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Levels of Rat Cellular RNA Homologous to Either Kirsten Sarcoma Virus or Rat Type-C Virus in Cell Lines Derived from Osborne-Mendel Rats

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DNA transcripts from V-NRK and RT21c rat type-C viruses were found to differ in their sequence homology to Kirsten and Harvey sarcoma viruses. V-NRK DNA transcripts consistently had homology to Kirsten and Harvey sarcoma virus, whereas RT21c DNA transcripts did not. To explain the differences, the nucleic acids and structural proteins of the two type-C viruses, released from each of two cell lines derived from Osborne-Mendel rats, were analyzed by molecular hybridization and competition radioimmunoassays. The p30 and p12 structural proteins of the two viruses were found to be highly related immunologically. In the V-NRK virus preparation, two sets of distinct RNA sequences were found in approximately equal amounts. One set is homologous to Ki-SV, and the other homologous to RT21c. In contrast, the RT21c virus preparation was found to contain a different ratio of these sequences. In this case the RT21c-like RNA sequences are present in 100-fold excess as compared to the additional Ki-SV specific sequences. Both NRK and RT21c cells contain in their DNA the full complement of Ki-SV homologous sequences, but NRK cells express much higher levels of these Ki-SV sequences in their RNA. These additional sequences, not homologous to RT21c, which are detected in uninfected NRK cellular RNA or V-NRK rat virus, could also be detected in the 60-70S RNA from a Moloney mouse type-C virus released from the NRK cells infected with the Moloney type-C virus. The results suggest that type-C viruses released from NRK cells incorporate species of RNA present in NRK cells which are homologous to Kirsten and Harvey sarcoma viruses. Either these sequences are of cellular origin, or rat cells contain two endogenous viruses with completely distinct nucleic acid sequences.

Previous studies of the Kirsten strain and Harvey strain of murine sarcoma viruses have indicated that each sarcoma virus contains two distinguishable sets of nucleic acid sequences (1, 15, 16). In each case one set of sequences was found which is homologous to the mouse type-C virus used in the original isolation of the sarcoma virus. For example, the Kirsten sarcoma virus (Ki-SV) was found to contain nucleic acid sequences homologous to the Kirsten strain of murine erythroblastosis virus, and the Harvey sarcoma virus (Ha-SV) was found to contain sequences homologous to the Moloney strain of mouse leukemia virus.

In addition, in both the sarcoma viruses rat genetic information was detected using DNA transcripts synthesized in endogenous reactions containing a rat type-C virus, V-NRK (2). The experiments thus indicated that both the Ki-SV and Ha-SV arose through recombination or reassortment between mouse type-C viruses and nucleotide sequences found in rat cells.

The current studies were undertaken to examine in greater detail the rat genetic information detected with the DNA transcripts from the V-NRK rat virus. To do this, we have examined two isolates of rat type-C virus spontaneously released from two different cell lines derived from Osborne-Mendel rats. DNA transcripts were synthesized from the RT21c virus, a type-C virus derived from a thymus cell line from an Osborne-Mendel rat (4). These DNA transcripts were found to contain no detectable homology to the Kirsten or Harvey strain of sarcoma virus. The DNA transcripts from the V-NRK rat virus, spontaneously released from NRK cells, a cell line also derived from an Osborne-Mendel rat, contain all the sequences detected in the RT21c virus, and in addition contain a set of sequences homologous to the

Kirsten strain and Harvey strain of murine sarcoma virus. The origin of the additional information, its quantitation in the two virus preparations, and the mechanism by which it is present in the V-NRK virus is discussed.

MATERIALS AND METHODS

Cells. All cells were grown in Dulbecco modification of Eagle medium with 10% calf serum (Colorado Serum Co.). The normal rat kidney (NRK) cell is derived from a non-inbred strain of Osborne-Mendel rat, as originally described by Duc-Nguyen et al. (5). The RT21c cell line, derived from an inbred Osborne-Mendel rat, was originally described by Cremer et al. (4) and was kindly supplied by Edwin Lennette, California State Department of Health, Berkeley, Calif. The source of the NIH 3T3 cell line (7) and various nonproducer sarcoma virus transformed NIH 3T3 cells also has been described (1). The NRK cell spontaneously producing endogenous rat type-C virus, V-NRK, was the gift of George Todaro and Raoul Benveniste, National Cancer Institute (2). The mink lung fibroblast, CCL 64, was obtained from the American Type Culture Collection (6). A nonproducer Ki-SV transformed mink lung fibroblast was obtained by serial dilution of filtered sarcoma virus stocks and isolation of transformed cells in Falcon microtest II plates as previously described (6, 16).

Viruses. The Moloney leukemia virus which was grown as indicated below in either NIH 3T3 or NRK cells was the gift of Robert Bassin, National Cancer Institute. The RT21c rat virus was obtained from the RT21c cell line, and the V-NRK virus from the NRK cell line. Neither rat virus has yet been successfully cloned in a non-rat cell line and therefore biological purity of either is difficult to be certain of. The biochemical characteristics of DNA probes from the viruses is dealt with below. Subgroup C Feline (FeLV) type-C virus was obtained from Padman Sarma, National Cancer Institute (13). The origin of the Kirsten strain of murine erythroblastosis virus (Ki-MuLV) has been described in earlier publications (16). All viruses were concentrated and purified from tissue culture supernatant medium by continuous flow centrifugation in sucrose density gradients.

