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Nature of Rous Sarcoma Virus-Specific RNA in Transformed and Revertant Field Vole Cells

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Cytoplasmic and polyribosomal RNAs from Rous sarcoma virus-transformed and phenotypically reverted field vole cells were fractionated by rate-zonal sedimentation and hybridized with a ^{3}H-labeled complementary DNA viral probe to determine the size classes of virus-specific RNA present in these cell types. In contrast to Rous sarcoma virus-infected permissive avian cells, only two of three discrete species of virus-specific RNA were detected in the cytoplasm of these vole cells. These included genome-length 35S RNA and a 21S RNA. However, viral 28S RNA, routinely detected in the cytoplasm of productively infected avian cells, could not be found in cytoplasmic RNA from vole cells. In addition, a low-molecular-weight viral RNA sedimenting less than 16S was detected in both infected avian and vole cells. Because of its heterogeneity this latter species is most likely generated from the intracellular degradation of the larger viral RNAs. Both the viral 35S and 21S RNA were also found to be associated with total polyribosomes from these vole cells. Studies were also performed to determine the distribution of both total viral genomic and sarcoma-specific RNA sequences among the size classes of fractionated total polyribosomes. In both vole cell types the majority of cytoplasmic viral RNA sequences were also associated with polyribosomes and were similarly distributed among the size classes of total polyribosomes. Sarcoma-specific sequences were present on both the 35S and 21S RNA species. These data suggest that the expression of the viral transforming gene in revertant field vole cells may be controlled at some stage subsequent to translation of the viral RNA.

Initiation and maintenance of neoplastic transformation in avian sarcoma virus-infected cells are dependent upon the continuous expression of the viral transforming gene designated *src* (1, 16, 23, 31). The mechanism(s) by which the genetic expression of this viral gene is regulated has been the subject of several recent investigations (3, 9, 10, 18, 20), but for the most part remains unknown. In one report a considerable reduction in the amount of sarcoma-specific RNA was observed in reverted avian sarcoma virus-infected hamster cells compared to transformed cells (10). It was concluded that the expression of the viral transforming gene is regulated, at least in part, at the transcriptional level. In contrast to these studies, we have recently obtained evidence suggesting that the genetic expression of the viral transforming gene might also be controlled at some stage subsequent to the transcription of viral sarcoma-specific RNA (18). This conclusion was derived from a comparative analysis of the transcriptional expression of the Rous sarcoma virus (RSV) genome in transformed field vole (*Microtus agrestis*) cells and their phenotypically reverted subclones. To date, we have shown that the entire biologically functional viral genome (one to two viral equivalents per diploid cell genome) is present in revertant and transformed vole cells (8). Furthermore, these sequences are at least transcriptionally active, since similar concentrations of virus-specific RNA can be found in both cell types (20). More importantly, we have not been able to detect any significant differences in either the extent or the amount of transcription of virus-transforming gene sequences in revertant and transformed vole cells, suggesting that the mechanism of viral sarcoma gene regulation must be occurring at some level beyond that of transcription (18). Moreover, neither transport nor polyadenylation is responsible for reversion, since no detectable differences in either the subcellular distribution of these RNA sequences or their content of polyadenylic acid can be found in these cells (18, 20). However, it is nevertheless conceivable that the reversion phenomenon is a reflection of either differences in the structure of viral RNAs or their ability to

associate with polyribosome and participate as mRNA.

In an effort to distinguish between these possibilities and ultimately elucidate the mechanism of reversion, we have continued our analysis of the nature of RSV-specific RNA in these revertant and transformed vole cells. Further impetus for these studies was provided by the recent characterization of several species of virus-specific RNA in productively infected avian cells (6, 15, 31). Three predominant species have been identified: a genome-length 35 to 39S RNA, which contains all four of the known viral genes (*gag*, core structure proteins; *pol*, reverse transcriptase; *env*, envelope glycoproteins; and *src*, required for neoplastic transformation), and two smaller viral RNAs sedimenting at 28S and 21S. The 28S RNA encodes for the *env* and *src* genes, whereas only the *src* gene sequences are present on the 21S RNA. Since the *gag* and *pol* gene sequences are present only on genome-length RNA, it appears most likely that this viral RNA functions as mRNA for the translation of these viral genes while the 28S and 21S species serve as mRNA for the *env* and *src* genes, respectively (7, 24–26, 31).

In this study we have examined the size of RNA present in the cytoplasm and associated with polyribosomes from transformed and revertant vole cells that contain viral genomic and sarcoma-specific gene sequences. These studies were performed by fractionating cytoplasmic and polyribosomal RNAs by rate-zonal centrifugation and determining the profiles of virus-specific RNA by hybridizing gradient fractions with either a viral ^{3}H-labeled complementary DNA (cDNA) probe or a probe specific for the viral transforming gene sequences ($cDNA_{src}$). To further characterize the association of these virus-specific RNA sequences with polyribosomes, their distribution among the size classes of total polyribosomes was also investigated.

