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Sarcoma-Virus-Related RNA Sequences in Normal Rat Cells

(rat type C virus/sarcoma specific RNA/30S subunit)

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ABSTRACT **A rat type C virus spontaneously activated from the NRK (normal rat kidney) cell line was found to have two major size classes of viral RNA subunits sedimenting at 35 and 30 S. Virus-producing cells contained both RNA species, while normal "virus-free" rat cells contained primarily virus-specific 30S RNA species. A DNA transcript, specific for Kirsten sarcoma virus, prepared from virus activated in nonproducer BALB/c cells originally transformed by Kirsten sarcoma virus and rendered specific for the virus by absorption of sequences related to mouse helper virus hybridized only with the 30S RNA species of virus-producing rat cells and normal rat cells. These findings are consistent with the hypothesis that sarcoma-specific nucleic acid sequences in Kirsten sarcoma virus emerged through a process that incorporated some portions of 30S RNA species from rat cells (either normal or virus-producing) into the Kirsten leukemia virus during passage *in vivo* of that virus. The virus designated M-MSV(RaLV), which originally derived from tumor induced by Moloney sarcoma virus (M-MSV) in rats, contained 35S RNA species of rat type C viruses and 30S RNA species specific for both rat and mouse viruses. It appears striking that for these two animal species, sarcoma-virus-specific information resides on a 30S subunit.**

Three isolates, previously considered to be murine sarcoma viruses, have been studied by nucleic acid hybridization techniques during the past 2 years. Two of these isolates, the Harvey (1) and Kirsten (2) strains, arose on rat passage of mouse leukemia viruses (Moloney and Kirsten strains, respectively), and the third, the Moloney strain (3), arose on mouse passage of the Moloney leukemia virus (M-MuLV). Based on minimal hybridization between rat and mouse type C viruses (4, 5), it was possible to show that the Kirsten and Harvey sarcoma viruses derived their sarcoma-specific nucleic acid sequences from the rat type C virus (4, 6), while the Moloney strain (M-MSV) was exclusively of mouse origin (4, 5).

We have previously reported preparation of KiSV-specific DNA transcripts that did not hybridize with RNA of helper mouse virus (7,27). Such transcripts detected a 30S RNA species in nonproducer mouse cells transformed by KiSV, while helper virus RNA subunits were of distinct hybridization specificity and sedimented at 35 S. In the present studies, a rat type C virus genome was found to have two major size classes of viral RNA subunits sedimenting at 35 S and 30 S. Further analysis of rat type C virus-specific RNA in normal or spontaneously virus-producing rat cells has now shown KiSV-specific sequences associated with a 30S RNA in both cells, whereas virus-producing rat cells produce an additional 35S RNA subunit that is not related to KiSV.

Abbreviations: KiSV, Kirsten sarcoma virus; MuLV, murine leukemia virus; M-MSV, Moloney sarcoma virus; RaLV, endogenous rat type C virus; R-MuLV, Rauscher murine leukemia virus; standard saline–citrate, 0.15 M NaCl–0.015 M Na_3 citrate; $NaDodSO_4$, sodium dodecyl sulfate; Me_2SO, dimethyl sulfoxide.

MATERIALS AND METHODS

Cells and Media. The following rat cell lines were used: NRK-9 (RaLV-NRK), a normal rat kidney (NRK) cell line producing endogenous rat type C virus (RaLV) (8); MSB-1 (9), a rat cell transformed by M-MSV producing M-MSV-(RaLV) (5); a Fisher rat embryo cell (5053) provided by Dr. A. Freeman (Children's Hospital, Akron, Ohio) (10); 78A1 cell, a rat embryo fibroblast transformed by M-MSV and producing M-MSV(MuLV) (11); and NRK cells producing KiSV(MuLV) [KiSV(MuLV)-NRK]. The virus was obtained from Dr. V. Klement, USC Medical School, Los Angeles, Calif.

The following mouse cells were used: BALB/3T3 (A-31), and a derivative K-234, a BALB/3T3 cell line transformed by KiSV (NP cell) (12, 13); M-58-2, a mutant cell line derived from the NP cell after short-term treatment with 5′-bromodeoxyuridine (BrdU) (14, 15); 58-2T, a cell line established from a slowly growing tumor in a BALB/c mouse that was inoculated with M-58-2 cells (7); Ki-MuLV-producing NIH/3T3 cell (Ki-MuLV-NIH/3T3), obtained from Dr. V. Klement (Ki-MuLV had not been passaged in rat); and JLS-V9 infected with Rauscher strain of MuLV (R-MuLV) (16). A nonproducer hamster cell transformed by M-MSV (HT-1) was also used (17).

