JOURNAL OF VIROLOGY, Feb. 1978, p. 518-526
0022-538X/78/0025-0518$02.00/0

Vol. 25, No. 2
Printed in U.S.A.

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

RICHARD A. KRZYZEK,[1] ALAN F. LAU,[1] PETER K. VOGT,[2] AND ANTHONY J. FARAS[1]*

Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455,[1] and Department of Microbiology, University of Southern California Medical School, Los Angeles, California 90033[2]

Received for publication 29 August 1977

Hybridization analysis of RNA from transformed clones of Rous sarcoma virus (RSV)-infected field vole cells and revertant subclones indicated the presence of similar amounts of viral-specific RNA in both cell types. Employing both a relatively uniform and representative complementary DNA probe and genome-length complementary DNA, we have demonstrated that the majority of RSV proviral DNA is transcribed into viral-specific RNA in both transformed and revertant clones. The viral-specific RNA is present in several size classes, the largest of which is genome-length 35S RNA. Studies on the intracellular distribution of viral-specific RNA indicate that both transformed and revertant cells contain viral RNA in their cytoplasm. Furthermore, the bulk of viral-specific nucleotide sequences are also associated with polyribosomes in both cell types. These data indicate that, contrary to previously reported studies with other retrovirus-revertant cell systems, the entire RSV provirus DNA is transcribed into viral RNA similarly in both transformed and revertant vole cells. Since we have previously demonstrated similarities in sarcoma-specific viral RNA in both revertant and transformed vole cells, these data collectively indicate that the loss of the transformed phenotype does not reflect changes in transcription of all or part of the RSV provirus, or the processing, transport, or polyribosome association of viral-specific RNA representing the entire RSV genome.

Transformation of mammalian cells by RNA tumor viruses in vitro leads to the establishment of stably transformed cell lines. In some instances, clones of these transformed cell lines have reverted at relatively high frequencies to the morphologically normal phenotype (3, 11, 20). Several laboratories have initiated studies on the mechanism(s) by which the genetic expression of the viral genome is regulated in revertant cells. To date, these studies suggest that several different mechanisms, rather than one specific mechanism, may be responsible for the phenomenon of reversion in these various cell systems. For example, in several instances a modification of cellular genes was suggested to be responsible for reversion (21, 24, 28), whereas in other instances the loss of the transformed phenotype in the revertant cell type was attributed to the specific loss of the transforming viral genome (12). In yet another study, control of viral gene expression in revertant cells was apparently regulated, at least in part, at the transcriptional level, since considerable differences in the amount of virus-specific RNA could be detected between the transformed and revertant phenotype in most of the clones studied (3, 6, 7, 22).

We have recently initiated studies on an avian retrovirus-transformed field vole cell line and revertant subclones obtained from these transformed cells. On the basis of all available evidence to date, it appears that the entire biologically active avian retrovirus genome is present in both transformed and revertant subclones of field vole cells, indicating that the loss of the transformed phenotype is not a result of a deletion of part, or all, of the viral genome from these cells (5, 17). Furthermore, we have recently demonstrated that at least a portion of the Rous sarcoma virus (RSV) provirus is transcribed into RNA in both transformed and revertant cells, since sarcoma gene-specific RNA can be identified in both cell types (17). Since it is entirely conceivable that coordinate expression of more than one viral gene may be required for transformation of cells by RSV, we have extended our initial studies to determine whether the entire RSV genome is expressed in both transformed and revertant cells. Contrary to other RSV-transformed/revertant cell sys-

tems studied to date (6, 7), we demonstrate in this report that not only do revertant and transformed vole cells contain similar amounts of viral-specific RNA, but, more importantly, the total viral genome is present as 35S RNA in both cell types. We further report that no significant differences in the amount of viral-specific RNA can be detected either in the nucleus or cytoplasm or associated with polyribosomes in transformed and revertant field vole cells, suggesting that the regulatory phenomenon probably does not involve either selectivity of viral RNA transport from the nucleus to the cytoplasm or formation of viral-specific polyribosomes. Thus, it appears that in RSV-transformed vole cells the genetic expression of the viral genome is controlled at some stage, either during or subsequent to translation of viral-specific mRNA. This level of regulation may be responsible for both phenotypic transformation and nonpermissiveness of viral replication in these vole cell cultures.

