JOURNAL OF VIROLOGY, Nov. 1974, p. 1262-1267

Vol. 14, No. 5
Printed in U.S.A.

Sarcoma and Helper-Specific RNA Tumor Virus Subunits in Transformed Nonproducer Mouse Cells Activated to Produce Virus by Treatment with Bromodeoxyuridine

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Received for publication 8 July 1974

A tumor line (58-2T) was established from a slowly growing tumor in a BALB/c mouse inoculated with M58-2 cells. The latter clonal cell line was isolated after bromodeoxyuridine treatment as a flat variant from nonproducer BALB/3T3 cells transformed by the Kirsten sarcoma virus. The 58-2T cells produced type C virus with two discrete virus-specific RNA species. One of the species, which was probably an endogenous virus RNA subunit, had a sedimentation coefficient of 35*S* as the largest major subunit, and had sequences *similar* to Rauscher leukemia virus RNA based on nucleic acid hybridization. The other RNA species had 30*S* as the largest major subunit and corresponded to Kirsten sarcoma virus-specific RNA. These two RNA species formed heterogeneous, 60 to 70*S*, high-molecular-weight RNA in virions.

DNA transcripts (58-2T DNA) from the activated virus contained base sequences complementary to Rauscher leukemia virus and Kirsten sarcoma virus. The Kirsten sarcoma virus-specific DNA sequences (58-2TS) were purified from 58-2T DNA by eliminating RLV-specific sequences.

A nonproducer (NP) BALB/3T3 cell line transformed by the Kirsten strain of murine sarcoma virus (KiSV) can be induced to produce both sarcoma and helper type-C (nontransforming) viruses by treatment with 5'-bromodeoxyuridine; (1, 8, 9). Recent studies have shown that KiSVs which are propagated on normal rat kidney cells contain two size classes of RNA subunits; one subunit (molecular weight (mol wt) 2.5×10^6) corresponds to the helper, Kirsten murine leukemia virus (Ki-MuLV), and the other subunit (mol wt 2.3×10^6) corresponds to KiSV (11). A 30*S* subunit RNA, which appeared to correspond to the 2.3×10^6-mol wt KiSV-specific RNA, was also found in pseudotype virions containing an excess (based on bioassay) of the Moloney strain of murine sarcoma virus and in NP hamster cells transformed by this virus, whereas the subunit RNA of Rauscher leukemia virus (RLV) which was used as a helper virus for the murine sarcoma virus preparation had a sedimentation coefficient of 35*S* (N. Tsuchida et al., Virology, in press).

In this paper, we report studies of the size and hybridization specificity of viral RNA subunits synthesized by KiSV-transformed NP cells induced by 5'-bromodeoxyuridine to produce both sarcoma and helper viruses.

MATERIALS AND METHODS

Cells and viruses. The BALB/3T3 (A-31) and a derivative (K-234) which was transformed by KiSV (referred to as NP cell) were originally obtained from G. Todaro and S. Aaronson (2, 3). A flat-appearing mutant cell line (designated M58-2) was derived by cloning from the NP cell after short-term treatment with 5'-bromodeoxyuridine (8, 9). The cell line 58-2T was established from explants of a slowly growing tumor in a BALB/c mouse inoculated with M58-2 cells which continuously produced virus (not requiring further treatment with bromodeoxyuridine. The 58-2T cells appeared to produce several times more virus than the original M58-2 cells, as based on investigation of RNA-dependent DNA polymerase activities in culture media, and thus were used for characterization of viral RNA. RNA from M58-2 and 58-2T cells was indistinguishable in hybridization patterns as described in this report. The following cell types were used in various aspects of this work: JLS-V9 infected with (RLV; 18); a BALB/c cell infected with RLA; HT-1, a NP hamster cell transformed by Moloney isolate of murine sarcoma virus; and Fisher rat embryo cells (5053), provided by A. Freeman (Children's Hospital, Akron, Ohio). KiSV was obtained from Vaclav Klement, (University of Southern California Medical School, Los Angeles, Calif.) and propagated on normal rat kidney cells. P97, a hamster cell transformed by Rous sarcoma virus, was obtained from Flow Laboratories, Inc. (Rockville, Md.). All cells were grown in Eagle medium with 10% fetal bovine serum and antibiotics.

