described in the text. After analysis fractions were assayed for hot TCA insoluble radioactivity. (●——●) no puromycin, (O---O) plus puromycin. (B) Incubation of unlabeled polyribosomes with and without puromycin as described for panel A followed by incubation with ^{125}I-IgG and analysis of the mixtures as described in Figure 1. (●——●) anti-FeLV binding without puromycin, (O---O) anti-FeLV binding of puromycin treated mixture. (C) A_{254} polyribosomes without puromycin. (D) A_{254} puromycin treated polyribosomes.



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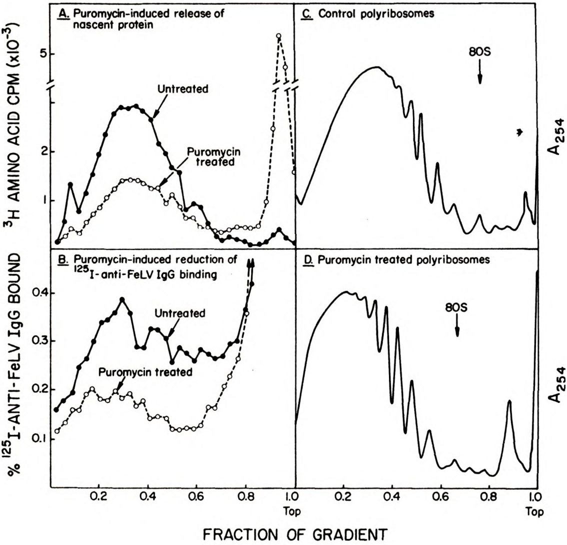

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However, under these conditions 55 to 60% of the ^{3}H-amino acids, which were incorporated in a one min labeling period, were released from polyribosomes (Figure 3A). Further, the ^{125}I-anti-FeLV IgG binding decreased by 55 to 58% for the puromycin treated polyribosomes. This concommitant release and reduction strongly suggests that the IgG bound to nascent virus-specific proteins.

Characterization of the Anti-FeLV Binding: Since anti-p30 (I) did not bind and since anti-p30 (II) had a low amount of binding, characterization of the binding with a monospecific IgG was not possible. As an alternative method for characterization of the polyribosomal sites, absorption experiments were performed with soluble viral proteins or p30. These experiments were performed to determine if the absorption could be seen as a 'competition' with polyribosomal nascent protein. As seen in Figure 4A, increasing amounts of soluble viral proteins decreased the binding from a level of almost 0.4% with no competing protein, to 0.06 to 0.09% with 32 μg protein which was the highest a amount used. Preincubation with p30 also decreased the binding (Figure 4B). The uncompeted binding level of 0.45% could be reduced to 0.1 to 0.13% with 24.8 μg of p30 in the preincubation mixture. Increasing the amount of p30 to almost 50 μg did not further reduce the binding (data not shown). For both sets of analyses increasing the amount of viral protein in the preincubation mixture decreased the binding of anti-FeLV IgG in relation to the amount of protein used.

Virus-Specific RNA within Polyribosomes: Hybridization experiments with the FeLV-R DNA were performed to correlate the binding of

Figure 4. Sedimentation analysis of absorbed ^{125}I-anti FeLV IgG: polyribosome mixtures. Polyribosomes were incubated with ^{125}I-anti-FeLV IgG as described in the text. Analyses were performed as described in Figure 1. (A) Anti-FeLV IgG binding to polyribosomes after absorption with total FeLV proteins; (1. ●——●) no FeLV protein, (2. O---O) 0.4 μg protein, (3. ▲——▲) 4.0 μg protein, (4. ■——■) 20 μg protein, (5. △——△) 32 μg protein (6. □---□) NRS Igg. (B) Anti-FeLV IgG binding to polyribosomes after absorption with purified FeLV p30; (1. ●——●) no p30, (2. O---O) 1.0 μg p30, (3. ▲——▲) 5.2 μg p30, (4. ■——■) 10.3 μg p30, (5. △——△) 24.8 μg p30, (6. □---□) NRS IgG.

