

Partial Transcription of Murine Type C Viral Genomes in BALB/c Cell Lines

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The mouse cell line, BALB/c 3T3, and its derivatives transformed either spontaneously or by treatment with a variety of external agents, were analyzed for cytoplasmic RNA complementary to DNA products prepared from the Kirsten strain of murine sarcoma-leukemia virus, and from an endogenous type C virus of BALB/c 3T3. Although none of these cell lines spontaneously releases complete type C virions, they all contain RNA which is partially homologous to a portion of the 35S RNA isolated from these viruses. The parental cell line, BALB/c 3T3, contains a low level of viral-related RNA, and there is an increased amount of this RNA in some of the transformed cells. The RNA detected represents only a fraction of the viral RNA found in virus-producing cells. The formation of RNA:DNA hybrids was detected by equilibrium centrifugation in Cs_2SO_4 density gradients and by analysis with a single-strand-specific nuclease from *Aspergillus oryzae*. Viral DNA products prepared either from an endogenous reaction with whole virus in the presence of actinomycin D or from purified 70S viral RNA as template using avian myeloblastosis virus DNA polymerase yield comparable data. In addition, all of the BALB/c lines examined produce detectable levels of murine type C virus group-specific antigen.

Type C viruses, as a class, contain single-stranded 70S RNA (15) and an RNA-dependent DNA polymerase (9, 37). DNA synthesized by the RNA-dependent DNA polymerase on the viral RNA template is a sensitive and specific probe for detecting viral nucleic acid sequences, and has been used to detect viral-related RNA in avian and mammalian cells infected with and producing RNA tumor viruses (8, 20, 25), and in cells transformed by RNA tumor viruses but not producing virus (12, 20, 40).

Several lines of evidence indicate that the genetic information coding for type C RNA viruses can be present in vertebrate cells without release of complete viral particles. For example, virus-free clonal cell lines can release infectious virus either spontaneously (1, 33, 38), or after treatment with various chemical and physical agents (3, 22, 27). These biological observations have been supported by molecular hybridization studies which demonstrate the presence of nucleic acid sequences homologous to the type C viral RNA in the DNA of many normal cells (10, 42). Recently, Hayward and Hanafusa (21) have reported the detection of avian type C virus-specific RNA in virus non-

producing chicken cells that contained the avian tumor virus group-specific antigen. It has been demonstrated further by radioimmunoassay that all murine cell lines examined contained low levels of one of the structural virion proteins, the group-specific (gs) antigen (29). Whether this low level of expression represented complete virus synthesis by a few cells or partial expression of the viral genome was not clear from these studies.

The current studies were undertaken to examine the extent of transcription of the type C viral genome in an uninfected murine cell line, BALB/c 3T3, and in derivatives of this line which are transformed either spontaneously, by radiation, methylcholanthrene, a DNA virus (SV40), B77 strain of avian sarcoma virus, or by Kirsten strain of sarcoma virus. None of these cell lines produces detectable extracellular type C virus as determined by a supernatant viral reverse transcriptase assay (31) or by a cocultivation infectivity assay with a sensitive indicator cell line (NIH/3T3) (Lieber and Todaro, in press).

The hybridization of virus-specific, radioactive DNA to cellular or viral RNA was measured

by sedimentation in Cs_2SO_4 density gradients and by hydrolysis of the products of the reaction mixture after the addition of a single-strand-specific nuclease purified from *Aspergillus oryzae* (5, 36).

MATERIALS AND METHODS

Cells. Cells were grown as monolayer cultures in Dulbecco's modification of Eagle minimal essential medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.). BALB/c 3T3 clone A31 is a contact-inhibited murine cell line (2). S2CL3 is a morphologically transformed cell line derived from a spontaneously transformed variant of A31; it continuously produces high titers of endogenous mouse type C virus (38). K-A31 clone 234 is a Kirsten sarcoma virus transformed nonproducer cell line derived from A31 (4); B77-A31 (obtained from Peter Vogt, Los Angeles, Calif.) is the B77 avian sarcoma virus transformant of A31; MC5-5 (obtained from Y. Ikawa, Bethesda, Md.) is a subclone of A31 transformed in vitro by methylcholanthrene (personal communication). R4 is a survivor of 1,200 rads of X irradiation which has the transformed phenotype (30). SVT2 is A31 transformed by SV40 virus (2). TuT3 is a transformed cell line derived from a tumor which appeared 8 months after the injection of 10^7 A31 cells into a BALB/c mouse (Aaronson and Todaro, unpublished data). BALB/c 3T12-3 was derived from the same embryo cells as BALB/c 3T3 but was continually transferred at high cell densities; it is spontaneously transformed and tumorigenic (2). SV-A clone 80 (SV80) is an SV40 transformed human diploid skin fibroblast cell line (39).