All cells were grown in Eagle's medium with 10% (v/v) fetal bovine serum and antibiotics.

Preparation of RNA. Cell RNA was prepared as described (18), by the hot phenol method. The concentration was determined by the orcinol reaction (19).

Synthesis of Virus-Specific DNA. Viral [^{3}H]DNA was prepared by the endogenous RNA-directed DNA polymerase reaction with RaLV, R-MuLV, M-MSV(MuLV), Ki-MuLV, and the virus produced by 58-2T cells, 58-2T virus. These viruses were purified as described (11). Reaction mixtures, generally 5 ml, contained 1–2 ml of purified virus solution (0.1–1.0 mg/ml of protein), 0.1 M glycine–NaOH buffer, (pH 8.0), 10 mM dithiothreitol, 0.05 mM each of dATP, dGTP, and dCTP, 1.25 mCi of [^{3}H]dTTP (40–50 Ci/mmol, New England Nuclear Corp.), 0.03 M NaCl, 1 mM $MnCl_2$, 0.01% Nonidet P-40 (NP-40), and 100 μg/ml of actinomycin D (Sigma Chemical Co., St. Louis, Mo.). Enzyme reactions were carried out at 37° for 16–18 hr and stopped by addition of

ethylenediaminetetraacetic acid (EDTA) to a concentration of 10 mM, NaCl to 100 mM, and sodium dodecyl sulfate ($NaDodSO_4$) to 0.5%. Viral [^{3}H]DNA was extracted with phenol as described (20), and precipitated by 2 volumes of ethanol in the presence of yeast tRNA (50 μg/ml) at $-20°$ overnight. The precipitates were dissolved in 0.5 ml of 0.1 concentration of standard saline–citrate (standard saline–citrate = 0.15 M NaCl–0.015 M Na_3 citrate) and treated with 0.05 volume of 2 M NaOH at 80° for 30 min to destroy residual RNA. After neutralization with 0.05 volume of 3 M NaH_2PO_4, the DNA product was dialyzed at 4° against 0.1 concentration of standard saline–citrate. KiSV-specific [^{3}H]DNA (58-2TS DNA) was prepared as follows: 58-2T [^{3}H]DNA (7, 27) was incubated with RLV 70S RNA (2 μg/ml) in twice the concentration of standard saline–citrate and 40% formamide at 45° for 48 hr. After hybridization, single-stranded [^{3}H]DNA (about 50% of input counts) was separated from the hybridized [^{3}H]DNA by differential batch elution on hydroxyapatite (21). More than 80% of the [^{3}H]DNA thus purified hybridized with 58-2T cell RNA and NP cell RNA, while no significant hybridization was observed with R-MuLV RNA and BALB/3T3 cell RNA.

Preparation of High-Molecular-Weight RNA of ^{32}P-Labeled RaLV. RaLV was labeled with ^{32}P and high-molecular-weight was purified as described (22). Since high-molecular-weight RNA of [^{32}P]RaLV forms a heterogeneous peak at 60–70 S (27), RNA sedimenting at 50–80 S was used for subunit RNA analysis.

Hybridization. Increasing amounts of RNA were incubated with 500 cpm of [^{3}H]DNA in 100 μl of twice the concentration of standard saline–citrate at 66° for 20 hr. The extent of hybridization was determined with a single-strand-specific S1 nuclease as described (22). S1 nuclease-resistant radioactivities (3.4% and 8.0% of the input counts for RaLV [^{3}H]DNA and 58-2TS [^{3}H]DNA, respectively) in a sample without RNA were subtracted from radioactivities in all other samples.

