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Type C Particle-Positive and Type C Particle-Negative Rat Cell Lines: Characterization of the Coding Capacity of Endogenous Sarcoma Virus-Specific RNA

EDWARD M. SCOLNICK,* DAVID WILLIAMS, JEAN MARYAK, WILLIAM VASS, ROBERT J. GOLDBERG, AND WADE P. PARKS

Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Maryland 20014

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Various rat cell lines have been analyzed for expression of endogenous RNA homologous either to RT21C, a typical rat type C virus, or to Kirsten sarcoma virus. Cells have been found that express either (i) high levels of RNA homologous to RT21C rat type C virus and low levels of RNA homologous to Kirsten sarcoma virus (RT21C^{high},sarc^{low}) or (ii) high levels of RNA homologous to Kirsten sarcoma virus and low levels of RNA homologous to typical rat type C virus (sarc^{high},RT21C^{low}). The properties of these two classes of cell lines have been compared. Each type of cell contains an equal amount of the expressed RNA on polysomes. Cell lines that are RT21C^{high} produce abundant rat p30 and p12 structural proteins and release rat type C particles containing viral RNA and reverse transcriptase into supernatant fluids from these cultures. Cell lines that are sarc^{high},RT21C^{low} have no detectable rat viral p12 protein and no p30 protein immunoreactive in even broad interspecies radioimmunoassays, and do not release type C particles into the supernatant from the cultures. When the particle-negative cell lines are superinfected with heterologous mouse or woolly type C viruses or are producing typical rat type C virus particles, the endogenous sarcoma virus-specific RNA is secreted from these cells. The sarcoma virus-specific RNA can be transcribed in complementary DNA in endogenous reverse transcriptase reactions carried out in vitro with such virus preparations. However, exposure of cells that are permissive to the helper virus with the particles containing sarcoma virus-specific RNA has not yet resulted in cell transformation or in the synthesis of these RNA sequences. The results suggest: (i) that the first step in the genesis of sarcoma viruses involves the packaging of this expressed sarcoma virus-specific RNA in helper viral particles; (ii) that efficient transmission of the sarcoma virus-specific RNA requires additional events; and (iii) that the formation of a stable sarcoma virus by recombination between the helper viral genome and part of the rescued sarcoma virus-specific RNA is a much less common event than the rescue process itself.

Two RNA-containing type C viruses with the ability to transform fibroblasts in cell culture have been isolated from rats. One of these, Kirsten sarcoma virus (KiSV), was formed by passage of Kirsten murine leukemia virus (Ki-MuLV) in Wistar-Furth (W/Fu) rats (13). The other, Harvey sarcoma virus (HaSV), was formed by passage of Moloney leukemia virus in Chester Beatty rats (10). In each case, the sarcoma virus has been found to be a recombinant between the mouse type C virus that was passaged in the rat and additional rat genetic information (1, 2, 16, 22, 25, 26). Because the gain of rat genetic information by recombination (17) was accompanied in two independent cases by acquisition of the ability to transform fibroblasts in cell culture, earlier studies have focused on the characterization of the rat genetic sequences acquired in the recombination process. These studies revealed that many preparations of rat type C viruses contain two distinct sets of RNA sequences in varying proportions (23, 24). Certain cultures, such as the RT21C rat cell line or a W/Fu rat cell line, released rat type C particles with a 50- to 100-fold excess of typical rat type C viral information as compared with the second set of sequences, namely those homologous to the Kirsten and Harvey sarcoma viruses. Other cultures producing rat type C viral particles, such as the XC or V-NRK cultures, contained approximately equal proportions of the typical rat type

C viral nucleic acid sequences and sarcoma virus-specific sequences. No cultures were found that produced a large excess of the sarcoma virus-specific sequences in the absence of the typical rat type C viral sequences (1, 23).

In additional studies, the sarcoma virus-specific sequences were found to be present in multiple copies in several uninfected rat cellular DNAs (1, 22, 23), to be inducible in certain cells with halogenated pyrimidines to high levels of expression (23), and to be able to be specifically rescued by type C viruses that were released from the appropriate rat cell cultures (9, 23, 24). Recently, Anderson and Robbins confirmed these observations and reported that a series of rat tumors had widely different levels of the two sets of sequences (1).

The current studies have quantitated in several cell lines derived from rat tumors the relative levels of the sarcoma virus-specific RNA and the typical rat type C viral RNA in an effort to gain insight into the coding properties of the sarcoma virus-specific RNA. Cell lines have been found that produce: (i) low levels of either set of sequences; (ii) high levels of both sets of sequences; (iii) high levels of the sarcoma virus-specific RNA ($sarc^{high}$) and low levels of the rat type C viral RNA ($RT21C^{low}$); and (iv) high levels of the rat type C viral RNA ($RT21C^{high}$) and low levels of the sarcoma virus-specific RNA ($sarc^{low}$). The properties of these different cell lines have been analyzed in detail, and the results indicate several important differences between $sarc^{high}$, $RT21C^{low}$ and $sarc^{low}$, $RT21C^{high}$ cell lines.

MATERIALS AND METHODS

Cells and viruses. The cells used were as follows. The normal rat kidney cell (NRK) line, derived from an Osborne-Mendel rat, has been previously described by Duc-Nguyen (5). A uterine carcinoma cell induced by 7,12-dimethylbenzanthracene treatment of a Sprague-Dawley rat has been described by Sekiya et al. (27) and was the kind gift of S. Sekiya, Chiba, Japan. He has designated this cell HTP (27), for "high tumor producing." WR123 cells, a fibroblast cell line derived from a testicular carcinoma of a Fisher rat, was the gift of Jack Parker of Microbiological Associates, Inc. This cell line was recently noted by Anderson and Robbins (1) to contain high levels of RNA homologous to KiSV. The IR162 fibroblast cell line was derived from a plasmacytoma from a Belgian rat. The plasmacytoma was the gift of Henry Metzger, National Institute of Arthritis, Metabolism, and Digestive Diseases. A fibroblast derived from a Fisher rat embryo, designated FRE clone 2, was the gift of Robert Huebner, National Cancer Institute. Two cell lines releasing rat type C virus, the RT21C cell line, derived from the thymus of an Osborne-Mendel rat, and the W/Fu cell line, derived from a Wistar-Furth rat, have been described (23, 24).

A dog thymus cell line (Cf_2th), a dog kidney culture (MDCK), and a mink lung fibroblast cell line were obtained from the American Type Culture Collection. A human tumor cell line, RD, derived from a human rhabdomyosarcoma, has been described by McAllister et al. (14). A nonproducer mink cell transformed by KiSV has been previously described (11, 24). A nonproducer dog kidney cell line transformed by HaSV virus was obtained by rescuing a rat cell nonproducer cell transformed by HaSV with an amphotropic strain of murine type C virus obtained from Janet Hartley, National Institute of Allergy and Infectious Diseases. The virus mixture released from the rat cells was transmitted to MDCK canine kidney cells, and nonproducer foci were obtained at limiting dilutions of the virus using Falcon Microtest II plates as previously described (2). This cell line is referred to as Ha-dog clone 8.