MATERIALS AND METHODS

Reagents, cells, and virus. The sources and preparation of most of the pertinent materials have been previously described (10, 16). Various strains of RSV (B77, Prague C of subgroup C and subgroup D) were either propagated in our laboratory and purified as previously described (2, 9), or obtained through the Office of Logistics and Resources, National Cancer Institute. The latter was obtained as virus pellets which were banded several times in 25 to 55% sucrose before use. The infection of an established cell line from the European field vole, *Microtus agrestis*, with RSV and the isolation of transformed and revertant clones were as previously described (17). Reverted subclones not only exhibited morphological properties similar to normal cells but grew with similar plating efficiencies both on plastic and in agar (R. A. Krzyzek, et al., submitted for publication). All clones of transformed cells and subclones of revertant cells employed in these studies contained the complete viral genome, since the entire infectious RSV genome could be rescued from transformed and revertant cells by fusion with permissive cells and transfection of avian cells with DNA obtained from both cell types (17). Hybridization analysis of transformed and revertant vole-cell DNA with RSV RNA or complementary DNA (cDNA) probes indicated that both cell types contained similar genome equivalents of viral-specific DNA (one to two viral equivalents per diploid genome) (5). As is the case for all mammalian cells studied to date, no RSV-specific DNA was detected in uninfected field vole cells (1, 30). 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 viral RNA. RNA was extracted from virus with sodium dodecyl sulfate (SDS)/phenol at room temperature and subsequently fractionated into 70S RNA and free, low-molecular-weight RNA by rate-zonal sedimentation (10).

Isolation of total, nuclear, cytoplasmic, polyribosome-associated, and poly(A)-containing RNA from field vole and avian cells. The procedures for the isolation of total, nuclear, cytoplasmic, and polyribosome-associated RNA from field vole and avian cells have been previously described (17). The extent of nuclear breakage and leakage of RNA into cytoplasmic fractions during the fractionation procedure was determined by previously published procedures (31). 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 was the 45S RNA precursor of 28S and 18S ribosomal RNA. Less than 1% of this species could be detected in the cytoplasm after fractionation. Nuclei were also tested for cytoplasmic contamination. No obvious cytoplasmic material was present in the nuclei preparation, as determined by phase-contrast microscopy. Furthermore, nuclear RNA preparations contained less than 5% of 18S ribosomal RNA, which is indicative of cytoplasmic contamination (23).

Polyadenylic acid [poly(A)]-containing RNA was isolated from total cellular RNA by affinity chromatography on polyuridylic acid [poly(U)]-Sepharose as previously described (26). Approximately 4 to 8 mg of RNA was applied to the column (0.5 by 60 cm) in loading buffer (0.1 M NaCl–0.01 M EDTA–0.01 M Tris-hydrochloride [pH 7.4]–0.2% SDS at room temperature. The column was washed with 10 ml of this buffer, and poly(A)-containing RNA was eluted with formamide buffer (90% formamide–0.01 M EDTA–0.01 M Tris-hydrochloride [pH 7.4]–0.2% SDS) and subsequently concentrated by ethanol precipitation. Of the total RNA applied to the column, approximately 1 to 2% attached to poly(U)-Sepharose and, therefore, contained poly(A) sequences.

Preparation of cytoplasmic RNA from transformed and revertant field vole cells for rate-zonal sedimentation. Cells used as a source of cytoplasmic RNA were harvested from roller bottles by scraping and 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 resuspended 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 dissolved in TE buffer (0.01 M Tris-hydrochloride [pH 7.4]–0.01 M EDTA).

The extent of nuclear RNA contamination of the cytoplasmic fraction during the isolation procedure was determined as described above and was found to be insignificant.

The conditions employed for rate-zonal centrifugation were essentially those described by McKnight and Schimke (19). Approximately 200 μg of cytoplasmic RNA from transformed and revertant 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 0°C. The RNA was layered directly onto a 15 to 30% (wt/vol) linear sucrose gradient made in 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, then collected by centrifugation and resuspended in 60 μl of TE buffer. A constant volume of each fraction, generally 10 to 25 μl, was dried down in a vacuum desiccator, suspended in 10 μl of buffer containing 0.01 M Tris-hydrochloride (pH 7.4), 0.6 M NaCl, 0.001 M EDTA, single-strand [^{3}H]cDNA (0.06 ng, 1,500 cpm), and 1.0 mg of yeast tRNA per ml, sealed in 30-μl microcaps, and incubated for 70 h at 68°C. Hybrid formation was detected by incubation with S1 nuclease as described below.

Synthesis of viral cDNA probes. The conditions for the synthesis of virus-specific DNA in vitro with detergent-activated RSV (B77, Prague C) were described by Stehelin et al. (27). The specific activity of the [^{3}H]cDNA probe prepared under these conditions was approximately 2.4×10^7 cpm/μg.

The representative [^{3}H]cDNA probe was prepared by a procedure yielding cDNA that protected at least 70% of radiolabeled 70S RNA from RNase hydrolysis at DNA/RNA ratios of 4:1. In this procedure, cDNA was synthesized with detergent-disrupted virus in the presence of calf thymus primer as described by Taylor et al. (29) and purified by selective hybridization to viral RNA. This enrichment step consisted of hybridizing cDNA to viral RNA at the specific ratio of DNA/RNA required to give maximum protection. The DNA/RNA hybrids were separated from unhybridized, single-stranded DNA by fractionation on hydroxyapatite, and the RNA moiety was removed by alkaline hydrolysis. Radiolabeled, genome-length DNA transcripts were prepared from endogenous reactions containing detergent-disrupted RSV and purified by rate-zonal sedimentation in alkaline sucrose as previously described (15, 29).

