0022-538X/78/0025-0115$02.00/0
JOURNAL OF VIROLOGY, Jan. 1978, p. 115-123

Vol. 25, No. 1
Printed in U.S.A.

Identification of a 30S RNA with Properties of a Defective Type C Virus in Murine Cells

RICHARD S. HOWK,[1] DAVID H. TROXLER,[2] DOUGLAS LOWY,[3] PETER H. DUESBERG,[4] AND EDWARD M. SCOLNICK[2]*

Laboratory of Tumor Virus Genetics[2] and Dermatology Branch,[3] National Cancer Institute, Bethesda, Maryland 20014; Meloy Laboratories, Rockville, Maryland 20850[1]; and University of California at Berkeley, Berkeley, California 94720[4]

Received for publication 22 August 1977

A novel species of 30S RNA has been detected in a variety of mouse cell lines. The 30S RNA is specifically packaged by helper-independent type C viruses propagated in such cells. Nucleic acid hybridization detects no homology between the 30S RNA and the genomic RNA of helper-independent mouse type C viruses. The properties of the 30S RNA suggest that it is a defective endogenous mouse type C virus and that it is analogous to a previously described class of defective endogenous rat type C virus, which has been shown previously to be the progenitor of Kirsten and Harvey murine sarcoma viruses.

During the past three years, we have described a novel class of vertically transmitted type C viral-like information present in rats (25-27). This viral-like RNA was discovered because a portion of it was incorporated into Kirsten sarcoma virus and Harvey sarcoma virus by recombination with replicating mouse type C viruses, which were passaged in rats (2, 26, 33). Although this rat RNA showed no homology, detectable by nucleic acid hybridization, to typical helper-independent rat type C viruses, we classified it as type C RNA based on certain similarities to typical endogenous mammalian type C viruses (5, 17, 25, 27). (i) The viral genes are present in multiple copies in normal rat DNA; (ii) the RNA is inducible to high levels of expression with halogenated pyrimidines; (iii) when replicating in rat cells expressing this RNA, type C viruses, but not type B or D viruses, can package this RNA with a high degree of specificity; (iv) like tumor virus RNA, the packaged 30S subunit of this RNA forms multimeric structures and is found in the 50 to 70S viral RNA complex when viral RNA is prepared from the pseudotyped virus complexes; and (v) the multimeric form of this rat RNA has an RNA primer associated with it and is transcribed into cDNA in endogenous reverse transcriptase reactions (27; H. A. Young, T. Y. Shih, M. O. Weeks, and E. M. Scolnick, manuscript submitted for publication). In contrast to cells producing helper-independent endogenous type C viruses, cells expressing this novel class of rat viral-like RNA do not produce type C viral particles, nor do they express type C structural proteins detectable even in broad interspecies radioimmunoassays (27).

The current studies were designed to characterize an apparently similar class of type C viral-like RNA, which has been found in NIH 3T3 and SC-1 mouse cells, two cell lines that have not been shown to release a murine type C viral particle, even after treatment with halogenated primidines. Initially, it was observed that when a cloned isolate of the Kirsten strain of murine type C virus (Ki-MuLV) was grown in NIH 3T3 cells, the viral RNA released from the cell could be resolved into two components (38S and 30S) on acrylamide gels (9). The 38S component was thought to represent Ki-MuLV, but the significance of the smaller (30S) component was unresolved. In this paper, we present evidence that this 30S RNA component represents a novel class of endogenous viral RNA, which is expressed at relatively high levels in certain clones derived from NIH 3T3 cells as well as in other mouse-derived cell lines. This novel RNA is packaged in virions released from the cells after the cells are infected with helper-independent type C viruses. Our studies demonstrate that another species, in addition to the rat, harbors a distinct class of type C viral-like genetic information that cannot be readily recognized by techniques designed to isolate replicating viruses, but can be detected by molecular assays designed to take advantage of the fact that this RNA can be specifically pseudotyped by helper-independent type C viruses.

MATERIALS AND METHODS

Cells and viruses. (i) Uninfected cells. The following uninfected cell lines were used: SC-1, a cell line permissive for the growth of ecotropic murine type C viruses, was obtained from Janet Hartley,

NIAID (National Institute of Allergy and Infectious Diseases), Bethesda, Md. (13). Three clonal cell lines derived from the NIH 3T3 cell line (14) were used: (i) NIH 3T3 clone 2, a contact-inhibited cell line derived from the cell line originally described by Jainchill et al. (14); (ii) NIH clone 4, a cell line that was derived by cloning cells from a piled-up area of the NIH 3T3 monolayer and which shows much less contact inhibition than clone 2 (see below); and (iii) NIH clone 10_{TK^-} (thymidine kinase minus), a cell line derived from the original NIH 3T3 line by selecting for cells resistant to 100 μg of bromodeoxyuridine (BUdR) per ml. This cell was the gift of Robert Goldberg, NCI (National Cancer Institute, Bethesda, Md.). Other cell lines used included: BALB/c 3T3 cells; FRE clone 2 rat cells; NRK rat cells; a dog thymus cell (Cf_2th); a canine kidney cell (MDCK); and a mink lung cell (CCL 64), the sources of which have been described previously (27, 31).

(ii) Sarcoma virus-transformed nonproducer cells. Nonproducer cells transformed by the Kirsten and Harvey strains of murine sarcoma virus, namely BALB/KiSV, mink/KiSV, and MDCK/HaSV have been described (27, 31). A dog cell nonproductively transformed by the p30+ strain of Moloney sarcoma virus, MDCK/S^+L^- Mo-SV; a rat cell transformed by Abelson virus, NRK/Ab-LV; a rat cell transformed by a naturally occurring sarcoma virus of BALB/c mice (22), NRK/BALB-SV; a rat cell infected with the woolly sarcoma virus, NRK/Wo-SV; and a rat cell nonproductively infected with the Friend strain of spleen focus-forming virus, SFFV-NRK clone 1, have been described previously (20, 31). A hamster cell nonproductively infected with the FBJ strain of murine sarcoma virus was the gift of J. Levy, University of California.