MATERIALS AND METHODS

Reagents, cells, and virus. The sources and preparation of most of the pertinent materials have been previously described (13, 17, 20). Various strains of RSV (B77, Prague, subgroup C; Schmidt-Ruppin, subgroup D) were either propagated in avian embryo fibroblasts in our laboratory and purified as previously described (2, 12), or obtained through the Office of Logistics and Resources, National Cancer Institute. Virus obtained from the latter source was banded several times in 25 to 55% sucrose before use. The infection of an established cell line from the European field vole, *M. agrestis*, with Schmidt-Ruppin strain of RSV and the isolation of transformed and revertant clones were as previously described (18). Compared to the transformed clones, the revertant clones closely resembled uninfected vole cells in many biological and biochemical properties. These include morphology, ordered pattern of growth, efficiency of plating, basal level of cyclic AMP, the degree of stimulation of adenylate cyclase with (-)isoproterenol, the amount of fibronectin, and the organization of microfilaments comprising the cytoskeleton (A. Lau et al., manuscript in preparation). Field vole cells were grown in Eagle modified minimum essential medium supplemented with 10% calf serum, 10% tryptose phosphate broth, 0.12% sodium bicarbonate, 2 mM glutamine, 20 U of penicillin per ml, and 20 μg of streptomycin per ml.

Purification of radiolabeled viral RNA. ^{32}P-labeled RSV (B77 strain) was prepared by labeling freshly transformed cultures of avian embryo fibroblasts with 1 mCi of carrier-free [^{32}P]orthophosphate (New England Nuclear Corp.) per ml in phosphate-free medium containing dialyzed calf serum. RNA was then extracted from purified virus with Pronase–sodium dodecyl sulfate (SDS)–phenol and subsequently fractionated into 70S RNA and free, low-molecular-weight RNA by rate-zonal sedimentation (13). 35S RNA, free of any 70S-associated low-molecular-weight RNA, was prepared as described (13).

Preparation of cytoplasmic RNA from avian and field vole cells. Cells used as a source of cytoplasmic RNA were harvested from roller bottles by scraping and then washed three times with phosphate-buffered saline and once with RSB buffer (0.01 M Tris-hydrochloride [pH 8.3]–0.01 M NaCl–0.0015 M $MgCl_2$). The cell pellets were suspended in RSB buffer containing 5 mM *N*-ethylmaleimide at approximately 5×10^7 cells per ml, incubated at 4°C for 10 min, and then disrupted by 20 strokes of a Dounce homogenizer. The nuclei and cell debris were removed by successive centrifugations at $700 \times g$ for 10 min and $10,000 \times g$ for 5 min. The cytoplasmic fraction was adjusted to 0.05 M sodium acetate (pH 5.1), 0.01 M EDTA, and 1% SDS, and then extracted three times at 56°C with an equal volume of phenol saturated with 0.05 M sodium acetate (pH 5.1) and 0.01 M EDTA. The cytoplasmic RNA was precipitated twice in the presence of 0.2 M sodium acetate with 2 volumes of ethanol, collected by centrifugation at $13,000 \times g$ for 30 min, and suspended in TE buffer (0.01 M Tris-hydrochloride [pH 7.4]–0.01 M EDTA). The extent of nuclear breakage and leakage of RNA into cytoplasmic fractions during the fractionation procedure was determined by previously published procedures (30). This entailed pulse-labeling cells for short periods of time (45 min) and analyzing the pulse-labeled nuclear and cytoplasmic RNA by polyacrylamide gel electrophoresis (data not shown). Under these conditions of labeling, the predominantly labeled RNA species is the 45S RNA precursor of 28S and 18S ribosomal RNA. Less than 1% of this species could be detected in the cytoplasm after fractionation.

Preparation of polyribosome-associated RNA from transformed and revertant field vole cells. Total polyribosome-associated RNA was isolated as described by Gielkins et al. (14) from cells grown in roller bottles. To maximize yields of polyribosomes, medium was changed 2 to 3 h before harvest and cycloheximide was added to all buffers used in har-

vesting and disruption of cells. Cells were scraped, washed three times with phosphate-buffered saline containing cycloheximide (200 μg/ml), and disrupted by suspension in buffer containing 0.05 M Tris-hydrochloride (pH 8.5), 0.225 M KCl, 0.008 M $MgCl_2$, 0.002 M dithiothreitol, and 2% Nonidet P-40 with 50 U of heparin and 200 μg of cycloheximide per ml. Nuclei and cell debris were removed by centrifugation, and the 13,000 × *g* postnuclear supernatant was layered onto a discontinuous sucrose gradient consisting of 8 ml of 1 M sucrose and 4 ml of 2 M sucrose in TMKH buffer (0.05 M Tris-hydrochloride [pH 7.8]–0.005 M $MgCl_2$–0.080 M KCl with 10 U of heparin per ml). Polyribosomes were pelleted in an SW27 rotor at 25,000 rpm for 21 h at 4°C, suspended in buffer containing 0.05 M sodium acetate (pH 5.1), 0.010 M EDTA, and 1% SDS, and then extracted with phenol as described above. In control experiments, treatment of the postnuclear supernatant with 0.03 M EDTA for 15 min at room temperature before centrifugation resulted in the release of 90% of the virus-specific RNA sequences from cellular polyribosomes.

Fractionation of total polyribosomes by rate-zonal sedimentation was performed by layering 2.5 to 3.0 ml of the postnuclear supernatant material onto a 35-ml 10 to 40% (wt/vol) sucrose gradient in TMKH buffer. Gradients were centrifuged in an SW27 rotor at 25,000 rpm for 140 min at 4°C, fractions were collected, and the optical density at 254 nm was determined by using a recording spectrophotometer. Gradient fractions were then pooled as indicated in Fig. 3, polyribosomes were precipitated with ethanol, and RNA was extracted with phenol as described above. The final RNA preparations were suspended in 200 μl of TE buffer.