The normal rat kidney line (NRK) has been described previously (14). Virus transformed NRK cells included a Kirsten sarcoma virus transformed nonproducer, K-NRK clone 32 (4), and a line transformed by Kirsten sarcoma virus which produces both the Kirsten strain of leukemia virus and Kirsten sarcoma virus.

Viruses. The preparation of Kirsten sarcoma virus (Ki-SV) also containing Kirsten murine leukemia virus (Ki-MuLV), and that of Ki-MuLV alone have been described previously (11). S2CL3 virus was obtained from supernatant fluids of S2CL3 cultures by isopycnic banding. Avian myeloblastosis virus (AMV) was obtained from J. Beard, Durham, N.C. These viruses were concentrated and purified as previously described (31).

Extraction of viral RNA. 70S viral RNA was extracted from purified virions by disruption with 1.0% sodium dodecyl sulfate (SDS) and purified by velocity sedimentation in a 15 to 30% sucrose density gradient as described previously (32). 35S viral RNA was isolated after velocity sedimentation of 70S RNA which had been heated at 65 C for 3 min in 0.01 M Tris-hydrochloride, pH 7.4, 0.1 M NaCl, 0.001 M EDTA.

Synthesis and purification of viral ^3H -DNA. The endogenous reverse transcriptase reaction from detergent-disrupted type C virus was used to synthesize ^3H -thymidine-labeled DNA in the presence of ac-

tinomycin D (50 $\mu\text{g}/\text{ml}$) (11). The specific activity of the ^3H -DNA was 2.0×10^7 counts/min per μg . Viral ^3H -DNA was also prepared (with identical incubation conditions) by using 70S RNA isolated from Kirsten sarcoma and leukemia viruses as a template in the presence of purified AMV RNA-dependent DNA polymerase. A 6- μg amount of 70S RNA resulted in the incorporation of 7 pmol of ^3H -TMP (2×10^5 counts/min) into the DNA product during a 1-h incubation period at 37 C.

^3H -DNA prepared from an endogenous reaction of Ki-SV-(MuLV) virus in the presence of actinomycin D was also annealed with a saturating amount of 35S RNA isolated from the Ki-SV-(MuLV) virions. The resulting RNA:DNA hybrid was digested with the single-strand-specific nuclease (S_1) isolated from *A. oryzae* (5, 36). This treatment eliminates any DNA sequences which do not hybridize with viral RNA. The RNA:DNA hybrid remaining after treatment with nuclease S_1 was deproteinized with SDS and phenol, and treated with 0.5 N KOH at 41 C for 24 h to hydrolyze RNA. The remaining DNA product was neutralized, dialyzed, and concentrated by lyophilization. The recovery of radioactivity indicates that approximately 5 to 15% of the nucleotide sequences could have been discarded.

Extraction of cytoplasmic RNA. Cells were harvested with a rubber policeman, pelleted by low-speed centrifugation, and washed several times with phosphate-buffered saline (PBS). The washed cells were suspended in three volumes of 0.05 M Tris, pH 8.3, 5 mM magnesium acetate, and 0.04 M sodium chloride, homogenized in a Potter-Elvehjem homogenizer, and centrifuged at $10,000 \times g$ twice to remove nuclei and whole cells. The supernatant fluid was adjusted to 0.1% SDS and extracted at room temperature with chloroform, distilled, neutralized phenol containing 10% m-cresol, and with isoamyl alcohol (24:24:1 vol/vol/vol). The RNA solutions were then extracted four times with ether to remove the phenol, lyophilized, and dialyzed exhaustively against 0.01 M Tris-hydrochloride, pH 7.4, 0.1 M NaCl, 10^{-4} M EDTA, and stored at -20 C at concentrations in excess of 10 mg/ml. Two grams (wet weight) of cells yielded approximately 4 to 6 mg of cytoplasmic RNA. Analysis of the optical density profile of this RNA on Cs_2SO_4 gradients revealed the presence of less than 1% DNA; the preparations were not treated with DNase.