Viruses. (i) Ecotropic murine viruses. The following clonal isolates of murine ecotropic type C viruses were used: (i) F-MuLV, the Friend strain of type C helper MuLV; (ii) WN1802N and WN1802B, endogenous ecotropic viruses isolated from BALB/c mice; and (iii) Moloney MuLV clone 2, an ecotropic clone of Mo-MuLV. Methods used to clone such replicating type C viruses have been described (31, 32). Each MuLV was propagated on FRE clone 2 rat cells or cells derived from the NIH 3T3 cell line as indicated below.

(ii) Xenotropic murine type C viruses. Three isolates of murine xenotropic virus, Balb virus 2, ATS-124, and NZB virus, were propagated in Cf_2th dog cells (16). A recombinant between ecotropic Moloney virus and xenotropic virus, Mo-MuLV clone 83, which has been described previously (32), was propagated in mink cells. The 1504A amphotropic MuLV, obtained from Janet Hartley, NIAID, was grown in Cf_2th cells.

(iii) Other viruses. The woolly leukemia virus (WoLV) was grown in FRE clone 2 cells. M432B, a type B retrovirus isolated from *Mus cervicolor*, was the gift of Robert Callahan, NCI (8). Two type C viruses from *Mus cervicolor* were the gift of Raoul Benveniste, NCI (4); cervicolor I was propagated on rabbit cells (SIRC), and cervicolor II was propagated on NIH clone 4 cells.

All cells were grown in Dulbecco modified Eagle medium containing either 10% calf serum or 10% fetal calf serum. Cells were routinely assayed for mycoplasma and found to be negative.

Preparation of cellular RNA and DNA. Total cellular RNA was prepared by extraction with phenol and chloroform:isoamyl alcohol, followed by treatment of the nucleic acids by RNase-free DNase I (Worthington Biochemicals) (3). DNA was prepared by the procedure of Marmur and was sonically treated by procedures previously described (19) to an average size of 5.5S for use in studies on nucleic acid reassociation kinetics. [^{3}H]thymidine-labeled mouse cellular DNA was prepared similarly from BALB 3T3 cells, and the unique sequence fraction was isolated by hydroxyapatite chromatography after reassociation to a C_0t of 200 mol · s/liter (7).

Preparation of [^{3}H]cDNA. Several different [^{3}H]cDNA's were used in these studies. (i) A [^{3}H]cDNA was prepared from the purified 30S viral RNA subunit; details are given below. (ii) The preparation of $cDNA_{SFFV}$, which hybridizes to xenotropic MuLV or to certain MuLV that are recombinants with xenotropic viruses, but not to ecotropic MuLV, has been described in a previous publication (31). (iii) [^{3}H]cDNA was prepared from sucrose density gradient-purified Mo-MuLV clone 83 grown in mink cells in an endogenous reverse transcriptase reaction by using calf thymus DNA fragments as primers (30). The following fractions of this cDNA representing different regions of the viral genome were prepared as described previously (20): (i) A cDNA representing sequences contained in the *gag* gene, designated $cDNA_{gag}$ (20, 22); (b) a cDNA representing sequences that are located at the 3′ end of the viral genome, designated $cDNA_{3'}$ (32); and (c) a cDNA representing sequences that code for parts of the *pol* and *env* genes of Mo-MuLV clone 83, designated $cDNA_{pol,env}$ (20). All cDNA's were labeled with [^{3}H]dCTP to specific activities of 2×10^7 cpm/μg of DNA.

Hybridization reactions. All of the following reaction mixtures were incubated at 66°C and contained in 0.05 ml: 0.02 Tris-hydrochloride (pH 7.5), 0.75 M NaCl, 5×10^{-4} M EDTA, approximately 2,000 trichloroacetic acid-insoluble counts per minute of [^{3}H]-cDNA, and either RNA or DNA, as indicated in the appropriate legends. Hybridizations were analyzed as a function of either RNA concentration and time (6), DNA concentration and time (7), or volume of supernatant of cultures and time (23). Hybridizations were analyzed with the use of S1 nuclease as described previously (3, 15).

RESULTS

Preparation of $cDNA_{30S}$. To synthesize $cDNA_{30S}$, we prepared 60 to 70S viral RNA from Mo-MuLV propagated in the TK^- clone of NIH cells (Fig. 1A). In earlier studies (21, 31), we had found that smaller subunits of various defective viruses sediment more slowly in the 60 to 70S viral RNA complex than the RNA of the helper virus. Therefore, the RNA from the leading edge (fractions 2 to 4) and trailing portion (fractions 8 to 10) of the 60 to 70S peak were pooled separately. Each pool was heat de-

