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Analysis of Cytoplasmic RNA and Polyribosomes from Feline Leukemia Virus-Infected Cells†

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Cytoplasmic virus-specific RNA and polyribosomes from a chronically infected feline thymus tumor cell line, F-422, were analyzed by using in vitro-synthesized feline leukemia virus (Rickard strain) (R-FeLV) complementary DNA (cDNA) probe. By hybridization kinetics analysis, cytoplasmic, polyribosomal, and nuclear RNAs were found to be 2.1, 2.6, and 0.7% virus specific, respectively. Size classes within subcellular fractions were determined by sucrose gradient centrifugation in the presence of dimethyl sulfoxide followed by hybridization. The cytoplasmic fraction contained a 28S size class, which corresponds to the size of virion subunit RNA, and 36S, 23S, and 15 to 18S RNA species. The virus-specific 36S, 23S, and 15 to 18S species but not the 28S RNA were present in both the total and polyadenylic acid-containing polyribosomal RNA. Anti-FeLV gamma globulin bound to rapidly sedimenting polyribosomes, with the peak binding at 400S. The specificity of the binding for nascent virus-specific protein was determined in control experiments that involved mixing polyribosomes with soluble virion proteins, absorption of specific gamma globulin with soluble virion proteins, and puromycin-induced nascent protein release. The R-FeLV cDNA probe hybridized to RNA in two polyribosomal regions (approximately 400 to 450S and 250S) within the polyribosomal gradients before but not after EDTA treatment. The 400 to 450S polyribosomes contained three major peaks of virus-specific RNA at 36S, 23S, and 15 to 18S, whereas the 250S polyribosomes contained predominantly 36S and 15 to 18S RNA. Further experiments suggest that an approximately 36S minor subunit is present in virion RNA.

The synthesis of oncornavirus proteins has been extensively examined in recent years. By using immunological techniques, 60,000- to 76,000-dalton intracellular precursors have been detected for the structural proteins of avian myeloblastosis virus (63, 64), various murine oncornaviruses (1, 25, 52, 60), and feline leukemia virus (FeLV) (37, 38). Furthermore, the immunological detection of even larger intracellular virus-specific proteins of 200,000 to 300,000 daltons (1, 60) suggests the possibility that at least in murine oncornavirus-infected cells, some of the viral proteins are synthesized as larger precursors. 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 intracelluar virus-specific species (14, 49, 58). It has been suggested that the intracellular viral genomic subunits function as viral mRNA (13). Since species of subunit size are present on polyribosomes (3, 9, 13, 14, 49, 51), and since RNA from these various oncornaviruses directs the synthesis of virus-specific products in vitro (18, 28, 35, 42, 47, 65), this hypothesis is probably correct. However, the presence on polyribosomes of smaller-than-subunit-size virus-specific RNA (3, 14, 17, 18, 51) and the ability of these smaller species to direct the synthesis of virus-specific products both in vitro (18, 41) and after microinjection (53, 61) also suggest an mRNA function.

The major Rickard's FeLV (R-FeLV) virion subunit RNA sediments as 28S RNA in sucrose gradients containing 99% dimethyl sulfoxide (Me_2SO) (7, 10). These subunits are further unique in that they do not have a modified 5'-terminal cap (57) as reported for avian (15, 27, 54) and murine (5, 44) oncornavirus RNA subunits.

Because of the unusual properties of R-FeLV virion subunit RNA, it became important to examine the intracellular virus-specific RNA in R-FeLV-producing cells. We were especially in-

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terested in determining whether smaller-than-subunit-size RNA species were present and whether they were of comparable size to those found in murine and avian oncornavirus-infected cells. In this study, R-FeLV-specific intracellular RNA and polyribosomes from a chronically infected thymus tumor cell line (F-422) were analyzed to: (i) quantitate virus-specific RNA within the subcellular fractions of infected cells; (ii) identify the size classes of virus-specific RNA found intracytoplasmically; and (iii) examine cellular polyribosomes for the presence of both virus-specific RNA and nascent protein. Further, since larger-than-subunit-size virus-specific RNA was detected intracellularly, experiments were also performed to reexamine virion RNA for the presence of larger subunits.

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MATERIALS AND METHODS

Cells and virus. The chronically infected feline thymus tumor cell line, F-422, which produces R-FeLV, was used as the source of virus and cells. This cell line, grown in suspension culture, was propagated as previously described (20). 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% McCoy 5A–60% Leibowitz L15 with 15% fetal calf serum.

Isotopic labeling. Viral or cellular RNA labeled with [^{3}H]- or [^{14}C]uridine was obtained by incubating cells in fresh medium containing 5.0 μCi of [^{3}H]uridine per ml (New England Nuclear Corp.; 40 to 50 Ci/mmol) or 0.2 μCi of [^{14}C]uridine per ml (New England Nuclear Corp.; >50 μCi/mmol) at a cell density of 2×10^6/ml for 4 h or less.

Viral RNA labeled with ^{32}P was obtained by incubating cells in Swim phosphate-free medium for 1 h followed by resuspension in fresh Swim phosphate-free medium containing 0.1 mCi of ^{32}P per ml (Amersham/Searle; 26 Ci/mg of P, as orthophosphate, in dilute HCl), 0.5 mg of amphotericin B per ml (P-L Biochemicals), and 1% Me_2SO (66). These cells were incubated for 4 h, followed by collection of the culture medium and resuspension of the cells in fresh Swim phosphate-free medium for 2 additional h. Both collections of culture medium were pooled and clarified at 10,000 rpm for 10 min in the Sorvall GSA rotor.

Cells labeled for 1 min with ^{3}H-labeled amino acids were obtained by incubation in amino acid-deficient medium containing 25 μCi of ^{3}H-labeled amino acid mixture per ml (New England Nuclear Corp.) at a cell density of 50×10^6/ml. After 1 min at 37°C, the pulse was ended by addition of the cells to partially frozen medium. ^{14}C-amino acid-labeled virus was prepared as described (20).

Preparation of virus. All culture supernatants used were from cells grown in fresh medium for 4 h or less. Virus was prepared from this material by either of two methods. In the first, clarified medium was (i) layered over 5 ml of 20% (vol/vol) glycerol in 0.06 M NaCl–0.05 M Tris-hydrochloride (pH 8.5), for rapid collection of virus used in the synthesis of DNA, or (ii) layered over 5 ml of 20% (vol/vol) glycerol in 0.1 M NaCl–0.1 M Tris-hydrochloride (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 h. In the second method, FeLV was prepared from culture medium by a discontinuous sucrose gradient centrifugation as described (37).

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 (PBS), and repelleted. Cells were allowed to swell in hypotonic buffer (reticulocyte standard buffer [RSB]) (0.1 M Tris [pH 7.4]–0.01 M NaCl–1.5 mM $MgCl_2$) at 4°C for 10 to 15 min. 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 $950 \times g$ for 5 min. 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.