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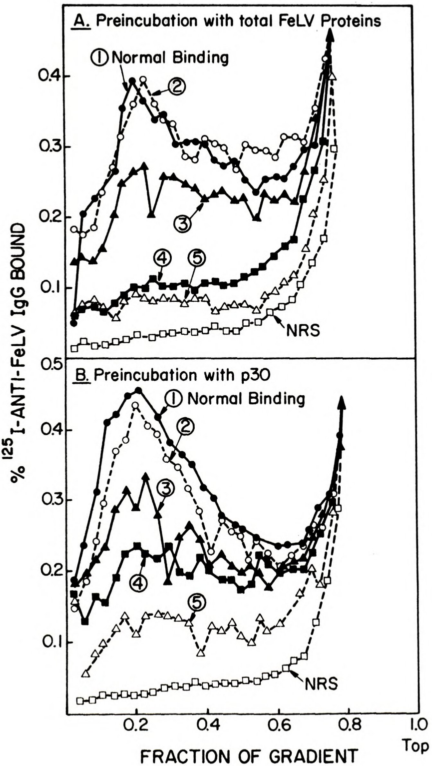

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anti-FeLV to nascent virus-specific protein with the presence of virus-specific RNA within polyribosomes. The hybridization analyses of polyribosome fractions are presented in Figure 5. The FeLV-R DNA hybridized to RNA from two regions within the polyribosome area of the gradient (Figure 5A). The fastest sedimenting region, designated I, contained the approximately 400 to 450S polyribosomes; whereas, the region designated II contained the approximately 250S polyribosomes. The DNA also hybridized to RNA from a third region within this gradient, designated III. The material present here had a sedimentation value of approximately 80S.

The EDTA treated polyribosome gradient only contained hybridizing material with values of less than 80S, designated IV (Figure 5B). There was no significant hybridization to the polyribosomal regions of the EDTA treated polyribosome gradient.

The size classes of virus-specific RNA within the designated regions of the gradients in Figure 5 were determined by velocity sedimentation in the presence of 99% DMSO. An analysis of total polyribosomal RNA is presented in Figure 6A. Three species with sedimentation values of 35S, 23S, and 18S were present. The same three species were also present in the RNA after the polyribosomes were pelleted through 2.0 _M_ sucrose (Figure 6B). The RNA from region I, the fastest sedimenting polyribosomes, contained three species of virus-specific RNA with sedimentation values of 33S, 22S, and 17S (Figure 6C). Region II only contained virus-specific RNA of 34S and 18S (Figure 6D). Region III from the untreated polyribosome gradient (Figure 6E) and the

Figure 5. Polyribosomal location of virus-specific RNA. Untreated and EDTA treated (final concentration-25 mM EDTA) polyribosomes were analyzed as described in Figure 1. RNA was prepared and hybridization performed with the FeLV-R DNA as described in the text. The amount of RNA was plotted as relative virus-specific RNA, using the relationship described by Fan and Baltimore (12), where 50% hybridization has a relative value of one. (A) Untreated polyribosomes, (●---●) virus-specific RNA, (———) A_{254}. (B) EDTA treated polyribosomes, (●---●) virus-specific RNA, (———) A_{254}.

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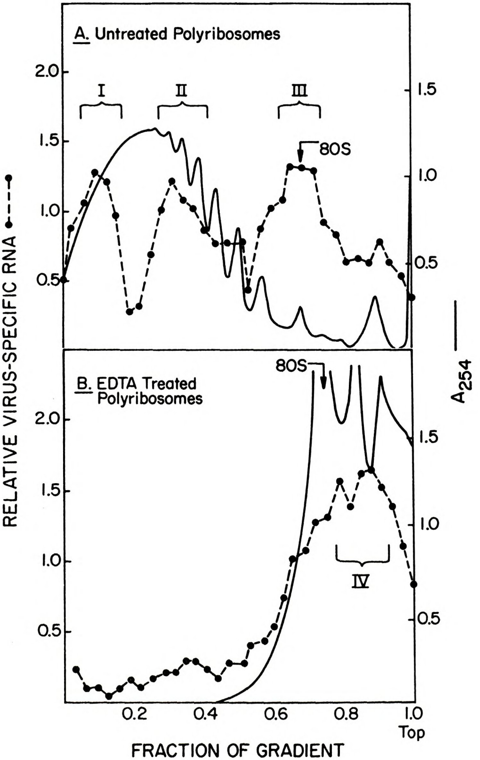