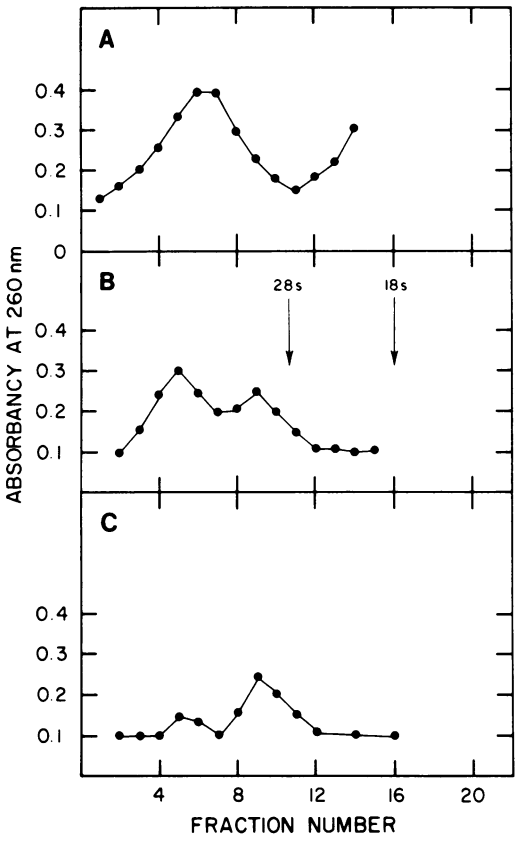

FIG. 1. *Preparation of viral RNA subunits. An 8-h collection of virus was prepared from 10 liters of culture fluid from NIH TK⁻ cells infected with Mo-MuLV clone 2, a cloned ecotropic isolate of Moloney leukemia virus (32). The virus was purified by continuous-flow centrifugation on sucrose gradients and disrupted at 37°C for 5 min with 1% sodium dodecyl sulfate–0.2% diethylpyrocarbonate. The RNA was sedimented for 16 h at 15,000 rpm at 15°C in a linear 15 to 30% sucrose gradient containing 0.02 M Tris · hydrochloride (pH 7.8), 0.10 M sodium chloride, 0.001 M EDTA (TNE buffer) (A). Fractions 2 through 4 and 8 through 10 from two such gradients were pooled and concentrated by ethanol precipitation. Each pool was denatured at 80°C for 3 min in TE buffer and sedimented in a similar gradient but for 18 h at 25,000 rpm. The positions of the markers are from parallel gradients containing [³H]ribosomal RNA.*

natured and resedimented in a second sucrose gradient (Fig. 1B and C). From the faster-sedimenting side of the 60 to 70S RNA, two peaks of RNA were observed (1B), one at approximately 38S and one at 30S. The 38S peak was in slight excess. From the slower-sedimenting side of the 60 to 70S viral RNA, again two peaks were obtained (Fig. 1C), but the predominant peak was now at 30S. The RNA from the 30S peak was then reverse transcribed to cDNA with partially purified AMV reverse transcriptase and calf thymus DNA fragments as primers (30, 31).

Hybridization specificity of $cDNA_{30S}$. The cDNA prepared from the purified 30S RNA was then tested for its hybridization specificity against a variety of RNAs. $cDNA_{30S}$ hybridized to the 30S RNA with a 1/2 C_rt of approximately 4.6×10^{-2} mol · s/liter, and the $cDNA_{30S}$ protected 50% of ^{32}P-labeled 30S RNA at 1:1 molar ratios and 88% at 5:1 molar ratios by using S1 nuclease to assay for hybrids. The $cDNA_{30S}$ did not hybridize to RNA from heterologous cells infected with a variety of ecotropic or xenotropic murine type C viruses or an amphotropic murine type C virus, or to the 38S RNA obtained from Fig. 1B (Table 1). To insure that adequate amounts of RNA were being tested in Table 1, we hybridized in parallel the three fractions of cDNA from Mo-MuLV clone 83, which detect different regions of the genome of helper-independent murine type C viruses (32). In each case, the cDNA from Mo-MuLV clone 83 hybridized appreciably to each of these replicating

TABLE 1. *Distinction between cDNA's from murine viruses and 30S RNA*

Source of RNA tested	% hybridization[a] with cDNA from: Mo-MuLV clone 83 grown in mink cells			30S NIH RNA
	3′ end	Pol, Env	Gag	
Dog cells infected with:				
ATS-124	38	38	38	<5
NZB virus	36	36	38	<5
Balb virus 2	35	35	38	<5
AMT 1504A	46	50	44	<5
Uninfected Cf_2th	<5	<5	<5	<5
Rat cells infected with:				
WN 1802B	33	33	33	<5
WN 1802N	38	33	34	<5
WN 1802NB	38	36	38	<5
F-MuLV clone 201	45	45	35	<5
Mo-MuLV clone 83	100	100	100	<5
Mo-MuLV clone 2	100	100	100	<5
Uninfected FRE clone 2	<5	<5	<5	<5
Prepared from Mo-MuLV:				
30S RNA	<5	<5	<5	100
38S RNA	100	100	100	<5

[a] Each hybridization reaction contained approximately 2,000 trichloroacetic acid counts per minute of cDNA. A 100% hybridization ranged from 1,500 to 1,800 cpm hybridized for each probe and was assayed with RNA from infected cells to saturating values at C_rt values of up to 5,000 mol · s/liter. With the 38S and 30S, RNA hybridization was performed to C_rt values of 3×10^{-1} mol · s/liter. The 38S was from Fig. 1B and 30S RNAs were from Fig. 1C.

murine viruses. In addition, these cDNA's hybridized to the 38S RNA but not to the 30S RNA. Therefore, these results indicated that the 30S subunit had little, if any, homology to helper-independent murine type C viruses. In addition, RNAs from nonmurine cells transformed by several mammalian sarcoma viruses were tested. These cells are noted above, and the viruses included Ki-SV, Ha-SV, Balb-SV, Wo-SV, Mo-SV, Ab-LV, SFFV, and FBJ viruses. None of these RNAs hybridized to the $cDNA_{30S}$ even at C_rt values of 5,000 mol · s/liter.

Origin of 30S RNA. To investigate the intracellular origin of the 30S RNA, the $cDNA_{30S}$ was hybridized to total cellular RNA from NIH clone 4. The results are shown in Fig. 2, along with hybridization of the same cellular RNA to the three probes from Mo-MuLV clone 83 and to the $cDNA_{SFFV}$, which detects sequences in the *env* gene region of murine xenotropic virus (31, 32). The $cDNA_{30S}$ hybridized relatively rapidly to this cellular RNA with a 1/2 C_rt in the range of 40 to 60 mol · s/liter. Each of the other cDNA's representing different sequences in ecotropic and xenotropic murine type C viruses hybridized much more slowly and failed to saturate the probes at C_rt values of greater than 1,000 mol · s/liter. The results indicated that these NIH murine cells had much higher levels of the RNA homologous to the $cDNA_{30S}$ than RNA of either xenotropic or ecotropic viruses. Importantly, each of these cDNA's hybridized to RNA from heterologous cells infected with ATS-124, the murine xenotropic virus isolated from NIH Swiss mice.