Rate-zonal sedimentation analysis of cytoplasmic and polyribosome-associated RNA from avian and field vole cells. The conditions employed for rate-zonal centrifugation were essentially those described by McKnight and Schimke (22). Approximately 100 to 200 μg of RNA from avian and field vole cells in 300 μl of TE buffer containing 1% SDS was heat denatured by incubation at 65°C for 10 min and then cooled rapidly to 4°C. The RNA was layered directly onto a 15 to 30% (wt/vol) linear sucrose gradient made in a buffer containing 0.01 M Tris-hydrochloride (pH 7.4), 0.005 M EDTA, and 1% SDS, and centrifuged in an SW41 rotor at 25,000 rpm for 16 h at 25°C. The gradients were collected from the bottom, and RNA in each fraction was precipitated in the presence of 0.2 M sodium acetate and 100 μg of yeast tRNA with 2 volumes of ethanol, collected by centrifugation, and suspended in 60 μl of TE buffer. A constant volume of each fraction (10 to 25 μl) was dried down in a vacuum desiccator and hybridized with radiolabeled viral cDNA as described below.

Synthesis of viral cDNA probes. Conditions for the synthesis of virus-specific DNA in vitro with detergent-activated RSV (B77 strain) were described by Stehelin et al. (29). The specific activity of the [^{3}H]cDNA probe prepared under these conditions was approximately 2.4 × 10^7 cpm/μg. In addition, a representative [^{3}H]cDNA probe ($cDNA_{rep}$) that protected at least 70% of radiolabeled RSV 70S RNA from RNase hydrolysis at DNA/RNA ratios of 4:1 was prepared as previously described (20). The [^{3}H]cDNA probe specific for the transforming gene sequences ($cDNA_{src}$) of RSV (Prague C strain) was also prepared as described (18). The transformation-specific nature of this probe was confirmed by demonstrating that $cDNA_{src}$ hybridizes to transforming sarcoma virus RNA but not to nontransforming leukosis virus RNA (19).

Hybridization analysis. Hybridization of virus-specific [^{3}H]cDNA with cellular RNA from avian and field vole cells was performed in 10-μl reaction mixtures containing 0.01 M Tris-hydrochloride (pH 7.4), 0.6 M NaCl, 0.001 M EDTA, single-strand [^{3}H]cDNA (1,500 cpm, 0.06 ng), 1.0 mg of yeast tRNA per ml, and the indicated amount of RNA. Reaction mixtures were sealed in 30-μl microcaps and incubated for 24 to 70 h at 68°C. The fraction of cDNA hybridized was determined by treatment of samples with single-strand specific S1 nuclease (*Aspergillus oryzae*) (21) in buffer containing 0.03 M sodium acetate (pH 4.5), 0.3 M NaCl, 0.003 M $ZnCl_2$, and 10 μg of denatured calf thymus DNA. In some instances results of hybridization were expressed as a function of C_rt (concentration of RNA in moles per liter × time of incubation in seconds) (21).

RESULTS

Size of virus-specific RNA present in the cytoplasm of RSV-infected avian and vole cells. To determine the size classes of virus-specific RNA present in the cytoplasm of Schmidt-Ruppin RSV-infected avian cells and revertant and transformed vole cells, cytoplasmic RNA was extracted from confluent cell cultures and fractionated by rate-zonal centrifugation. Virus-specific RNA was identified by annealing each fraction with a constant amount of labeled RSV cDNA, and the percentage of cDNA hybridized was measured. Four species of virus-specific RNA were detected in the cytoplasm of productively infected avian cells (Fig. 1A). These included genome-length 35S RNA and two smaller species of virus-specific RNA sedimenting at 28S and 21S, which have also recently been identified by others (15, 31). In addition, we also observed a viral RNA, sedimenting between 10 and 13S, that is similar in size to the low-molecular-weight viral RNA reported by Brugge et al. (6). The relative amounts of 35S, 21S, and 10 to 13S RNAs were similar to each other but were greater than that observed for the 28S RNA.

Cytoplasmic RNA extracted from two transformed (Fig. 1B and C) and two revertant clones of vole cells (Fig. 1D and E) revealed species of virus-specific RNA similar to that observed in infected avian cells with the exception that no discrete class of viral 28S RNA could be detected. The 21S RNA was generally the predom-