Oligo(dT)-cellulose chromatography of cytoplasmic RNA. Poly(A)-containing RNA was isolated from total cytoplasmic RNA by oligo(dT)-cellulose chromatography by using procedures described previously (34). Typically, 0.3 to 1.0% of the cytoplasmic RNA applied was bound to the dT-cellulose column.

Hybridization reactions. Approximately 2,000 counts/min (0.1 ng) of enzymatically synthesized DNA was incubated with either viral or cytoplasmic RNA in 10^2 - to 10^7 -fold excess for 48 to 72 h at 41 C in 0.20-ml reaction mixtures containing 0.015 M Tris-hydrochloride, pH 7.4; 0.15 M sodium chloride; 5×10^{-4} M EDTA; 0.1% SDS, and 38% formamide.

Analysis of RNA:DNA hybrids by Cs_2SO_4 centrifugation and by hydrolysis with purified S_1 nuclease. The hybrids were detected by these two

methods exactly as described previously (11). Nuclease S_1 degrades unhybridized single-stranded DNA to trichloroacetic acid-soluble radioactivity, but does not degrade RNA:DNA hybrids (11, 25). Thus, the appearance of trichloroacetic acid-precipitable radioactivity after hybridization to RNA can be used to measure the formation of RNA:DNA hybrids. Percentage of hybridization is defined as the acid-precipitable radioactivity resistant to nuclease S_1 , divided by the total acid-precipitable radioactivity present in the reaction measured in the absence of S_1 . The fact that single-stranded RNA as well as DNA is a substrate for the enzyme must be considered in the design of hybridization experiments involving nuclease S_1 . Usually, mixtures of ^3H -DNA product and the RNA that are being annealed are maintained at -70°C while identical mixtures are incubated at 41°C , and both are hydrolyzed together at the end of the hybridization. The presence of up to 2 mg of RNA raised the amount of non- S_1 degradable ^3H -DNA product by about 1 to 5% (20–100 counts/min) over that of product degraded in the absence of RNA. Because of its ease of preparation (5, 36), its tolerance to high salt concentrations and low levels of formamide (5, 11, 25, 36), and its stability during storage (11), S_1 nuclease also has certain advantages over the single-strand-specific nucleases which have been isolated from mung bean sprouts (6) and from *Neurospora crassa* (26).

Mouse type C virus group-specific antigen assay. The radioimmunoprecipitation assay for the murine intraspecies-specific (gs 1) antigen was performed as previously described (35). The total cell extracts were adjusted to a protein concentration of 10 to 50 mg/ml, and dialyzed against 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2.

Purification of AMV polymerase. AMV polymerase was purified by phosphocellulose chromatography from purified virus as described for the murine type C virus polymerase by Ross et al. (31).

RESULTS

Hybridization of viral ^3H -DNA products to cytoplasmic RNA. Table 1 lists the cell lines examined for the presence of cytoplasmic RNA homologous to murine type C viral RNA. Except for S2CL3 and the Ki-SV-(MuLV)-producing NRK cells, none of these cell lines produce type C virus particles detectable by either a supernatant viral reverse transcriptase assay of 100-fold concentrated cell culture supernatant fluids (31) or by a sensitive biological assay such as rescue of the sarcoma genome after cocultivation with transformed nonproducer NIH/3T3 or NRK cell lines.