Size of Virus-Specific RNA. The whole cell RNA was treated with dimethylsulfoxide (Me_2SO) as described (21) and layered on a 15–30% sucrose gradient in NTE buffer [0.01 M Tris·HCl (pH 7.0), 0.1 M NaCl, 0.001 M EDTA] containing 0.5% $NaDodSO_4$. The RNA was centrifuged for 5–6 hr at 36,000 rpm at 20° in the SW-41 rotor in a Spinco model L2-65 ultracentrifuge. After centrifugation, fractions were collected from the bottom of the tube. Absorbancy at 260 nm was measured and RNA precipitated with two volumes of ethanol in the presence of 50 μg/ml of yeast tRNA. RNA collected by low speed centrifugation was dissolved in 0.1 ml of 0.1 concentration of standard saline–citrate. A portion of each fraction was hybridized with [^{3}H]DNA and the extent of hybridization was assayed as described above.

RESULTS

RaLV [^{3}H]DNA Hybridizations. RaLV [^{3}H]DNA transcript was hybridized with increasing amounts of RNA of the following cells (Fig. 1A): RaLV-producing cells (RaLV-NRK and MSB-1); KiSV transformed cells (58-2T and NP cells); normal rat cells [NRK and Fisher rat embryo cell (5053)]; normal BALB/3T3 cells; and Ki-MuLV-NIH/3T3 cells. About 20% of the RaLV [^{3}H]DNA hybridized with RNA of cells carrying the KiSV genome (Fig. 1A), suggesting that a

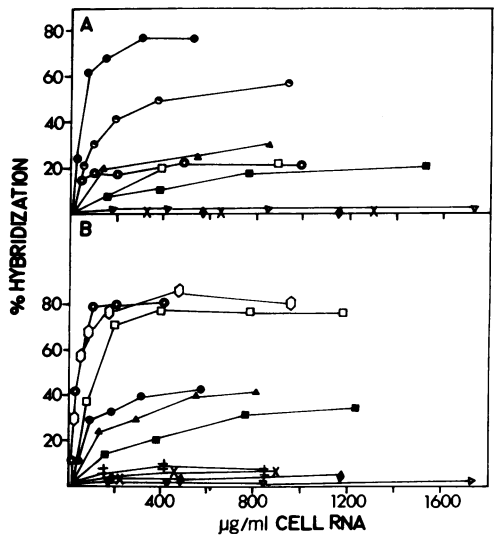

FIG. 1. Hybridization-saturation experiments with [^{3}H]DNA and various cell RNAs. (A) RaLV [^{3}H]DNA (500 cpm) was hybridized with increasing amounts of 58-2T cell RNA (○); KiSV-BALB/3T3 cell RNA (□); RaLV-NRK cell RNA (●); MSB-1 cell RNA (◑); NRK cell RNA (▲); Fisher rat embryo cell (5053) RNA (■); BALB/3T3 cell RNA (×); M-MSV-hamster (HT-1) cell RNA (◆); and Ki-MuLV-NIH/3T3 cell RNA (▽). After hybridization at 66° for 20 hr, the extent of hybridization was determined as described in *Materials and Methods*. (B) 58-2TS [^{3}H]DNA (250 cpm) was hybridized with increasing amounts of 58-2T cell RNA (○); KiSV-BALB/3T3 cell RNA (□); RaLV-NRK cell RNA (●); Ki-MSV(MuLV)-NRK cell RNA (○); NRK cell RNA (▲); Fisher rat embryo cell (5053) RNA (■); BALB/3T3 cell RNA (×); M-MSV-hamster (HT-1) cell RNA (◆); Ki-MuLV-NIH/3T3 cell RNA (▽), and R-MuLV-producing JLS-V9 cell RNA (‡).

portion of the RaLV genome is homologous to the KiSV genome, which is consistent with the previous findings of Scolnick *et al.* (4). A slightly higher level of hybridization was detected with RNAs of normal rat cells. These cells were not producing virus at the time of assay. RNA from Ki-MuLV-producing cells did not significantly hybridize with RaLV [^{3}H]DNA, although 175 μg/ml of this RNA preparation half-saturated Ki-MuLV [^{3}H]DNA. Similarly, no detectable hybridization was observed with the M-MSV-transformed hamster cell (HT-1) RNA. These two results are also consistent with previous findings (4). RNA of MSB-1, a rat cell transformed with M-MSV and producing M-MSV(RaLV) (5), hybridized with the RaLV-NRK [^{3}H]DNA transcript, reaching saturation at 50–60% of the level obtained with RaLV-NRK RNA.