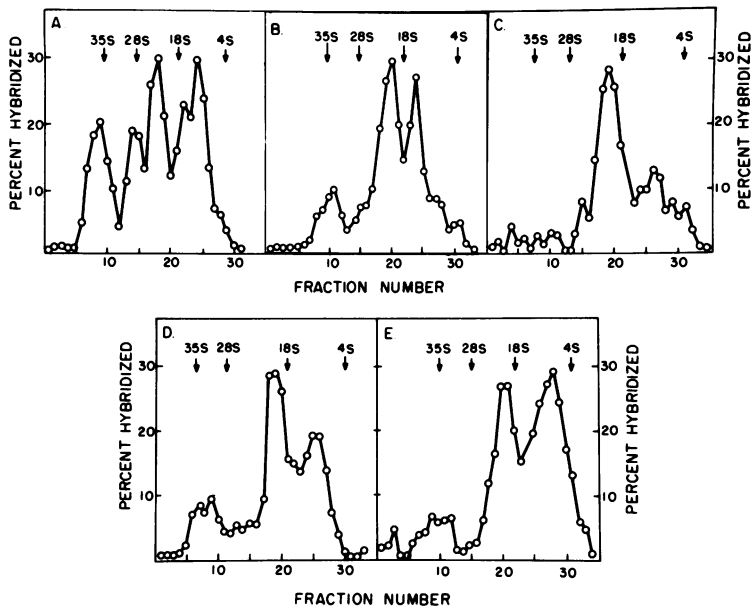

FIG. 1. *Size of virus-specific RNA in the cytoplasm of RSV-infected avian and vole cells. Cytoplasmic RNA was extracted from Schmidt-Ruppin RSV-infected chicken embryo fibroblasts and clones of transformed and revertant vole cells as described in the text. Approximately 100 to 200 μg of heat-denatured cytoplasmic RNA (65°C, 10 min) was centrifuged in a 15 to 30% (wt/vol) linear sucrose gradient containing 0.01 M Tris-hydrochloride (pH 7.4), 0.005 M EDTA, and 1% SDS in an SW41 rotor at 25,000 rpm for 16 h at 25°C. Radiolabeled RSV 35S RNA and field vole 28S and 18S RNAs were sedimented in a parallel gradient to provide markers. Gradients were collected from the bottom, and RNA in each fraction was precipitated with ethanol and suspended in TE buffer. An equal volume from each fraction (10 to 25 μl) was hybridized with an RSV [^{3}H]cDNA probe (1,500 cpm) at 68°C for 24 to 70 h as described in the text. Hybridization was measured by resistance of the [^{3}H]cDNA probe to hydrolysis with S1 nuclease. The percent hybridization was corrected for the intrinsic S1 nuclease resistance of the viral probe (7%). (A) Productively infected chicken embryo fibroblasts; (B) transformed vole clone 1; (C) transformed vole clone 22; (D) revertant vole clone 4; (E) revertant vole clone 866.*

inant size class of viral RNA, whereas the concentration of 35S RNA was either greatly diminished compared to infected avian cells (Fig. 1A) or, in one instance, barely discernible (Fig. 1C). Since we have previously shown that the entire RSV proviral genome is transcribed in all four vole cell clones (20), the inability to detect a discrete viral 35S RNA in transformed clone 22 (Fig. 1C) could possibly result from the substantially lower amount of virus-specific RNA present in this clone compared to the others (20). Nevertheless, in contrast to all other RSV-infected mammalian cells to date (5, 10), the vole cells are unique in that genome-length viral RNA is found in the cytoplasm.

A heterogeneous low-molecular-weight viral RNA was present in varying amounts in all four vole cell clones, with the greatest relative concentration found in revertant clone 866 (Fig. 1E) and the least in transformed clone 22 (Fig. 1C). This RNA was somewhat larger (16S compared to 10 to 13S) and less heterogeneous in transformed clone 1 (Fig. 1B) than that found in other vole cell clones (Fig. 1C, D, and E) and productively infected avian cells (Fig. 1A). A similar 12 to 15S viral RNA has also been identified in RSV-infected hamster cells (10). Since it is conceivable that low-molecular-weight viral RNA represents breakdown of the higher-molecular-weight viral RNA during extraction, we have performed experiments to examine the effect of our isolation procedure on the integrity of viral RNA. Immediately after disruption of either Schmidt-Ruppin RSV-infected avian cells or transformed vole cells by Dounce homogenization, ^{32}P-labeled viral 35S RNA was added to each cell homogenate, which was then subjected to the manipulations performed during isolation of cytoplasmic RNA. Figure 2 shows the analysis of these RNAs by rate-zonal centrifugation. Identical profiles were obtained with both the control RNA, which was not subjected to this treatment, and the RNA samples that were processed by the above procedure. These results suggested that the small viral RNA was not generated during extraction of RNA from cells.

Since substantially lower amounts of this RNA are found on polyribosomes from vole cells (see below, Fig. 4), and because of its heterogenous size distribution, it appears most likely that this RNA is the product of viral RNA degradation that is normally occurring within the cell. However, these results do not rule out the possibility that a portion of this RNA may represent the transcription product from a unique portion of the proviral genome.

Distribution of virus- and sarcoma-specific RNA sequences among the size classes of total polyribosomes. We have previously demonstrated that as much, or slightly more, virus-specific RNA and, more importantly, sarcoma-specific RNA sequences are associated with total cellular polyribosomes of revertant than with transformed vole cells (18, 20). These results suggested that virus-specific RNA present in revertant vole cells had a similar capacity to direct the synthesis of viral proteins, including the product of the viral sarcoma gene. However, since this RNA was isolated from pelleted total polyribosomes, it was not possible to determine if there were any differences in the size of the polyribosomes that contained virus-specific RNA. It was conceivable that virus-specific RNA present in revertant cells is preferentially associated with smaller size classes of polyribosomes than in transformed cells, which would suggest that viral gene expression might be controlled at some stage during the initiation of translation. To investigate this possibility, total cellular polyribosomes from both cell types were fractionated by rate-zonal sedimentation, and RNA was extracted from pooled regions of the gradient. The distribution of total virus- and sarcoma-specific RNA sequences among the size classes of polyribosomes was determined by annealing a constant amount of RNA from each gradient fraction with either a [^{3}H]cDNA viral probe (Fig. 3A) or a [^{3}H]cDNA probe specific for the viral transforming gene sequences (cDNA$_{src}$) (Fig. 3B), respectively. The results indicated that the majority of virus- (Fig. 3A) and sarcoma-specific (Fig. 3B) RNA sequences present in the cytoplasm of revertant and transformed vole cells were associated with all size classes of total polyribosomes. A similar distribution of these sequences among the size classes of polyribosomes was observed for both cell types. Furthermore, the larger polyribosomes contained the bulk of polyribosome-associated total virus- and sarcoma-specific RNA. Addition of EDTA to polyribosome preparations from both vole cell types, prior to sedimentation, resulted in both polyribosome dissociation and the displacement of virus-specific RNA from the polyribosomes (Fig. 3C), a requisite for RNA functioning as mRNA (28).