The [^{3}H]cDNA probe specific for the transforming gene sequences ($cDNA_{src}$) of RSV (Prague C strain) was also prepared as previously described (17, 27).

Hybridization analysis. Hybridization of viral-specific [^{3}H]cDNA probe to viral RNA was performed in a 10-μl reaction mixture 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), 1.0 mg of yeast tRNA per ml, and the indicated amount of RNA. The reaction mixtures were sealed in 30-μl microcaps and incubated for either 24 or 70 h at 68°C. Hybrid formation was detected by treatment of samples with single-strand specific S1 nuclease (*Aspergillus oryzae*) (18) 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 per ml. Digestions were performed for 2 h at 50°C, followed by the addition of 40 μg of calf thymus DNA and precipitation with 10% trichloroacetic acid. The results of hybridization analysis were expressed as a function of C_rt (concentration of RNA in moles per liter × time of incubation in seconds) (18).

RESULTS

Extent of RSV proviral DNA transcribed into RNA in transformed and revertant vole cells. The identification of the total infectious RSV genome in both revertant and transformed field vole cells indicated that neither phenotypic reversion nor nonpermissiveness of virus replication was due to a deletion or point mutation affecting the activity of RSV genes in either cell type (17). Furthermore, previous studies employing a cDNA probe specific for the RSV transforming gene sequences ($cDNA_{src}$) indicated that sarcoma-specific RNA was present in similar amounts in transformed and revertant vole cells, indicating that reversion was not the result of any major change in transcription of the viral sarcoma gene (17). Because it was conceivable that viral genes, in addition to the sarcoma gene, may also have some function in transformation of vole cells by RSV, we proceeded to determine whether similar extents of transcription of RSV proviral DNA occurred in both transformed and revertant vole cells.

Measurement of the proportion of the viral genome transcribed into RNA in transformed and revertant field vole cells was accomplished by hybridization analysis, which involved annealing radiolabeled, single-stranded cDNA, synthesized by the DNA polymerase of RSV, to RNA extracted from several clones of transformed and revertant field vole cells. The majority of the cDNA probe prepared by standard procedures (13) from disrupted virions of RSV was not uniform with respect to representation of viral sequences (4), and thus would not provide a very sensitive means of determining the extent of RSV proviral DNA transcription. We have therefore developed techniques that allow for either the enrichment of a representative cDNA probe ($cDNA_{rep}$) or the synthesis of genome-length cDNA. These cDNA probes were employed to determine the proportion of the viral genome transcribed into viral RNA in transformed and revertant field vole cells. From the data presented in Table 1, identical extents of hybridization were observed with RNA from both transformed and revertant cells under conditions employed to achieve maximum hybridization. Although the extent of hybridization of viral-specific RNA to the $cDNA_{rep}$ probe was

TABLE 1. *Genetic complexity of viral-specific RNA from RSV-transformed and revertant field vole cells*[a]

Cell type	$cDNA_{rep}$[b] (%)		$cDNA_{genome\ length}$[c] (%) total cellular RNA
	Total cellular RNA	Polysomal RNA	
Transformed clone 1	74.9	73.7	94.7
Transformed clone 22	71.8	81.2	85.2
Revertant clone 4	75.9	74.3	97.2
Revertant clone 866	72.6	72.6	96.6
Transformed clone 1 + revertant clone 866	77.5		
Normal vole cells	10.3		11.6
Vole liver	10.7		10.0
RSV-infected duck cells	77.8		96.9

[a] Total cellular and polysomal RNA were isolated from normal, transformed, and revertant field vole cells and RSV-infected duck embryo fibroblasts, as described in the text. Hybridization to total cellular and polysomal RNA was performed at a C_rt of 2×10^4 in 0.6 M NaCl with 1,200 cpm of cDNA (0.13 ng). Hybridization to the combination of transformed (clone 1) and revertant (clone 866) RNA was conducted at a C_rt of 4×10^4. Hybrid formation was determined using the single-strand specific S1 nuclease (18). Values represent the mean of duplicate determinations.

[b] The representative cDNA probe ($cDNA_{rep}$) was prepared in an endogenous reaction that was supplemented with calf thymus primer DNA (29; A. Lau, R. A. Krzyzek, and A. Faras, submitted for publication). The cDNA protected at least 70% of ^{32}P-labeled 70S RSV RNA against digestion by RNase at a molar ratio of DNA to RNA of <4:1. Approximately 75% of the cDNA hybridized to RSV 70S RNA at a C_rt of 1. Self-hybridization of the $cDNA_{rep}$ probe was 7%.

[c] cDNA, the length of the viral genome, was synthesized in an endogenous reaction and purified by rate-zonal sedimentation in alkaline sucrose (15). Intrinsic resistance of the $cDNA_{genome\ length}$ probe was 9.9%.

not 100%, no additional hybridization was observed when RNAs from both transformed and revertant field vole cells were included together in the hybridization mixture, indicating that similar regions of the viral genome were transcribed in both cell types. Furthermore, these values were similar to those obtained from an RSV-infected permissive avian cell or purified viral 70S RNA, indicating that the amount of hybridization observed with transformed or revertant field vole cell RNA was the maximum that could be achieved with this particular viral-specific cDNA probe. Even greater extents of hybridization were obtained with the genome-length cDNA probe, substantiating the presence of most, if not all, of the viral genomic sequences in both cell types (Table 1).