Preparation of RNA. Total cell RNA freed of DNA was prepared by hot phenol extraction and digestion with pancreatic DNase I as described previously (16). For size analysis, RNA was treated with dimethyl sulfoxide as described previously (17). For hybridization saturation experiments and to remove any possible residual DNA, cellular RNA was further purified by centrifugation in Cs_2SO_4 (ρ = 1.256) for 60 h at 20 C in a Spinco SW41 rotor at 33,000 rpm. Under these conditions RNA sedimented to the bottom of the tube, and DNA around ¼ of the tube length from the bottom. The supernatant was carefully removed and the RNA at the bottom of the tube was dissolved in water and dialyzed against 1 × SSC (0.15 M NaCl and 0.015 M Na_3 citrate), precipitated by 2 volumes of ethanol, collected by centrifugation, and redissolved in 0.1 × SSC. Viral high-molecular-weight (HMW) RNA was isolated as described previously (17). RNA concentrations were determined by the orcinol reaction (6) and gave values which agreed with those calculated from absorbancy at 260 nm (23 A_{260} unit = 1 mg of RNA/ml).

Preparation of ^{3}H-labeled viral DNA. ^{3}H-labeled viral DNA was prepared by the endogenous RNA-directed DNA polymerase reaction using purified RLV and virus synthesized by 58-2T cells (58-2T virus) as described previously (N. Tsuchida et al., J. Exp. Med., in press). Reaction mixtures (generally 5 ml) contained: 1 to 2 ml of purified virus (0.1 to 1.0 mg of protein per ml); 0.1 M glycine-sodium hydroxide buffer (pH 8.0); 10 mM dithiothreitol, 0.05 mM dATP, dGTP, and dCTP; 1.25 mCi of [^{3}H]TTP (40 to 50 Ci/mmol; New England Nuclear, Boston, Mass.), 0.03 M NaCl; 1 mM $MnCl_2$; 0.01% Nonidet 40 (NP-40), and 100 μg of actinomycin D (Sigma Chemical Co., St. Louis, Mo.) per ml. Enzyme reactions were at 37 C for 16 to 18 h and stopped by adding EDTA to a reaction concentration of 10 mM, NaCl to 100 mM, and sodium dodecyl sulfate to 0.5%. The ^{3}H-labeled viral DNA was extracted with phenol as described previously (7), and precipitated by 2 volumes of ethanol in the presence of 50 μg of yeast tRNA per ml at −20 C for 18 h. The precipitates were dissolved in 0.5 ml of 0.1 × SSC, and treated with 0.05 volumes of 2 N NaOH at 80 C for 30 min to destroy residual RNA. After neutralization with 0.05 volumes of 3 M NaH_2PO_4, the DNA product was dialyzed at 4 C against 0.1 × SSC. [^{3}H]DNA thus purified has a sedimentation coefficient of 4 to 5S, and the specific activity averaged 1.8×10^7 to 2.6×10^7 counts per min per μg of DNA, as calculated from the specific activity of [^{3}H]TTP, assuming equimolar amounts of the four deoxyribonucleotides. The transcripts used protected 80% of ^{32}P-labeled viral 70S RNA from ribonuclease digestion after hybridization with 18 times excess of [^{3}H]DNA. Five to seven percent of double-stranded DNA remained after phenol extraction. After phenol extraction, [^{3}H]DNA was treated with RNase A at 37 C, with 20 μg of [^{3}H]DNA per ml, for 30 min in 0.1 × SSC. Five to seven percent of the radioactivity was eluted with 0.4 M sodium phosphate buffer from hydroxylapatite (17), indicating double-stranded structure.