Total polyribosomes were prepared from the cytoplasmic extract containing 50 μg of sodium heparin per ml, 0.2% sodium deoxycholate, and 0.2% NP-40. This suspension was centrifuged at $27,000 \times g$ for 5 min, and the supernatant was removed, applied to a column (18 by 1.5 cm) of Sepharose 2B previously equilibrated in RSB, and chromatographed (11). The excluded material, containing the polyribosome fraction almost devoid of ribosomal subunit and ribosomal monomer material (see Fig. 5 and 6), 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 $950 \times g$ for 5 min and twice in RSB by the same procedure.

RNA extraction. RNA was extracted from pelleted material other than nuclei by the TNE-9 sodium dodecyl sulfate (SDS)-phenol procedure as previously described (7). 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 (19).

Polyacrylamide gel electrophoresis. Electrophoresis of nucleic acids in 2% polyacrylamide–0.5% agarose composite gels was performed as previously described (7).

Velocity sedimentation analysis of RNA. Neu-

tral sucrose gradient analysis of viral RNA and velocity sedimentation of 5 to 20% (wt/vol) sucrose gradients in the presence of 10 mM LiCl, 1 mM EDTA, and 99% Me_2SO was performed as described (7). RNA from Me_2SO gradient fractions used in hybridization experiments was collected by ethanol precipitation in the presence of 0.2 M NaCl and 50 μg of carrier yeast RNA per ml.

Preparation of R-FeLV cDNA. The endogenous RNA-directed DNA polymerase reaction was used to prepare radioactive DNA complementary to FeLV RNA (cDNA). A modification of the reaction mixture described by Rothenberg and Baltimore (45) was used. It contained 50 μg of actinomycin D per ml, 50 mM Tris-hydrochloride (pH 8.3), 60 mM NaCl, 30 mM $MgCl_2$, 0.013% NP-40, 5 mM each dCTP, dATP, and dGTP, 0.1 mM TTP (generally 115 μCi of [^{3}H]TTP per ml; I.C.N. Pharmaceuticals; 40 Ci/mmol), and virus at 1.5 to 3.0 mg of protein per ml. The mixture was incubated at 37°C for 12 to 16 h, followed by making the sample 0.2 M sodium acetate, 20 mM Tris-hydrochloride (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 (45), chromatographed on Sephadex G-50, incubated for 4 h at 37°C in 0.4 M NaOH, neutralized, and precipitated in 67% ethanol at −20°C. The specific activity of the cDNA probe was 218 cpm/pmol.

RNA-DNA hybridization. Cellular or viral RNA was incubated with ^{3}H-labeled FeLV cDNA probe (generally 1,000 to 1,500 cpm) in a hybridization mixture consisting of (final concentrations) 0.01 M Tris-hydrochloride (pH 7.2), 0.4 M NaCl, 0.05% SDS, 0.25 mM EDTA, 0.075 mg of calf thymus DNA per ml, and 0.15 mg of yeast RNA per ml at 66°C as described (50). The reactions were assayed for DNA hybrid formation by the use of a crude preparation of S1 nuclease (32). The crude nuclease was prepared by extraction of 8 g of α-amylase (Sigma Chemical Co.) into 100 ml of nuclease buffer (25 mM potassium acetate [pH 4.5]–0.1 mM $ZnSO_4$–0.1 M NaCl [55]), followed by clarification of the extract. The extract was made 50% glycerol and stored at −20°C. Two milliliters of nuclease buffer containing 0.0125 ml of crude nuclease preparation per ml was added to each reaction, incubated at 45°C for 30 min, precipitated with trichloroacetic acid, collected on 0.45-nm filter disks (Millipore Corp.), and assayed for radioactivity as previously described (20).

Oligo(dT)-cellulose chromatography. Oligodeoxythymidylic acid [oligo(dT)]-cellulose chromatography was performed with oligo(dT)-cellulose (Collaborative Research, T3) by a modification of the procedure described (43). Polyadenylic acid [poly(A)]-containing RNA was bound in 0.5 M KCl–20 mM Tris (pH 7.5) and eluted in 20 mM Tris (pH 7.5).

Preparation of antisera and IgG fractions. Rabbit anti-FeLV (Tween 80–ether-disrupted virus) and normal rabbit serum (NRS) were prepared as described (20). All sera were subjected to two successive precipitations with 40% saturated ammonium sulfate followed by extensive dialysis against 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. (24) 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 of ^{125}I (New England Nuclear Corp.; carrier-free, 17 Ci/mg) per iodination reaction containing 2 mg of protein. After iodination, the proteins were extensively dialyzed against several changes of PBS. Specific activities ranged from 1.5×10^4 to 2.4×10^4 cpm per μg of protein.

Determination of protein concentrations. The method of Lowry et al. (30) was used to determine protein concentrations, using bovine serum albumin as the standard.

Binding of ^{125}I-labeled IgG preparations to polyribosomes. Binding of the labeled IgG's was performed by direct addition of the preparations to purified polyribosomes (generally 5 to 8 μg of ^{125}I-labeled IgG per unit of absorbancy at 260 nm of polyribosomes). For direct binding experiments the mixtures were incubated at 4°C for 45 min. An NRS ^{125}I-labeled IgG–polyribosome mixture and an unlabeled NRS IgG–polyribosome mixture (control for degradation of polyribosomes under the conditions of incubation and analysis) were run with each analysis.

Analysis of ^{125}I-labeled IgG–polyribosome mixtures was performed on 20 to 45% (wt/vol) linear sucrose gradients in RSB. Gradients were centrifuged at 40,000 rpm for 1.25 h at 4°C in the SW50.1 rotor (Beckman). Equal fractions were collected and counted directly in an autogamma spectrometer (Packard).

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 R-FeLV cDNA, synthesized in the presence of 50 μg of actinomycin D per ml, was also found to be single stranded, based on its being rendered greater than 98% trichloroacetic acid soluble after treatment with S1 nuclease (55).

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. The native product contained a very high proportion of slow-migrating material (Fig. 1A). This material represented DNA product complexed with viral RNA, which, after alkali treatment to destroy the RNA, migrated faster as heterogeneous smaller species (Fig. 1B). A small amount of the product migrated with an estimated molecular weight of 2.0×10^6 (an equivalent sedimentation value of approximately 30S). There were present a number of species migrating in the molecular weight range of 1.7×10^5 to 4.5×10^5 (9 to 15S). However, the majority of the DNA migrated with estimated molecular weights of less than 0.8×10^5 (less than 6S).

To assess the extent and uniformity of the transcription, the [^{3}H]cDNA product was hybridized to ^{32}P-labeled viral RNA at various DNA/RNA ratios, and the hybrids formed were assayed for RNase resistance (16). The cDNA product protected 32.5% of the viral genome at a DNA/RNA ratio of 1 (Fig. 2). 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 cDNA product 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 indicate nonuniform transcription.