Figure 5

Figure 6. Sizes of FeLV-R-specific polyribosomal RNA. RNA from polyribosomes obtained by sepharose 2B chromatography, polyribosomes pelleted through 2.0 M sucrose after sepharose 2B chromatography, and regions I, II, III, and IV from the gradients described in Figure 5 were prepared and analyzed as described in the text. Arrows indicate internal ribosomal marker RNA in panels (C), (D), (E), (F) and parallel markers in panels (A) and (B). Gradient fractions were analyzed for virus-specific RNA as described in Figure 5. (A) Sepharose 2B chromatography purified polyribosomal RNA, (B) RNA from polyribosomes prepared as in A which were pelleted through 2.0 M sucrose before RNA extraction, (C) Region I RNA from the gradient described in Figure 5A, (D) Region II RNA from the gradient described in Figure 5A, (E) Region III RNA from the gradient described in Figure 5A, (F) Region IV RNA from the gradient described in Figure 5B.

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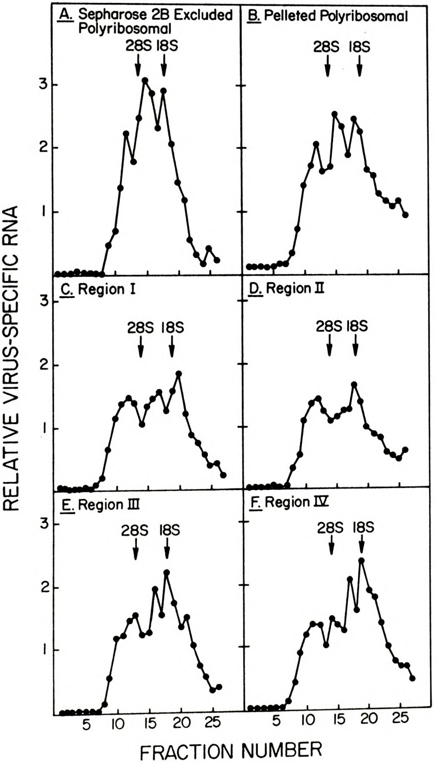

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designated region IV from the EDTA treated gradient (Figure 6F) contained a peak of virus-specific RNA at a value of 28S in addition to the three peaks at 34S, 22S, and 18S. The region II 34S RNA was present as a shoulder to the faster sedimenting side of the 28S species and there was also present RNA sedimenting at less than 18S (Figure 6E).

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DISCUSSION

The identification of polyribosomes involved in FeLV-R protein biosynthesis was performed by using two independent approaches; binding with labeled specific antibody preparations and hybridization with FeLV-R ^{3}H-DNA. Each approach has independently allowed us to analyze the virus-specific components of interest.

Antibody preparations have been successfully used as probes for nascent determinants in a variety of eukaryotic systems to study the biosynthesis of specific proteins (4,27,28,31,36). In most of these cases the protein under study was a major biosynthetic product of the cell type studied. Despite the relatively low amounts of oncornavirus proteins synthesized in infected cells, we were able to detect significant ^{125}I-anti-FeLV IgG binding to F-422 cell polyribosomes. In contrast, anti-p30 IgG I did not bind and anti-p30 II had little binding. It is possible that the affinity of anti-p30 I antibody, which was prepared by injection of rabbits with renatured guanidine HCl-agarose chromatography purified p30, is much less than the antibody prepared against whole FeLV which was not denatured with guanidine HCl. Although this anti-p30 reacts in immunodiffusion (14) and immunoprecipitation (25) with FeLV p30, in the experiments described here it could be possible that the integrity of the initial antibody-nascent protein complexes was not maintained during the velocity sedimentation procedure.

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The very low level (0.02 to 0.03%) of NRS IgG radioactivity associated with polyribosomes suggests that there is no nonspecific association of IgG protein with polyribosomes. In further confirmation of this observation, the preincubation of polyribosomes with an excess of NRS IgG did not reduce the subsequent binding of anti-FeLV (Figure 2).