KiSV-Specific [^{3}H]DNA Hybridizations. If common sequences are present in KiSV and RaLV genomes, a reciprocal relationship should exist between the two viruses. To answer the question, KiSV-specific DNA was prepared as described (7, 27; see *Materials and Methods*). This DNA did not significantly hybridize with M-MSV-transformed hamster cell RNA or with R-MuLV or Ki-MuLV mouse cell RNA. This transcript did hybridize with RNAs of cells carrying the KiSV genome [58-2T, KiSV-BALB/3T3, and KiSV(MuLV)-NRK] to a similar extent (80% of input counts) (Fig. 1B). KiSV-specific [^{3}H]DNA hybridized to a similar extent (35–45%) with RNAs of rat cells either producing RaLV or not.

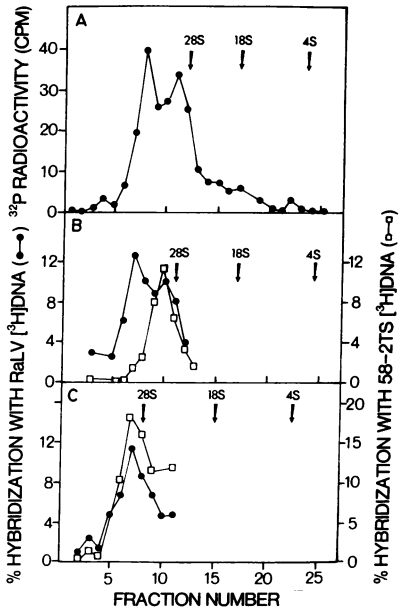

FIG. 2. Rate zonal sedimentation patterns of viral and virus-specific cell RNA. (A) High-molecular-weight RNA of [^{32}P]RaLV mixed with NRK cell RNA and heated at 70° for 1 min in 0.1 concentration of standard saline–citrate and centrifuged in a 15–30% sucrose gradient containing NTE buffer and 0.5% $NaDodSO_4$ with SW41 rotor at 36,000 rpm for 5 hr. After centrifugation, fractions were collected and absorbancy was measured at 260 nm to determine the positions of 28S and 18S ribosomal RNA (arrows). Aliquots of each fraction were mixed with 10 ml of Aquasol (New England Nuclear Corp.) and radioactivities were measured in a Beckman Scintillation counter. (B) RaLV-NRK cell RNA and (C) NRK cell RNA were isolated, treated with Me_2SO, and centrifuged on 15–30% sucrose gradients as described in *Materials and Methods*. After centrifugation, fractions were collected, absorbancy at 260 nm was measured, and RNA was precipitated. Collected RNA was dissolved in 0.1 ml of 0.1 concentration of standard saline–citrate. Ten microliters of each fraction from RaLV-NRK cell RNA and 20 μl of each fraction from NRK cell RNA were hybridized with 1000 cpm of RaLV [^{3}H]DNA (●). Ten microliters of each fraction from RaLV-NRK cell RNA and 40 μl of each fraction from NRK cell RNA were hybridized with 500 cpm of 58-2TS [^{3}H]DNA (□). The extent of hybridization was measured as described in *Materials and Methods*. 1.2 A_{260} units of RaLV-NRK cell RNA and 1.2 A_{260} units of NRK cell RNA were loaded on each gradient.

The results in Fig. 1A and B suggest that a portion of the KiSV genome is homologous to a portion of the RaLV genome. Normal rat cells synthesize RNA hybridizable both to KiSV-specific and RaLV [^{3}H]DNAs.

Size of RaLV Subunit RNA with Sequences Homologous to KiSV. High-molecular-weight RNA of ^{32}P-labeled RaLV was isolated and heated at 70° for 1 min in 0.1 concentration of standard saline–citrate. As shown in Fig. 2A, RaLV RNA subunits were found to be composed of 35S and 30S RNA subunits. To examine which RNA subunit has sequences homologous to the KiSV genome, RaLV-producing rat cell RNA and NRK cell RNA were isolated, treated with Me_2SO to dissociate RNA, and centrifuged in a sucrose gradient. After fractionation, constant volumes of each fraction were hybridized with KiSV-specific [^{3}H]DNA and RaLV [^{3}H]DNA (Fig. 2B and C). Two RaLV-specific RNA molecules sedimenting at 35 S and 30 S were detected in the RaLV-NRK cell. However, KiSV-specific [^{3}H]DNA hybridized only with the 30S fraction. In contrast to the RaLV-NRK cells, NRK cells synthesized primarily the 30S RaLV-specific RNA and the 30S KiSV-specific RNA (Fig. 2C). A similar result was obtained with Fisher rat embryo cells (5053).