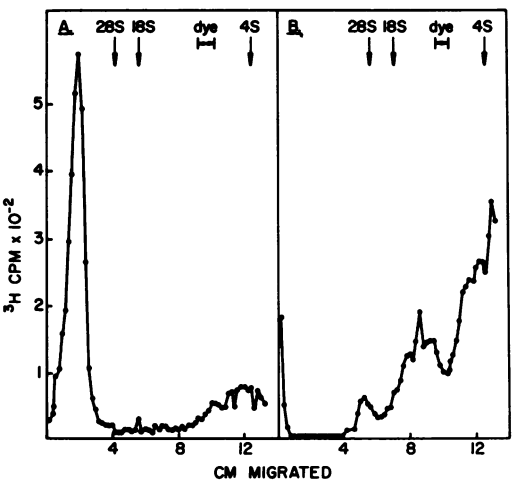

FIG. 1. *Polyacrylamide gel electrophoresis of the product of the FeLV endogenous RNA-directed DNA polymerase reaction. Electrophoresis of the nucleic acids extracted and purified from the reaction mixture described in the text was performed in 2.0% polyacrylamide–0.5% agarose composite gels as described (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 RNAs were run in parallel.*

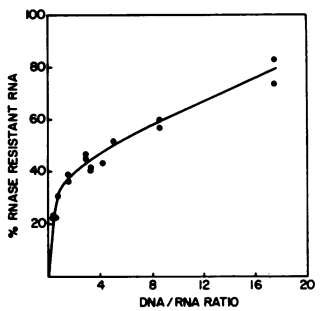

FIG. 2. *Hybridization of ^{32}P-labeled FeLV 28S subunit RNA with varying concentrations of single-stranded ^{3}H-labeled FeLV cDNA. The specific activity of the RNA was 158 cpm per pmol of nucleotide, and that of the DNA probe, prepared as described for Fig. 1B, was 218 cpm per pmol of nucleotide. After incubation of the reaction mixtures for 48 h at 66°C, samples were digested with pancreatic RNase A (50 μg/ml) in 0.3 M NaCl for 30 min at 37°C (16). The percentage of ^{32}P-labeled 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%.*

Amount of intracellular virus-specific RNA. Experiments were performed to determine the rates of hybridization of various intracellular RNA fractions from F-422 cells with the R-FeLV cDNA. RNA-DNA hybridization in RNA excess can be performed to determine the value of $C_rt_{1/2}$, the product of the concentration of RNA and the time at half-saturation (2). The relative concentration of virus-specific RNA in those fractions tested can be estimated by comparison of the $C_rt_{1/2}$ obtained for the viral RNA (13, 49). These experiments were performed with either varied time or concentration, with essentially identical results obtained by both methods. The amount of viral DNA hybridized is plotted as a log function of C_rt (Fig. 3). By comparison with the viral $C_rt_{1/2}$ for 28S FeLV subunit RNA, unfractionated cytoplasmic RNA, nuclear RNA, and total polyribosomal RNA contained 2.1, 0.7, and 2.6% virus-specific RNA, respectively (Table 1). Under these conditions, cytoplasmic RNA from uninfected feline embryo lung fibroblasts (FLF-3) did not hybridize to the FeLV cDNA probe (Fig. 3). Maximum hybridization for the viral, cytoplasmic, nuclear, and polyribosomal RNA ranged from 76 to 84%.

The relative distribution of virus-specific sequences was determined from a comparison of

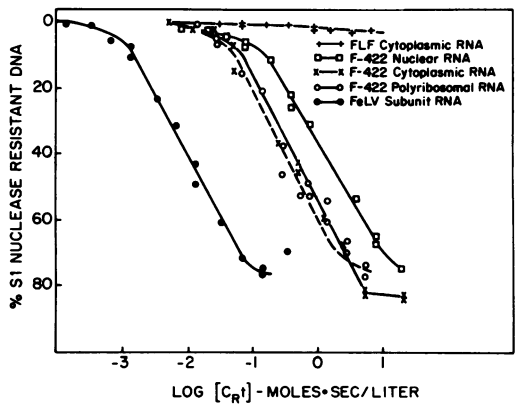

FIG. 3. *Relative concentrations of virus-specific RNA in subcellular fractions from F-422 cells. The relative concentrations were determined by hybridization of RNA from the various subcellular fractions and FeLV 28S subunit RNA at varying concentrations with ^{3}H-labeled FeLV DNA (1,400 cpm per reaction mixture) for 20 h in the reaction mixture described in the text. Hybrid formation was detected by S1 nuclease digestion as described in the text.*

TABLE 1. *FeLV-specific RNA in F-422 cells*

Subcellular fraction	Relative virus-specific RNA (%)[a]	Distribution of cellular RNA (%)[b]	Distribution of virus-specific RNA[c]
Cytoplasm	2.1	77.8	0.91
Nuclei	0.7	22.3	0.09
Polyribosomes	2.6	29.7	0.43

[a] From Fig. 3.

[b] Determined by measuring RNase-sensitive, trichloroacetic acid-precipitable radioactivity in subcellular fractions after labeling cells with [^{3}H]uridine for 14 h.

[c] Expressed as [(relative virus-specific RNA in subcellular fraction)(distribution of RNA in subcellular fraction)]/[(cytoplasmic relative virus-specific RNA)(cytoplasmic distribution) + (nuclear relative virus-specific RNA)(nuclear distribution)].

the distribution of cellular RNA within the subcellular fractions with the relative virus-specific values (Table 1). The cytoplasmic and nuclear fractions contained 91 and 9% of the virus-specific sequences, respectively, while 43% of the total sequences were associated with polyribosomes.

Integrity of cytoplasmic RNA during extraction. To determine whether the extraction procedures used had any effect on the stability of RNA from subcellular fractions, control mixing experiments were performed. Virion 28S RNA, labeled with [^{3}H]uridine, was added to extracts, and the RNA was purified and analyzed on the denaturing gradients. Viron 28S RNA was not affected by incubation with cytoplasmic extracts for 0.5 or 1.35 h, conditions required for preparation of cytoplasmic extracts and polyribosomes, respectively (data not shown).