Synthesis of virus-specific DNA. Endogenous (no exogenous template or primer) reverse transcriptase reactions from sucrose density gradient banded viruses were used as the source of all viral DNA transcripts. Actinomycin D (Calbiochem, Los Angeles, Calif.) at 40 to 60 μg/ml was included in all reactions. Details of the preparation of the [^{3}H]DNA probes or unlabeled DNA transcripts have been given in earlier publications (1, 15, 16). A virus preparation containing the Ki-SV and the subgroup C FeLV was obtained by rescuing a Ki-SV-transformed mink nonproducer cell with the subgroup C FeLV. A tritiated DNA transcript was obtained from the Ki-SV (FeLV) virus mixture from a 1-h endogenous reverse transcriptase reaction. The [^{3}H]DNA transcript from this reaction was sedimented in a glycerol gradient using the velocity sedimentation method of Schlom and Spiegelman (14). A broad peak of [^{3}H]DNA sedimenting between 50 and 70*S* in the gradient was obtained. Since Maisel et al. (8) reported that Ki-SV-specific RNA was smaller than that of replicating murine type-C viruses, the slower sedimenting half of the broad peak of [^{3}H]DNA attached to high-molecular-weight RNA was pooled. This [^{3}H]DNA preparation was treated with 0.20 N NaOH for 60 min at 37 C and dialyzed extensively against distilled water before use (1). The [^{3}H]DNA transcript thus obtained from the Ki-SV (FeLV) virus preparation was found to be highly enriched for Ki-SV-specific sequences; 95% of the hybridizable counts in the [^{3}H]DNA transcript were found to be homologous to Ki-SV. Thus, by rescuing the Ki-SV virus with a non-murine type-C virus and velocity sedimentation of the [^{3}H]DNA transcript, we obtained a [^{3}H]DNA transcript highly enriched for Ki-SV sequences. These Ki-SV sequences are partly mouse type-C viral (16) and partly rat genetic information (16); the probe used here allows us to measure the rat-specific component of the Ki-SV virus.

Isolation of 60-70S RNA. The isolation of the 60-70*S*[^{3}H] was as previously described and the isolation of unlabeled 60-70*S* RNA was carried out by similar procedures (12). To be sure that hybridization detected with the various unlabeled RNAs was not due to contaminating DNA in the preparations, control hybridizations were done with RNA that had been treated with 0.2 N NaOH for 60 min at 37 C and then dialyzed. In each case, the hybridization with the RNA was destroyed by this alkali treatment.

Hybridization. The conditions for hybridization and analysis of either [^{3}H]DNA-RNA or [^{3}H]RNA-DNA hybridization reactions with the use of S1 nuclease has also been previously reported (1, 15). Total cellular RNA was extracted by a chloroform-phenol procedure as detailed earlier (1). Exact conditions for each hybridization are given in the legends to the appropriate tables and figures. All hybridizations are for 36 h and are extents of reactions.

Purification of rat viral proteins. The p30(gs) protein of V-NRK or RT21c rat (9) virus was purified by procedures previously described for the purification of the p30 murine or primate type-C viruses (10). Purification of the low molecular proteins (p10, p12) from V-NRK and RT21c was accomplished by agarose gel column chromatography in 6 M guanidine hydrochloride also by methods previously described (17, 20). The calibration of the guanidine hydrochloride chromatography and sodium dodecyl sulfate gel electrophoresis will be reported elsewhere. The purity of all proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Each protein was found to be at least 90% pure as judged by densitometer tracings of acrylamide gels.

Radioimmunoassays for rat viral proteins. The sera used to precipitate iodinated rat p30 or p12 proteins were obtained from Roger Wilsnak, Huntington Research Laboratory, Baltimore, through the Resources and Logistics Segment of the Virus Cancer Program. Sera were prepared in goats against Tween-ether disrupted rat type-C virus obtained from a Wistar-Furth rat (P. Sarma, personal communication). The iodination of the rat viral proteins and details of the competition radioimmunoassays were identical to conditions used in earlier work for prepar-

ing iodinated mouse virus structural polypeptides, and details of the competition assays also are as in earlier publications (10, 11). Details of each competition assay are given in the appropriate tables.

RESULTS

The relationship between two rat type-C virus isolates and the Kirsten and Harvey strains of murine sarcoma viruses was compared by hybridization of ^{3}H viral 60-70*S* RNA with an excess of nonradioactive single-stranded DNA transcript synthesized from endogenous reverse transcriptase reactions (Table 1). As previously noted (15, 16), transcripts from V-NRK rat virus hybridized well to the virus preparations from mouse cells containing either the Kirsten strain or Harvey strain of murine sarcoma viruses. Reciprocally, the DNA transcripts from the Kirsten strain of sarcoma virus or the Harvey strain of sarcoma virus hybridized to the 60-70*S* [^{3}H]RNA obtained from the V-NRK producing cultures. Approximately 25% of the 60-70*S* [^{3}H]RNA counts derived from the V-NRK-releasing culture are protected by the DNA probes for either the Kirsten strain or Harvey strain of sarcoma viruses. These results are in agreement with earlier studies which showed that both Ki-SV and Ha-SV contain rat genetic information (15, 16; cited in 18; Tsuchida et al. Proc. Nat. Acad. Sci. U.S.A., in press; Klement and Roy-Burman, submitted for publication).