Viruses. The sources of rat type C virus used in these experiments were either the RT21C cell line, the W/Fu cell line, or the V-NRK culture (24). As previously described (23, 24), the W/Fu and RT21C cultures release approximately a 50-fold excess of typical rat type C sequences over KiSV-homologous sequences; the V-NRK culture releases approximately equal quantities of the RT21C sequences and the KiSV-homologous sequences in the supernatant fluids from this cell. The woolly leukemia virus was obtained from the supernatant fluid of RD cells or from NC37 human lymphocytes (19). An amphotropic strain of murine type C virus was obtained from Janet Hartley of the National Institute of Allergy and Infectious Diseases and is a mouse type C virus capable of growing in both NIH 3T3 cells and various nonmurine cells, and therefore has been designated as amphotropic. It was propagated in NIH 3T3 cells for preparation of the complementary DNAs (cDNAS) and in mink cells for the preparation of viral RNA. Another virus preparation, a pseudotype of KiSV, was obtained by rescuing the mink nonproducer of KiSV with a subgroup C strain of feline type C virus (FeLV) by procedures previously described (24). This virus complex, KiSV-FeLV, was used to prepare the KiSV-specific cDNA (24). The rat and mouse cells were propagated in Dulbecco's modification of Eagle medium with 10% calf serum, obtained from either GIBCO or Colorado Serum Co. The other cells were propagated in Dulbecco's modification of Eagle medium with 10% fetal calf serum, obtained from GIBCO. All cells were monitored for mycoplasma by both aerobic and anaerobic techniques and found to be free of contaminating mycoplasma species.

Preparation of cDNA probes. A tritiated cDNA probe from either the RT21C virus or the woolly leukemia virus grown in NC37 cells, or from the amphotropic murine type C virus grown in NIH 3T3 cells, was prepared from virus preparations banded one time in sucrose density gradients, under reaction conditions previously described (2, 23, 24). The cDNA specific for the rat sequences of KiSV was prepared by hydroxyapatite chromatography, also

as previously described (23). Briefly, the probe was hybridized to FeLV and Ki-MuLV 70S RNA to remove helper sequences in the virus mixture released from mink cells producing KiSV-FeLV. Tritium-labeled probes were prepared with [^{3}H]dCTP (22 Ci/mmol) at concentrations of 10^{-4} M, and unlabeled cDNA probes were prepared at concentrations of cold dCTP of 5×10^{-3} M. The tritium-labeled probes protected 60% of homologous ^{32}P-labeled RNAs at 1:1 molar ratios and 80% of homologous RNAs at 3.0:1 molar ratios.

Preparation of RNA. (i) Cellular RNA. Total cellular RNA was prepared from cells by extraction with Sarkosyl and cesium chloride as described by Glisin et al. (8). Yields of approximately 1 to 3 mg of RNA per g of packed cells were obtained.

(ii) ^{32}P-labeled viral RNA. Viral RNA labeled with ^{32}P was prepared by incubating cell cultures with carrier-free ^{32}P (New England Nuclear Corp.) in phosphate-free Dulbecco medium with 10% calf serum at 500 μCi/ml. Cultures were first incubated with the ^{32}P-containing medium for 16 h, and the supernatant fluid was discarded. Fresh ^{32}P-containing medium was added, and three collections of virus-containing fluid were harvested at 3-h intervals. Each 3-h collection was clarified at 3,000 rpm just after harvest of the fluid and stored at 4°C. The maximum time of storage at 4°C was 6 h. The ^{32}P-labeled viral particles were then concentrated by centrifugation through a 30% sucrose solution containing 0.01 M Tris-hydrochloride, pH 7.2, and 10^{-3} M EDTA onto a cushion of 60% sucrose; centrifugation was performed in an SW27 rotor at 25,000 rpm at 4°C for approximately 14 to 18 h. The virus was aspirated from the 60% cushion of sucrose, diluted approximately fourfold with a 0.01 M Tris-hydrochloride, pH 7.2, and 10^{-3} M EDTA, and concentrated again by centrifugation at 100,000 $\times$ g for 1.5 h at 4°C. The pelleted virus was disrupted at room temperature with 1.0 ml of a solution containing 1% sodium dodecyl sulfate (SDS) and 0.2% (vol/vol) diethylpyrocarbonate in 0.01 M Tris-hydrochloride pH 7.2. The disrupted virus was applied to a 15 to 30% sucrose gradient containing 0.01 M Tris-hydrochloride, pH 7.2, and 10^{-3} M EDTA and centrifuged at 39,000 rpm in an SW41 rotor for 2 h at 15°C. The 70S RNA was collected by puncturing the tubes from below and collecting approximately 25 equal fractions. The 70S RNA consistently migrated in fractions 8 through 12 in the gradient. For preparation of 35S RNA in sucrose gradients, the 70S RNA from the initial sucrose gradient was precipitated at −20°C with 2 volumes of ethanol in the presence of 15 μg of yeast RNA per ml. The ethanol-precipitated RNA was collected by centrifugation at 27,000 rpm for 30 min at 4°C and redissolved in a buffer containing 0.01 M Tris-hydrochloride, pH 7.2, and 0.01 M EDTA. The RNA solution was heated for 2 min at 80°C and recentrifuged through a 15 to 30% sucrose gradient for 6 h at 39,000 rpm at 15°C. Under these conditions, the 35S RNA was in fractions 9 through 13 out of the 25 fractions in the sucrose gradient. [^{3}H]uridine-labeled RNA was prepared identically except that cultures were labeled with 100 μCi of [5,6-^{3}H]uridine per ml (specific activity, 50 Ci/mmol).

(iii) Viral RNA. Unlabeled 70S viral RNA was prepared by disruption of virus preparations with 1% SDS and centrifugation in 15 to 30% sucrose gradients by the same procedures as used for labeled viral RNA.

Polyacrylamide gel electrophoresis. Acrylamide gel electrophoresis of ^{32}P-labeled viral RNA was carried out in agarose-acrylamide gels (4, 20) containing 2.2% (wt/vol) acrylamide and 0.5% (wt/vol) agarose; the acrylamide solution was a 20:1 ratio of acrylamide and biacrylamide. The electrophoresis buffer contained 0.089 M Tris-hydrochloride, pH 8.3, 0.0025 M EDTA, and 0.089 M boric acid. Samples were applied to 100-cm gels in 0.10 ml which contained: the labeled viral RNA; 12% (wt/vol) sucrose; the electrophoresis buffer noted above; and 0.03% (wt/vol) bromophenol blue as marker. The gels were electrophoresed at 4°C for approximately 4 to 4.5 h at 3 mA/gel. Slices of 1 mm in thickness were cut and dissolved at 60°C for 16 h in 0.40 ml of 30% hydrogen peroxide. The dissolved slices were counted with 10 ml of Aquasol (New England Nuclear Corp.). For recovery of the RNA from gels, the disrupted gel slices were eluted instead at 55°C for 16 h in a solution containing 0.01 M Tris-hydrochloride, pH 7.5, 0.4 M lithium chloride, 10^{-3} M EDTA, and 0.2% (vol/vol) SDS (16). The eluted samples were passed through a Gelman filter to remove solid acrylamide and then passed over a G-25 medium Sephadex column equilibrated in distilled water to remove water-soluble acrylamide. Samples were then lyophilized before use. Recovery of counts by either the hydrogen peroxide or the lithium chloride elution was over 60% of the counts initially applied to the gel.