Size of virus-specific RNA in cytoplasmic extracts and on polyribosomes. RNA was extracted directly from F-422 cytoplasmic extracts as described in Materials and Methods. After sedimentation in a 5 to 20% (wt/vol) sucrose gradient containing 99% Me_2SO, the RNA was precipitated from fractions and analyzed for virus-specific RNA, using ^{3}H-labeled FeLV cDNA probe. The major cytoplasmic species present sedimented with a value of 28S (Fig. 4A). There was also present faster-sedimenting material with a value of 36S. 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). Smaller amounts of hybridizable RNA sedimenting at approximately 23S and less than 18S were also present, but not resolved as separate peaks in the presence of a large 28S peak. Due to the denaturing conditions of the analysis, virus-specific RNA of undenatured size

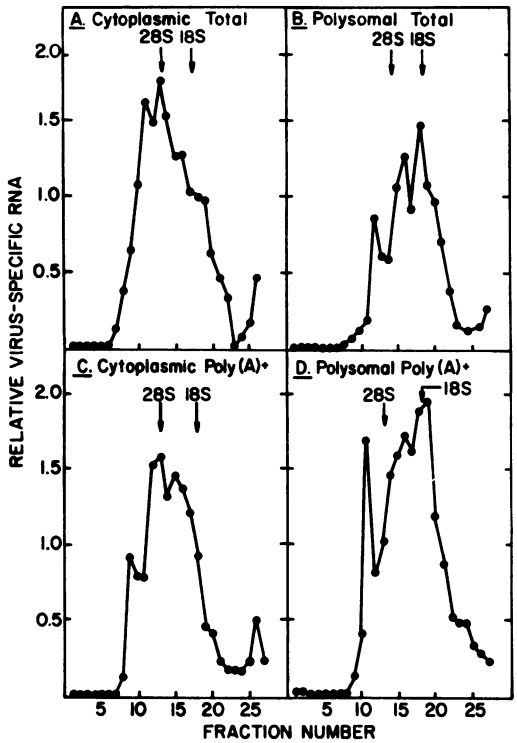

FIG. 4. *Sizes of cytoplasmic and polyribosomal virus-specific RNA, as determined by analysis under denaturing conditions. RNA extracted from cytoplasm or polyribosomes purified by Sepharose 2B chromatography as described in the text was analyzed on sucrose gradients containing 99% Me_2SO by the method previously described (7). RNA in panels (C) and (D) was first chromatographed on oligo(dT)-cellulose described in the text. After centrifugation, gradients were fractionated, processed, and assayed for virus-specific RNA as described in the text. Arrows indicate internal marker positions. The amount of RNA was plotted as relative virus-specific RNA, using the relationship described by Fan and Baltimore (13), where 50% hybridization has a relative value of 1. (A) 8.1 μg of total cytoplasmic RNA; (B) 4.3 μg of polyribosomal RNA; (C) 22 μg of cytoplasmic poly(A)-containing RNA; (D) 17 μg of polyribosomal poly(A)-containing RNA.*

(50 to 60S) would not be detected. The RNA detected would include subunits from virion precursor particles present in the cytoplasm as well as nonvirion virus-specific RNA.

Polyribosomes from F-422 cells were purified by the Sepharose 2B exclusion method as described (11), extracted, and analyzed for virus-specific RNA as described for the cytoplasmic extract RNA. There were three areas of hybridizing RNA, with average values of 36S, 23S, and 15 to 18S (Fig. 4B). The two slower-sedimenting areas were more discernible as separate peaks

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 Me_2SO gradient analysis (data not shown) and could be released from polyribosomes by EDTA treatment (Fig. 5). It is notable that there appeared to be no peak of hybridizing material at a value of 28S in this polyribosomal RNA.

Poly(A)-containing cytoplasmic and polyribosomal RNA. To further characterize the virus-specific RNA species detected, the cytoplasmic and polyribosomal RNAs were subjected to oligo(dT)-cellulose chromatography before Me_2SO gradient analysis. Cytoplasmic poly(A)-containing virus-specific RNA (Fig. 4C) contained three RNA species, with average sedimentation values of 36S, 28S, and 23S. The 36S and 28S species were present in the total cytoplasmic RNA fraction (Fig. 4A) described above. However, oligo(dT)-cellulose chromatography increased the relative proportion of the 23S species (Fig. 4C) when compared with the total extract (Fig. 4A). Since only approximately 66% of R-FeLV virion 28S RNA contains poly(A) (7), this relative increase would be expected if the oligo(dT)-cellulose chromatography procedure selected only the poly(A)-containing intracellular 28S RNA. Although hybridizable poly(A)-containing RNA was present in the 15 to 18S region, it was not resolved as a separate peak (Fig. 4C).

Polyribosomes contained three predominant species of poly(A)-containing virus-specific RNA (Fig. 4D), which sedimented with values of 36S, 23S, and 15 to 18S, as was the case for total polyribosomal virus-specific RNA (Fig. 4B).

Binding of anti-FeLV ^{125}I-labeled IgG to purified polyribosomes. Initial experiments were performed to determine whether rabbit anti-FeLV IgG preparations would bind to nascent virus-specific proteins (Fig. 5A). The binding profile of the NRS IgG is also included, as well as an absorbance profile of the polyribosomes. The NRS IgG binding was at a very low background level of approximately 0.02 to 0.03% throughout the polyribosome region of the gradient, suggesting that there was very little nonspecific association of IgG protein with polyribosomes. Anti-FeLV IgG bound at a much higher level, ranging from 0.25 to 0.32% in the

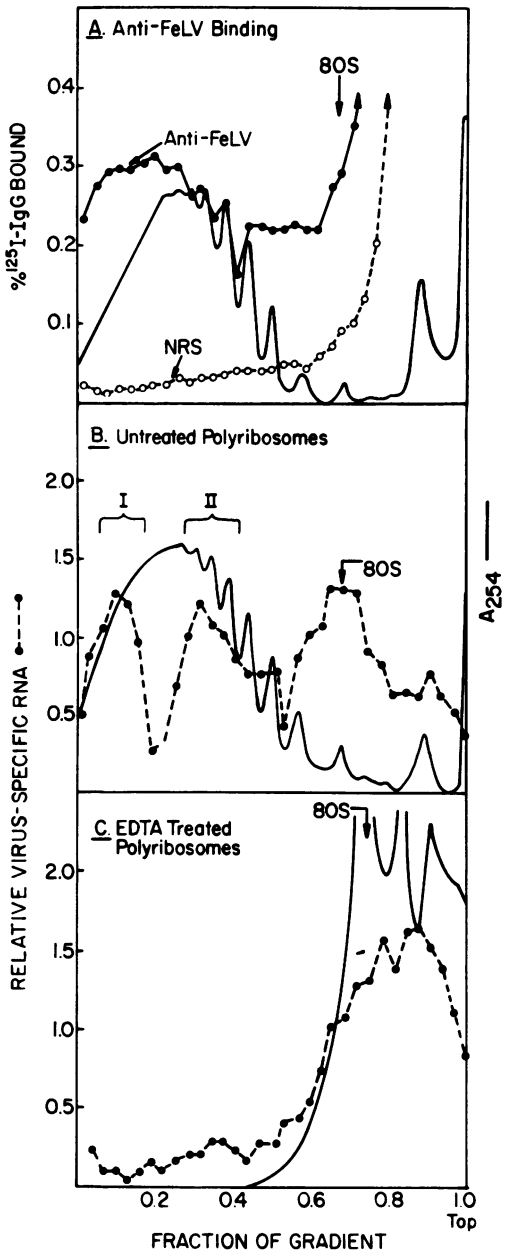

FIG. 5. *Polyribosomal location of virus-specific nascent protein and RNA. (A) F-422 polyribosomes purified by Sepharose 2B chromatography were incubated with anti-FeLV and NRS IgG as described in the text. Analysis was performed on parallel 4.8-ml, 20 to 45% (wt/vol) linear sucrose gradients centrifuged at 40,000 rpm in an SW50.1 rotor for 1.25 h. The radioactivity in each fraction of the gradient was expressed as the percentage of the total ^{125}I-labeled IgG used in the entire gradient. Symbols: (●) Rabbit anti-FeLV IgG; (○) NRS IgG; (——) total polyribosomes at optical density of 254 nm. (B) Untreated polyribosomes and (C) EDTA (final concentration, 25 mM)-treated polyribosomes were centrifuged as described above. RNA was prepared from gradient fractions as described in the text, and hybridization was performed with the R-FeLV cDNA as described in the legend to Fig. 4. Symbols: (●) Virus-specific RNA; (——) optical density of 254 nm.*

polyribosome region and as high as 0.45% in subsequent experiments. This binding was highest in the regions containing the fastest-sedimenting polyribosomes (Fig. 5A, 6B, and 7), with a sedimentation coefficient of approximately 400S as calculated by the method of McEwen (31).