Size of viral-specific RNA in transformed and revertant vole cells. Since hybridization analysis with both the representative cDNA probe and genome-length cDNA suggested that the bulk of the viral genomic sequences were present in both transformed and revertant vole cells, we performed studies to determine whether the entire 35S viral RNA genome could be detected in both cell types. Cytoplasmic RNA was isolated from confluent vole cell cultures and fractionated by rate-zonal centrifugation. The viral-specific RNA was identified by annealing each fraction with a constant amount of labeled RSV cDNA, and the percentage of cDNA hybridized was measured. Profiles of viral-specific RNA from both transformed and revertant cells are presented in Fig. 1. Both cell

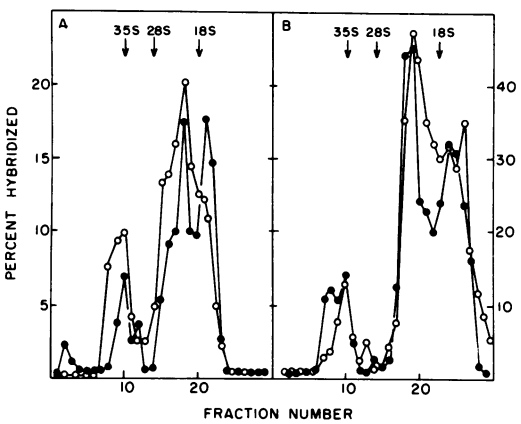

FIG. 1. *Rate-zonal sedimentation of viral-specific RNA present in the cytoplasm of transformed and revertant field vole cells. Cytoplasmic RNA was extracted from cells of transformed clone 1 and revertant subclone 4 as described in the text. Approximately 200 μg of cytoplasmic RNA was heat denatured (65°C, 10 min) and 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. Labeled RSV 35S RNA and field vole 28S and 18S RNAs were sedimented in a parallel gradient to provide markers. The gradients were collected from the bottom, and RNA in each fraction was precipitated and resuspended in 60 μl of TE buffer. Samples of 10 and 25 μl from each gradient fraction of transformed and revertant cells, respectively, were removed and hybridized with either an RSV [^{3}H]cDNA probe or an RSV [^{3}H]$cDNA_{src}$ probe 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. (A) Transformed clone 1; (B) revertant subclone 4. Symbols: ●, RSV cDNA; ○, RSV $cDNA_{src}$.*

types exhibited several size classes of viral-specific RNA, including genome-length 35S RNA. A predominant 21S RNA was found as well as material sedimenting at 12S to 15S. These size classes of viral RNA have also been identified recently in transformed hamster cells; however, no 35S RNA could be detected in the revertant hamster cell lines tested (7). Since the 12S to 15S viral RNA in the hamster transformed/revertant cell system was thought to represent the breakdown of higher-molecular-weight viral-specific RNA, it is conceivable that this size class of viral RNA in the vole cell system also reflects some breakdown of viral RNA during extraction. Nevertheless, the demonstration of 35S RNA in both transformed and revertant vole cells suggests that the entire virus genome is transcribed in both cell types. Other studies from our laboratory have demonstrated that sarcoma virus-specific RNA is present in similar quantities in transformed and revertant vole cells (17). Hybridization of a cDNA probe specific for the RSV transforming gene sequences ($cDNA_{src}$) indicates that sarcoma-specific sequences can be demonstrated in both 35S and 21S viral RNA. Thus, sarcoma-specific sequences are contained within identical size classes of viral-specific RNA in both transformed and revertant vole cells.

Quantitation of total and poly(A)-containing RSV-specific RNA in transformed and revertant field vole cells. Although the bulk of RSV nucleotide sequences were present in both transformed and revertant vole cells, it was conceivable that viral-specific RNA was present in different amounts in these cell types, as in the RSV-transformed/revertant hamster cell system (7). Furthermore, although we have previously shown that transformed and revertant vole cells contain similar amounts of sarcoma-specific RNA (17), it was also conceivable that, since several size classes of viral-specific RNA are present in these cells, the amount of sarcoma-specific RNA differs from the amount of other viral RNAs present in transformed and revertant cells. To determine whether the amount of viral-specific RNA was the same or different in transformed and revertant cells, RSV cDNA was annealed to increasing quantities of total RNA extracted from both cell types (Fig. 2). The $C_rt_{1/2}$ was determined for the viral-specific RNA from transformed and revertant cells, and the quantity of virus-specific RNA per cell was computed relative to the $C_rt_{1/2}$ for RSV 70S RNA (Table 2) (18). Appreciable viral-specific RNA was observed in both subclones of revertant field vole cells (Fig. 2, Table 2). Interestingly, revertant subclone 866 contained amounts of viral-specific RNA similar to those in transformed clone 1, while revertant subclone 4 contained only slightly less viral-specific RNA than this transformed clone. However, both re-