Preparation of polysomes. Polysomes from HTP cells were prepared by a modification of procedures previously described (7). Cells were scraped into cold phosphate-buffered saline and washed three times by resuspension and centrifugation in phosphate-buffered saline. They were then suspended in a buffer containing 0.025 M Tris-hydrochloride pH 7.6, 0.025 M sodium chloride, 0.005 M magnesium chloride, and 0.14 M sucrose (polysome buffer). Approximately 5 to 7 volumes of this buffer was used per ml of packed cells. To the cell suspension, both Triton X-100 and deoxycholate were added to a final concentration of 0.7% (vol/vol), and the cells were then homogenized with six strokes in a loose-fitting Dounce homogenizer. Nuclei were removed by centrifugation at 5,000 rpm for 5 min at 4°C, and 6.0 ml of the polysomes was collected by centrifugation in an SW41 rotor at 39,000 rpm for 16 h through a 4.0-ml column of 1.0 M sucrose onto a 2.0-ml 2.5 M sucrose cushion prepared in polysome buffer. The polysomes were aspirated from the 2.5 M sucrose cushion and passed over a Sepharose 4B column equilibrated in polysome buffer without sucrose in order to remove the sucrose. They were then spun in a 10 to 50% (wt/vol) sucrose gradient in an SW41 rotor at 15,000 rpm at 4°C for 16 to 18 h. Fractions of 0.5 ml were collected by puncturing the tubes from below. The optical density of each fraction was determined, and appropriate fractions were pooled as indicated later in Results. For treatment with EDTA, the Sepharose 4B fractions were first treated at 4°C for 10 min with 10^{-2} M EDTA and then spun

on the 10 to 50% sucrose gradient with parallel control preparations.

Radioimmunoassay of viral proteins. The p30 proteins of either RT21C virus or the Moloney strain of mouse type C virus, or the p12 protein of the RT21C rat virus, were purified by procedures previously described (19, 24). The proteins were iodinated by the chloramine-T method to specific activities of between 5×10^3 to 2×10^4 cpm/μg of protein. Double-antibody radioimmunoassays were performed in 0.50-ml reaction mixtures containing: 0.01 M potassium phosphate buffer, pH 7.4; 0.01% (vol/vol) Triton X-100 (TP buffer); 0.01 M EDTA; 0.02% (vol/vol) normal goat serum; and 200 μg of bovine serum albumin per assay. All components used in the assay were diluted in TP buffer before use. The assay was initiated by adding to each tube 0.15 ml of TP buffer and 0.05 ml of unlabeled antigen followed by 0.10 ml of a primary antibody, noted in the appropriate legends. The primary antibody and putative competing antigen were incubated in this 0.3-ml volume at 37°C for 2 h. A 10-ml aliquot of the iodinated antigen was then added to deliver approximately 50,000 cpm per assay. Reaction mixtures were then incubated for an additional hour at 37°C. The last addition of 0.10 ml of second antibody was then made; the second antibody used in these experiments was pig anti-goat serum used at a 1:8 dilution in all assays. The reaction was incubated for an additional hour at 37°C and then at 4°C for 12 to 16 h. The precipitate was recovered by centrifugation at 2,500 rpm at 4°C for 20 min. The supernatant was aspirated, and the pellets were washed at 4°C in buffer containing 0.1% Triton X-100, 0.05% (vol/vol) deoxycholate, 0.05 M Tris-hydrochloride, pH 7.5, 0.10 M sodium chloride, 10^{-3} M EDTA, and 0.05% (vol/vol) normal rabbit serum. The washed pellets were recentrifuged at 2,500 rpm at 4°C for 20 min, and the pellets were counted in an LKB gamma counter. Competition assays were performed at dilutions of primary antibody that precipitated approximately 20 to 30% of a given iodinated antigen. The dilutions of primary antibody used against the different viral antigens are indicated in the legends to Fig. 2 and Fig. 3.

Hybridization reactions. RNA-[^{3}H]DNA hybridization reactions were performed by procedures previously described and assayed with the use of S1 nuclease (2). Details are given in the appropriate legends. Hybridization kinetics were analyzed as a function of RNA concentration and time and expressed as C_rt (moles·second per liter) as suggested by Birnsteil et al. (3). [^{3}H]RNA-DNA hybridization experiments were carried out by procedures previously described with 0.3 to 1.0 ng of ^{32}P-labeled viral RNA with specific activity of 2×10^6 to 4×10^6 cpm/μg and with 100 ng of unlabeled cDNA. Hybridization was also analyzed by using S1 nuclease as fully detailed previously (24).

Hybridization to viral RNA in the supernatant fluids from cell cultures was carried out as previously detailed and analyzed as a function of V_0t (milliliters × hour) as suggested by Ringold et al. (21). To ensure the comparability of experiments, identical numbers of cells were seeded in comparisons between supernatant fluids from different cells.

RESULTS

Cell lines with high KiSV-homologous RNA and low rat type C viral RNA. Several rat cells were examined for relative levels of RNA homologous to KiSV or RT21C rat virus. Three cell lines derived from different strains of rats were found to contain high levels of RNA homologous to KiSV and low levels of RNA homologous to RT21C rat virus. The results of RNA-[^{3}H]DNA hybridization experiments performed on the total cellular RNA from such cells are shown in Fig. 1. In Fig. 1A, a uterine carcinoma cell, designated HTP, was analyzed for the relative levels of the two sets of sequences. Only low levels of the RT21C-like sequences were found in these cell lines, and the $^1/_2C_rt$ was not achieved at levels of greater than 10^3 mol·s per liter. In contrast, the cells contained very high levels of KiSV-homologous sequences, with a $^1/_2C_rt$ value of approximately 7×10 to 8×10 mol·s per liter. Similarly, a cell line derived from a testicular carcinoma of a Fisher rat, WR123, and a cell line derived from a plasmacytoma of a Belgian rat had low levels of the RT21C sequences and high levels of the sarcoma virus-specific sequences. The $^1/_2C_rt$ value for the KiSV-homologous sequences in WR123 cells was approximately 100 to 200 mol·s per liter, and for the IR162 cell it was approximately 200 mol·s per liter. In contrast and as a reference for these cells, the results with RT21C cells are shown in Fig. 1B. As previously reported, these cells had low levels of the sarcoma virus-specific sequences but high levels of the typical rat type C viral nucleic acid sequences. Importantly, the $^1/_2C_rt$ value for the typical rat type C viral nucleic acid sequences in these cells was approximately 100 to 200 mol·s per liter, a value in close agreement with the values for the sarcoma virus-homologous RNA found in the HTP, WR123, and IR162 cells. In fact, the levels of the sarcoma virus-specific RNA in HTP cells were slightly greater than the levels of the typical rat type C viral sequences in RT21C cells.

Protein analysis of cell lines. The same four cell lines were analyzed for various structural proteins of mammalian type C viruses by competition radioimmunoassays (Fig. 2 and 3). In Fig. 2, the various rat cells were analyzed in competition radioimmunoassays in three separate radioimmunoassays for the p30 protein. In Fig. 2A, an assay is shown that demonstrates the radioimmunoassay between the iodinated rat p30 and a goat serum prepared against RT21C rat virus. In Fig. 2B, an interspecies assay is shown between an iodinated mouse p30 protein and a goat serum prepared against FeLV (19). In Fig. 2C, an assay is shown be-