Specificity and nature of the polyribosomal binding sites. The specificity of binding was further examined by preincubation of polyribosomes with an excess of unlabeled NRS IgG, followed by incubation with ^{125}I-labeled anti-FeLV. The binding of anti-FeLV to polyribosomes preincubated with NRS IgG was the same as the binding of anti-FeLV to polyribosomes preincubated with buffer alone (data not shown).

A mixing experiment was performed to determine whether soluble viral proteins could nonspecifically adsorb to polyribosomes and cause artificial binding of ^{125}I-labeled IgG that would not be due to antibody reaction with nascent virus-specific protein. The labeled viral protein was incubated with polyribosomes and analyzed as described. Radioactivity was not associated with the polyribosome region of the gradient (data not shown). Further, unlabeled viral protein added to the cytoplasmic extract during polyribosome purification did not increase the binding of ^{125}I-labeled anti-FeLV IgG compared with 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-labeled anti-FeLV IgG binding. The conditions of release without disaggregation of polyribosomes were established as a modification of the method described by Blobel and Sabatini (4). Disaggregation of the ribosomal-mRNA complexes, which would be

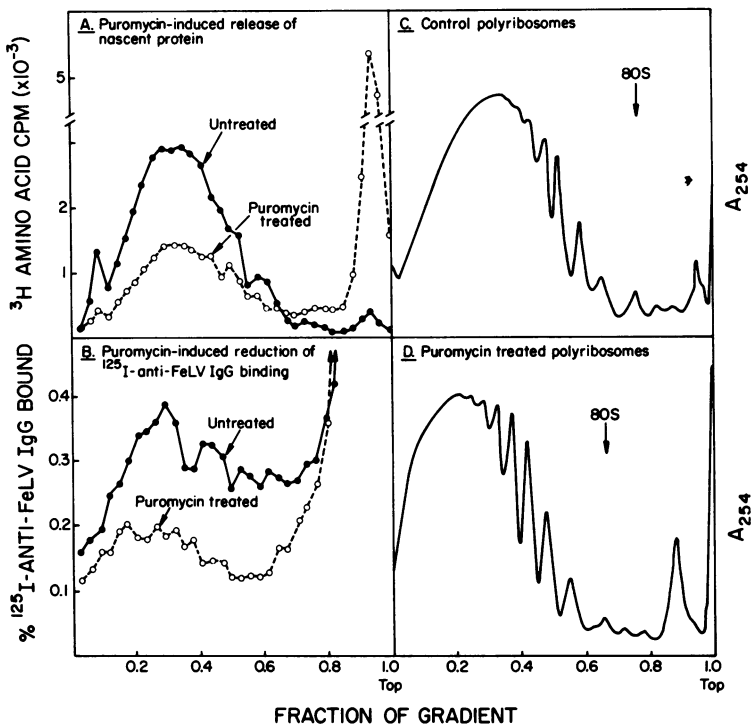

FIG. 6. *Partial release of nascent protein and partial reduction of anti-FeLV binding by puromycin treatment. The method described by Blobel and Sabatini (4) was modified for use in partial release of nascent proteins by puromycin without polyribosomal disaggregation. (A) Polyribosomes from cells labeled for 1 min with ^{3}H-amino acids were prepared as described, and samples were incubated with or without (final concentrations) 10 mM puromycin and 50 mM KCl (total monovalent cation concentration, 60 mM) at 0°C for 45 min. After sedimentation analysis as described in the legend to Fig. 5, fractions were assayed for hot trichloroacetic acid-insoluble radioactivity. Symbols: (●) No puromycin; (○) plus puromycin. (B) Incubation of unlabeled polyribosomes with and without puromycin for 15 min as described for panel (A), followed by incubation with ^{125}I-labeled IgG for an additional 30 min and analysis of the mixtures as described in the legend to Fig. 5A. Symbols: (●) Anti-FeLV binding without puromycin; (○) anti-FeLV binding of puromycin-treated mixture. (C) Absorbancy at 254 nm of polyribosomes without puromycin. (D) Absorbancy at 254 nm of puromycin-treated polyribosomes.*

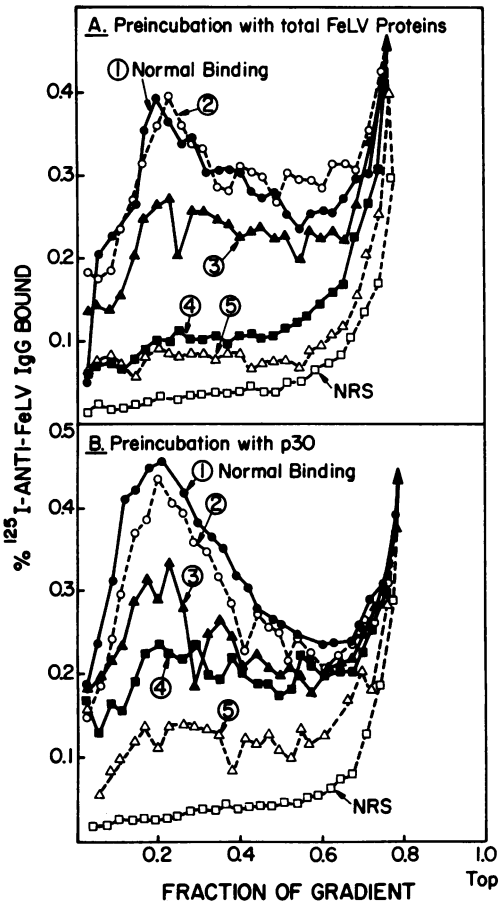

FIG. 7. *Sedimentation analysis of polyribosome-absorbed ^{125}I-labeled anti-FeLV IgG mixtures. Polyribosomes were incubated with ^{125}I-labeled anti-FeLV IgG preparations, which were absorbed as follows. Varying amounts of soluble FeLV proteins (0.4 to 32 μg) or FeLV p30 (1.0 to 50 μg) were incubated with a constant amount of ^{125}I-labeled anti-FeLV IgG at 37°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, and analyses were performed as described in the legend to Fig. 5A. (A) Anti-FeLV IgG binding to polyribosomes after absorption with total FeLV proteins: (1, ●) no FeLV protein; (2, ○) 0.4 μg of protein; (3, ▲) 4.0 μg of protein; (4, ■) 20 μg of protein; (5, △) 32 μg of protein; (6, □) NRS IgG. (B) Anti-FeLV binding to polyribosomes after absorption with GuHCl-agarose chromatography-purified FeLV p30: (1, ●) no p30; (2, ○) 1.0 μg of p30; (3, ▲) 5.2 μg of p30; (4, ■) 10.3 μg of p30; (5, △) 24.8 μg of p30; (6, □) NRS IgG.*