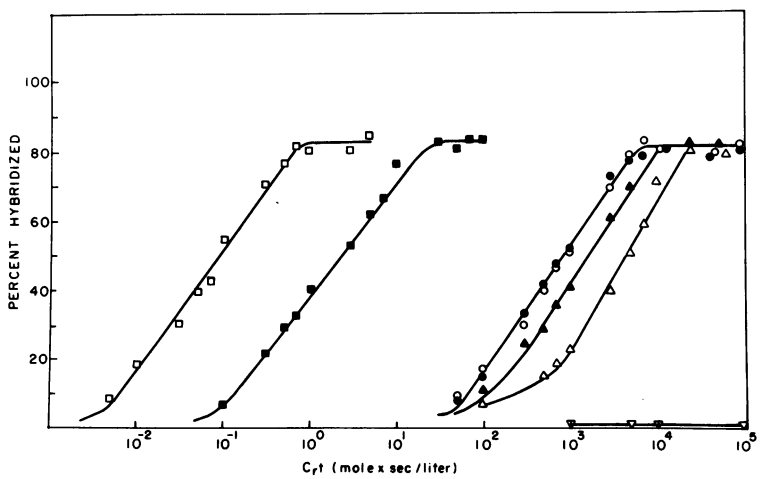

FIG. 2. *Total RSV-specific RNA in transformed and revertant field vole cells. Viral 70S RNA and total cellular RNA were prepared and hybridized with single-stranded [^{3}H]cDNA (0.06 ng, 1,500 cpm), as described in the text. Hybridization reactions containing viral 70S RNA and avian cell RNA were carried out for 24 h at 68°C, and those containing field vole RNA were carried out for 70 h. The percentage of hybridization was corrected for the intrinsic S1 nuclease resistance of cDNA (7%). The fraction of [^{3}H]cDNA hybridized is presented as a function of C_rt (product of RNA concentration and length of hybridization). Each point represents the average of three separate determinations. Symbols:* □, *viral 70S RNA;* ■, *RSV-infected duck cells;* ○, *RSV-transformed field vole clone 1;* △, *RSV-transformed field vole clone 22;* ▲, *revertant field vole subclone 4;* ●, *revertant field vole subclone 866;* ▽, *uninfected field vole.*

TABLE 2. *Quantitation of RSV RNA in transformed and revertant field vole cells*[a]

RNA source	Morphology[b]	$C_rt_{1/2}$[c]	Genome equivalents per 2.5×10^{-5} μg of RNA[d]
70S RNA	—	5×10^{-2}	—
RSV-infected duck cells	T	1.2	7.75×10^4
Normal field vole cells	NT	—	—
Vole liver	—	—	—
Transformed vole cells (clone 1)	T	5×10^2	4.80×10^2
Revertant vole cells (clone 866)	NT	5×10^2	4.80×10^2
Transformed vole cells (clone 22)	T	3×10^3	0.80×10^2
Revertant vole cells (clone 4)	NT	1×10^3	2.35×10^2

[a] Total cellular RNA was isolated from normal, transformed, and revertant field vole cells and RSV-infected duck embryo fibroblasts as described in the text.

[b] T, Transformed phenotype; NT, nontransformed phenotype.

[c] $C_rt_{1/2}$ and maximum hybridization values were derived from the data shown in Fig. 1. The $C_rt_{1/2}$ is the value at which 50% of the cDNA is hybridized to RNA.

[d] Genome equivalents were derived from the relationship: viral 35S RNA equivalent per cell = ($C_rt_{1/2}$ of RSV RNA/$C_rt_{1/2}$ of total cell RNA) × (weight of RNA per cell/5×10^{-12}), where 5×10^{-12} is the weight of one molecule of RSV 35S RNA, as described by Deng et al. (6). The data are presented as the concentration of viral RNA per 2.5×10^{-5} μg, which is the amount of total RNA present per transformed cell.

vertant subclones 866 and 4 contained more viral-specific RNA than transformed clone 22. Therefore, in contrast to revertant RSV-infected hamster cells (6, 7), it appears that revertant vole cells do not contain significantly reduced levels of viral-specific RNA as compared with transformed cells. As expected, no RSV-specific RNA can be detected in normal field vole cells (Fig. 2).

Since transformed and revertant cells exhibit different growth properties, it was conceivable that hybridization analysis of total RNA from these cell types may not reveal differences in the amount of viral-specific RNA, because of differences in the amount of ribosomal RNA present in transformed and revertant cells. For instance, ribosomal RNA constitutes the bulk of the cell RNA present in eucaryotic cells, and, therefore, slight differences in the concentration of ribosomal RNA in transformed and revertant cells would affect the $C_rt_{1/2}$ values computed for the hybridization of viral-specific RNA to the cDNA probes. To address this problem, we performed similar hybridization analysis on the poly(A)-containing fraction of cellular RNA purified by affinity chromatography on poly(U)-Sepharose (26). Removal of ribosomal RNA from the RNA preparations did not affect the rates of hybridization of viral-specific RNA from the transformed field vole cells relative to the revertant cells (Fig. 3B). Although the overall $C_rt_{1/2}$ of poly(A)-containing viral-specific RNA was lower than that of total cell RNA because of the substantial reduction of ribosomal RNA in the RNA preparation, the overall rates of hybridization of poly(A)-containing viral RNA from revertant and transformed cells exhibited patterns similar to total RNA from these cell types.