In contrast, the RT21c isolate shows no detectable homology to either Kirsten or Harvey strains of sarcoma virus. The RT21c DNA transcripts can protect essentially the entire complement of homologous RNA sequences (88%), but protect only 62% of the V-NRK high-molecular-weight RNA. Since V-NRK protects equally both V-NRK [^{3}H]RNA and RT21c [^{3}H]RNA, V-NRK must contain all of the sequences present in RT21c and additional sequence which hybridize to the Ki-SV and Ha-SV.

Analysis of Ki-SV sarcoma virus-specific sequences in V-NRK. To further investigate the sequence differences between the V-NRK rat virus and the RT21c rat virus, [^{3}H]DNA transcripts were synthesized from RT21c, V-NRK, and from Ki-SV (Feline type-C virus coated Ki-SV in mink cells prepared as described in Materials and Methods). Each [^{3}H]-DNA transcript was hybridized to varying amounts of total cellular RNA from NRK cells, V-NRK cells, RT21c cells, and Ki-SV transformed nonproducer mink cells (Fig. 1 and 2). RT21c [^{3}H]DNA transcript (Fig. 1B) hybridizes with RNA in uninfected NRK cells; however, a much higher level of hybridizable RNA is found in NRK cells releasing the V-NRK rat virus. The cellular RNA from RT21c cells releasing its virus hybridizes with [^{3}H]RT21c DNA to the same final extent as the cellular RNA from the NRK cell releasing the V-NRK virus. Consistent with the results in Table 1, the RT21c probe does not detect homologous RNA in the Kirsten nonproducer cell.

In Fig. 2, the same cellular RNAs are hybridized to the Ki-SV-specific [^{3}H]DNA transcript. The uninfected NRK cell hybridizes well to the Ki-SV-specific probe, indicating that NRK cells not releasing endogenous rat type-C virus contain relatively high levels of sequences homologous to Ki-SV. Surprisingly, the same cell releasing virus, V-NRK, contains comparable levels per microgram and comparable final extents of

TABLE 1. *DNA excess hybridization to viral RNAs*[a]

DNA transcript from virus	Cell virus grown in	Hybridization of 60-70*S* [^{3}H]RNA (%)					
		RT21c	V-NRK	Ki-MuLV	Ki-MuLV/ Ki-SV	Mo-MuLV	Mo-MuLV-Ha-SV
RT21c	Rat	88	62	<3	<3	<3	<3
V-NRK	Rat	84	88	<3	29	<3	33
Ki-MuLV	Mouse	<3	<3	93	61	nd[b]	nd
Ki-MuLV/Ki-SV	Mouse	<3	25	88	86	nd	nd
Mo-MuLV	Mouse	<3	<3	nd	nd	98	72
Mo-MuLV/Ha-SV	Mouse	<3	25	nd	nd	96	94

[a] Each hybridization reaction contained in 0.10 ml: 0.4 M NaCl; 10^{-4} M EDTA; 0.02 M Tris-hydrochloride, pH 7.2; 0.10% sodium dodecyl sulfate; 30 μg of yeast RNA; 1,000 to 1,500 trichloroacetic acid-precipitable counts per min of 60-70*S* [^{3}H]RNA (1 to 5 $\times$ 10^6 counts/min per μg); and DNA transcripts up to 200 ng. Hybridization was carried out at 66 C for 24 to 36 h, and assayed as previously described with S1 nuclease (1, 15). The mouse cells used were NIH 3T3 cells. The DNA transcripts and 60-70*S* [^{3}H]RNA were from virus preparations grown in the same cell.

[b] nd = not done.

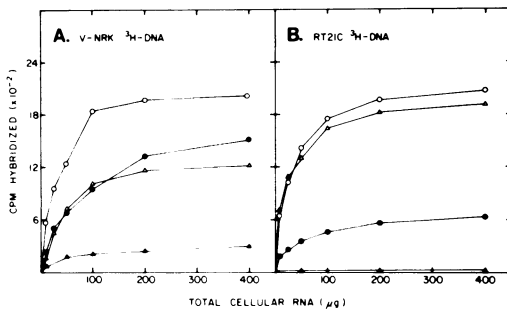

FIG. 1. *Hybridization with V-NRK or RT21c [³H]DNA. Each hybridization reaction of 0.2 ml contained approximately 3,000 trichloroacetic acid-precipitable counts per min of [³H]DNA. Conditions of hybridization are as described in Table 1. S_1 nuclease was used to assay for hybridization as previously described (1). (A) V-NRK [³H]DNA; (B) RT21c [³H]DNA. Maximal hybridization represents approximately 80% of the input counts. Background is 30 counts/min in the absence of hybridization.* (○) *V-NRK, total cellular RNA;* (●) *NRK, total cellular RNA;* (△) *RT21c, total cellular RNA;* (▲) *Ki-Mink, total cellular RNA.*