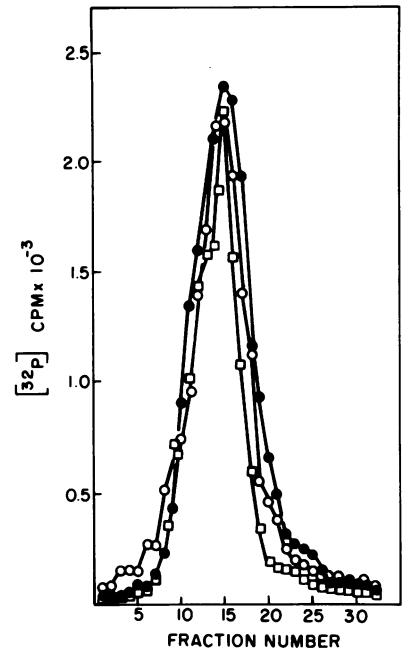

FIG. 2. *Effect of extraction procedure on the integrity of viral RNA. Approximately 50,000 cpm of ^{32}P-labeled RSV 35S RNA was added to suspensions of either productively infected chicken embryo fibroblasts or cells of transformed vole clone 1 immediately after disruption by Dounce homogenization; cells were then subjected to the procedure for the isolation of cytoplasmic RNA as described in the text. Radiolabeled viral RNA processed in this manner was then heat denatured prior to rate-zonal sedimentation, and gradients were collected as described in Fig. 1. To determine the profiles of labeled viral RNA, gradient fractions were precipitated with 10% trichloroacetic acid in the presence of 40 μg of calf thymus DNA, and the precipitates were collected on glass fiber filters and counted in a liquid scintillation spectrometer. As a control, labeled viral RNA that was not exposed to the extraction procedure was sedimented in a parallel gradient. Symbols: (●) control ^{32}P-labeled RSV RNA; (□, ○) ^{32}P-labeled RSV RNA added to disrupted suspensions of productively infected chicken embryo fibroblasts and cells of transformed vole clone 1. respectively.*

Size of virus-specific RNA associated with total polyribosomes from transformed and revertant voles. To determine which size classes of virus-specific RNA present in the cytoplasm of vole cells are associated with total polyribosomes, cytoplasmic extracts from transformed and revertant vole cells were prepared by detergent lysis with Nonidet P-40, and polyribosomes were isolated by pelleting through a discontinuous sucrose gradient. The extracted

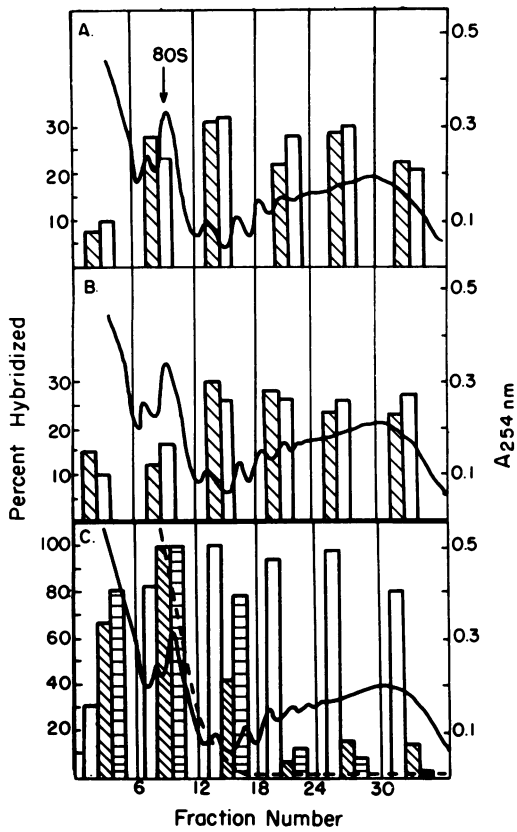

FIG. 3. *Distribution of virus-specific RNA sequences among fractionated total polyribosomes from transformed and revertant vole cells. (A) and (B) Cytoplasmic extracts were prepared from transformed and revertant vole cells by detergent treatment, and total polyribosomes were fractionated by rate-zonal sedimentation in a 10 to 40% (wt/vol) sucrose gradient as described in the text. The fractions were collected, their optical density at 254 nm was determined, and the RNA was pooled as indicated by the vertical lines. After extraction with phenol, an equal concentration of RNA from each pooled region was hybridized with either an RSV [^{3}H]cDNA or a RSV [^{3}H]cDNA$_{src}$ probe at C$_o$t's of 0.7×10^3 and 0.35×10^3, respectively. Hybridization was measured by resistance of the RSV [^{3}H]cDNA probes to S1 nuclease and corrected for the intrinsic S1 nuclease resistance of the viral probes (7 to 10%). (A) RSV [^{3}H]cDNA; (B) RSV [^{3}H]cDNA$_{src}$. Symbols: (——), absorbance at 254 nm; □ and ▩, percent hybridization obtained with RNA from transformed clone 1 and revertant clone 866R, respectively. (C) Cytoplasmic extracts prepared from transformed and revertant vole cells were divided into two equal portions. To one portion EDTA was added to 25 mM, and the preparation was incubated at room temperature for 15 min. Both samples were then subjected to rate-zonal sedimentation and processed as described above. After ethanol precipitation, extracted RNA from each pooled region was suspended in 200 μl of TE buffer, and an equal volume of sample was hybridized with an RSV [^{3}H]cDNA probe. Values for percent hybridization were normalized to 100% (maximum hybridization observed ranged from 35 to 40%). Symbols: (- ——) and (- - -), absorbance at 254 nm of untreated and EDTA-treated preparations, respectively; ▤ and ▩, percent hybridization obtained with EDTA-treated preparations from revertant vole clone 866 and transformed vole clone 1, respectively; □, percent hybridization obtained with untreated preparations from either transformed vole clone 1 or revertant vole clone 866.*