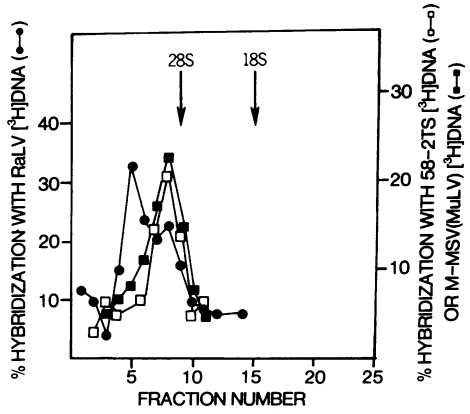

FIG. 3. Rate zonal sedimentation of virus-specific RNA in MSB-1 cells. MSB-1 cell RNA was isolated, treated with Me_2SO, and centrifuged in a 15–30% sucrose gradient as described in *Materials and Methods*. After centrifugation, fractions were collected, absorbancy at 260 nm was measured, and RNA was precipitated. Collected RNA was dissolved in 0.1 ml of 0.1 concentration of standard saline–citrate. Thirty microliters of each fraction were hybridized with 1000 cpm of RaLV [^{3}H]DNA (●), 40 μl of each fraction with 500 cpm of 58-2TS [^{3}H]DNA (□), and 20 μl of each fraction with 500 cpm of M-MSV(MuLV) and [^{3}H]DNA (■). 2.2 A_{260} units of MSB-1 cell RNA was loaded on a gradient.

MSB-1 cells also synthesized 35S and 30S RNA detected by RaLV [^{3}H]DNA (Fig. 3). As in RaLV-NRK cells, KiSV-specific [^{3}H]DNA detected only 30S RNA. It is noteworthy that the M-MSV(MuLV) [^{3}H]DNA probe also detected a 30S RNA species. Since (*a*) this DNA probe did not significantly hybridize with KiSV-transformed cell RNA (Tsuchida, unpublished data), (*b*) the KiSV-specific DNA probe did not significantly hybridize with RNA from cells transformed by M-MSV, and (*c*) RaLV [^{3}H]DNA did not hybridize to RNA from M-MSV-transformed cells, two distinct hybridization specificities are detected in the 30S fraction.

Size of Viral RNA Subunit having Sequences Homologous to RaLV Genome in Cells Carrying the KiSV Genome. In other publications (7, 27), we reported that KiSV-specific RNA sediments at 30 S in cells carrying the KiSV genome. The sedimentation rate of RNA hybridizable with RaLV [^{3}H]-DNA was studied with 58-2T cells (Fig. 4A), KiSV-BALB/3T3 cells (Fig. 4B), and KiSV(MuLV)-NRK cells (Fig. 4C). The 58-2T cell, which was derived from nonproducer KiSV-BALB/3T3 cells after BrdU treatment, synthesizes 35S R-MuLV-specific RNA and also 30S KiSV-specific RNA (Fig. 4A). As shown in these figures, the RNA with sequences homologous to the RaLV genome sedimented at 30 S in all three cell lines. The sedimentation value coincided with the 30S KiSV-specific RNA, confirming that a portion of the 30S KiSV genome RNA has sequences homologous to a portion of the RaLV genome.

DISCUSSION

Reciprocal hybridization experiments between RaLV and KiSV have shown, in agreement with previous findings (4),