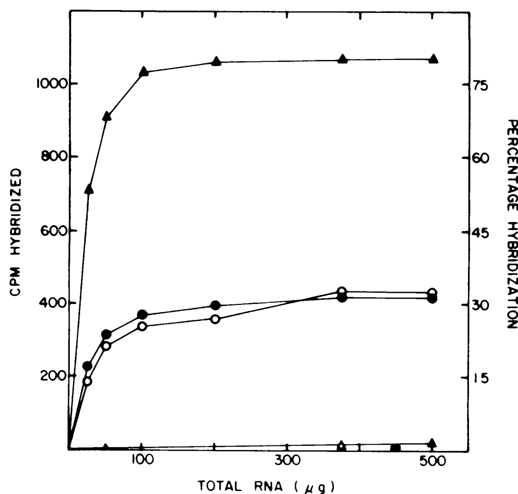

FIG. 2. *Hybridization with Ki-SV-specific [³H]-DNA. Each hybridization reaction of 0.20 ml contained 1,400 trichloroacetic acid-precipitable counts per min of the [³H]DNA specific for Ki-SV as described.* (▲) *Ki-Mink, total cellular RNA;* (■) *Mink, total cellular RNA;* (●) *NRK, total cellular RNA;* (○) *V-NRK, total cellular RNA;* (△) *RT21c, total cellular RNA.*

hybridizable RNA. With the Ki-SV [³H]DNA, the Kirsten nonproducer transformed mink cell hybridized to a greater final extent than that achieved with either of the rat cell RNAs possibly due to the Ki-MuLV component of Ki-SV (1).

Although the Ki-SV sequences were easily detected in the NRK and V-NRK RNA, RT21c cellular RNA at 500 μg of total cellular RNA barely hybridized to detectable levels to the Ki-SV-specific probe, indicating that the RT21c cell expresses relatively few sequences homologous to KiSV.

The results with the V-NRK [³H]DNA probe are shown in Fig. 1A. Total RNA from NRK cells hybridizes with the V-NRK [³H]DNA probe. In addition, RT21c cellular RNA hybridizes relatively well to the V-NRK [³H]DNA probe. The cellular RNA from the V-NRK culture releasing rat virus hybridizes to a final higher level of hybridization than that achieved with the cellular RNA from RT21c alone or the cellular RNA from the NRK cell before its releasing endogenous rat virus.

Quantitation of Ki-SV and RT21c sequences in various viruses. These results suggested that the V-NRK [³H]DNA product was a mixture of sequences, part of which were contained in RT21c, and part of which were contained in the Ki-SV [³H]DNA probe (Fig. 2). To test this hypothesis, a mixing experiment was performed (Table 2) in which the V-NRK [³H]DNA transcript was hybridized to NRK cellular RNA, RT21c cellular RNA, or a mixture of the two. The results indicate that a mixture of the NRK and RT21c RNAs, neither of which alone can saturate the V-NRK probe, together give a final level of hybridization equal to that achieved with the V-NRK cellular RNA.

To quantitate the RT21c and Ki-SV sequences in various viruses, a series of 60-70*S* RNAs were prepared from V-NRK virus, RT21c virus, Moloney leukemia virus growing in NIH 3T3 cells, and Moloney leukemia virus growing in NRK cells not releasing the V-NRK virus. The results are shown in Fig. 3 of hybridizations with the same RNAs to [³H]RT21c DNA, [³H]Ki-SV DNA, or [³H]V-NRK DNA. The RT21c viral RNA (Fig. 3A), although giving detectable hybridization with V-NRK

TABLE 2. *Distinguishable sequences in V-NRK [³H]DNA*[a]

Cellular RNA added (μg)	Hybridized (counts/min)
None	25
NRK (300)	847
RT21c (400)	892
NRK + RT21c (300 + 400)	1575
V-NRK (600)	1610

[a] Each hybridization reaction contained in 0.20 ml approximately 2,000 trichloroacetic acid-precipitable counts per min of V-NRK [³H]DNA. Conditions and analysis by S1 nuclease are as described in Table 1.