Specificity of pseudotyping of 30S RNA. To investigate whether the RNA detected in NIH cells was specifically packaged by type C viruses replicating in NIH cells, we examined the cellular RNA and supernatant RNA from uninfected NIH clone 4 cells, and NIH clone 4 cells superinfected with either F-MuLV, a type C virus, or M432B, a type B virus (Fig. 3 and 4). With the $cDNA_{30S}$, no sedimentable RNA was detected in the supernatant of uninfected NIH clone 4 cells even at V_ot values of 10^2 ml · h. However, high levels of the 30S RNA were detected in the supernatant medium when NIH clone 4 cells were superinfected with F-MuLV (Fig. 3B). Importantly, replicating F-MuLV packages this RNA to approximately the same degree as it packages its own RNA as a result of the release of virions from infected cells (Fig. 3A versus B). In contrast, when M432B type B virus is grown in NIH clone 4 cells, whereas high levels of M432B RNA were released from the cell (Fig. 4B), the $cDNA_{30S}$ detected much lower levels of homologous 30S RNA released into the supernatant. Based on the measured intracellular ratios of M432B RNA and RNA homologous to $cDNA_{30S}$, this type B virus rescued its own RNA 100-fold more efficiently than it rescued the 30S RNA. When other type C viruses, namely, *Mus cervicolor* II, WN 1802N, Ki-MuLV, or Mo-MuLV clone 2, were propagated in the NIH clone 4 cells, each type C virus rescued and purified the RNA homologous to the $cDNA_{30S}$ with an efficiency equal to that with which each purified its own viral RNA (data not shown). The results indicate that type C viruses, when grown on NIH clone 4 cells, can efficiently package the indicated 30S RNA, whereas a type B virus does not. These results and analogous observations previously made on endogenous viral RNA in rat cells (12) indicate that the 30S RNA species found in NIH clone

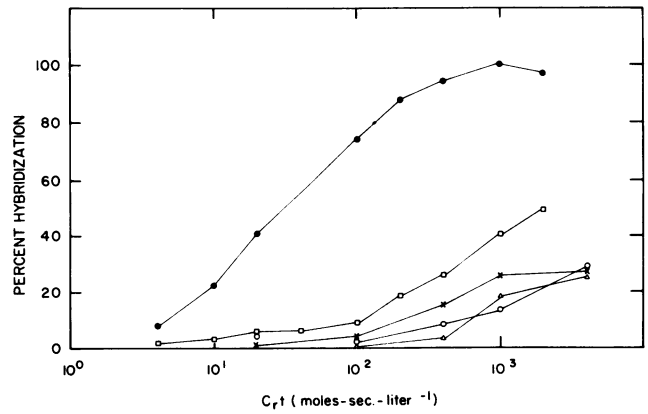

FIG. 2. *Viral RNA levels in NIH mouse cells. Total cellular RNA from NIH clone 4 cells was hybridized to approximately 2,000 trichloroacetic acid counts per minute of the indicated [^{3}H]cDNA's. Hybridizations were assayed with the use of S1 nuclease; background in the absence of added RNA was less than 50 cpm. Symbols: $cDNA_{30S}$ (●); $cDNA_{SFFV}$ (×); from Mo-MuLV clone 83: cDNA $_{gag}$ (□); $cDNA_{pol,env}$ (△); $cDNA_{3'}$ (○).*

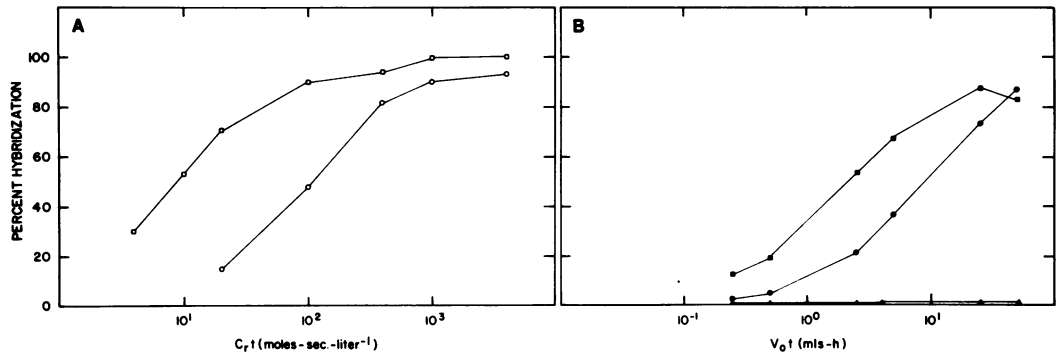

FIG. 3. *Rescue of 30S sequences by a type C virus replicating in NIH mouse cells. NIH clone 4 cells were infected with F-MuLV propagated in FRE clone 2 rat cells. The culture was grown for approximately 2 weeks, including one subcultivation of the cells. At that time, five 75-cm^2 flasks were seeded with 3 × 10^6 NIH clone 4 cells or 3 × 10^6 clone 4 cells infected with F-MuLV. Supernatant fluid was collected from the flasks over the next 24 h, clarified at 3,000 rpm at 4°C for 10 min, and then centrifuged at 100,000 × g for 2 h. Total cellular RNA was extracted from the cells after the collection of supernatant fluid. RNA was extracted (25) from the concentrated supernatants and suspended in 1.0 ml of distilled water after extensive dialyses. The solution was then hybridized to either cDNA$_{30S}$ or cDNA from F-MuLV grown in FRE rat embryo cells (31). The reassociation kinetics are plotted as a function of C$_r$t intracellularly and V$_o$t extracellularly (6, 23). Symbols: (A) Intracellular RNA: cDNA$_{30S}$ (○); cDNA$_{F\text{-}MuLV}$ (□). (B) Extracellular RNA: cDNA$_{30S}$ (●); cDNA$_{F\text{-}MuLV}$ (■); supernatant from uninfected NIH cells with cDNA$_{30S}$ (▲).*