Quantitation of nuclear, cytoplasmic, and polyribosome-associated viral-specific RNA in transformed and revertant field vole cells. Similar hybridization analyses were also performed on various fractions of transformed and revertant field vole cell RNA to determine the intracellular distribution of viral-specific RNA sequences. Preparations of both cell types were separated into cytoplasmic and nuclear fractions, and the RNA extracted from both fractions was analyzed for viral-specific RNA (Fig. 3C, D). On the basis of the $C_rt_{1/2}$ analysis, it appeared that no significant differences in the amount of viral-specific RNA could be detected in the cytoplasm of transformed and revertant field vole cells (Fig. 3C). As with total RNA from these cells, revertant subclone 866 contained equivalent amounts of cytoplasmic and nuclear viral-specific RNA as in transformed clone 1, whereas both revertant subclones contained more viral RNA than transformed clone 22. A similar distribution of viral-specific RNA between revertant and transformed cells was observed when RNA isolated from polyribosomes was tested for the amount of viral-specific RNA (Fig. 3A).

DISCUSSION

We have previously demonstrated that revertant subclones of RSV-infected field vole cells contain amounts of sarcoma-specific RNA similar to transformed cells (17). In this communication, we demonstrate that the entire RSV proviral DNA is actively transcribed into viral-specific RNA in both transformed and revertant field vole cells. This conclusion is based upon several observations obtained from our analysis of the structural features of viral-specific RNA from these cell types. First, genetic complexity analysis of viral RNA indicated that similar extents of the viral genome were transcribed in both transformed and revertant vole cells. According to our hybridization analyses with either a relatively representative cDNA probe or genome-length cDNA transcripts, it appears that most, if not all, of the viral-genomic nucleotide sequences can be identified in both cell types. Second, size analysis of viral RNA obtained from

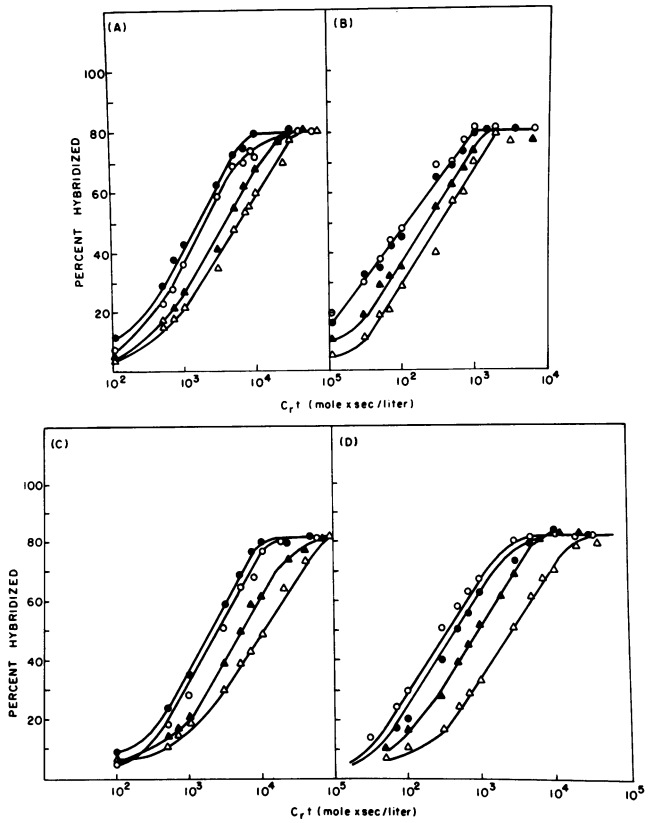

FIG. 3. *Quantitation of poly(A)-containing, polyribosome-associated, cytoplasmic, and nuclear viral-specific RNA in transformed and revertant field vole cells. Poly(A)-containing, polyribosome-associated, cytoplasmic, and nuclear RNAs were prepared as described in the text and hybridized with single-stranded [^{3}H]cDNA (0.06 ng, 1,500 cpm). Hybridizations were performed for 70 h at 68°C. Each point represents an average of three separate determinations. (A) Polyribosome-associated RNA; (B) poly(A)-containing RNA; (C) cytoplasmic RNA; (D) nuclear RNA. Symbols:* ○, *RSV-transformed field vole clone 1;* △, *RSV-transformed field vole clone 22;* ▲, *revertant field vole subclone 4;* ●, *revertant field vole subclone 866.*

transformed and revertant cell types indicated that genome-length 35S viral RNA was present in both cell types. In addition to data on the size and genetic complexity of viral RNA, we have also demonstrated that similar concentrations of viral-specific RNA were present in transformed and revertant cells. Since the bulk of RSV genomic sequences can be detected in transformed and revertant vole cells, phenotypic reversion does not appear to result from a reduction in the expression of other viral genes that may be important in addition to the sarcoma gene for transformation. Thus, since no differences in either the amount, the genetic complexity, or the size of viral RNA can be detected between transformed and revertant vole cells, reversion cannot be attributed to a change in either the extent of transcription of RSV proviral DNA or the amount of viral-specific RNA in these cells.