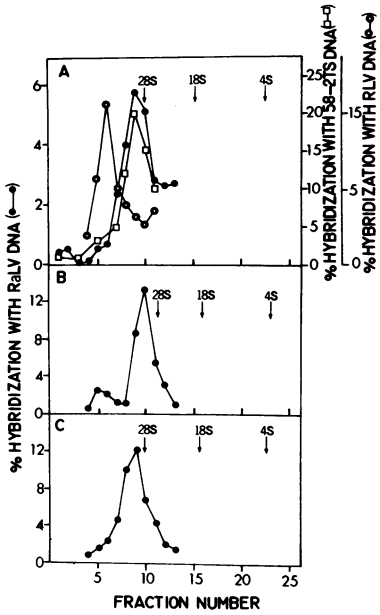

FIG. 4. Rate zonal sedimentation patterns of cells transformed by KiSV genome. (A) 58-2T cell RNA, (B) KiSV-BALB/3T3 cell RNA, and (C) KiSV(MuLV)-NRK cell RNA were isolated, treated with Me_2SO, and centrifuged in a 15–30% sucrose gradient as described in *Materials and Methods*. After centrifugation, fractions were collected, absorbancy at 260 nm was measured, and RNA was precipitated. Collected RNA was dissolved in 0.1 ml of 0.1 concentration of standard saline–citrate. Twenty microliters, 30 μl and 10 μl of each fraction of 58-2T cell RNA were hybridized with 500 cpm of R-MuLV [^{3}H]DNA (○), 500 cpm of 58-2TS [^{3}H]DNA (□), and 1000 cpm of RaLV [^{3}H]-DNA (●), respectively. Thirty microliters and 20 μl of each fraction of KiSV-BALB/3T3 cell RNA and KiSV(MuLV)-NRK cell RNA, respectively, were hybridized with 1000 cpm of RaLV [^{3}H]DNA (●). 1.5, 1.0, and 1.6 A_{260} units of 58-2T cell RNA, KiSV-BALB/3T3 cell RNA, and KiSV(MuLV)-NRK cell RNA, respectively, were loaded on sucrose gradients.

that certain rat type C viruses have nucleic acid sequences in common with the Kirsten sarcoma virus. The common sequences are specifically those retained in mouse cells carrying the KiSV genome and are associated with a 30S subunit RNA molecule. RNA molecules of this size which share sequences with KiSV are found in virus-producing rat cells and normal rat cells in culture, in addition to the nonproducer mouse cells. The rat type C viruses studied have, in addition to a 30S RNA subunit, an RNA subunit with a sedimentation coefficient of 35 S, which is also found in virus-producing rat cells. The 35S RNA species was not found in mouse cells carrying the KiSV genome or in normal rat cells and, thus, is not related to KiSV. Current experiments do not indicate whether the 35 and 30S RNAs in RaLV share common sequences not detected by the KiSV transcript, nor is the precise relationship among the 30S RNAs detected in KiSV and RaLV yet established. The striking point is that normal cultured rat cells produce RNA molecules that share sequences with the KiSV genome and have the same unusual size characteristics (30S) of molecules associated with the oncogenic effects of a type C virus. We also find it striking that 30S RNA molecules are found in nonproducer cells transformed by M-MSV (ref. 22 and present study), although these sequences have not shown relationship to KiSV by current hybridization methods. Sequence analysis may reveal some degree of similarity not detectable by relatively stringent hybridization techniques.

The crucial question that has been raised concerns the biologic relevance of these 30S molecules in normal cells; they may represent preformed molecules that need some cellular activating step(s) to produce oncogenic effects, as predicted by the oncogene theory (23), or as proposed by Temin (24, 25), molecules in the process of rapid evolutionary change that have the potential of becoming oncogenic molecules or, ultimately, portions of a viral genome. These considerations would seem to dictate a thorough comparison of the 30S molecules found in virions and normal cells. The presence of sarcoma-virus-related RNA in normal rat cells should be considered in evaluating studies on the mechanism of cell transformation by external agents, e.g., chemicals and x-ray. It should be noted that there exists the possibility of quantitative or qualitative differences in synthesis or processing of this RNA species by different treatments.

The virus isolated from MSB-1 cells, previously designated as M-MSV(RaLV) based on immunologic (26) and molecular hybridization methodology (5), appears to synthesize at least three distinct virus-related RNA subunits; 35S of RaLV, 30 S related to KiSV or RaLV, and 30 S of M-MSV. However, the possibility that a 30S RNA molecule with both KiSV- and M-MSV-related sequences exists has not yet been excluded. The distribution of these three distinct sequences in the virion population is currently unknown.

In relation to the main theme of this communication, the retention of 30S M-MSV-specific RNA in the original nonproducer rat tumor parallels the situation found in M-MSV hamster tumors and KiSV-transformed mouse cells where 30S virus-specific RNAs are also found (7, 22, 27).

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