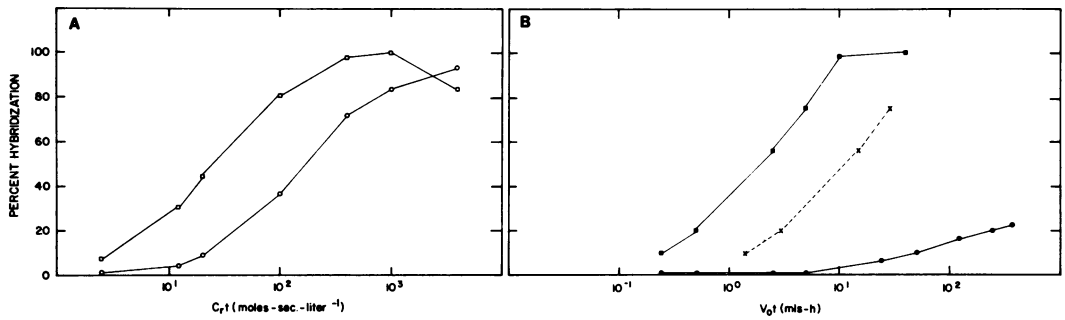

FIG. 4. *Lack of rescue by type B virus of NIH 30S RNA. Supernatant and cellular RNAs were obtained as indicated in the legend to Fig. 3. (A) Intracellular RNA: cDNA$_{30S}$ (○); cDNA$_{M432B}$ (□). (B) Extracellular RNA: cDNA$_{30S}$ (●); cDNA$_{M432B}$ (■); (×) indicates theoretical curve with cDNA$_{30S}$ if ratio of it to M432B RNA was the same as the intracellular ratio.*

4 cells can be classified as type C viral RNA.

Reverse transcription of rescued RNA. Since the novel 30S RNA was packaged in a type C virus grown in NIH cells, we examined a cDNA product synthesized from one such virus, F-MuLV, grown in these cells. We performed these studies to determine if cDNA probes synthesized from helper-independent viruses grown in mouse cells would be "contaminated" by these sequences and thus potentially give confusing results with regard to nucleic acid homology between different mouse helper-independent viruses (Table 2). The cDNA from this virus preparation hybridized to a high degree to RNA from uninfected NIH cells and to the purified 30S RNA as opposed to RNA from a rat cell. This cDNA probe was then hybridized to RNA from F-MuLV-infected rat cells, and the hybridized and unhybridized portions were separated by hydroxyapatite chromatography (29). The resultant two fractions were retested for their hybridization properties. Fraction II, the unhybridized portion, still hybridized well to NIH cellular RNA and the 30S subunit, but hybridized poorly to RNA from F-MuLV-infected rat cells. Fraction III, the hybridized portion eluting at 0.46 M phosphate, hybridized well to RNA from NRK/F-MuLV but poorly to NIH RNA. The results indicate that the cDNA from viruses grown in NIH cells are composed of two largely nonhomologous fractions, one homologous to the replicating helper virus (F-MuLV) and one to the rescued 30S RNA.

Copy number in DNA. A property of endog-

TABLE 2. *Reverse transcription of rescued RNA from F-MuLV grown in NIH cells*

RNA tested	% Hybridization with cDNA fraction[a]		
	I (Unfractionated)	II (0.12 M phosphate)	III (0.46 M phosphate)
NIH clone 4	40	100	8
30S	35	90	8
NRK	10	<5	<5
NRK/F-MuLV	100	12	100

[a] The cDNA from F-MuLV grown in NIH clone 4 was hybridized to RNA from NRK cells infected with F-MuLV. The hybridized and unhybridized portions were separated by hydroxyapatite chromatography and then retested as indicated. Each reaction contained approximately 2,000 trichloroacetic acid counts per minute of such indicated cDNA, and hybridizations were performed and analyzed as detailed in footnote *a*, Table 1. The 30S RNA is from Fig. 1C.

enous mammalian type C viruses is that they are reiterated in the DNA of the species of their origin (5). Therefore, by using the $cDNA_{30S}$ described above, we examined the copy number of homologous sequences in cellular DNA from NIH clone 4 cells (Fig. 5). The $cDNA_{30S}$ hybridizes more rapidly to NIH cellular DNA than does the unique-sequence [^{3}H]DNA prepared from mouse cells. The 1/2 C_0t of approximately 40 to 60 mol · s/liter was obtained with the $cDNA_{30S}$, whereas the unique sequence control was in the range of 800 to 900 mol · s/liter. The results indicate that the sequences detected by $cDNA_{30S}$ are reiterated approximately 10 to 15× compared with the average of unique-sequence cellular genes and that multiple copies of the viral-like DNA are integrated into the host cell DNA.

Inducibility of sequences. Some aspects of the regulation of these sequences were next examined, as shown in Fig. 6A and B. Three types of mouse cells were examined: BALB 3T3, SC-1, and various clones of NIH cells. A clone of NIH cells, clone 2, which is more contact inhibited than clone 4, had lower basal levels of the RNA (Fig. 6B). When this cell was treated with BUdR (17), an eightfold increase in the RNA could be detected. Interestingly, a TK$^-$ clone of NIH cells, clone 10, derived by chronic treatment over 6 months with increasing doses of BUdR, had even higher RNA levels with a 1/2 C_rt of 10 to 20 mol · s/liter.

The levels of homologous RNA were examined in SC-1 cells and in BALB 3T3 cells (Fig. 6A). Both cells had detectable basal levels, and BUdR treatment led to an increase in the RNA in both cells. In this particular experiment a 7- to 8-fold increase was achieved in SC-1, whereas almost a 50-fold increase was achieved in BALB 3T3. The results indicate that BUdR treatment leads to an increase in the RNA homologous to the $cDNA_{30S}$ in each of the three types of cells.

A further aspect of the regulation of this RNA is shown in Fig. 7A and B. The levels of RNA in BALB mouse cells transformed by Ki-SV were no higher than those in BALB 3T3 cells, indicating that transformation per se does not lead to increased levels of RNA. When these cells were treated with either iododeoxyuridine (IUdR) or cycloheximide (10), a massive increase in the RNA homologous to the $cDNA_{30S}$ was achieved with IUdR, but less than a twofold change occurred with cycloheximide. When the same RNAs were examined with a cDNA from Balb virus 2 grown in dog cells, the levels of RNA detected with this probe rose comparably with either IUdR or cycloheximide induction. The regulation of this novel RNA is thus different from the regulation of xenotropic viral RNA in these cells.