polyribosomal RNA was then fractionated by rate-zonal sedimentation, and profiles of virus-specific RNA were determined by hybridizing each fraction with either a [^{3}H]cDNA viral probe (Fig. 4A and C) or a [^{3}H]cDNA$_{src}$ probe (Fig. 4B and D). Hybridizations performed with the [^{3}H]cDNA$_{src}$ probe indicated that both the viral 21S and 35S RNAs were associated with total polyribosomal RNA from transformed (Fig. 4A) and revertant vole clones (Fig. 4C). As observed for cytoplasmic RNA extracted from these vole cell types (Fig. 1), the predominant species of virus-specific RNA associated with polyribosomes was the 21S RNA. The relative concentration of these viral RNAs was similar in both cell types. Hybridization analysis performed with [^{3}H]cDNA$_{src}$ revealed that viral sarcoma-specific RNA sequences were present on both 35S and 21S RNAs. The relative concentration of the low-molecular-weight virus-specific RNA that comprised a large portion of viral cytoplasmic RNA (Fig. 1) was greatly reduced in preparations of polyribosomal RNA.

Analysis of virus-specific RNA present in retransformed revertant vole cells. We have recently been able to obtain spontaneous retransformants of revertant vole cells. This was accomplished by maintaining the revertant vole clones at high saturation densities for extended periods of time, which allowed the selection of a population of cells that now exhibited the morphological and growth features of the transformed vole cells. Since we have previously demonstrated that reversion of transformed vole cells to the normal cell phenotype is not accompanied by any significant differences in either the extent and amount of transcription of the viral genome or the association of these RNA sequences with polyribosomes (18, 20), it was of interest to examine whether any differences in these properties could be detected between revertant vole cells and their derived retransformed subclones. To quantitate the amount of total virus- and sarcoma-specific RNA sequences that are present in the cytoplasm and associated with total polyribosomes from revertant and retransformed vole cells, cytoplasmic

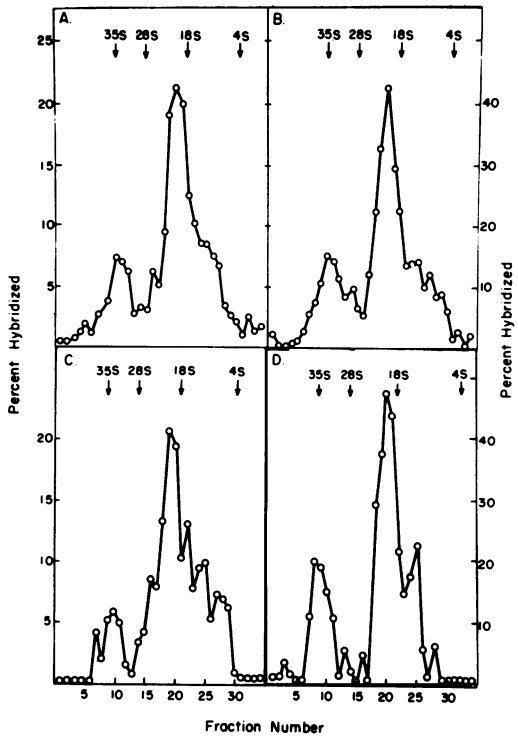

FIG. 4. *Size of virus-specific RNA associated with cellular polyribosomes of transformed and revertant vole cells. Polyribosome-associated RNA was extracted from transformed and revertant vole cells as described in the text. Approximately 200 μg of heat-denatured RNA was fractionated by rate-zonal sedimentation as described in Fig. 1. Samples of 15 μl from each gradient fraction of transformed and revertant cells were hybridized with 1,500 cpm of either an RSV [^{3}H]cDNA probe (A and C) or an RSV [^{3}H]cDNA$_{src}$ probe (B and D) at 68°C for 70 h as described in the text. Hybridization was measured by resistance of the [^{3}H]cDNA probes to hydrolysis with S1 nuclease and corrected for the intrinsic S1 nuclease resistance of the viral probes (7 to 10%). (A) and (B), transformed vole clone 1; (C) and (D), revertant vole clone 866.*