reflected by an increase in absorbance in the slower-sedimenting part of the gradient due to release of ribosomal subunits from polyribosomes (4), did not occur to a significant extent (Fig. 6C, D). However, 58% of the ^{3}H-labeled amino acid radioactivity, which was incorporated during a 1-min labeling period, was released from polyribosomes, and ^{125}I-labeled anti-FeLV IgG binding was decreased by 57% after puromycin treatment (Fig. 6A, B). This concomitant nascent protein and antibody binding reduction strongly suggests that the IgG bound to nascent virus-specific proteins.

To further characterize the polyribosomal binding sites and confirm their virus specificity, competition experiments were performed with soluble viral proteins and purified p30 to determine whether added proteins could compete with nascent protein and reduce the specific binding. Increasing amounts of soluble virion proteins decreased the binding in the 400S polyribosome region from an uncompeted level of almost 0.4% to near background levels of 0.06 to 0.09% with 32 μg of protein, the highest amount used (Fig. 7A). Increasing p30 also decreased the binding, but not as extensively, since 50 μg (data not shown) did not reduce binding further than the level of 0.1 to 0.14% obtained with 24.8 μg (Fig. 7B). These data indicate that most (i.e., approximately 70 to 75%) of the virus-specific nascent antigenic determinants were p30. Since this anti-FeLV serum also contains significant anti-p15 and some anti-p11 (20), the vast majority of the anti-FeLV reactivity involves virus-specific proteins.

Virus-specific RNA within different size classes of polyribosomes. Hybridization experiments with the R-FeLV cDNA were performed across polyribosome gradients to correlate the binding of anti-FeLV to nascent virus-specific protein RNA within polyribosomes. The R-FeLV cDNA hybridized to RNA from two regions within the polyribosome area of the gradient (Fig. 5B). The fastest-sedimenting region, I, contained the approximately 400 to 450S polyribosomes, whereas region II contained the approximately 250S polyribosomes. There was no significant hybridization to the polyribosomal regions of the EDTA-treated polyribosome gradient (Fig. 5C).

The size classes of virus-specific RNA within the two polyribosome regions of the gradient in Fig. 5B were determined by velocity sedimentation in the presence of 99% Me_2SO followed by hybridization. The RNA from region I, the fastest-sedimenting polyribosomes, contained the same three species of virus-specific RNA that were found in total polyribosomes, with sedimentation values of 36S, 23S, and 15 to 18S (Fig. 8A). Region II contained discernible virus-specific RNA peaks of 36S and 15 to 18S (Fig. 8B).

Analysis of virion RNA. R-FeLV virion

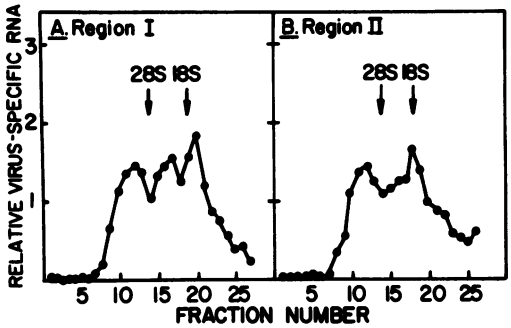

FIG. 8. *Sizes of R-FeLV-specific polyribosomal RNA. RNA from polyribosomal regions I and II of the gradient described in the legend to Fig. 5B were prepared and analyzed as described in the text. Arrows indicate internal ribosomal marker RNA in all panels. Gradient fractions were analyzed for virus-specific RNA as described in the legend to Fig. 4. (A) Region I RNA from the gradient described in the legend to Fig. 5B; (B) region II RNA from the gradient described in the legend to Fig. 5B.*

RNA contains only a 28S subunit as detected by labeling alone (7; Fig. 9B). However, the finding of a 36S virus-specific RNA species in intracellular RNA raised questions about its possible origin and the possibility that a low level of a similar-size species might exist in virion RNA. In attempts to detect and more completely resolve a possible >28S virion RNA species, R-FeLV 50 to 60S RNA (Fig. 9A) was analyzed by velocity sedimentation on a shallower gradient with subsequent hybridization under conditions of excess RNA and time sufficient to saturate the input cDNA probe (Fig. 9C). The 28S subunit was detectable by hybridization with R-FeLV [^{3}H]cDNA in coincidence with the viral subunit labeled with [^{14}C]uridine, as expected. Additionally, virus-specific RNA of approximately 35S, which cosedimented with poliovirus RNA, was detected by hybridization with the R-FeLV cDNA probe (Fig. 9C). This experiment suggests that R-FeLV contains an additional minor subunit in addition to the major 28S subunit RNA. Presently, the 35S virion subunit and the 36S intracellular RNA are assumed to be equivalent, since the size difference is within the range of resolution attainable in these experiments.

Because the above analysis was done with considerable RNA excess and time to assure detection of a minor species clearly not detectable by labeling (Fig. 9B), the equal height of the cDNA peaks in Fig. 9C reflects saturation of the probe rather than equal amounts of 28S and 36S RNA. Therefore, the relative proportion of these two species in virion RNA was determined by hybridization kinetics analysis. R-