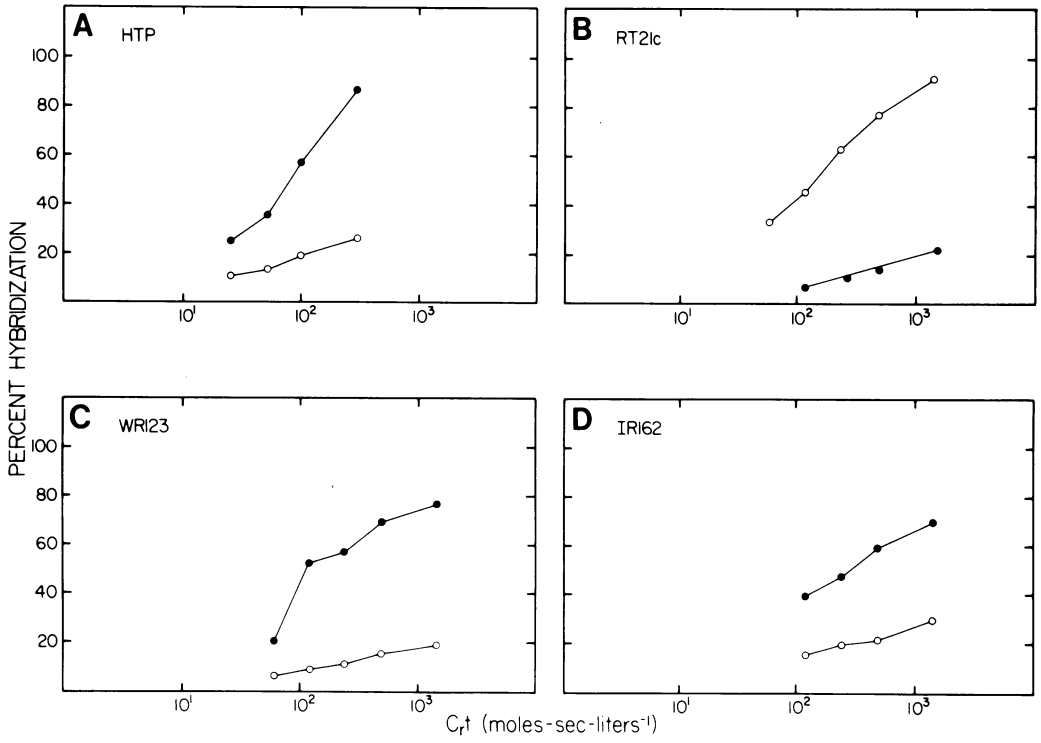


FIG. 1. *Hybridization to various rat cell RNAs with cDNA from RT21C or Kirsten sarcoma virus. Each hybridization reaction was incubated at 66°C for varying periods of time and contained in 0.05 ml: 0.02 M Tris-hydrochloride, pH 7.5, 0.6 M sodium chloride, 10^{-4} M EDTA, 0.05% SDS, 1.0 μg of calf thymus DNA, and 100 μg of each of the indicated cellular RNAs. Reaction mixtures were incubated for varying periods of time, and hybridization was assessed with the use of S1 nuclease as previously described (2). Reactions contained approximately 1,500 trichloroacetic acid-precipitable cpm of either [^{3}H]cDNA, and background in the absence of RNA was less than 75cpm. One hundred percent hybridization with each probe was approximately 1,200 acid-precipitable cpm, and all values have been normalized to this 100%. Preparation of the probe is detailed in Materials and Methods. (A) Uterine carcinoma cell line, designated HTP (27); (B) RT21C cells; (C) WR123 Fisher rat cells; (D) IR162 Belgian rat cells. Symbols: (○) RT21C probe; (●) Kirsten sarcoma virus-specific probe.*

tween iodinated mouse p30 protein and a goat serum prepared against RD114 endogenous feline virus. In each assay, RT21C cells were found to contain high levels of immunoreactive protein. Positive reactions in each assay were obtained with as little as 0.5 to 1 μg of cellular protein, and complete displacements were obtained over a range of 10 to 100 μg of protein in the assays. In the three cell lines HTP, WR123, and IR162, virtually no detectable p30 protein could be found even with up to 100 μg of cellular protein in the assay. Thus, the three cell lines with the high levels of this sarcoma virus-specific RNA had at least a 100-fold deficiency in immunoreactive p30 protein compared with RT21C cells. The results are in contrast to the comparable C_rt values for the respective virus-specific RNAs in these cells.

In Fig. 3, a similar analysis was performed on these four cell lines, using the p12 protein from the RT21C rat type C virus. Again, the RT21C cell line (and in other studies the W/Fu cell line) reacted well in this assay. However, neither of the rat cell lines containing the high levels of the KiSV-specific RNA was reactive in this assay. In addition, neither a mink cell transformed by KiSV nor a dog cell transformed by HaSV was found to contain detectable levels of rat p12 protein. The results indicate that the rat cell lines producing low levels of the typical rat type C viral RNA and high levels of the sarcoma virus-specific RNA do not contain either immunoreactive mammalian type C viral p30 protein or rat type C viral p12 protein.

Analysis of polysomes in HTP cells. Since HTP cells were found to contain high levels of the sarcoma virus-specific RNA and yet no de-

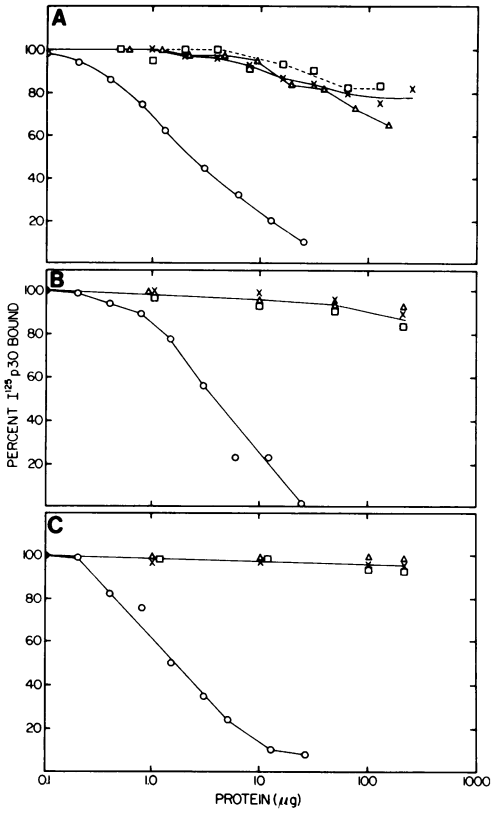


FIG. 2. *Radioimmunoassay of rat cells for mammalian p30 antigen. (A) ^{125}I-labeled rat type C viral p30 and a 1:3,200 dilution of goat serum prepared against RT21C rat virus. Sensitivity of assay for 10% competition was 0.5 ng of rat p30. (B) ^{125}I-labeled mouse (Moloney) type C viral p30 and a 1:25,600 dilution of goat serum made against feline leukemia virus (19). Sensitivity of assay for mouse, cat, and rat p30 assay for 10% competition was 1.0 ng and for woolly p30, 2.0 ng. (C) ^{125}I-labeled mouse type C viral p30 and a 1:6,400 dilution of a goat serum prepared against RD114 virus. Sensitivity of assay for RD114 or baboon viral p30 was 0.5 ng. Sensitivity for mouse, cat, or rat p30 was 1.0 to 2.0 ng. Symbols: (○) RT21C cells; (△) HTP cells; (×) IR162 cells; (□) WR123 cells.*

tectable immunoreactive proteins, the RNA from these cells was analyzed to see whether it was present on polysomes in HTP cells. In parallel experiments, the RNA from RT21C cells was analyzed to see whether it was on polysomes. A typical polysome profile prepared without and with treatment with EDTA is shown in Fig. 4. A broad peak of optical density was seen in the dense region of the sucrose gradient; this peak disappeared after the polysomal preparation was treated with EDTA (or, in experiments not shown, with puromycin). The optical density seen in fractions 6 through 14 of Fig. 3 moved to the top of the tube in fractions 21 through 26.