and polyribosomal RNAs were hybridized with either a [^{3}H]cDNA$_{rep}$ probe or a [^{3}H]cDNA$_{src}$ probe, and the kinetics of hybridization were determined. Both the rates and extents of hybridization of the [^{3}H]cDNA$_{rep}$ probe were identical for the corresponding fractions of cytoplasmic (Fig. 5A) and polyribosomal RNAs (Fig. 5C) prepared from these vole cell types. Moreover, when cytoplasmic (Fig. 5B) and polyribosomal RNAs (Fig. 5D) were hybridized with the [^{3}H]-cDNA$_{src}$ probe, identical rates and extents of hybridization were also obtained. Similar results were observed when these analyses were performed with RNAs extracted from a retransformed subclone of revertant clone 4 and also another retransformed subclone of revertant clone 866 (data not shown). Furthermore, the same size classes of virus-specific RNA were detected in the cytoplasm and associated with total polyribosomes from both cell types (compare Fig. 1E and 4C with Fig. 6A and B). Thus, it appears that neither reversion of transformed vole cells to the normal cell phenotype nor retransformation of revertant vole cells is accompanied by any significant changes in either the amount and extent of viral genome transcription or the size of the transcribed RNAs and their association with cellular polyribosomes.

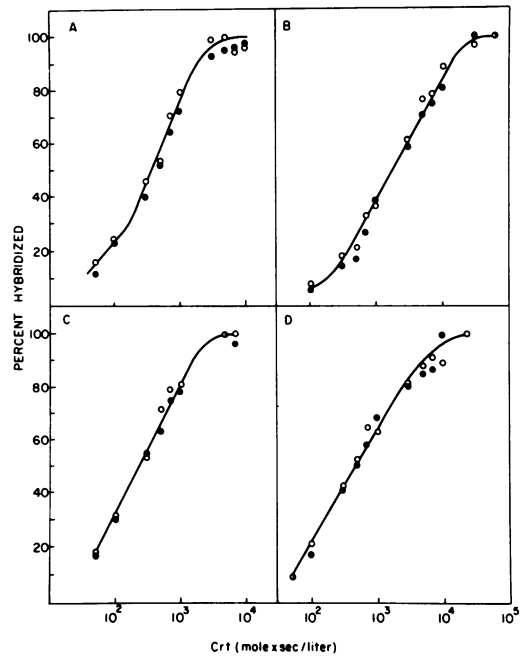

FIG. 5. *Quantitation of virus-specific RNA sequences present in the cytoplasm and associated with polyribosomes of a revertant vole clone and a derived retransformed subclone. Cytoplasmic and polyribosome-associated RNAs were prepared from revertant vole clone 866 and retransformed subclone 866-1 as described in the text and hybridized with 1,500 cpm of either [^{3}H]cDNA$_{rep}$ (A and C) or [^{3}H]cDNA$_{src}$ (B and D). The fraction of [^{3}H]cDNA hybridized is presented as a function of C$_o$t (concentration of RNA in moles per liter × time of incubation in seconds) (22). Values for percent hybridized were corrected for the intrinsic S1 nuclease resistance of the viral probes (7 to 10%) and were then normalized to 100% (actual hybridization averaged 80 to 85% and 65 to 70% for the viral [^{3}H]cDNA$_{rep}$ and [^{3}H]cDNA$_{src}$ probes, respectively). (A) and (B) cytoplasmic RNA; (B) and (D) polyribosome-associated RNA. Symbols: (○) revertant vole clone 866; (●) retransformed vole subclone 866-1.*

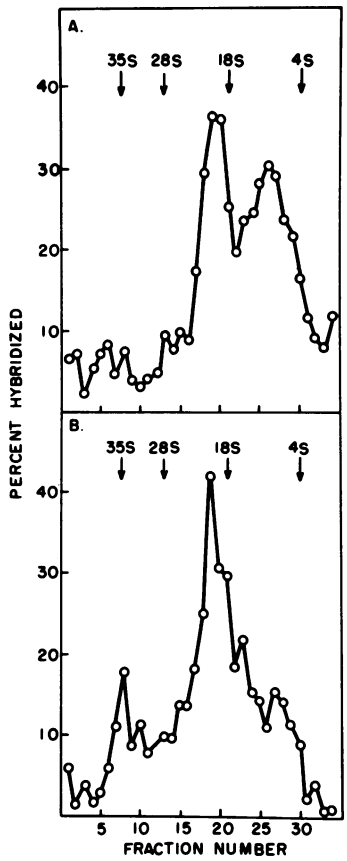

FIG. 6. *Size of virus-specific RNA present in the cytoplasm and associated with polyribosomes of retransformed vole cells. Cytoplasmic and polyribosome-associated RNAs were extracted from retransformed vole clone 866-1 as described in the text. Approximately 200 μg of each RNA preparation was heat denatured and resolved by rate-zonal sedimentation as described in Fig. 1. Samples of 15 μl were removed from each gradient fraction and hybridized with an RSV [^{3}H]cDNA probe at 68°C for 70 h as described in the text. (A) Cytoplasmic RNA; (B) polyribosome-associated RNA.*