Since transformation of nonpermissive cells with RSV depends upon the continuous presence of some viral gene product(s) (14), it appears that the regulation of expression of the viral gene sequences in the revertant cell type must be occurring post-transcriptionally. The studies reported in this communication on the structure and intracellular location of viral-specific RNA suggest that neither the lack of polyadenylation nor transport of viral RNA from the nucleus to the cytoplasm is responsible for the loss of the transformed phenotype in revertant subclones. Studies of the intracellular distribution of RSV RNA indicate that both transformed and revertant vole cells contain viral-specific RNA in the cytoplasm at concentrations similar to those observed with total cell RNA. Furthermore, no differences in the amount of poly(A)-containing viral RNA could be detected in these cell types. Since no major size differ-

ences in virus-specific RNA could be detected in revertant and transformed vole cells, these data suggest that processing of viral RNA is probably completed in both cell types. However, further work on the structure (polyadenylation, methylation, capping, splicing, etc.) and sequence organization of the individual viral mRNA species will be required before this conclusion can be rigorously substantiated and the precise nature of the post-transcriptional regulatory mechanisms can be elucidated.

Since we can detect viral-specific RNA associated with polyribosomes in revertant subclones, it is likely that viral RNA is translated in these cells. Preliminary radioimmunoassay studies of RSV-specific proteins in transformed and revertant vole cells substantiate this supposition (A. Lau, unpublished data). Studies are currently in progress to determine the proportion of viral RNA that is translated into viral-specific proteins in both transformed and revertant field vole cells. It is conceivable that both the nonpermissiveness of virus replication in RSV-infected vole cells and the loss of the transformed phenotype during reversion reflect the absence or reduced rate of processing of polyproteins (8, 25) or the lack of modification of viral proteins subsequent to cleavage.

ACKNOWLEDGMENTS

We thank S. Kanellos and D. Nelson for excellent technical assistance, C. Thull for typing this manuscript, and H. Varmus for his critical evaluation of this manuscript. We also thank J. Gruber and the Office of Program Resources and Logistics, Viral Cancer Program, Division of Cancer Cause and Prevention, National Cancer Institute, for supplying us with valuable reagents.

This investigation was conducted under contract NO1-CP-61055 within the Virus Cancer Program of the National Cancer Institute. R.K. and A.L. are currently postdoctoral fellows supported by NIH fellowship CA 05231 and training grant CA 09138 from the National Cancer Institute, respectively.