Properties of NIH clones. Growth properties of the NIH clones with varying levels of RNA homologous to the $cDNA_{30S}$ were analyzed and summarized in Table 3. NIH 3T3 clone 2 had the lowest levels of RNA and failed to grow in agar. NIH clone 10 had 10- to 20-fold higher levels of this RNA species and grew well in agar. Clone 4 was intermediate in RNA and formed a few small colonies in soft agar. These colonies have not yet been examined to see if they have higher levels of this species of RNA than the mass culture derived from NIH clone 4. Importantly, the 1/2 C_rt for the novel species of RNA in NIH clone 10 is comparable to the 1/2 C_rt for Mo-MuLV RNA in the infected cell. But the NIH clone 10 cells did not release viral

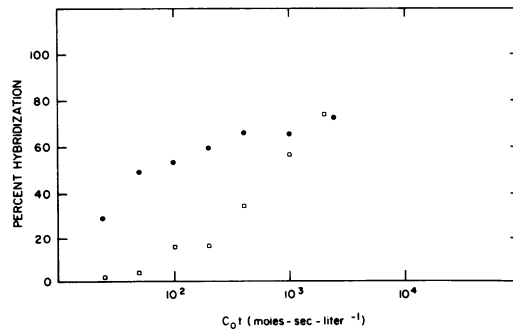

FIG. 5. *Copy number of sequences homologous to $cDNA_{30S}$ in mouse cell DNA. Unlabeled DNA from NIH clone 4 cells was hybridized to either $cDNA_{30S}$ (●) or ^{3}H-labeled unique-sequence mouse cellular DNA (□). Each reaction mixture contained approximately 2,000 trichloroacetic acid counts per minute of labeled DNA. Annealing was analyzed with S1 nuclease and plotted as a function of DNA concentration and time (7).*

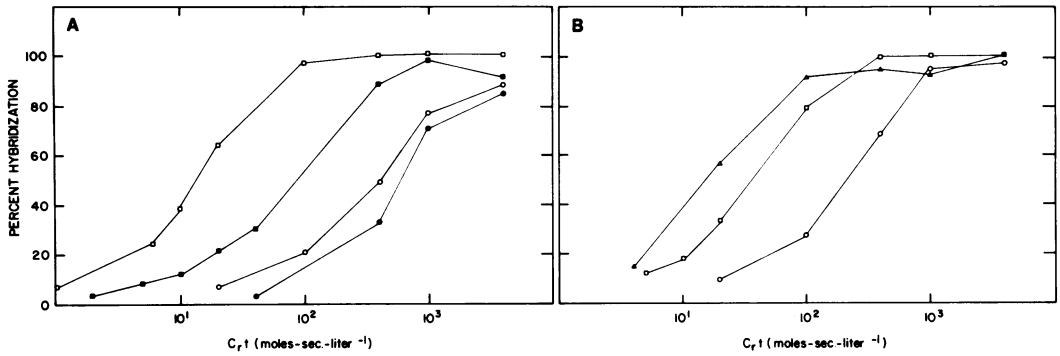

FIG. 6. *Induction by BUdR of viral-like sequences in various mouse cells. BALB 3T3 cells, SC-1 cells, or NIH clone 2 cells were seeded at 3 × 10^6 cells per 75-cm² flask. After 24 h later, BUdR was added to a final concentration of 20 μg/ml and left in the culture for 48 h. The BUdR-containing medium was then removed and the cells were refed with fresh medium for an additional 48 h. RNA was then extracted and hybridized to the $cDNA_{30S}$. (A) Control BALB 3T3 (○); plus BUdR (□); control SC-1 cells (●); plus BUdR (■). (B) Control NIH clone 2 cells (○); plus BUdR (□); NIH clone 10 TK⁻ cells chronically propagated on 100 μg of BUdR (△) per ml.*

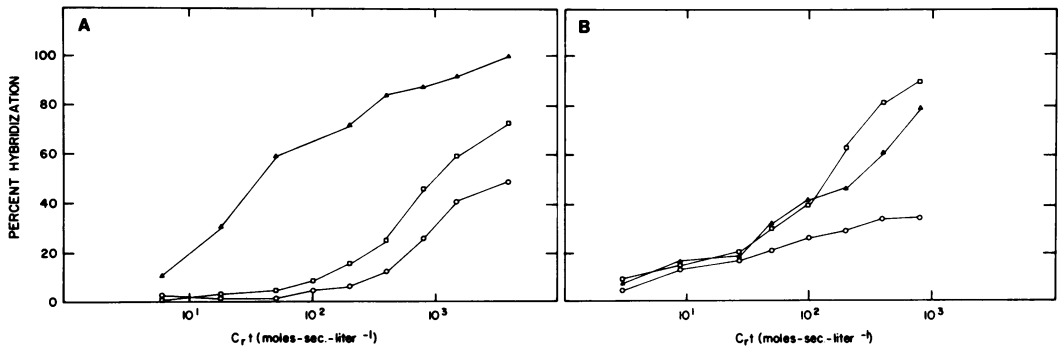

FIG. 7. *Comparative induction of xenotropic viral RNA and RNA homologous to $cDNA_{30S}$. BALB/Ki-SV cells were seeded at 3 × 10^6 cells per 75-cm² flask and treated either with IUdR (20 μg/ml) as indicated in the legend to Fig. 5 or with 10 μg of cycloheximide per ml for 4 h. RNA was extracted after 24 h from the cycloheximide-treated cultures (10). (A) $cDNA_{30S}$ versus RNA from: control (○); cycloheximide-treated (□); IUdR (△). (B) cDNA from Balb virus 2 propagated in dog thymus cells. Control (○); cycloheximide-treated (□); IUdR-treated (△).*

particles containing the RNA, whereas the superinfected cells did.