A number of experiments were performed to determine that the binding observed was to virus-specific nascent proteins and not artifacts due to non-specific binding. In gamma globulin synthesizing cells, non-specific IgG binding may occur through the Fc portion binding to ribosomes (7). Also soluble antigen has been shown to increase specific antibody binding by adsorption to polyribosomes (11). The mixing experiment described in Figure 2 and the addition of soluble FeLV proteins to the cytoplasmic extract during polyribosome isolation allowed us to determine that soluble FeLV proteins added to polyribosomes will not become associated with the polyribosomes. In addition, the experiments described in Figure 3 confirms the nascent character of the antigens which bind anti-FeLV IgG. We were able to partially release nascent protein from purified polyribosomes (Figure 3D) by treatment with puromycin and low salt concentration. The release of nascent virus-specific protein should be the same as the release of total nascent protein. This idea was confirmed by the very similar anti-FeLV binding decrease and nascent protein release between puromycin treated and untreated polyribosomes (Figure 3A and 3B). The results of this experiment combined with the results of the analyses presented in Figure 2 demonstrate that anti-FeLV IgG bound to nascent virus-specific

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proteins. Vecchio et al. (39) used immunoprecipitation of labeled polyribosomes with unlabeled specific antiserum to detect nascent Moloney-murine leukemia virus (M-MuLV) proteins. Polyribosomes at approximately 350S contained the nascent M-MuLV-specific proteins. Although we observed antibody binding through a broad range of polyribosome sizes, the peak of antibody binding containing nascent FeLV-R specific protein is approximately 400S. This agrees closely to the size of polyribosomes containing M-MuLV-specific nascent protein.

The anti-FeLV binding was further characterized by absorption experiments using total FeLV protein or purified p30 (Figure 4A and 4B). This procedure made possible the determination of most of the virus-specific determinants to which the anti-FeLV bound. Total viral protein reduced the binding close to the level of NRS IgG binding whereas both 24.8 μg and 50 μg (not shown) of p30 only reduced the binding to approximately 0.14%. This suggests that the remaining antibody recognizes some viral determinants not contained in 'nascent p30'. Since this remaining level is much less than the uncompeted binding, approximately 0.45%, it appears that most of the anti-FeLV IgG binding is due to recognition of 'nascent p30' determinants.

By hybridization with FeLV-R ^{3}H-DNA, the polyribosomal area of polyribosome gradients were shown to contain two major regions of virus-specific RNA (Figure 5A). The fastest sedimenting region, 400 to 450S. correlates well with the peak binding of the anti-FeLV IgG. Since the anti-FeLV IgG binding encompasses a broad range of the polyribosomal area, there is further correlation of the binding to the 250S

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polyribosomal area which also contains a peak of FeLV-R specific RNA. From the combination of these two analyses, it is not possible however, to determine which of the two polyribosomal size classes detected by hybridization contains the major functional FeLV-R specific RNA template(s). The combined hybridization and immunological analyses do suggest that the detected RNA is virus-specific mRNA since both virus-specific nascent protein and EDTA-releasable RNA are present in the polyribosomal size classes observed. The polyribosomal location of FeLV-R specific RNA is similar to the 300 to 350S size observed for M-MuLV (13,39), and the 350S size for Rauscher-MuLV (R-MuLV) (15). In one case however, M-MuLV-specific RNA was associated with 150 to 200S polyribosomes (12).

It was previously shown that FeLV-R infected cell polyribosomes purified by sepharose 2B chromatography contained three species of virus-specific RNA which sediment at 36S, 23S, and 18S (6) and similar data are included for comparison (Figure 6A). The same three size classes have been detected in polyribosomes purified by the more standard method of pelleting through 2.0 M sucrose (Figure 6B), and also in the fastest sedimenting class of polyribosomes (Figure 6C). The 250S polyribosomes do not contain the 23S species, which is the only difference between the virus-specific RNA from this size class and the fastest sedimenting polyribosomes. Although the fast sedimenting polyribosomes from R-MuLV infected cells are enriched for 36S RNA (15), the fastest sedimenting FeLV-R infected cell polyribosomes are not enriched for the largest species of intracellular RNA.