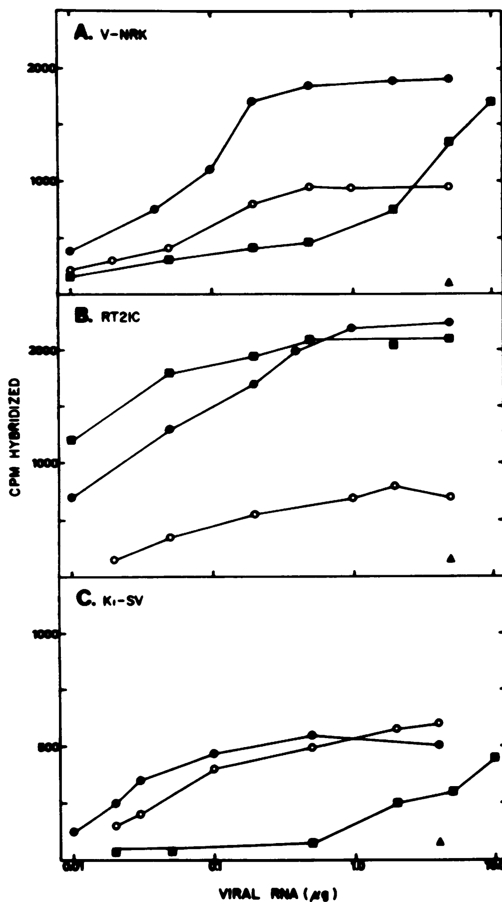

FIG. 3. *Quantitation of Ki-SV and RT21c sequences in various viral RNAs. Each [^{3}H]DNA was hybridized to viral RNAs and the counts/min hybridized were analyzed with S1 nuclease. (A) V-NRK [^{3}H]-DNA; 2,500 trichloroacetic acid-precipitable counts per min input; maximum hybridization approximately 75%; (B) RT21c [^{3}H]DNA; 2,500 trichloroacetic acid-precipitable counts per min input; maximum hybridization approximately 75%; (C) Ki-SV [^{3}H]DNA; 1,400 trichloroacetic acid-precipitable counts per min input; maximal hybridization approximately 80%. (●) V-NRK viral RNA; (■) RT21c viral RNA; (O) viral RNA from Moloney MuLV growing in NRK cells; (△) viral RNA from Moloney MuLV growing in NIH 3T3 cells.*

[^{3}H]DNA at low levels of RNA (0.01 to 0.05 μg), plateaus over the range of 0.1 to 1.0 μg of viral RNA, and does not quite achieve saturation of the V-NRK probe even at 10 μg of RT21c viral RNA. In contrast, with the V-NRK [^{3}H]DNA and V-NRK viral RNA, saturation is achieved at 0.2 to 0.5 μg of the V-NRK viral RNA. Importantly, the high-molecular-weight RNA from Moloney virus growing in NRK cells, hybridizes well to the V-NRK [^{3}H]DNA. The final extent of hybridization nevertheless is less than that achieved with the RNA of the V-NRK virus, and resembles the result seen with the V-NRK [^{3}H]DNA and cellular RNA from NRK cells (Fig. 1A).

When the same RNAs were hybridized to the RT21c [^{3}H]DNA, a different pattern was obtained (Fig. 3B). The RT21c viral RNA saturated the RT21c [^{3}H]DNA probe at 0.2 to 0.5 μg of RNA; this level is between 30- to 100-fold less than that necessary to achieve saturation of the V-NRK [^{3}H]DNA probe with the same RT21c viral RNA preparation. The V-NRK viral RNA also hybridized well to the RT21c [^{3}H]DNA probe, saturating it with between 0.4 and 1.0 μg of viral RNA, levels approximately equal or somewhat higher than that needed to saturate the V-NRK probe. The Moloney virus RNA prepared from the Moloney virus grown in rat cells also hybridized to the RT21c [^{3}H]DNA probe, to a level somewhat higher than that seen with Moloney virus grown in NIH 3T3 cells.

In Fig. 3C, the Ki-SV-specific [^{3}H]DNA transcript is hybridized to the same RNAs. This probe readily hybridized to V-NRK RNA and to RNA from the Moloney virus grown in the NRK cells, indicating that RNA from these viruses grown in NRK cells contained similar information in their high-molecular-weight RNA. The RT21c viral RNA, on the other hand, did not hybridize to detectable levels to the Ki-SV probe until almost 1.0 μg of viral RNA was added and did not quite saturate the Ki-SV probe even at 10 μg of RT21c viral RNA.

Thus, the RT21c viral RNA contains two components, a component which hybridizes readily and saturates at low levels (0.2 to 0.5 μg), the RT21c [^{3}H]DNA, and an RNA which is present at approximately 50- to 100-fold lower levels. This second component hybridizes to the Ki-SV-specific [^{3}H]DNA transcript. In contrast the V-NRK RNA appears to be a more equal mixture of the two RNAs, the one found in the Ki-SV, and the other one found in excess in the RT21c virus. The data also indicate that a heterologous virus, the Moloney type-C virus, growing in NRK cells, but not growing in NIH 3T3 cells, contains RNA with homology to the Ki-SV-specific [^{3}H]DNA transcript. In contrast that the RNA present in V-NRK with homology to Ki-SV is the same as the RNA in the Moloney leukemia virus released from the uninfected NRK cells. Since this RNA is present in uninfected NRK cells, it seems that either a rat or mouse type-C virus when released from NRK cells can incorporate RNA with homology to Ki-SV.

DNA-DNA hybridization studies with [^{3}H]DNA Ki-SV sequences. The Ki-SV-specific [^{3}H]DNA transcript was hybridized to cellular DNA from RT21c, NRK, and V-NRK cells, and, as a negative control, calf thymus DNA. The results in Table 3 indicate that the final extent of hybridization with the Ki-SV-specific [^{3}H]DNA transcript is equal with the RT21c-, V-NRK-, and NRK-derived cellular DNAs, indicating that the full complement of Ki-SV specific sequences detected with this transcript is present in both RT21c cells and NRK cells. Therefore, the differences in Ki-SV sequences in cellular RNA between RT21c and V-NRK measured earlier (Fig. 2) would most likely be explained in differing expression in the two cell lines.