Preparation of viral HMW RNA. For studies of viral RNA subunits, virus labeled with either [^{3}H]uridine or ^{32}P was prepared from the peak fractions (1.14 to 1.16 g of virus per ml) in 15 to 60% sucrose gradients, and HMW RNA was extracted as described previously. The ^{3}H-labeled 58-2T virus formed a peak at 1.15 g of virus per ml. Unlabeled, viral HMW RNA from 58-2T virions (58-2T HMW RNA) was isolated by the same method for labeled viral HMW RNA, except that regular media were used. Labeled, 58-2T viral RNA formed broad peaks at 60 to 70S and 4 to 5S. Labeled and unlabeled 58-2T HMW RNA was collected from the 40 to 80S region of a 15 to 30% sucrose gradient, where ^{32}P-labeled RLV RNA was used for the reference marker in a separate gradient.

Hybridization. For hybridization saturation experiments, increasing amounts of RNA were incubated with [^{3}H]DNA (500 counts/min) in 100 μliters of 2 × SSC at 66 C for 20 h. The extent of hybridization was determined using a single-strand-specific S-1 nuclease (15) as described previously (N. Tsuchida et al., Virology, in press). S-1 nuclease-resistant radioactivity (5 to 10% of the input counts) in a sample without RNA was subtracted from radioactivities of all other sample.

RESULTS

RLV-specific RNA sequences synthesized by 58-2T cells. 58-2T cells were tested for virus-specific RNA. Increasing amounts of RNA were hybridized with RLV DNA transcript, which was found to be adequate for this purpose as based on comparison of cells actively producing RLV with the activated cell (58-2T cell; Fig. 1). RNA from the JLS-V9 (RLV) and the 58-2T cell lines protected the [^{3}H]DNA probe to a similar extent; in both cases, the final percent-

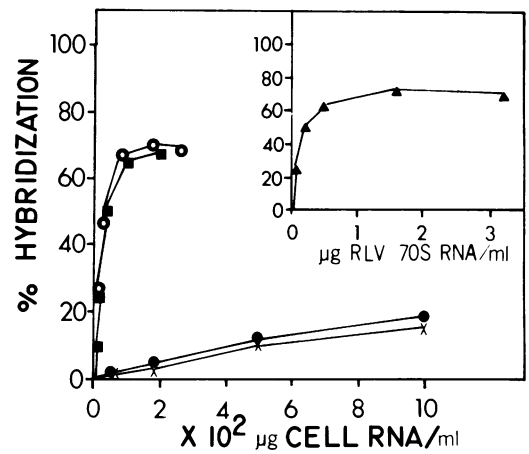

FIG. 1. *Hybridization-saturation of RLV [^{3}H]DNA with various RNAs. A sample of RLV [^{3}H]DNA (500 counts/min) was hybridized with: increasing amounts of RLV-producing cell RNA (○); 58-2T cell RNA (■); NP cell RNA (●); BALB/3T3 cell RNA (×); and RLV 70S RNA (▲; inset).*

age of hybridization of the viral DNA was similar to that obtained with homologous RLV 70S RNA (Fig. 1 inset). A low level of hybridization was obtained with RNA from NP and BALB/3T3 cells. The final percentage of hybridization (65 to 70%) was similar for both cell lines at $C_rt = 46$.

Detection of KiSV-specific sequences and isolation of KiSV-specific DNA. DNA transcripts (58-2T DNA) of the virus induced in 58-2T cells by bromodeoxyuridine were made and used to detect KiSV-specific sequences. This DNA detected KiSV-specific RNA in NP cells (Fig. 2). However, the extent of hybridization either with NP cell RNA- or RLV-producing cell RNA was only about half of the maximum obtained with 58-2T cell RNA.