A single-stranded ^3H -DNA product synthesized in the presence of actinomycin D by the endogenous reaction of the RNA-directed DNA polymerase from virus preparations containing Ki-MuLV and Ki-SV was annealed to cellular or viral RNA in the presence of formamide as described in Materials and Methods. Figure 1 shows representative data from these initial studies obtained after digestion of the RNA:DNA hybrids with S_1 nuclease. S2CL3 cells, and transformed NRK cells producing Kirsten leukemia and sarcoma viruses contained RNA which hybridized to the DNA made from virus preparations containing Ki-SV and Ki-MuLV. Saturating levels were achieved with 60 μg of cytoplasmic RNA, and represented 68% of the total input ^3H -DNA radioactivity. A similar level of hybridization is also obtained with 0.5 μg of 70S RNA extracted from these viruses (data not shown). Higher levels of homology have not been obtained presumably because of

TABLE 1. Properties of cell lines examined for extent of transcription of murine type C viral RNA

| Species | Cell line | Phenotype | Derivation ^a |
|-------------------|----------------------|------------------------------------|---|
| BALB/c mouse | 3T3 clone A31 | Contact-inhibited | Continually transferred at high cell densities DNA virus (SV40) Avian sarcoma virus Kirsten sarcoma virus X irradiation survivor Methylcholanthrene A31 tumor developed in BALB/c mice Spontaneous |
| | 3T12-3 | Transformed | |
| | SVT2 ^b | Transformed | |
| | B77-A31 ^b | Transformed | |
| | K-A31 ^b | Transformed | |
| | R4 ^b | Transformed | |
| | MC5-5 ^b | Transformed | |
| TuT3 ^b | Transformed | | |
| | S2CL3 ^b | Transformed-type C virus-producing | |
| Rat | NRK clone 2 | Contact-inhibited | Kirsten sarcoma virus Kirsten sarcoma virus |
| | K-NRK | Transformed | |
| | Ki-SV-(MuLV)-NRK | Transformed-type C virus-producing | |
| Human | SV80 | Transformed | DNA virus (SV40) |

^a See also Materials and Methods.

^b All derived from A31.

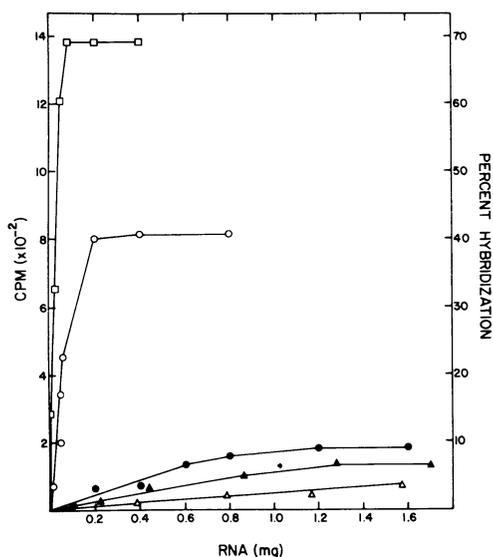


FIG. 1. Detection of RNA:DNA hybrids with S_1 nuclease. The ^3H -DNA product (2,000 acid counts/min) was prepared from Ki-SV/Ki-MuLV; \square , cytoplasmic RNA from transformed NRK cells producing Ki-SV/Ki-MuLV or from S2CL3 "producer" cells; \circ , RNA from K-A31; \bullet , RNA from SVT2; \blacktriangle , RNA from TuT3; \triangle , RNA from A31 cells. The radioactivity shown represents ^3H -DNA product which is resistant to digestion by nuclease S_1 ; the zero time (-70°C) values have been subtracted (see Materials and Methods).

the presence of some hybridized regions of the ^3H -DNA product which are digested by nuclease S_1 (11). RNA extracted from the Kirsten sarcoma virus transformed nonproducer (K-A31) hybridizes to 41% of the Ki-SV-(MuLV) DNA probe. SVT2 RNA to about 9.0%, TuT3 RNA about 6.0%, and BALB/c 3T3 clone A31 RNA yields approximately a 2% hybridization level.

To examine the specificity of these initial data, DNA copies of viral RNA were also prepared, in the presence of actinomycin D, from the endogenous BALB/c virus (S2CL3) and from a sarcoma virus-free stock of Kirsten murine leukemia virus. To rule out the possibility that cellular RNA, possibly present in the virion, might be transcribed into DNA, a DNA product was also synthesized from purified Ki-SV-(MuLV) 70S RNA template which had been isolated on a sucrose density gradient. This 70S viral RNA was incubated in the presence of actinomycin D with AMV polymerase that had been purified by phosphocellulose chromatography. An additional method was employed to limit the amount of nonviral spe-

cific hybridization. ^3H -DNA product prepared from an endogenous reaction of Ki-SV-(MuLV) virus was annealed with a saturating amount of 35S RNA isolated from the Ki-SV-(MuLV) virions as described in Materials and Methods. The resulting RNA:DNA hybrid was then digested with the single-strand-specific nuclease (S_1) to eliminate any nonhybridized DNA sequences. The remaining RNA:DNA hybrid was deproteinized, and the RNA was hydrolyzed.