Reverse transcription of rat sequences. Earlier studies and those shown in Fig. 1A indicated that a [^{3}H]DNA transcript prepared from V-NRK rat virus released from NRK cells contained the sequences that were detected in the Ki-SV. To see if the sequences could also be detected in a [^{3}H]DNA transcript from the Moloney mouse type-C virus released from NRK cells, the following experiment was performed. The [^{3}H]DNA prepared from Moloney MuLV grown in NRK cells was hybridized to the cellular RNA from uninfected NRK cells. The DNA that did not hybridize was digested with the S1 nuclease, and the DNA resistant to S1 nuclease was recovered by phenol extraction, alkali treatment, and extensive dialysis (3). This cycled [^{3}H]DNA transcript was then hybridized to a variety of cellular RNAs and the results are shown in Table 4. The recycled [^{3}H]DNA transcript hybridized well to RNA from NRK cells or from Kirsten or Harvey nonproducer NIH cells, but poorly to RNA from Moloney sarcoma virus transformed NIH cells or NIH cells producing Ki-MuLV or NIH cells producing Moloney MuLV. The results indicate that rat sequences are present in Moloney high-molecular-weight RNA in Moloney virus grown in NRK cells, and that these sequences can be transcribed into [^{3}H]DNA in endogenous reverse transcriptase reactions. It seems likely that the V-NRK [^{3}H]DNA transcripts which detect the Kirsten sarcoma-specific sequences derive these sequences from the uninfected NRK cells when the endogenous rat virus is released from the NRK cells in a manner analogous to the way the Moloney virus released from the rat cells also derives these rat sequences. Since RT21c and V-NRK do not readily infect other rat cells or heterologous cells, it was not readily possible to directly test the genetic stability of the sequences associated with each virus.

TABLE 3. *Hybridization of KI-SV-specific [^{3}H]DNA to cellular DNAs*[a]

Cellular DNA	Hybridized (counts/min)
None	10
Calf thymus	25
RT21c	495
NRK	485
V-NRK	520

[a] DNA-DNA hybridization was performed as previously described (11) at a C_0t of 5×10^4 (mol/s per liter) with 800 trichloroacetic acid-precipitable counts per min per reaction mixture.

TABLE 4. *Hybridization with absorbed [^{3}H]DNA from Moloney MuLV grown in NRK cells*[a]

Cellular RNA	Hybridized (counts/min)
None	22
NRK	893
Ki-NIH	475
Ha-NIH	494
Mo-NIH	88
NIH(Mo-MuLV)	83
NIH(Ki-MuLV)	99

[a] Each hybridization reaction contained 1,000 counts/min. Conditions and analysis are as described in Table 1. Each cellular RNA was present at 500 μg per reaction mixture. Ki-NIH, Ha-NIH, and Mo-NIH indicate the Kirsten, Harvey, and Moloney sarcoma virus-transformed NIH nonproducer cells. NIH(Mo-MuLV) indicates NIH 3T3 cells producing Moloney MuLV and NIH(Ki-MuLV) indicates NIH 3T3 cells producing Ki-MuLV. To prepare the adsorbed probe, approximately 500,000 trichloroacetic acid-precipitable counts per min of cDNA were hybridized to saturating levels (500 μg/ml) of NRK cellular RNA. Approximately 10 to 20% of the counts/min were resistant to subsequent digestion by S1 nuclease in the presence of the RNA but less than 2% in the absence of RNA. The S1-resistant counts were recovered by phenol extraction and the RNA was removed by treatment of the counts with 0.2 N NaOH for 2.0 h at 37 C. The final recovery was 50,000 trichloroacetic acid-precipitable counts per min out of the initial 500,000 counts per min.

Immunological comparison of rat viruses. To further quantitate the amounts of rat RNA and rat virus proteins in the various viruses used in these studies, the structural proteins of V-NRK and RT21c were examined for cross-reactions as shown in Table 5. The virus preparation or purified polypeptides obtained from the viruses were measured by competition radioimmunoassay for the level of either rat type-C p30, rat p10, or p12 low-molecular-weight polypeptides, or mammalian p30 interspecies (gs3)

TABLE 5. *Radioimmunoassays of rat type-C viruses*

Competing antigen	Antigen levels in different competition assays[a]			
	V-NRK p30	V-NRK p12	Inter-species (gs-3)	MuLV p30(gs)
V-NRK	40	19	33	<0.1
RT21c	120	45	110	<0.1
V-NRK p30	100	<0.1	120	
V-NRK p12		100		
RT21c p12		85		
MuLV (Moloney)			750	600

[a] Antigen levels are expressed as micrograms per milliliter of the various preparations of competing antigens. Competition radioimmunoassays were performed as previously described by a double antibody precipitation (10,11). Each assay contained approximately 10,000 counts/min of ^{125}I antigen and limiting first antibody to precipitate 30 to 50% of the ^{125}I antigen. For the V-NRK p30 protein, a goat antisera (see Materials and Methods) at a 1:3,000 dilution was used and for the p12 antigen a goat serum at a 1:200 dilution was used. A rabbit anti-goat immunoglobulin G was used in excess as the source of the second antibody. For the gs-3 assay, ^{125}I-MuLV p30 and IS8 serum were used as previously described (10). For the MuLV p30 assay, a rabbit antisera prepared as previously described was used (10). Other details are in earlier publications (10, 11).