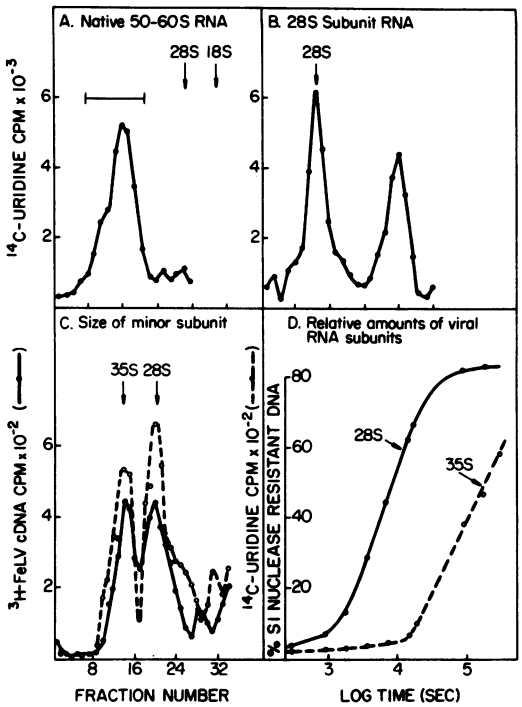

FIG. 9. *Identification of the minor R-FeLV virion RNA subunit. (A) Neutral velocity sedimentation of R-FeLV 50 to 60S RNA labeled with [^{14}C]uridine as described in the text. (B) Velocity sedimentation of R-FeLV 50 to 60S RNA labeled with [^{3}H]uridine on a sucrose gradient containing 99% Me_2SO as described in the legend to Fig. 4. (C) Velocity sedimentation and hybridization of R-FeLV RNA. The [^{14}C] uridine-labeled 50 to 60S RNA from the gradient illustrated in panel (A), as indicated by the bar, was ethanol precipitated in the presence of [^{14}C]uridine-labeled poliovirus 35S RNA. The resuspended RNA was analyzed on a sucrose gradient containing 99% Me_2SO as described in the text and modified to include a 7.5 to 20% gradient and 18 h of centrifugation. The ^{14}C radioactivity of half of each fraction was determined directly, while the other half was used in hybridization with R-FeLV [^{3}H]DNA, under conditions of RNA excess and time sufficient to saturate the input DNA. (D) Relative amounts of 35S and 28S viral RNA. R-FeLV [^{3}H]DNA was hybridized to viral RNA from the pooled center three fractions of 28S and 35S peaks of a gradient similar to that described in panel (C). The amount of DNA hybridized is plotted as a log function of the time of hybridization.*

FeLV cDNA was hybridized to equal volumes of each RNA, which was pooled from center fractions of the two symmetrical peaks from the denaturing gradients (Fig. 9C) to minimize cross-contamination. The amount of DNA hybridized is plotted as a log function of the time of hybridization (Fig. 9D). By comparison of the $t_{1/2}$ for each size of RNA, it was determined that 4% or

less of the virion RNA subunits were 35S. The value obtained can only be taken as an upper limit because, despite the precaution mentioned above, any contamination of the 35S peak by the leading edge of the 28S peak would result in an artificially high estimate.

DISCUSSION

In this study we have used complementary single-stranded viral cDNA to study cytoplasmic FeLV-specific RNA. The major qualifications of this approach concern the uniformity and completeness of the transcript. The majority of the native DNA transcript migrates as material remaining bound to the high-molecular-weight RNA template (Fig. 1). This result has been noted by others and is assumed to represent a faithful transcript of the template (21). Most of the denatured product migrates as small molecules (Fig. 1B). The results of the template protection experiment (Fig. 2) suggest that not all R-FeLV RNA sequences are present in the DNA probe. DNA probes of this quality can be used in analyzing intracellular RNA (13), but it should be emphasized that certain intracellular virus-specific RNA sequences may not be detected.

Recently, larger and more uniform transcripts of oncornavirus RNA have been obtained (8, 26, 45). Even though the conditions of synthesis used here are similar to those described by Rothenberg and Baltimore (45), high uniformity and complete transcription were not obtained. Since this work was completed, published results suggest that more uniform transcription occurs under conditions of restricted magnesium ion (46).

The value of approximately 2% R-FeLV-specific RNA in unfractionated F-422 cytoplasmic extracts (Table 1) was higher than the values of 0.2 to 1.0% most commonly reported for Moloney murine leukemia virus (M-MuLV)- or Rous sarcoma virus (RSV)-infected cells (3, 13, 14, 22, 29, 49, 62). Similarly, the estimate of 2.6% R-FeLV-specific polyribosomal RNA (Table 1) was significantly higher than the levels of 0.05 to 0.58% reported for Rauscher murine leukemia virus (R-MuLV) (17) and M-MuLV (14, 62) polyribosomal RNA. The F-422 line is one of the higher oncornavirus-producing cell lines, and the higher levels of R-FeLV-specific RNA may be related to the apparently high viral synthetic rate (20). Because of these higher levels of virus-specific RNA, the FeLV system used in this study offers an important advantage as sources of intracellular RNAs for subsequent analysis (Thomason et al., submitted for publication).

Because of the appearance of virus-specific RNA in four specific areas of the Me_2SO gradients, we will use the average RNA size designations of 36S, 28S, 23S, and 15 to 18S in subsequent discussion of intracellular R-FeLV-specific RNA. Since the FeLV system has an additional 28S RNA that differs from that in the murine and avian systems, the resultant crowding of the Me_2SO gradient makes the simultaneous resolution of all four peaks difficult in some cases. However, when all the information now available concerning intracellular R-FeLV-specific RNA is considered, the existence of four RNA species is clear. The presence of a 28S cytoplasmic RNA (Fig. 4A, C) similar in size to the major virion RNA subunit (7; Fig. 9B) is expected. Furthermore, the clear demonstration of the largest RNA as a minor subunit of the virion RNA on a shallower gradient (Fig. 9) again suggests that a similar-sized species should be found in the cell. The apparent absence of the 28S RNA from the polyribosome fractions (Fig. 4B, D), although interesting in itself, is of additional value because it permits a clearer resolution of the 36S as well as the 23S and 15 to 18S species. We have since achieved better separation of the four peaks in longer aqueous low-salt preparative gradients (Thomason et al., submitted). The evidence that the 23S and 15 to 18S species are not degradation products of the larger RNA is quite clear. First, under the RNA extraction conditions used, added virion 28S RNA was not degraded at all in control experiments addressing this point (data not shown). Second, we now have evidence (Thomason et al., submitted) that (i) the two smaller RNA species have capped 5′ termini; (ii) the cap-to-nucleotide ratio is close to what would be expected for 23S and 15 to 18S molecules; and (iii) the penultimate bases of the cap structures are different for each RNA, thereby ruling out degradation as a factor in the appearance of these RNAs.

The presence of smaller-than-genomic-subunit-size RNA in unfractionated cytoplasm has been reported in M-MuLV-infected cells (13, 58, 59), RSV-infected cells (29, 49), and RD-114 cells (36). Although the exact sizes of these smaller cytoplasmic RNA molecules vary among the virus systems studied, their presence as two separate size classes appears to be a general phenomenon, which, as a result of this study, is extended to include FeLV 23S and 15 to 18S cytoplasmic RNAs.

The detection of three distinct R-FeLV-specific RNA species (36S, 23S, and 15 to 18S) on F-422 polyribosomes (Fig. 4B, D) is in part similar to the findings by Gielkens et al. (17) of 36S, 21S, and 14S R-MuLV-specific RNA on polyribosomes. They further showed that 21S and 36S RNAs were the major poly(A)-containing intracellular species (18). The one important

difference is that the 36S R-MuLV polyribosomal RNA is the same size as R-MuLV virion subunit RNA, whereas the R-FeLV 36S polyribosomal species is not the same size as the R-FeLV 28S major virion subunit RNA, as will be discussed later.