These results (RLV RNA and NP cell RNA having saturated the 58-2T viral probe only to about half the value of the homologous reaction, and NP cell RNA and normal BALB/3T3 cell RNA having hybridized with RLV DNA to similar low levels) suggested the possibility that 58-2T DNA was composed of KiSV- and RLV-specific sequences. To test this possibility, 58-2T DNA (380,000 counts per min per ml) was hybridized with saturating amounts of RLV HMW RNA (2 μg of 58-2T DNA per ml) in 2 × SSC and 40% formamide at 45 C for 48 h. The unhybridized, single-stranded [^{3}H]DNA (about 50% of input) was separated from the hybridized DNA by differential batch elution on hydroxyapatite (17). Hereafter, the unadsorbed [^{3}H]DNA will be referred to as 58-2TS DNA. The results of hybridization of 58-2TS DNA with increasing concentrations of cell and viral RNA's is shown in Fig. 3. Eighty percent of the 58-2TS probe hybridized with 58-2T cell and

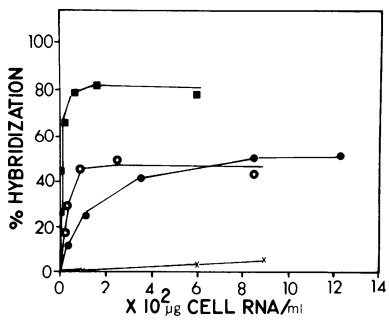

FIG. 2. *Hybridization-saturation of 58-2T [^{3}H]DNA with various RNAs. A sample of 58-2T [^{3}H]DNA (500 counts/min) was hybridized with: increasing amounts of 58-2T cells RNA (■); RLV-producing cell RNA (○); NP cell RNA (●); BALB/3T3 cell RNA (×).*

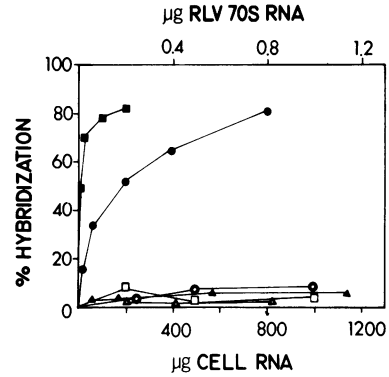

FIG. 3. *Hybridization of 58-2TS [^{3}H]DNA product with increasing amounts of various RNAs. [^{3}H]DNA (500 counts/min) was hybridized with RNA isolated from: the 58-2T cell (■); NP cell (●); BALB/3T3 cell (○); HT-1 cell (▲); P97 cell (△); and RLV HMW RNA (□).*

NP cell RNA, but no significant hybridization was detected with RLV RNA or normal BALB/3T3 cell RNA. Neither Rous sarcoma virus-transformed hamster cell (P97) nor hamster cell (HT-1;) transformed by Maloney murine sarcoma virus RNA was capable of protecting this probe from S-1 nuclease digestion. Since 200 μg of HT-1 cell RNA per ml was sufficient to allow detection of Maloney murine sarcoma virus-specific RNA sequences (7, 17), the absorption procedure resulted in the preparation of a DNA product specific for KiSV RNA.

Characterization of 58-2T viral RNA. A sample of [^{3}H]labeled 58-2T virion HMW RNA was prepared from 58-2T virion RNA. After 40-80S 58-2T virion RNAs were pooled, they were concentrated by ethanol precipitation and mixed with ^{32}P-labeled RLV HMW RNA. The mixture was analyzed on a sucrose gradient. The sedimentation constant of RLV RNA was taken to be 70S (10). The 58-2T RNA (Fig. 4, A) appeared heterogeneous as compared to RLV, since the RNA had a major peak at 60S with a skewed distribution toward the 70S region of the gradient. When the HMW RNAs were denatured by heat (70 C in 0.1 × SSC) and cosedimented with 58-2T cell RNA, the RLV RNA was converted to the expected 35S (N. Tsuchida et al., Virology, in press) whereas 58-2T RNA again appeared heterogeneous (Fig. 4, B). A clear peak at approximately 30S and higher (shoulder at ~35S) components were also clearly seen. Lower-molecular-weight components (around 20S) may have been degradation components of 30 to 35S RNA, since in other experiments no significant peak was observed, and the amount was much lower.