immunological reactivity. The results indicate that the virus preparations containing V-NRK rat virus and RT21c rat virus contain approximately equal levels of the rat-specific p30 protein and interspecies gs3 reactivity. Thus the predominant p30 proteins present in both rat virus preparations show a strong cross-reaction between each other; in studies not shown complete displacement was observed between the RT21c p30 and the ^{125}I p30 from V-NRK. In addition, there was a two-way cross-reaction between the p12 low-molecular-weight protein and p10 (data not shown) obtained from the V-NRK virus and the RT21c rat virus or RT21c p12 viral protein. These proteins in earlier experiments (17, 20) have been shown to be highly type specific and would be expected to detect differences in the V-NRK and RT21c rat virus if these two virus preparations were not closely related immunologically. The results thus indicate that the V-NRK and RT21c rat virus are closely related immunologically and quantitatively indicate that the V-NRK rat viral proteins can be accounted for by a virus which completely cross-reacts with RT21c. However, these results do not rule out an additional rat type-C virus which does not cross-react immunologically with the RT21c, p30, or p12 polypeptide.

Packaging of Ki-SV sequences. To further explore the nature of the packaging of the rat RNA found in the Moloney virus preparation released from NRK cells, we next examined V-NRK, Moloney virus growing in NRK cells, and the RT21c virus for their RT21c-like viral RNA sequences, their Ki-SV viral-like sequences, and the amount of rat p30 and rat p12 structural polypeptides. The results are shown in Table 6. The Moloney virus growing in the NRK cell contained relatively more of the Kirsten sarcoma-specific sequences and less of the RT21c-specific sequences. However, for a given amount of Kirsten sarcoma-specific RNA sequences in the Mo-MuLV preparation compared to the V-NRK virus, the Moloney virus growing in NRK cells does not contain detectable p12 rat polypeptide and only low levels of rat p30 structural polypeptide. The results suggest that the majority of the Kirsten sarcoma-specific sequences present in the Moloney virus growing in NRK cells are not packaged in rat structural proteins which cross-react immunologically with the V-NRK p 30 or V-NRK p12 proteins, and suggest instead that this RNA is packaged in the Moloney virus released from the NRK cells.

DISCUSSION

The current studies were undertaken to investigate the origin of the rat sequences detected in the Ka-SV and Ha-SV sarcoma viruses (15, 16; cited in 18; Klement and Roy-Burman, submitted for publication; Tsuchida et al., in press). Initial experiments indicated that DNA transcripts synthesized from two separate isolates of rat-C virus, each of which was derived from

TABLE 6. *Quantitation of rat RNA and proteins in various viruses*[a]

Virus preparation	RNA homologous to (μg/ml)		Rat p30 (μg/ml)	Rat p12 (μg/ml)
	RT21c	Ki-SV		
V-NRK	20-50	20-50	502	64
Moloney MuLV (NRK)	1-3	10-20	4	<2
RT21c	20-40	0.2-0.6	924	180

[a] The levels of rat type-C proteins were determined by competition radioimmunoassays with ^{125}I V-NRK p30 or p12 proteins as described in Table 5. The levels of RNA that hybridize to [^{3}H]DNA from RT21c and Ki-SV were determined by calibration curves like those shown in Fig. 3. Each virus preparation was phenol extracted for RNA and the RNA was suspended in a volume equal to the initial volume of virus. Hybridization was tested on varying volumes of the RNA solutions until saturation of either probe was achieved, and the amount of RNA to achieve saturation was then calculated, and expressed as the RNA concentration in micrograms per milliliter.

cell lines from Osborne-Mendel rats, differed in their ability to detect the rat sequences in Ki-SV and Ha-SV sarcoma virus. Whereas the DNA probes from the V-NRK rat virus consistently detected the rat sequences in Ki-SV and Ha-SV, DNA transcripts from RT21c consistently failed to detect these rat sequences. In immunological studies of the two rat viruses, a strong cross-reaction was observed between the p30 and p12 structural proteins of both the RT21c and V-NRK rat virus. Therefore, we explored in greater detail the nucleic acid sequences present in the two rat viruses and in the cellular RNA and DNA from the cells releasing them in an effort to find an explanation for the different hybridization results. To do this we used a Ki-SV-specific [^{3}H]DNA probe and an RT21c [^{3}H]DNA probe which did not hybridize with Ki-SV-specific sequences.

Using these two probes with nonoverlapping sequences, V-NRK virus preparations and RT21c virus preparations were found to contain in their high-molecular-weight RNA varying amounts of two distinguishable sets of RNA sequences. One set of sequences was homologous to the RT21c [^{3}H]DNA probe, and the other set of sequences was homologous to the Ki-SV-specific [^{3}H]DNA probe. V-NRK virus preparations contained approximately equal amounts of the RT21c-specific sequences and the Ki-SV-specific sequences. In contrast, RT21c virus preparations were found to contain approximately a 100-fold excess of the RT21c-like sequences over the Ki-SV-specific sequences. Correspondingly, the DNA transcripts from V-NRK contained sufficient amounts of the Ki-SV-specific sequences to detect these in both Ha-SV and Ki-SV, whereas the levels of the additional sequences in the RNA of RT21c virus preparations were too low to allow DNA probes synthesized from the RT21c virus to contain the Ki-SV or Ha-SV rat sequences. When we examined the DNA from RT21c cells and V-NRK cells we found the complete complement of the Ki-SV-specific sequences and, in studies not shown, the RT21c sequences in the DNA of both of these cells. The cellular RNA from V-NRK contained high amounts of the Ki-SV-specific sequences, whereas the RNA from RT21c cells contained barely detectable levels of the Ki-SV sequences. Thus, it appears that the production or degradation of the Ki-SV-specific RNA by the V-NRK cell and the RT21c cell is different. Experiments are in progress to analyze in detail the complete reassociation kinetics of the Ki-SV and RT21c probes to rat cellular DNA; such studies may help to resolve whether the Ki-SV sequences are of viral or cellular origin. It will be of interest to determine what factors regulate the expression of the Ki-SV-specific sequences, and whether or not we can increase the levels of Ki-SV-specific RNA in the RT21c cells. Also, it will be of interest to determine the expression of the Ki-SV versus the RT21c-specific sequences in various natural tissues and tumors from different strains of rats.