Table 2 summarizes the hybridization data obtained with the five ^3H -DNA probes and RNA extracted from the various murine cell lines. The data shown represent the average of several experiments performed with five different preparations of cellular RNA. Up to 2 mg of cytoplasmic RNA from each cell line was annealed to the ^3H -DNA probes.

All the murine cell lines tested contain RNA which hybridizes to the various ^3H -DNA probes used. BALB/c 3T3 clone A31 cytoplasmic RNA hybridized least well to all the various DNA products, with saturation levels ranging between 1.8 and 8.0%. The highest saturation value was obtained with the DNA probe prepared from the endogenous BALB virus (S2CL3), whereas the lowest was obtained with the DNA product that had been preannealed to 35S viral RNA.

The RNA from the majority of the transformed non-virus-producing cells saturated the various probes at a level two to five times greater than that from A31. This difference is most evident with the DNA probe that had been preannealed to 35S viral RNA and with the DNA probe prepared from disrupted Ki-SV-(Ki-MuLV) virus preparations. With the former probe, RNA extracted from BALB/c 3T12-3, R4, SVT2, TuT3, and B77-A31 cells saturate, respectively, 6.0, 7.0, 7.5, 5.5, and 6.0% of the ^3H -DNA product. With MC5-5 RNA there is no detectable increase in hybridization values over those obtained with A31 RNA, except when annealing this RNA to the DNA probe that was preannealed to 35S viral RNA. The hybridization values listed in Table 2 varied by approximately 20% of the percentages listed in the separate experiments.

The various ^3H -DNA products appear to be specific for murine type C information, since 55 to 72% of the nucleic acid sequences are homologous to the viral RNA present in Ki-SV-(Ki-MuLV). The DNA products were also tested for specificity by hybridizing them to cytoplasmic RNA extracted from a human cell line (SV80), rabbit liver, yeast, and a rat cell line, NRK clone 2. These RNAs recognized less than 1% of the information present in the various probes, as can be seen from Table 2. The ^3H -DNA

probes were also tested for hybridization to poly(A). This control was included to test for the possibility that a portion of the ^3H -DNA might have been transcribed from an adenine-rich segment of viral RNA. Poly(T) accounted for at most 0.7% of the information present in the DNA probes.

Hybridization with cytoplasmic RNA purified by oligo(dT)-cellulose chromatography. The data listed in Table 2 represent saturating hybridization values obtained by adding increasing amounts of cytoplasmic RNA (up to 2 mg) to the DNA probes, as shown in Fig. 1. Although this amount of RNA represents a 10^7 -fold excess over DNA, it was not obvious, because of the low levels of hybridization detected, that the extent of hybridization observed represented final saturating values to the ^3H -DNA probes.

To clarify this situation, some of the cytoplasmic RNA preparations were chromatographed on an oligo(dT)-cellulose column. As has been previously described for RNA extracted from

K-A31 cells (34), this procedure enriches for poly(A)-containing RNA sequences and eliminates ribosomal and transfer RNA from the preparations (7). Approximately 30 to 50% of the hybridizable RNA is not recovered in the poly(A) fractions, presumably because some of the viral RNA sequences become detached from the poly(A) regions and are therefore not retained. About 0.3 to 1.0% of the cytoplasmic RNA applied is recovered, and a 10- to 40-fold purification of sarcoma-specific RNA is achieved.