Size of RLV- and KiSV-specific RNA. The evidence that 58-2T viral RNA was heterogeneous raised the possibility that KiSV- and RLV-specific sequences might be found on different size subunits. The RLV and 58-2TS probes were used to test this hypothesis with a variety of RNAs. Viral HMW RNA (pooled material sedimented at 40 to 80*S* in a sucrose gradient) was isolated from unlabeled 58-2T virion, denatured by heat (70 C in 0.1 × SSC for 1 min), and sedimented in a sucrose gradient. Constant volumes of individual fractions were hybridized with the two DNA products. The major peak of RLV-specific sequences sedimented around 35*S* with a shoulder at 30*S*, whereas the major peak of KiSV-specific sequences sedimented at 30*S*, (Fig. 5).

The sizes of 58-2T virion subunit RNAs were compared to intracellular virus-specific RNA in 58-2T-, NP-, RLV-infected JLS-V9, and KiSV-(MuLV)-infected NRK cells. Total cellular RNA was treated with dimethyl sulfoxide to dissociate aggregates and was fractionated in sucrose gradients. A constant volume of each fraction was assayed for the amounts of virus-specific RNA (Fig. 6). The major peak of KiSV-specific RNA was detected at a sedimentation coefficient of 30*S* in NP cells, KiSV(MuLV)-infected NRK cells and 58-2T cells, whereas the major peak of RLV-specific RNA was detected at 35*S* in RLV-infected JLS-V9 cells. No significant RNA peak that was reactive with RLV DNA was observed in the 15 to 40*S* region

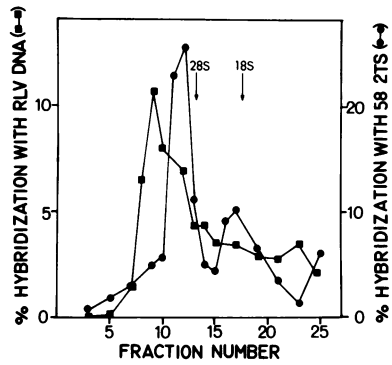

FIG. 5. *Rate-zonal sedimentation patterns of viral RNA subunits from 58-2T virus. Nonlabeled 58-2T virion HMW (40 to 80S) RNA was isolated, heated at 70 C for 1 min in 0.1 × SSC, layered on a 15 to 30% sucrose gradient in NTE buffer containing 0.5% sodium dodecyl sulfate, and centrifuged at 20 C in a Spinco SW41 rotor for 5 h at 39,000 rpm. In the same run, Fisher rat embryo cell RNA was sedimented in a different gradient as a reference marker. Fractions (0.5 ml) were collected from the bottom. Absorbancy was measured at 260 nm for cellular RNA. Viral RNA (58-2T) was precipitated with 50 μg of carrier yeast tRNA and dissolved in 100 μl of 0.1 × SSC. Samples of 50 and 25 μliters in each fraction were hybridized with 500 counts/min of RLV [^{3}H]DNA product (■), and 250 counts/min of 58-2TS [^{3}H]DNA product (●), respectively. After hybridization, the extent of hybridization was measured using S-1 nuclease as described previously (15). Direction of sedimentation is from right to left.*