To further explain the presence of the Ki-SV-specific sequences in the V-NRK virus, we examined also the cellular RNA of NRK cells (not releasing virus) and the high-molecular-weight RNA of a heterologous virus, the Moloney leukemia virus growing in NRK cells. Studies with the Ki-SV-specific [^{3}H]DNA indicated that the NRK cells, not releasing the endogenous rat virus, already contained relatively high levels of the Ki-SV-specific sequences. By growing the Moloney leukemia virus in these NRK cells, the Moloney leukemia virus released from such cells was also found to contain in its high-molecular-weight RNA high levels of the Ki-SV-specific RNA. In experiments not shown, this Ki-SV-specific RNA was present in approximately $\frac{1}{10}$ the amount as the Moloney-specific RNA in the same high-molecular-weight RNA. In addition to its presence in the Moloney high-molecular-weight RNA, a [^{3}H]DNA transcript from this Moloney virus preparation contained the rat sequences found in the Ki-SV. Thus, the Moloney virus released from NRK cells contained in the virus preparation an RNA with homology to Ki-SV which was capable of being transcribed in an endogenous reverse transcriptase reaction. It seems likely that the V-NRK endogenous rat virus, when it was released from NRK cells, also contained in its preparation, in a manner analogous to the Moloney leukemia virus growing in NRK cells, the same rat sequences homologous to Ki-SV and Ha-SV. In the case of the Moloney virus released from NRK cells, the question was explored as to whether or not the "rescued" rat sequences were present in the virus preparation in the form of a rat type-C virus or packaged in the Moloney virus structural proteins. Quantitative analysis of the Moloney virus-containing mixture suggested that the Kirsten-specific RNA was not packaged in rat structural proteins but instead was packaged in Moloney viral proteins. However, further work will be necessary before this question is resolved since we cannot completely exclude the presence, in the Moloney-NRK virus, of a rat virus which is immunologically or biochemically unrelated to

RT21c or V-NRK, analogous to FeLV and RD114 in cats (2) or MMTV and MuLV in mice (11).

The current studies raise several additional questions which must be resolved in future experiments. One of the major issues is whether or not the RNA detected in NRK cells which is homologous to Ki-SV and Ha-SV is of cellular or endogenous viral origin. The apparent encapsidation of an RNA present in the NRK cells by a leukemia virus released from these cells is analogous to the observation concerning the presence of globin mRNA in the 60*S* RNA of Friend leukemia virus released from a cell line making hemoglobin mRNA (J. Ross, Y. Ikawa, J. Gielen, S. Packman, and P. Leder (ed.), 7th Int. Symp. Comp. Leuk. Res., in press). being encapsidated by a type-C virus when that virus is released from a cell producing that cellular mRNA. When NRK cells spontaneously release their RT21c-like rat virus, the levels of RT21c-related RNA rise markedly in the cell. In contrast, the level of the Ki-SV-specific RNA in NRK cells does not increase when the cell spontaneously releases its endogenous rat virus. The example of the globin mRNA and this data might suggest that the Ki-SV-specific sequences are not of viral origin. However, we will have to learn what factors regulate the expression of the Ki-SV-specific sequences and more about the physical and chemical characteristics of the Ki-SV and RT21c sequences in NRK cells before the question can be clearly resolved.

Nevertheless, the results do indicate that the source of the Ki-SV- and Ha-SV-specific sequences earlier detected using the V-NRK [^{3}H]DNA transcripts are derived from the "rescue" of an RNA already present in NRK cells before the release of the NRK rat type-C virus. It will be of importance now to examine whether or not the rat-specific RNA contained in Moloney virus preparations released from NRK cells can be transmitted to a non-rat cell since the Ki-SV and Ha-SV have formed genetically stable recombinants with this rat information. Obviously, a high degree of biologic containment must be used in experiments to attempt transmission of these sequences to other species to determine their biologic role, if any, in transformation. The present work also suggests that information transfer as outlined in Temin's protovirus hypothesis (19) might proceed in some manner analogous to the observations about globin (J. Ross et al. (ed.), 7th Int. Symp. Comp. Leuk. Res., in press) or Ki-SV sequences and that experiments with DNA transcripts and/or 70*S* RNA from type-C virus should consider whether sequences measured in hybridization experiments represent viral or cellular information.

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lated and expressed as the RNA concentration in micrograms
ing us of the usefulness of mink cells for focus formation before the publication of their data (6).

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