The RNA from fractions 6 through 14 in the control gradient and in the EDTA-treated gradient, and the RNA in fractions 21 through 26 from each gradient, was analyzed with the sarcoma virus-specific cDNA for homologous sequences. The results of this hybridization analysis are shown in Fig. 5 and plotted as a function of RNA concentration and time. Fractions 6 through 14 from the control gradient had high levels of the sarcoma virus-specific RNA with a $^1/_2C_rt$ value of approximately 100 mol·s per liter. In the gradient treated with EDTA, a drastic reduction in the levels of the sarcoma virus-

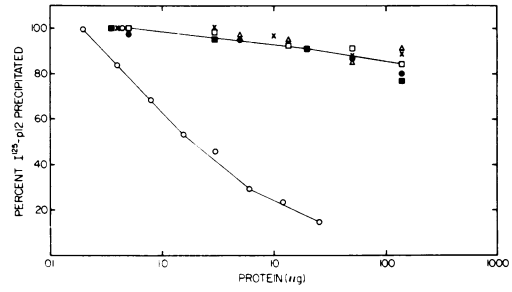


FIG. 3. *Immunoassay of rat cells for RT21C p12 protein. Each reaction contained 50,000 cpm of ^{125}I-labeled p12 protein and a 1:400 dilution of a goat serum prepared against RT21C virus. The sensitivity of the assay for 10% competition was 1.0 to 2.0 ng of p12 protein. Symbols: (○) RT21C cells; (△) HTP cells; (×) IR162 cells; (□) WR123 cells; (●) Ki-mink nonproducer cells; (■) Ha-dog nonproducer cells.*

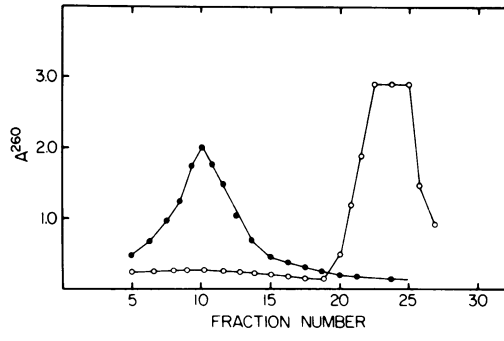


FIG. 4. *Preparation of polysomes from HTP cells. Polysomes were prepared from HTP cells as detailed in Materials and Methods. Symbols: (●) Polysome profile in the absence of EDTA; (○) profile after treatment of the preparation with EDTA. Fractions from 6 through 14 and 21 through 26 were pooled from each gradient and analyzed for hybridization in Fig. 5.*

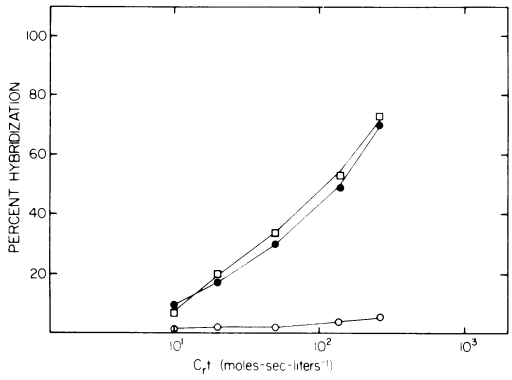


FIG. 5. *Hybridization to polysomal RNA from HTP cells. Each hybridization reaction of 0.05 ml was incubated at 66°C for varying periods of time and contained in 0.05 ml of ionic ingredients indicated in the legend to Fig. 1 and approximately 2.0 μg of RNA from each of the regions of the polysome gradient indicated in Fig. 4. Reactions were incubated for varying periods of time and analyzed for hybridization with S1 nuclease as previously described, using [^{3}H]cDNA specific for the rat sequences of Kirsten sarcoma virus as detailed in the legend to Fig. 1. Symbols: (●) Fractions 6 through 14 from the control HTP gradient; (□) fractions 21 through 26 from the plus-EDTA gradient; (○) fractions 6 through 14 from the plus-EDTA gradient. Fractions 21 through 26 from the control gradient gave no hybridization, and thus this symbol is omitted from the graph.*

specific RNA in this region of the gradient was seen. All of the hybridizable RNA was now recovered at the top of the gradient. Similar results were also seen if the polysomes from HTP cells were treated with puromycin instead of EDTA. The results indicate that the sarcoma virus-specific RNA in HTP cells is present on polysomes at high levels in the cells. In comparative experiments on HTP and RT21C cells, approximately 7% of the total hybridizable RNA in each cell was present on polysome preparations in each cellular extract. Thus, the results indicate that equal quantities of typical rat type C viral RNA are present on the polysomes of RT21C cells and sarcoma virus-specific RNA on the polysomes of HTP cells. The results suggest that the inability to detect immunoreactive type C viral proteins in the HTP cells is not due to an absence of the sarcoma virus-specific RNA from polysomes.

Analysis of supernatant fluids from RT21C and V-NRK cells. To further characterize the sarcoma virus-specific RNA, the RT21C culture and the V-NRK culture were analyzed for the size of the species of RNA detected in the supernatant of each of these cells. Intracellular sizing of these sequences (1, 28) had given heterogeneous profiles, presumably due to difficulties with cellular RNase. Therefore, 70S viral RNA labeled with [^{32}P]- or [^{3}H]uridine was prepared from each culture as detailed in Materials and Methods. This RNA was heat denatured and subjected to agarose-acrylamide gel electrophoresis (Fig. 6). The RT21C culture gave one distinct peak of tritium counts at a position migrating slightly faster than a 35S marker of Moloney leukemia viral RNA. In contrast, the V-NRK culture showed two resolvable peaks of radioactivity in the gel. One of these peaks was the same size as the peak in the RT21C culture, and the other peak was somewhat smaller.

To further identify the nature of the RNA species in each peak, the remainder of the RNA from the two peaks was hybridized to a cDNA probe prepared to either RT21C virus or KiSV as indicated in Table 1. Pool 1, representing the left-hand shoulder of peak 1 in the V-NRK culture, hybridized to the RT21C probe but not to the KiSV-specific probe. In contrast, pool 2 hybridized to the KiSV-specific probe. Because of trailing from peak 1, pool 2 also hybridized somewhat to the RT21C probe. The results indicate that peak 1 of the V-NRK culture con-

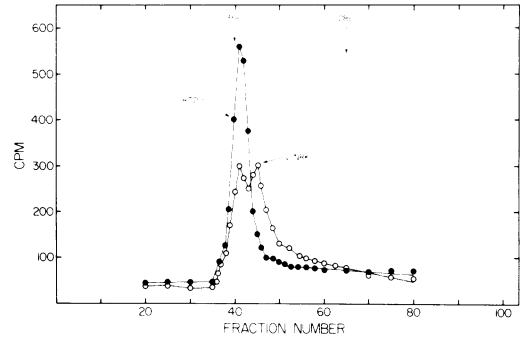


FIG. 6. *Gel electrophoresis of RT21C and V-NRK viral RNA. Polyacrylamide agarose gel electrophoresis was carried out as detailed in Materials and Methods. Approximately 25,000 cpm of [^{3}H]uridine-labeled 70S RNA of RT21C virus (●) was applied to the gel with 25,000 cpm of [^{32}P]RNA of V-NRK 70S RNA (○). Electrophoresis and elution of counts from gel slices was carried out as detailed in Materials and Methods. A 0.10-ml aliquot out of 1.0 ml of the elution buffer was sampled to locate the counts. Recovery of ^{32}P and ^{3}H counts from the gel was approximately 60% of the input counts. Parallel gels contained ^{32}P-labeled 70S RNA from Moloney leukemia virus co-mixed with [^{3}H]uridine-labeled rRNA to provide the markers indicated. In other experiments the Moloney viral RNA was found to be slightly larger than the RT21C viral RNA. Pool 1 represents fractions 38 through 40, and pool 2 represents fractions 46 through 50.*