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RNA from the non-polyribosomal region of these gradients contained a peak of 28S FeLV-R specific RNA. This size is equivalent to the FeLV-R genomic subunit RNA (5,9) and is not present in the polyribosomal regions (Figure 6C and 6D). In addition to the presence of the 28S RNA, the relatively low proportion of 34S RNA and the presence of virus-specific RNA of less than 18S (Figure 6E) may indicate that this region contains RNA produced by nucleolytic cleavage. Since EDTA-released virus-specific polyribosomal RNA also contained a peak of 28S (Figure 6F), it could be possible that the 28S size species is produced during translation or is an immediate post-translational cleavage product. From this data, we cannot develop a specific mechanism for the generation of the 28S RNA on polyribosomes. It is known that microsomal fractions from M-MuLV infected cells contain endonucleolytic activity which produces two smaller size RNA species from the 36S RNA (34). An activity of this type may be present on F-422 cell polyribosomes and may account for the presence of the 28S size in polyribosomal released RNA. It is not known whether the 36S or the 28S species contain the entire genomic capacity of FeLV-R. If the 28S RNA molecule represents the entire genome, then polyribosomal-associated cleavage may be the mechanism which generates a presumptive full length subunit size FeLV-R RNA from a 36S species. Alternatively, the 28S polyribosomal-released species may simply be an intermediate size degradation product of the 36S RNA.

Evidence for a mRNA function of the largest intracellular oncornavirus-specific RNA is found from a number of studies. Since oncornavirus subunit RNA is the same size and polarity as the largest

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intracellular species, it has been used in cell free protein synthesis. The Rous sarcoma virus (RSV), 30 to 40S RNA directed the cell free synthesis of a 75,000 to 80,000 dalton protein which was specifically precipitated with antiviral serum and contained tryptic peptides similar to the viral structural proteins (29,42). RNA from the murine oncornaviruses directed the cell free synthesis of viral structural protein precursor size products (16,20,24,30) besides extremely large proteins (20,24). Further, Mueller-Lantzsch et al. (23) have reported that 35S M-MuLV-specific RNA is the only size RNA obtained from polyribosomes containing M-MuLV p30 nascent determinants. This experiment and the cell free translation experiments suggest that the subunit size RNA can function as mRNA and that the low molecular weight structural protein precursors are probably the major synthetic products of this translation.

The smaller than genomic subunit size viral RNA molecules present on polyribosomes would serve an important function. Viral mRNA of these sizes would permit the separate or independent biosynthesis of specific viral products, when and if required in nonequimolar quantities. In mammalian cells transformed by Schmitt-Ruppin RSV, the 24S intracellular virus-specific RNA was shown to contain sequences specific for the RSV 'sarc' gene (2,8). By an analogous method the 24S intracellular virus-specific RNA from Rous associated virus-2 (RAV-2) infected cells was shown to contain the avian oncornavirus 'envelope' sequences (17). In this case, the 24S RNA could be either a heterogenous population or the two gene sequences are present within one molecule. Their results

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suggest that independent expression of oncornavirus genes can occur. The three size classes of R-MuLV-specific RNA found intracellularly have been translated in a cell free system (16). Discrete size products of 65,000 and 72,000 daltons were obtained using the 35S RNA, and 70,000 daltons using the 22S RNA. The 14S size class directed the synthesis of smaller products recognized by antiserum to R-MuLV. Further analysis of these R-MuLV products and products directed by other oncornavirus-specific intracellular RNAs will be necessary in order to understand the precise mode of oncornavirus gene expression.

The specific mRNA functions of the smaller 23S and 18S FeLV-R-specific species are not known. Experiments combining competition-hybridization and cell free translation of these smaller than genomic subunit size RNA will probably be required to determine the exact mRNA function of the intracellular FeLV-R-specific species. In addition, experiments will be required to determine whether the 34 to 36S intracellular RNA or the 28S size species contain the entire FeLV-R genomic capacity.

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