DISCUSSION

We have previously reported that reversion of transformed vole cells to the normal cell phenotype cannot be attributed to differences in the transcriptional expression of the RSV proviral genome and, more importantly, the viral transforming gene sequences, since the genetic complexity and concentration of these RNA sequences are similar in both transformed and revertant vole cells (18, 20). In both vole cell types, equivalent amounts of these viral RNA sequences are also polyadenylated and transported to the cytoplasm (18, 20). Moreover, retransformation of revertant vole cells is not accompanied by an increase in viral gene transcription. Collectively these data suggest that the expression of the viral transforming gene is regulated in vole cells at some level beyond that of transcription. One likely possibility would be at the level of viral RNA processing, whereby differences in the size and/or structure of viral RNA might be revealed. In an effort to shed light on this issue the present study was undertaken to analyze the structure of viral RNA present in transformed and revertant vole cells. The data presented in this communication suggest that both the transcription of the proviral genome and the processing of viral RNA in revertant and transformed vole cells occur by a similar mechanism, since identical species of cytoplasmic RNA can be identified in both vole cell types. The largest viral RNA species is genome length in size (35S RNA) and is generally found in similar concentrations in both vole cell types. Although the 35S RNA is present in the lowest amount of the viral RNA species detected in vole cells, its presence nevertheless constitutes the first direct demonstration of genome-length viral RNA in the cytoplasm of RSV-infected mammalian cells. In similar studies performed with RSV-infected hamster cells, 35S RNA was either absent or confined to the nucleus (5, 10). However, indirect evidence for cytoplasmic 35S RNA was provided by the detection of *gag* gene-coded precursor polypeptides in RSV-infected mammalian cells (11, 27). The 35S RNA species has been shown to be responsible for the *gag-pol* gene precursor protein products in cell-free translation systems in vitro (25, 26).

A 21S RNA containing sarcoma gene sequences has been detected in the cytoplasm of both revertant and transformed vole cells, suggesting that the viral transforming gene sequences are present on RNA species similar to that identified in permissively infected avian cells (15, 31). In most cases, the 21S RNA is the predominant species of viral RNA identified in vole cells. The detection of 21S and 35S RNAs associated with polyribosomes and their release from polyribosomes with EDTA suggests that these viral RNAs are functioning as mRNA in both vole cell types. Furthermore, since no significant differences were observed for either the qualitative or quantitative distribution of viral RNA sequences corresponding to these viral RNAs among the size classes of total polyribosomes, the functional capacity of these viral RNAs as mRNA appears to be identical in these vole cells. Preliminary radioimmunoassay studies of RSV structural proteins in transformed

and revertant vole cells substantiate this supposition for at least 35S RNA (A. Lau, R. A. Krzyzek, and A. J. Faras, unpublished data). Thus, these data are consistent with the control of viral gene expression in revertant vole cells at some stage subsequent to the translation of viral RNA.

This may also be true for the sarcoma gene sequences if they also are translated in the revertant vole cells. It is conceivable that reversion could result from an alteration in a host cell factor(s) which either can no longer participate in some essential post-translation modification (phosphorylation, methylation, glycosylation, etc.) of the viral sarcoma gene protein or, alternatively, cannot interact with this protein to induce cellular transformation. However, it is also possible that slight alterations either in the structure (methylation, capping, polyadenylation, splicing, etc.) or the sequence organization of 21S RNA, which would not appreciably affect the size of RNA but may affect its translation, is responsible for phenotypic reversion. Experiments are currently in progress to distinguish between these possibilities by directly determining the translational capacity of 21S RNA in transformed and revertant vole cells by quantitating the amount of viral sarcoma gene protein (4, 26) present in these cells.

In addition to the 21S and 35S RNAs, a heterogeneous low-molecular-weight viral RNA generally sedimenting between 10S and 13S is present in varying amounts of the cytoplasm of both infected avian and vole cells. Low-molecular-weight viral RNAs similar to the 10S to 13S species identified in vole cells have been reported in some studies of RSV-infected avian (6) and hamster cells (10) but not in others (5, 15, 31). The significance of this viral RNA species is presently unknown. Since we have shown that this viral RNA appears not to be generated by breakdown of larger viral RNA during extraction, and that its relative concentration is greatly reduced in polyribosomal RNA from vole cells, it may represent the products of viral RNA degradation or processing that is normally occurring within the cell.

In contrast to vole cells, infected avian cells also contain a cytoplasmic 28S RNA, which contains the genomic sequences of the *env* and *src* genes but apparently only functions as mRNA for the translation of the *env* gene (15, 31). The inability to detect 28S viral RNA both in the cytoplasm and nucleus of vole cells (R. Krzyzek, unpublished data) is not unique to this cell type but appears to be characteristic of all RSV-infected mammalian cells examined to date (5, 10). Apparently, either this viral RNA is not transcribed in detectable amounts or it is either rapidly degraded or abnormally processed in nonpermissive cells. We have previously reported evidence for the transposition of 5′ terminal genomic sequences onto the viral 28S and 21S RNAs in infected avian cells (19). Since we also observe these sequences on 21S RNA in vole cells (R. Krzyzek, unpublished data), it is conceivable that the processing of 28S RNA requires additional factors not present in mammalian cells. The restriction in the synthesis and processing of viral RNA is even more severe in other RSV-infected mammalian cells, since, unlike vole cells which in most cases contain cytoplasmic 35S RNA, this viral RNA is either absent or present only in the nucleus in other mammalian cells (5, 10). Of all the viral RNAs identified in infected avian cells, 21S RNA is the only viral RNA consistently detected in mammalian cells (5, 10). However the nonpermissiveness of RSV-infected mammalian cells is related not only to the synthesis of viral RNA but also to the improper processing of viral proteins (11, 27).

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ADDENDUM IN PROOF

Recent data from our laboratory employing immunoprecipitation and SDS-polyacrylamide gel electrophoresis demonstrate the presence of the *src* gene product ($pp60^{src}$) in both transformed and revertant vole cells, indicating that the 21S RNA representing the *src* gene sequences is being translated in both cell types.

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