LITERATURE CITED

1. **Baluda, M. A.** 1972. Widespread presence in chickens of DNA complementary to the RNA genome of avian leukosis viruses. Proc. Natl. Acad. Sci. U.S.A. **69**:576–580.
2. **Bishop, J. M., W. Levinson, N. Quintrell, D. Sullivan, L. Fanshier, and J. Jackson.** 1970. The low molecular weight RNAs of Rous sarcoma virus. I. The 4S RNA. Virology **42**:182–195.
3. **Boettiger, D.** 1974. Reversion and induction of Rous sarcoma virus expression in virus transformed baby hamster kidney cells. Virology **62**:522–529.
4. **Collett, M. S., and A. J. Faras.** 1975. In vitro transcription of DNA from the 70*S* RNA of Rous sarcoma virus: identification and characterization of various size classes of DNA transcripts. J. Virol. **16**:1220–1228.
5. **de la Maza, L. M., A. Faras, H. Varmus, P. K. Vogt, and J. J. Yunis.** 1975. Integration of avian sarcoma virus specific DNA in mammalian chromatin. Exp. Cell Res. **93**:484–487.
6. **Deng, C. T., D. Boettiger, I. Macpherson, and H. E. Varmus.** 1974. The persistence and expression of virus-specific DNA in revertants of Rous sarcoma virus-transformed BHK-21 cells. Virology **62**:512–521.
7. **Deng, C. T., D. Stehelin, J. M. Bishop, and H. E. Varmus.** 1977. Characteristics of virus-specific RNA in avian sarcoma virus-transformed BHK-21 cells and revertants. Virology **76**:313–330.
8. **Eisenman, R., V. M. Vogt, and H. Diggelmann.** 1974. Synthesis of avian RNA tumor virus structural proteins. Cold Spring Harbor Symp. Quant. Biol. **39**:1067–1075.
9. **Faras, A. J., and N. A. Dibble.** 1975. RNA-directed DNA synthesis by the DNA polymerase of Rous sarcoma virus: structural and functional identification of 4S primer RNA in uninfected cells. Proc. Natl. Acad. Sci. U.S.A. **72**:859–863.
10. **Faras, A. J., J. M. Taylor, J. P. McDonnell, W. E. Levinson, and J. M. Bishop.** 1972. Purification and characterization of the deoxynucleic acid polymerase associated with Rous sarcoma virus. Biochemistry **11**:2334–2342.
11. **Fischinger, P. J., S. Nomura, P. T. Peebles, D. K. Haapala, and R. H. Bassin.** 1972. Reversion of murine sarcoma virus transformed mouse cells: variants without a rescuable sarcoma virus. Science **176**:1033–1035.
12. **Frankel, A. E., D. K. Haapala, R. L. Newbauer, and P. J. Fischinger.** 1976. Elimination of the sarcoma genome from murine sarcoma virus transformed cat cells. Science **191**:1264–1266.
13. **Garapin, A. C., H. E. Varmus, A. J. Faras, W. E. Levinson, and J. M. Bishop.** 1973. RNA-directed DNA synthesis by virions of Rous sarcoma virus: further characterization of the templates and the extent of their transcription. Virology **52**:264–274.
14. **Graf, T., and R. R. Friis.** 1973. Differential expression of transformation in rat and chicken cells infected with an avian sarcoma virus *ts* mutant. Virology **56**:369–374.
15. **Junghans, R. P., P. H. Duesberg, and C. A. Knight.** 1975. *In vitro* synthesis of full-length DNA transcripts of Rous sarcoma virus RNA by viral DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **72**:4895–4899.
16. **Kieras, R. M., and A. J. Faras.** 1975. DNA polymerase of reticuloendotheliosis virus: inability to detect endogenous RNA-directed DNA synthesis. Virology **65**:514–523.
17. **Krzyzek, R., A. Lau, D. Spector, and A. Faras.** 1977. Post-transcriptional control of avian oncornavirus transforming gene sequences in mammalian cells. Nature (London) **269**:175–179.
18. **Leong, J.-A., A.-C. Garapin, N. Jackson, L. Fanshier, W. Levinson, and J. M. Bishop.** 1972. Virus-specific ribonucleic acid in cells producing Rous sarcoma virus: detection and characterization. J. Virol. **9**:891–902.
19. **McKnight, G. S., and R. T. Schimke.** 1974. Ovalbumin messenger RNA: evidence that the initial product of transcription is the same size as polysomal ovalbumin messenger. Proc. Natl. Acad. Sci. U.S.A. **71**:4327–4331.
20. **Macpherson, I. A.** 1965. Reversion in hamster cells transformed by Rous sarcoma virus. Science **143**:1731–1733.
21. **Nomura, S., P. J. Fischinger, C. G. Mattern, B. I. Gerwin, and R. J. Dunn.** 1973. Revertants of mouse cells transformed by murine sarcoma virus. II. Flat variants induced by fluorodeoxyuridine and colcemid. Virology **56**:152–163.
22. **Peebles, P. T., E. M. Scolnick, and R. S. Howk.** 1976. Increased sarcoma virus RNA in cells transformed by leukemia viruses: model for leukemogenesis. Science **192**:1143–1145.
23. **Penman, S.** 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. **17**:117–130.
24. **Renger, R., and C. Basilico.** 1972. Mutation causing temperature-sensitive expression of cell transformation by a tumor virus. Proc. Natl. Acad. Sci. U.S.A. **69**:109–114.
25. **Reynolds, F. H., Jr., C. A. Hanson, S. A. Aaronson, and J. R. Stephenson.** 1977. Type C viral *gag* gene

expression in chicken embryo fibroblasts and avian sarcoma virus-transformed mammalian cells. J. Virol. **23**:74–79.

26. **Staskus, K. A., M. S. Collett, and A. J. Faras.** 1976. Initiation of DNA synthesis by the avian oncornavirus RNA-directed DNA polymerase. Structural and functional localization of the major species of primer RNA on the oncornavirus genome. Virology **71**:162–168.
27. **Stehelin, D., R. V. Guntaka, H. E. Varmus, and J. M. Bishop.** 1976. Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses. J. Mol. Biol. **101**:349–365.
28. **Stephenson, J. R., R. K. Reynolds, and S. A. Aaronson.** 1973. Characterization of morphologic revertants of murine and avian sarcoma virus-transformed cells. J. Virol. **11**:218–222.
29. **Taylor, J. M., R. Illmensee, and J. Summers.** 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta **442**:324–330.
30. **Varmus, H. E., J. M. Bishop, and P. K. Vogt.** 1973. Appearance of virus-specific DNA in mammalian cells following transformation by Rous sarcoma virus. J. Mol. Biol. **74**:613–626.
31. **Varmus, H. E., R. V. Guntaka, C. T. Deng, and J. M. Bishop.** 1974. Synthesis, structure and function of avian sarcoma virus-specific DNA in permissive and nonpermissive cells. Cold Spring Harbor Symp. Quant. Biol. **39**:3874–3878.