DISCUSSION

In studies to elucidate the origin of the Kirsten and Harvey murine sarcoma viruses, a novel type of genetic information, which was related to the sarcoma virus-specific sequences of Ki-SV and Ha-SV, was detected in rats. This rat genetic information has been shown to have the following properties. (i) It is present in the DNA of rats in 20 to 40 copies per diploid genome; (ii) it is inducible with halogenated pyrimidines to high levels of expression; (iii) it is efficiently packaged by replicating type C viruses when such viruses are grown in rat cells that express high levels of this RNA; (iv) it is 30 to 32S in size and forms multimeric, presumably dimeric, complexes like tumor virus RNA; and (v) it is transmissible under special conditions in pseudotype form to certain cells (E. M. Scolnick, unpublished data). The properties of this genetic information permitted a classification of these genes as type C viral in nature despite the facts that (i) rat cells expressing the genetic information as RNA did not form type C particles or contain antigens of helper-independent type C viruses; and (ii) this rat genetic information had no detectable homology by hybridization to typical helper-independent rat type C viruses (25–27).

The current studies have identified genetic information in mouse cells that shares many characteristics with the previously described rat

TABLE 3. *Properties of clones of NIH mouse cells*[a]

Cell	1/2 C,t with:		Doubling time (h)	% Cloning efficiency in agar	Supernatant particles
	$cDNA_{30}S$	$cDNA_{Mo\text{-}MuLV}$			
NIH 3T3 clone 2	200	$>10^3$	19–20	$<10^{-4}$	–
NIH clone 4	40–100	$>10^3$	19–20	1	–
NIH clone 10 TK^-	10–20	$>10^3$	28	50–60	–
NIH TK^-/Mo-MuLV	10–20	10–20	28	50–60	+

[a] Growth in soft agar was performed as described previously (18). Doubling time was determined by following the growth rate of each cell in liquid medium over 7 days, with a media change on day 4. C,t values were determined by standard procedures as indicated in footnote *a*, Fig. 1. The "supernatant particle" status is defined (27) by hybridization analysis of supernatant fluids for viral RNA that is sedimentable at 100,000 × *g* for 2 h to V_0t values of 10^3 ml · h with a [^{3}H]$cDNA_{30S}$.

viral-like genes. Characteristics of this novel mouse genetic information are: (i) It is present in 10 to 20 copies per diploid genome in normal mouse DNA; (ii) it is inducible to high levels of expression in cells derived from either inbred or wild mice; (iii) it is packaged by type C viruses growing in mouse cells; (iv) it is 30S in size; and (v) it shares little, if any, homology to the xenotropic, amphotropic, or ecotropic helper-independent murine type C viruses. In addition, the mouse cell lines expressing only this class of type C-like RNA do not form viral particles even though high levels of RNA are detected in these cells. The 30S subunit may be the same molecular species of RNA previously reported in JLS-V9 mouse cells by Fan and Besmer (11) and P. Besmer, U. Olshevsky, and H. Fan (personal communication).

What is the significance of this type C viral-like genetic information now shown to be present in two mammalian species? In the case of the rat, a portion of this genetic information became incorporated presumably by recombination into two independently derived murine sarcoma viruses, Ki-SV and Ha-SV. Many lines of evidence suggest that the rat information alone or in combination with the 3′ end of Ki-MuLV plays an important role in the ability of the Kirsten sarcoma virus to transform fibroblasts (24, 28). In the case of the mouse genetic information, no such evidence exists to implicate it in transformation since we have not yet detected it in any mouse-derived transforming virus. Some cells that grow well in agar express the RNA at high levels. However, no causal relationship between the expression of the endogenous RNA and transformation has been demonstrated. Thus, the full physiological significance of this viral-like RNA cannot be assessed.

Nevertheless, the implications of the existence in two species of this type C-like genetic information can be further considered. In particular, is there a relationship in the evolution of type C viruses between this class of type C information and the type C information from which helper-independent type C virus particles are derived? It is possible that nucleic acid sequencing methods might show homology between these two classes of type C viral information, which cannot be detected by hybridization assays. Conversely, we have also considered the possibility that the 30S RNA might be complementary to helper-independent type C RNA and performed RNA · RNA annealing to test this. Thus far, no positive results have been obtained. In the mouse and rat cells examined, this information seems to be defective for particle formation, but this class of type C-like RNA might have evolved from helper-independent type C viruses in other species of rodents. As other possibilities, this type C-like information might play some role in the life cycle of replicating type C viruses, or this class of type C-like RNA might be related in some way to intracisternal A particles, which at various times have been both compared and contrasted to type C viruses. Lastly, it will be important to determine the comparative biology of this class of type C viral information to determine if it is present also in species of vertebrates from which helper-independent endogenous type C viruses have never been isolated. The ability of replicating type C viruses to pseudotype this class of RNA should provide a molecular approach to this question.

LITERATURE CITED

1. **Aaronson, S. A., G. Todaro, and E. M. Scolnick.** 1971. Induction of murine C-type virus from clonal lines of virus-free Balb/3T3 cells. Science 174:157–159.
2. **Anderson, G. A., and K. C. Robbins.** 1976. Rat sequence of the Kirsten and Harvey murine sarcoma virus genomes: nature, origin and expression in rat tumor RNA. J. Virol. 17:335–351.
3. **Benveniste, R., and E. M. Scolnick.** 1972. RNA in mammalian sarcoma transformed nonproducer cells homologous to murine leukemia virus. Virology 51:370–382.
4. **Benveniste, R. E., R. Callahan, C. J. Sherr, K. Chapman, and G. J. Todaro.** 1977. Two distinct endogenous type-C viruses isolated from the Asian rodent *Mus cervicolor*: conservation of virogene sequences in related rodent species. J. Virol. 21:849–862.