The very low level or possible absence of 28S intracellular RNA on polyribosomes (Fig. 4B, D) may reflect the lack of a 5'-terminal cap structure, as is the case for virion 28S RNA (57). There is now evidence that the rate of binding of mRNA to ribosomes at potassium ion concentrations close to those found in intact cells is strongly influenced by the m^7G group (67). Consistent with this interpretation is our observation that the 23S and 15 to 18S RNAs that are found on purified polyribosomes (Fig. 4B, D) both have 5'-terminal cap structures (Thomason et al., submitted). Although it is reasonable to assume that 36S RNA that is bound to polyribosomes (Fig. 4 and 8) will also be capped and that intracellular 28S RNA will exhibit the same unique lack of a cap structure as the virion 28S RNA, additional experiments are needed to resolve this question.

Antibody preparations have been successfully used as probes for nascent antigenic determinants in a variety of eukaryotic systems to study the biosynthesis of specific proteins (6, 39, 40, 48, 56). 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-labeled anti-FeLV IgG binding to nascent virus-specific proteins on F-422 cell polyribosomes (Fig. 5–7). Vecchio et al. (62) used immunoprecipitation of labeled polyribosomes with unlabeled virus-specific antiserum, which detected nascent M-MuLV proteins primarily in an approximately 350S region. Although we observed antibody binding through a broad range of polyribosome sizes, the region of highest antibody binding containing nascent R-FeLV-specific protein was approximately 400S. This agrees closely with the size of polyribosomes containing M-MuLV-specific nascent protein.

By hybridization with R-FeLV [^3H]cDNA, two major regions of the polyribosome gradients were shown to contain virus-specific RNA (Fig. 5B), which was subjected to size analysis (Fig. 8). The fastest-sedimenting 400 to 450S region correlated well with the peak binding of the anti-FeLV IgG. However, anti-FeLV IgG binding occurred over a broad range of the polyribosome gradient area, including the 250S polyribosomal area, which contained a peak of R-FeLV-specific RNA (Fig. 5B). From these data, it is not possible to determine whether just one or both polyribosomal size classes detected by hybridization contain the major functional R-FeLV-specific mRNA. The combined hybridization and immunological analyses do suggest that the RNA detected with the R-FeLV cDNA is virus-specific mRNA, since both virus-specific nascent protein and EDTA-releasable RNA were present in the polyribosomal size classes observed. There was an obvious lack of detectable virus-specific RNA between the 250S and 400 to 450S regions of the polyribosome gradient (Fig. 5A). Since nascent virus-specific antigenic determinants were present in this region (Fig. 5A, 6B, and 7), the lack of detectable RNA may be due to the nonuniformity of the R-FeLV cDNA probe. If there are viral sequences present in the gap region that are not highly represented in the DNA, they may not be detected. The virus-specific RNA in the 400 to 400S region sedimented at 36S, 23S, and 15 to 18S (Fig. 8A), as expected since the RNA from total polyribosomes had the same sedimentation coefficients (Fig. 4B, D). Although the possible absence of a distinct 23S peak of RNA from the 250S polyribosome region (Fig. 8B) is of potential interest, further analysis will be required to show that the 23S RNA is actually absent and not just reduced slightly in these analyses.

Since oncornavirus subunit RNA is the same size and polarity as the largest intracellular species, its messenger activity in cell-free protein synthesis may be considered analogous to that of the largest intracellular species. The RSV 30 to 40S RNA directed the cell-free synthesis of a 75,000- to 80,000-dalton protein similar to the intracellular structural protein precursor (42, 63–65). RNA from murine oncornaviruses directed the cell-free synthesis of viral structural protein precursor size products of 60,000 to 75,000 daltons (17, 28, 35, 47) in addition to extremely large proteins (greater than 160,000 daltons) (28, 35). Further, Mueller-Lantzsch et al. (33, 34) have reported that 35S M-MuLV-specific RNA is the only size of RNA obtained from polyribosomes containing M-MuLV p30 nascent determinants. These results suggest that subunit-size RNA can function as mRNA and that the precursors of the low-molecular-weight structural proteins are probably the major synthetic products of this translation.

The smaller-than-subunit-size viral RNA molecules present on polyribosomes of oncornavirus-infected cells (3, 9, 17, 18, 51), including the 23S and 15 to 18S R-FeLV-specific species (Fig. 4B, D; Fig. 8), could serve the important function of permitting the independent biosynthesis of viral gene products that are found in nonequimolar quantities. Consistent with this suggestion are the observations that the 24S intracellular

virus-specific RNA contains RSV "sarc" (3, 9) and avian oncornavirus "envelope" gene sequences (23), respectively. There is now direct evidence that R-MuLV and avian oncornavirus 21 to 24S intracellular RNA codes for the envelope glycoprotein as shown by translation either in cell-free systems (41) or by microinjection into oocytes (63) or into fibroblasts transformed by envelope-defective RSV (53) and identification of the product by immunoprecipitation or detection of infectious virus, respectively. Although the R-MuLV 14S RNA has been translated, identity of the products is not clear at this time (63).

The results presented here and other preliminary data lead to the following speculation about R-FeLV RNA metabolism in F-422 cells and the nature of the virion RNAs. R-FeLV from F-422 cells is 2.5 logs less infectious than virus from the original R-FeLV isolate passed in feline fibroblasts (A. Haberman and L. F. Velicer, unpublished data). Since the major virion 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. This notion is supported by the preliminary evidence (Fig. 9) that R-FeLV 50 to 60S RNA preparations contain a minor 35S subunit of a size similar to that of the largest intracellular species, as well as the major 28S subunit, but would require the yet unproven assumption that the 36S RNA is the full FeLV genome. The alternate idea is that the 28S RNA is the full-length genome RNA that sediments more slowly than MuLV genome RNA due to conformational differences and that the intracellular 36S RNA is actually an uncleaved precursor RNA. Although larger-than-genome RNA has been reported for virus-specific RNA from M-MuLV- (22) and RSV-infected cells (3), there is more recent evidence that a larger precursor MuLV RNA does not exist (12). Also, the largest nuclear species found are not more than 15% larger than the genomic subunit (3). Although our analysis does not permit an exact size determination of the 36S RNA, its molecular weight appears to be 50 to 70% larger than that of the 28S RNA. Furthermore, if 36S FeLV RNA is an uncleaved precursor of 28S RNA, it would have the properties of being detectable under steady-state conditions and present on polyribosomes and in virions, properties not reported for the largest intranuclear RSV RNA (3).

Further analysis of the cell-free translation products of the smaller-than-genomic-subunit-size RNAs found in avian, feline, and murine oncornavirus-infected cells, in comparison with know intracellular precursor proteins and virion proteins, will be needed to completely understand the role of the smaller RNAs in oncornavirus gene expression. In addition, cell-free translation, competition hybridization, and other studies will be required to determine whether the R-FeLV-specific 36S or 28S size species, which are both found intracellularly and in the virion, contain the entire R-FeLV genomic capacity.

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