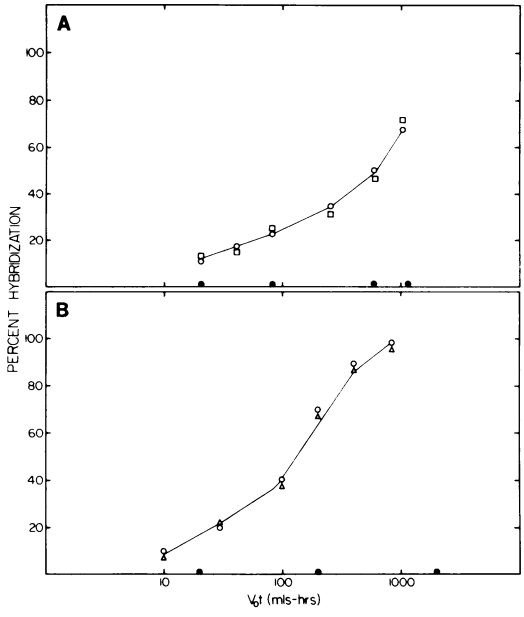
TABLE 1. *Hybridization to two peaks of V-NRK RNA*[a]

[32P]RNA	cpm hybridized			
	Input cpm	−cDNA	+cDNA	
			RT21C	KiSV
Pool 1 (fractions 38-40)	209	19	85	10
		20	95	18
Pool 2 (fractions 46-50)	207	14	44	50
		10	36	48

[a] Each reaction mixture was incubated at 66°C for 24 h and contained in 0.05 ml: 0.02 M Tris-hydrochloride, pH 7.2, 0.75 M sodium chloride, 10^{-4} M EDTA, 0.05% SDS, 1.0 μg of calf thymus DNA, 10 μg of yeast RNA, the indicated input of [32P]RNA, and 100 ng of cDNA prepared either to the RT21C type C virus or to the feline type C pseudotype of KiSV grown in mink cells as detailed in Materials and Methods. Hybridizations were analyzed with the use of S1 nuclease by procedures previously described (2).

tained only the RT21C sequences and peak 2 contained the sequences homologous to KiSV.

RNA rescued with heterologous type C viruses. To further identify the size of the KiSV-specific RNA, two of the cell lines indicated in Fig. 1, namely the HTP cell and the WR123 cell lines, were superinfected with type C viruses not of rat origin. In the case of WR123 cells, an amphotropic strain of mouse type C virus was used because it grew readily in these cells, and in the case of HTP cells a strain of woolly leukemia virus was used because it grew readily in HTP cells. The supernatants from the uninfected and infected cells were analyzed for sarcoma virus-specific RNA to ascertain whether particles containing the RNA were being produced from these cells (Fig. 7A, B). The uninfected WR123 cells failed to release sarcoma virus-homologous RNA sequences into the supernatant even at V_0t values of 1,000 to 2,000 ml × h. In contrast, cells superinfected



The pellets were extracted by phenol and chloroform extraction for putative viral RNA in the presence of 50 μg of yeast carrier RNA per ml (19). The extracted RNA was dialyzed extensively against 0.01 M Tris-hydrochloride, pH 7.2, and concentrated by lyophilization to 1.0 ml. Aliquots of 0.05 ml of each RNA were then assayed for hybridization for varying periods of time under conditions as indicated in the legend to Fig. 1. Each reaction mixture contained approximately 1,500 trichloroacetic acid-precipitable cpm of either the cDNA prepared to the amphotropic strain of murine type C virus grown in NIH 3T3 cells or the woolly leukemia virus grown in NC37 cells, or the KiSV-specific probe. The KiSV-specific probe had less than 3% hybridization with either amphotropic viral RNA or with woolly leukemia viral RNA; conversely, the woolly virus cDNA and the amphotropic viral cDNA had less than 5% hybridization with the cellular RNA from either WR123 or HTP cells compared with hybridization with their homologous RNAs. One hundred percent hybridization values were estimated for the amphotropic virus with RNA from that virus grown in a mink lung cell; 100% hybridization for the woolly viral probe was obtained with RNA from the human lymphocyte NC37 infected with woolly virus, and 100% hybridization for the KiSV-specific probe was with cellular RNA from either WR123 or HTP cells. (A) WR123 cells. Symbols: (●) Supernatant from WR123 uninfected cells versus KiSV cDNA; (○) supernatant from WR123 culture superinfected with amphotropic virus versus KiSV cDNA; (□) supernatant from WR123 cells superinfected with amphotropic virus versus amphotropic viral cDNA. (B) HTP cells. Symbols: (●) Supernatant fluid from uninfected HTP cells versus KiSV cDNA; (○) supernatant fluid from HTP cells superinfected with the woolly type C virus versus KiSV cDNA; (△) supernatant fluid from superinfected HTP cells versus woolly viral cDNA.

FIG. 7. *Hybridization to supernatant fluids from uninfected and infected Fisher rat and Sprague-Dawley rat cells. WR123 Fisher rat cells and HTP uterine carcinoma cells were superinfected with an amphotropic stain of murine type C virus and the woolly type C virus, respectively, as detailed in Materials and Methods. After the cells had been grown for approximately 2 weeks, the uninfected and infected cultures were seeded in 75-cm² Falcon flasks at approximately 5 × 10⁶ cells per flask. Fresh medium was added, and 24 h later approximately 600 ml of supernatant fluid was collected from each of the cultures. The supernatant fluid was clarified at 3,000 rpm immediately upon harvest and pelleted at 100,000 × g at 4°C for 2 h to pellet viral particles.*

with the amphotropic strain of murine type C virus had readily detectable levels of sarcoma-specific RNA even at V_0t values of 10 ml × h. Importantly, the producing culture contained equal quantities in the supernatant fluid of the mouse type C viral sequences and the sarcoma virus-homologous sequences.

The supernatant from the HTP cells, uninfected and infected with woolly leukemia virus, was similarly analyzed (Fig. 7B). Again, no viral RNA was detected in supernatants from the uninfected HTP cells even at high V_0t values. Again, the supernatant from the superinfected cell contained both the woolly leukemia sequences and the rescued sarcoma virus-homologous sequences. As in the WR123 culture, the woolly virus-producing HTP cells released approximately equal amounts of the woolly viral RNA and the sarcoma virus-specific RNA into the supernatant from the infected cells. Thus, neither the uninfected HTP cells nor the uninfected WR123 cells released the sarcoma virus-specific RNA in type C particles into the supernatant unless they were producing other type C viruses.