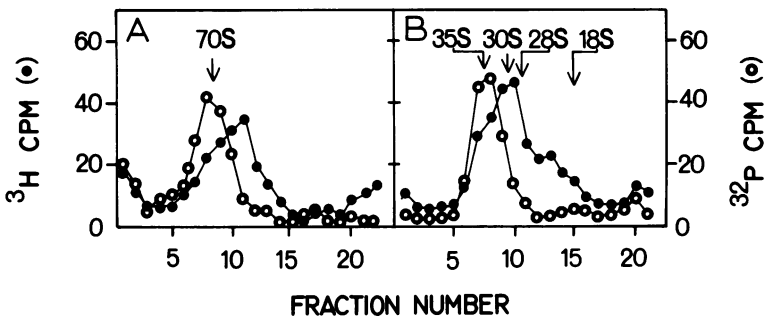

FIG. 4. *Rate-zonal-sedimentation patterns of viral RNA extracted from [^{3}H]uridine-labeled 58-2T virus. ^{3}H-labeled 58-2T HMW RNA and ^{32}P-labeled RLV HMW RNA were separately prepared in 0.1 × SSC. (A) 58-2T HMW RNA (736 counts/min) and RLV HMW RNA (335 counts/min) were mixed and centrifuged on a 15 to 30% sucrose gradient containing NTE buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 7.0), and 0.001 M EDTA) and 0.5% SDS (SW 41 rotor, 40,000 rpm at 20 C for 2.5 h). (B) 58-2T HMW RNA (736 counts/min) and RLV HMW RNA (223 counts/min) were mixed and heat-denatured at 70 C for 1 min. After cooling to 0 C, the sample was sedimented (SW41 rotor, 40,000 rpm at 20 C for 4.5 h) in a 15 to 30% sucrose gradient containing NTE buffer and 0.5% sodium dodecyl sulfate using 58 to 2T (18 and 28S) cell RNAs as reference markers. After centrifugation, fractions (0.5 ml) were collected from the bottom, and ultraviolet absorbancy was measured at 260 nm. Radioactivities were measured by adding 10 ml of Aquasol (New England Nuclear, Boston, Mass.; the pH was preadjusted by the addition of 50 ml of acetic acid and 80 ml of water per liter), using a Beckman scintillation counter. Recoveries of radioactivities in gradient (A) were 70% for ^{32}P (○) and 76% for ^{3}H (○), and those in gradient (B) were 97% for ^{32}P and 96% for ^{3}H. Direction of sedimentation is from right to left.*