5. **Benveniste, R. E., and G. J. Todaro.** 1974. Multiple divergent copies of endogenous C-type virogenes in mammalian cells. Nature (London) **242**:456–459.
6. **Birnsteil, M. L., B. H. Sells, and I. F. Purdom.** 1972. Kinetic complexity of RNA molecules. J. Mol. Biol. **63**:21–39.
7. **Britten, R. J., D. E. Graham, and B. R. Neufeld.** 1974. Analysis of repeating DNA by reassociation kinetics. Methods Enzymol. **29**:363–418.
8. **Callahan, R., C. J. Sherr, and G. J. Todaro.** 1977. A new class of murine retraviruses: immunological and biochemical comparison of novel isolates from *Mus cervicolor* and *Mus caroli*. Virology **80**:401–416.
9. **Duesberg, P. H., and E. M. Scolnick.** 1977. Murine type-C viruses propagated in mouse cells contain a 30S RNA subunit of unknown biological activity, in addition to the 38S subunit of the viral genome. Virology **83**:211–216.
10. **Dunn, C. Y., S. A. Aaronson, and J. R. Stephenson.** 1975. Interactions of chemical inducers and steroid enhancers of endogenous mouse type-C RNA viruses. Virology **66**:579–588.
11. **Fan, H., and P. Besmer.** 1975. RNA metabolism of murine leukemia virus. II. Endogenous virus-specific RNA in the uninfected BALB/c cell line, JLS-V9. J. Virol. **15**:836–842.
12. **Goldberg, R. J., R. Levin, W. P. Parks, and E. M. Scolnick.** 1976. Quantitative analysis of the rescue of RNA sequences by type C viruses. J. Virol. **17**:43–50.
13. **Hartley, J. W., and W. P. Rowe.** 1975. Clonal cell lines from a feral mouse embryo which lack host-range restrictions for murine viruses. Virology **65**:128–134.
14. **Jainchill, J. F., S. Aaronson, and G. Todaro.** 1969. Murine sarcoma and leukemia viruses. Assay using clonal lines of contact-inhibited mouse cells. J. Virol. **4**:549–553.
15. **Leong, J. A., A. C. Garapin, N. Jackson, L. Fanshier, N. Levinson, and J. M. Bishop.** 1972. Virus-specific ribonucleic acid in cells producing Rous sarcoma virus: detection and characterization. J. Virol. **9**:891–902.
16. **Levy, J. A.** 1973. Xenotropic viruses: murine leukemia viruses associated with NIH Swiss, NZB, and other mouse strains. Science **182**:1151–1153.
17. **Lowy, D. R., W. P. Rowe, N. Teich, and J. W. Hartley.** 1971. Murine leukemia virus: high-frequency activation in vitro by 5-iododeoxyuridine and 5-bromodeoxyuridine. Science **174**:155–156.
18. **Macpherson, I.** 1969. Clonal analysis of virus-induced cell transformation, p. 214–219. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York, N.Y.
19. **Marmur, J.** 1961. Isolation of DNA from microorganisms. J. Mol. Biol. **3**:208–218.
20. **Parks, W. P., R. S. Howk, A. Anisowicz, and E. M. Scolnick.** 1976. Deletion mapping of Moloney type-C virus: polypeptide and nucleic acid expression in different transforming virus isolates. J. Virol. **18**:491–503.
21. **Parks, W. P., and E. M. Scolnick.** 1977. In vitro translation of Harvey murine sarcoma viral RNA. J. Virol. **22**:711–719.
22. **Peters, R. L., L. S. Rabstein, R. VanVleck, G. J. Kelloff, and R. J. Huebner.** 1974. Naturally occurring virus of the BALB/c mouse. J. Natl. Cancer Inst. **53**:1725–1729.
23. **Ringold, G., E. Y. Lasfargues, J. M. Bishop, and H. G. Varmus.** 1975. Production of mouse mammary tumor virus by cultured cells in absence and presence of hormones: assay by molecular hybridization. Virology **65**:135–147.
24. **Scher, C., E. M. Scolnick, and D. Siegler.** 1975. Induction of erythroid leukemia by the Harvey and Kirsten sarcoma virus. Nature (London) **256**:225–227.
25. **Scolnick, E. M., R. J. Goldberg, and D. Williams.** 1976. Characterization of rat genetic sequences of Kirsten sarcoma virus: distinct class of endogenous rat type C viral sequences. J. Virol. **18**:559–566.
26. **Scolnick, E. M., E. Rands, D. Williams, and W. P. Parks.** 1973. Studies on the nucleic acid sequences of Kirsten sarcoma virus: a model for formation of a mammalian RNA-containing sarcoma virus. J. Virol. **12**:458–463.
27. **Scolnick, E. M., D. Williams, J. Maryak, W. Vass, R. J. Goldberg, and W. P. Parks.** 1976. Type C particle-positive and type C particle-negative rat cell lines: characterization of the coding capacity of endogenous sarcoma virus-specific RNA. J. Virol. **20**:570–582.
28. **Shih, T. Y., H. A. Young, J. M. Coffin, and E. M. Scolnick.** 1978. Physical map of the Kirsten sarcoma virus genome as determined by fingerprinting RNase T1-resistant oligonucleotides. J. Virol. **25**:238–252.
29. **Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt.** 1976. DNA related to the transforming genes of avian sarcoma virus is present in normal avian DNA. Nature (London) **260**:170–173.
30. **Taylor, J. M., R. Illmensee, and J. Summers.** 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta **442**:324–330.
31. **Troxler, D. H., J. K. Boyars, W. P. Parks, and E. M. Scolnick.** 1977. Friend strain of spleen focus-forming virus: a recombinant between mouse type C ecotropic viral sequences and sequences related to xenotropic virus. J. Virol. **22**:361–372.
32. **Troxler, D. H., D. Lowy, R. Howk, H. Young, and E. M. Scolnick.** 1977. The spleen focus-forming virus is a recombinant between ecotropic murine type-C virus, and the *env* gene region of xenotropic type-C virus. Proc. Natl. Acad. Sci. U.S.A., **74**:4671–4675.
33. **Tsuchida, N. R., R. V. Gilden, and M. Hatanaka.** 1974. Sarcoma-virus related RNA sequences in normal rat cells. Proc. Natl. Acad. Sci. U.S.A. **71**:4503–4507.