Ratios of helper sequences and rescued rat sequences. To further characterize the RNA rescued from Fisher rat cells with the amphotropic strain of murine type C virus, ^{32}P-labeled 70*S* viral RNA was prepared from the amphotropic virus grown in mink cells and from the amphotropic virus grown in WR123 cells. Each of these RNAs was heat denatured and run in agarose-acrylamide gels for preparation of the viral subunits. In repeated studies not shown, we were unable to separate in 2.2 or 2.4% agarose-acrylamide gels the subunit of the amphotropic virus itself from the subunit of the rescued rat sequences even if the 70*S* RNA was denatured and applied in 50% formamide. Also, when gels were run for shorter times, no additional peaks were seen between 35*S* and 10*S* on the gels. Therefore, to prove that the 35*S* subunit obtained from the gels contained the rat sequences, we analyzed both the 70*S* RNA and the 35*S* RNA for rat-related sequences by [^{32}P]RNA-excess DNA hybridization. To do this, we prepared a cDNA from the amphotropic virus grown in NIH mouse cells and a cDNA probe containing the sarcoma virus-related sequences from the V-NRK culture. Since the RT21C component of the V-NRK culture has no homology to mouse type C virus (24), we could analyze directly with this cDNA the [^{32}P]RNA prepared from the amphotropic virus grown in mink cells or the amphotropic virus grown in the sarc^{high},RT21C^{low} cells, WR123 (Table 2). The [^{32}P]RNA from the amphotropic virus grown in mink cells failed to hybridize the

TABLE 2. *Hybridization to 35S and 70S RNA from WR123 Fisher rat cells infected with mouse type C virus*[a]

RNA	cpm			
	Input	Hybridized		
		−cDNA	+cDNA	
			V-NRK	AMT
70*S*				
AMT mink	1,100	32	50	912
		44	46	880
AMT WR123	1,000	56	407	410
		65	423	444
35*S*				
AMT mink	1,000	30	48	760
		40	53	837
AMT WR123	1,100	48	388	369
		42	435	406

[a] Each hybridization reaction was incubated at 66°C for 24 h and contained in 0.05 ml: the ionic ingredients indicated in the legend to Table 1, the input ^{32}P-labeled viral RNA indicated in the table, and 100 ng of cDNA prepared from an endogenous reaction of the V-NRK viruses or amphotrophic murine type C virus as detailed in Materials and Methods. Hybridization was monitored with the use of S1 nuclease procedures previously described (2). The 35S and 70S RNAs were prepared as detailed in Materials and Methods and in Fig. 5. The hybridization values indicated in the table are individual determinations performed in duplicate in the same experiment. AMT mink refers to the amphotropic virus growing in mink cells, and AMT WR123 refers to that virus growing in the Fisher rat cell line WR123.

V-NRK cDNA containing the sarcoma virus-homologous sequences. In contrast, approximately 41% of the counts of the ^{32}P-labeled 70*S* RNA prepared from the amphotropic virus grown in the WR123 culture hybridized to the V-NRK cDNA probe. Furthermore, approximately 45% of the same RNA hybridized to a cDNA prepared to the amphotropic virus grown in NIH mouse cells. Thus, the proportion of the mouse and sarcoma virus RNA in the ^{32}P-labeled 70*S* RNA was approximately 1:1 as determined by excess DNA versus [^{32}P]RNA hybridization experiments. This result is in excellent agreement with the V_0t analysis shown with excess RNA and limiting [^{3}H]DNA hybridization experiments shown in Fig. 7. Furthermore, the 30–35*S* RNA subunit obtained by agarose-acrylamide gel analysis from the amphotropic virus produced in WR123 cells showed hybridization results identical to those obtained with the 70*S* viral RNA prepared from the virus produced from the cells. The results indicate that the virus produced by the Fisher rat cells infected with amphotropic mouse type

C virus contains a 1:1 ratio of sarcoma virus-specific and mouse viral sequences, and that the rat sequences are contained in a viral RNA the same size as the helper RNA as determined by agarose-acrylamide gel analysis.

Reverse transcription of rescued rat sequences. To further evaluate the ratio of the rat and mouse sequences from Fisher rat cells superinfected with amphotropic virus and HTP cells superinfected with woolly helper virus, the virus preparations prepared from these cells were used to prepare tritiated cDNA's in endogenous reverse transcriptase reactions. The probes from these endogenous reactions and a probe from each helper virus grown in non-rat cells were analyzed by tritiated DNA-excess RNA hybridization until saturating values of hybridization were achieved with various viral 70S RNAs (Table 3). The cDNA from the amphotropic virus grown in NIH cells hybridized well to its homologous 70S RNA but did not hybridize to detectable levels to RNA from either the woolly virus grown in human cells or the RNA from V-NRK or RT21C virus. In contrast, the probe prepared from the amphotropic virus grown in Fisher rat cells hybridized equally well to the amphotropic viral RNA and the RNA from the V-NRK culture.

Similar results were also obtained with probe prepared from the woolly virus grown in HTP rat carcinoma cells. The probe from this viral preparation contained approximately equal quantities of rat and woolly virus information at saturation, whereas the woolly virus grown in human cells contained no detectable hybridization to the rat viral RNA. Thus, the results indicate (i) that virus preparations prepared from WR123 and HTP cells contain equal quantities of the helper RNA and the rescued sarcoma virus-specific RNA in these particles, and (ii) that the cDNA probes in vitro prepared from these particles also contain equal quantities of the helper sequences and the rescued rat sequences. We cannot be certain, however, whether the two RNAs are contained as heterodimers in single virus particles or homodimers in separate particles, or whether any recombination between the RNAs has occurred.

TABLE 3. *In vitro reverse transcription of rescued rat sequences from Fisher or Sprague-Dawley rat cells*[a]

[^{3}H]cDNA from virus	Cell grown in:	Percent hybridization to 70S RNA from:			
		Woolly RD	AMT mink[a]	V-NRK	RT21C
AMT	NIH 3T3 (mouse)	<5	95	<3	<3
AMT	WR123 (rat)	<5	48	52	<10
Woolly	NC37 (human)	93	<5	<3	<3
Woolly	HTP (rat)	45	<5	45	<10

[a] Each hybridization reaction was performed with approximately 2,500 trichloroacetic acid-precipitable cpm of tritium-labeled cDNA from virus preparations indicated in the table. Hybridizations were carried out to C_rt values of 5 $\times 10^{-1}$ mol·s per liter with the indicated 60–70S viral RNA preparations and analyzed with S1 nuclease as described in Materials and Methods.

[b] AMT, Amphotropic.

An attempt was made to determine whether the rat sequences could be transmitted from either culture to a non-rat cell culture. The amphotropic viral preparation with the 1:1 ratio was transmitted to mink cells, which are highly permissive for growth of the amphotropic virus, and the woolly viral preparation was transmitted to RD cells, which are highly permissive for it. In each case, after approximately 12 cell passages, RNA homologous to the helper virus could easily be detected at a C_rt value of 10 mol·s per liter in infected cultures. However, in neither case could we detect sarcoma virus-specific RNA in the transmitted cells at C_rt values of 20,000 mol·s per liter, even though the virus preparations used in the transmission contained equal quantities of helper RNA and rescued sarcoma virus-specific RNA and even though in in vitro reverse transcriptase reactions equal proportions of the helper virus and sarcoma virus-homologous RNA were transcribed.

Summary of properties of rat cells. A summary of the properties of the rat cells with varying levels of expression of endogenous rat RNAs is shown in Table 4. As indicated earlier, RT21C and W/Fu cells contain high levels of RNA homologous to typical rat type C virus, produce abundant structural proteins of rat type C viruses, and produce type C particles in the supernatant of such cells containing viral reverse transcriptase and virus-specific RNA. The four cell lines HTP, WR123, IR162, and, as previously described, NRK (24) contain high levels of the sarcoma virus-specific RNA and low levels of the typical rat type C viral RNA. None of these cell lines produce particles in the supernatant of such cells as determined by V_0t analysis, nor do they synthesize intracellularly structural proteins immunoreactive with either rat virus or p30 protein of any known mammalian type C virus. A Fisher rat embryo cell line, FRE clone 2, used as a control for these experiments, produced low levels of either RNA and did not produce either particles or rat viral structural proteins. The V-NRK culture, as previously described, contains high levels of both sequences and produces particles and viral structural proteins. Thus the simple presence of the KiSV-specific RNA in rat cells does not preclude those cells from also producing typical rat type C viral particles.