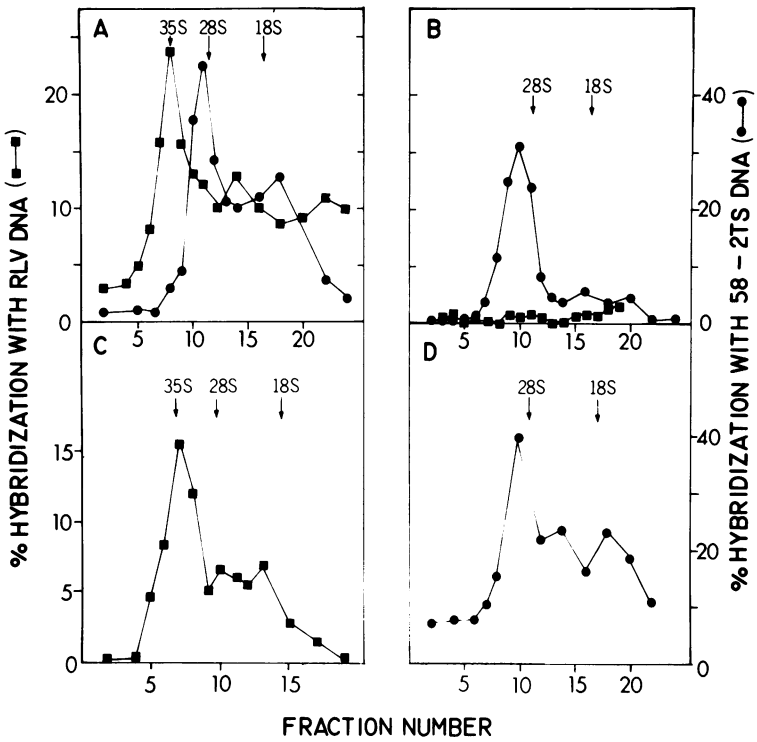

FIG. 6. *Rate-zonal sedimentation patterns of sarcoma- and leukemia-specific RNA in sucrose gradient. The cell RNA was isolated from: (A) 58-2T cell; (B) NP cell; (C) RLV-producing JLS-V9 cell; (D) KiSV(MuLV)-producing normal rat kidney cell and layered on 15 to 30% sucrose gradient in NTE buffer containing 0.5% sodium dodecyl sulfate and centrifuged at 20 C in a Spinco SW41 rotor for 6 h at 36,000 rpm. The amounts of RNA loaded on the gradients were: 1.6 A_{260} units for 58-2T cell RNA; 3.4 A_{260} units for NP cell RNA; 6.0 A_{260} units for RLV-producing JLS-V9 cell RNA; and 2.53 A_{260} units for KiSV(MuLV)-producing normal rat kidney cell RNA. Fractions (0.5 ml) were collected from the bottom. RNA was precipitated with 50 μg of carrier yeast tRNA and dissolved in 100 μliters of 0.1 × SSC. Smaller amounts of each fraction: A, 10 μliters of 58-2T cell RNA; B, 20 μliters of NP cell RNA; and D, 5 μliters of KiSV(MuLV)-producing cell RNA were hybridized with 58-2Ts [^3H]DNA product 250 counts/min. Also, smaller amounts of each fraction C, 5 μliters of RLV-producing JLS-V9 cell RNA; A, 40 μliters of 58-2T cell RNA; and B, 20 μliters of NP cell RNA were hybridized with RLV [^3H]DNA product (500 counts/min). After hybridization, the extent of hybridization was measured with S-1 nuclease. Direction of sedimentation is from right to left.*

of BALB/3T3 cell RNA (not shown) and NP cell RNA, under the hybridization conditions used.

DISCUSSION

At least two distinct virus-specific RNAs were synthesized in nonproducer BALB/c mouse cells that were transformed by KiSV and activated to produce virus. One of these (with a sedimentation coefficient of 35*S*) is closely related to RLV. This RNA is clearly distinct from Ki-MuLV RNA, based on the approximate 50% relationship between RLV and Ki-MuLV in reciprocal hybridization experiments (5; Tsuchida, unpublished data). Although RLV and activated BALB/c RNA appear essentially identical through direct hybridization, preliminary competition hybridization experiments carried out as previously described (16) show that 58-2T cell RNA shared 80% of RLV 70*S* RNA sequences, whereas RLV-infected JLS-V9 cell RNA shared more than 95% of such sequences. Thus, the RLV strain in our laboratory and the activated BALB/c virus were not completely identical. Nevertheless, the close relationship is noteworthy since RLV was isolated from BALB/c mice after multiple passages in this strain of an ascites tumor originating as a leukemia in Swiss mice (13).

The second RNA species corresponded to KiSV genome and had a sedimentation coefficient of 30*S*. The 30*S* KiSV species in the NP cell was distinct from RLV and thus far has been found only in KiSV-transformed mouse cells, whether or not actively producing virus

(N. Tsuchida et al., J. Exp. Med., in press). This RNA species was not found in RLV. Under the same conditions, Maloney murine sarcoma virus RNA sedimented at 30S (N. Tsuchida et al., Virology, in press), which was similar to the *S*-value of KiSV RNA.

DNA transcripts from the activated virus contain both RLV- and KiSV-specific sequences. This result led us to isolate KiSV-specific DNA by eliminating RLV-specific sequences from the 58-2T DNA. A technique for preparing the DNA probe of the entire sarcoma genome was obviously only appropriate when the sarcoma genome was distinguishable from the helper virus. In this particular case, the ease in distinction is based on the origin of KiSV-specific sequences from the rat (14). Most sarcoma viruses contain nucleic acid sequences partially homologous to helper viruses from the same species (4, 14). In such cases, virus preparations with excess sarcoma virus could be utilized to prepare specific probes (11; N. Tsuchida et al., Virology, in press).

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