TABLE 4. *Properties of rat cells with varying levels of RNAs homologous to RT21C virus or Kirsten sarcoma virus*[a]

Cell line	Supernatant type C particles	Type C viral structural proteins		RNA homologous to:	
		p30	p12	RT21C	Ki-SV
RT21C	+	+	+	High	Low
W/Fu	+	+	+	High	Low
HTP	−	−	−	Low	High
WR123	−	−	−	Low	High
IR162	−	−	−	Low	High
NRK	−	−	−	Low	High
FRE clone 2	−	−	−	Low	Low
V-NRK	+	+	+	High	High

[a] This table summarizes the hybridization and radioimmunoassay data presented in earlier tables and figures. The designation "high" refers to $^1/_2C_rt$ values between 80 and 160 mol·s per liter; the designation "low" refers to $^1/_2C_rt$ values of $>2 \times 10^3$ mol·s per liter. The designation "p30+" or "p12+" refers to at least a 20% competition in the radioimmunoassay with less than 50 μg of cellular protein in assays that can detect 1 ng of the designated viral structural proteins. The designation "supernatant type C particles" is defined in two ways: (i) as V_0t hybridization analysis for sarcoma virus sequences as presented in Fig. 7; and (ii) as sedimentable viral reverse transcriptase in the supernatant. In reverse transcriptase assays, approximately 10^6 RT21C or W/Fu cells were fed with 5 ml of culture fluid for a 24-h harvest. The medium was removed, clarified at 3,000 rpm for 10 min and sedimented for 2 h at 100,000 × *g*. The pellet was dissolved in 0.10 ml of 0.05 M Tris-hydrochloride, pH 7.5, 0.1 M sodium chloride, 1 mM dithiothreitol, and 0.1% Triton X-100. An aliquot of 0.01 ml was assayed for reverse transcriptase with poly(rA)·oligo(dt_{12-18}) as template as previously described with manganese as divalent cation (23). Positive cultures incorporated approximately 100 to 300 pmol of [^{3}H]TMP per ml of fluid in a 60-min reaction; negative cultures incorporated less than 3 pmol. No reverse transcriptase was detected in the negative cultures if they were assayed with magnesium as divalent cation.

DISCUSSION

Previous studies have indicated that the Kirsten and Harvey strains of murine sarcoma virus arose by recombination between mouse helper type C viruses and a novel type of rat genetic information (1, 16, 22–26, 28). A plausible hypothesis has seemed to be that the acquired rat genetic information coded for the function(s) that endowed these viruses with the ability to produce malignant transformation (1, 26). Thus, characterization of the properties of the rat-derived RNA and of cells producing the endogenous sarcoma virus-specific RNA has been undertaken. Earlier studies have indicated that the sarcoma virus-specific RNA shared three properties with known mammalian type C viruses: (i) presence in multiple copies in uninfected rat cellular DNA; (ii) inducibility with halogenated pyrimidines; and (iii) ability to be specifically rescued by type C viral particles produced from cells expressing the sarcoma virus-specific RNA (9, 23). These and earlier studies (1, 28) indicate that the size of the sarcoma virus-specific RNA is comparable to that of the subunit of the RT21C rat type C virus or the subunit of an amphotropic strain of mouse type C virus. Thus, by a fourth criterion, size, the sarcoma virus-specific RNA is similar in properties to typical mammalian type C viral RNA. Furthermore, since the endogenous sarcoma-specific RNA is larger than Harvey sarcoma virus (16), the results indicate that only a portion of the endogenous genome has been incorporated into the stable sarcoma virus.

In contrast to these similarities between endogenous sarcoma-specific RNA and type C viral sequences, the current studies have revealed significant differences in cells producing high levels of the respective RNAs. Cells producing high levels of typical type C viral RNA produce structural proteins that cross-react in assays for rat p12 and mammalian p30 radioimmunoassays. Supernatants from such cells produce type C particles containing viral reverse transcriptase and viral RNA. In contrast, four cell lines producing high levels of the sarcoma virus-specific RNA produce no p30 protein detectable even in broadly reacting p30 assays, no rat p12 protein, and no type C particles containing viral RNA or polymerase. Since we have not been able to purify the rat viral glycoprotein, we cannot exclude the possibility that the sarcoma virus-specific RNA codes for this protein. However, since the sarcoma virus-homologous RNA does not code for either the RT21C viral polymerase or gag protein, since it lacks homology to typical RT21C rat type C RNA, the possibility that the sarcoma virus RNA codes for the RT21C coat protein seems remote.

The absence of immunoreactive proteins cannot be explained by the RNA not being on polysomes, since both RT21C and HTP cells contained comparable amounts of their respective RNAs on polysomes of each cell. These results leave four main possibilities as the explanation of the results. Proteins might be produced and rapidly degraded and thus not be detectable. Protein precursors might be made which are not cleaved and lack determinants detectable in the assays used. Although the RNA is on polysomes, it may not be translated as an endogenous sequence. Or the class of

"type C-like" RNA may code for a class of proteins that do not immunologically cross-react with the proteins of the known class of particle-producing mammalian type C viruses. The implications of these alternatives are important to an explanation of the genesis of Kirsten or Harvey sarcoma virus. The first three alternatives might imply that the combination of the rat sequences and mouse viral sequences of KiSV or HaSV would allow protein(s) to be made or to accumulate in cells infected with these viruses that do not usually occur when the sarcoma virus sequences are endogenous. The fourth alternative would imply that this sarcoma virus RNA might code directly for protein(s) responsible for transformation even when expressed in uninfected rat cells. If this last alternative is true, then virus stocks obtained from either WR123 or HTP cells with a 1:1 ratio of helper virus RNA and the rescued endogenous sarcoma-specific RNA should be useful in identifying the coding functions of the RNA. Since the 5′ end of type C viral RNA apparently can be translated in vitro (12, 16, 29), it may be possible to use such stocks to identify proteins coded for by the 5′ end of this endogenous sarcoma virus-specific RNA.

Finally, the studies on the sarcoma virus-specific RNA in NRK cells (1, 23, 24) and now in HTP and WR123 cells suggest the following events in the genesis of a sarcoma virus: (i) expression of endogenous sarcoma virus-specific RNA and (ii) packaging of this RNA in heterologous viruses grown in these cells. Since reverse transcription of the RNA can occur in vitro, it will be of interest to follow the potential in vivo reverse transcription and later steps in the replicative cycle of the helper particles with the rescued sarcoma virus sequences when they are transmitted to new cells. Such studies should be useful in unraveling the genesis of sarcoma viruses, since the current studies clearly demonstrate that either synthesis, integration, or transcription of this DNA in newly infected cells must be a less common event, and probably the rate-limiting step in the genesis of a sarcoma virus. However, again, with stocks of virus such as those described herein with a 1:1 ratio of helper viral RNA and endogenous sarcoma virus-specific RNA, it may be possible to identify the rate-limiting step in formation of a sarcoma virus in cell culture.

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