Cytoplasmic RNA extracted from K-NRK, R4, SVT2, MC5-5, and SV80 cells was purified by dT-cellulose chromatography, and the RNA samples were tested for hybridization with all of the ^3H -DNA probes listed in Table 2. The results obtained with the DNA probe that was preannealed to 35S viral RNA are shown in Fig. 2. A 40- μg amount of dT-cellulose purified RNA from K-NRK cells saturates this DNA product, whereas about 10 times as much RNA is needed to saturate the same probe when this RNA is

TABLE 2. Hybridization of RNA with various ^3H -DNA viral probes

| RNA | Percentage of hybridization with DNA product ^a | | | | |
|----------------------|---|--|-------------------------------------|-------|---------|
| | Ki-SV-(Ki-MuLV) | | | S2CL3 | Ki-MuLV |
| | Endogenous product | 70S RNA plus AMV polymerase ^b | Preannealed to 35S RNA ^c | | |
| BALB/c 3T3 clone A31 | 3.0 | 5.0 | 1.8 | 8.0 | 5.0 |
| MC5-5 | 2.0 | 4.0 | 3.0 | 7.0 | 2.0 |
| BALB/c 3T12-3 | 7.5 | 11.0 | 6.0 | 10.5 | 9.5 |
| R4 | 14.0 | 12.0 | 7.0 | 10.0 | 9.0 |
| SVT2 | 9.0 | 17.0 | 7.5 | 12.0 | 9.0 |
| TuT3 | 6.0 | 11.5 | 5.5 | 12.0 | 11.0 |
| B77-A31 | 6.0 | 13.0 | 6.0 | 11.0 | 11.0 |
| K-A31 | 41.0 | 33.0 | 44.0 | 29.0 | 29.0 |
| K-NRK | 40.0 | 34.5 | 44.0 | 30.0 | 29.0 |
| Ki-SV-(MuLV)-NRK | 68.0 | 55.0 | 72.0 | 65.0 | 65.0 |
| S2CL3 | 66.0 | NT ^d | 69.0 | 60.0 | 62.0 |
| SV80 | 0.5 | 0.7 | 0.3 | 0.8 | NT |
| Rabbit liver | 0.5 | NT | NT | NT | 0.4 |
| Yeast | 0.2 | NT | 0.0 | 0.5 | 0.2 |
| NRK clone 2 | 0.6 | 1.0 | NT | NT | 0.6 |
| Poly (A) | 0.2 | 0.0 | 0.0 | 0.7 | 0.5 |

^a Approximately 2,000 counts/min (0.07 pmol of ^3H -dTMP incorporated into DNA) of each ^3H -DNA product was hybridized to the RNA solutions as described in Materials and Methods. The percentage of hybridization values listed represent average final saturation values to the DNA probes. Separate determinations did not vary by more than 20% of the percentages listed. Up to 2 mg of cytoplasmic RNA was added for each of the cell lines listed, except poly (A), where 0.1 mg was added. The background zero time values have been subtracted.

^b DNA product prepared with 70S Ki-SV-(MuLV) viral RNA as a template for purified AMV RNA-dependent DNA polymerase.

^c Ki-SV-(Ki-MuLV) endogenous DNA product which has been preannealed to 35S RNA isolated from the same virus, digested with S_1 nuclease, extracted with phenol, and treated with KOH to hydrolyze the RNA (see Materials and Methods).

^d NT, not tested.

not purified on the dT-cellulose column (see Fig. 1). RNA's extracted from SVT2, R4, and MC5-5 cells now demonstrate a clear saturating level, comparable to that obtained with crude cytoplasmic RNA, except that about one-tenth as much RNA is required. Finally, the addition of up to 260 μg of dT-cellulose-purified SV80 RNA does not result in hybridization to this ^3H -DNA probe. Thus, chromatography on dT-cellulose does not lead to the purification of a class of RNA (such as poly[A]) which would yield nonspecific results.

The fact that RNA from murine cells not producing virus saturates the ^3H -DNA probes at lower levels than observed for RNA from cells producing virus suggests that many of the viral sequences in ^3H -DNA product are not present in the RNA extracted from the cells not producing virus. Hybridizations performed with a mixture of RNAs extracted from several of the transformed murine cell lines (such as SVT2 and R4) did not raise the final saturation level above the highest value obtained with either RNA alone. Similar experiments revealed that the RNA extracted from the transformed cells does not contain information that is not already present in the cytoplasmic RNA of the Kirsten sarcoma virus nonproducer cell line, K-A31. For example, the addition of 1.5 mg of cytoplasmic RNA from either SVT2, R4, or TuT3 cells to 500 μg of K-A31 RNA resulted in the same final hybridization level of 44% with the DNA product that was preannealed to 35S viral RNA as is obtained when K-A31 RNA alone is added.

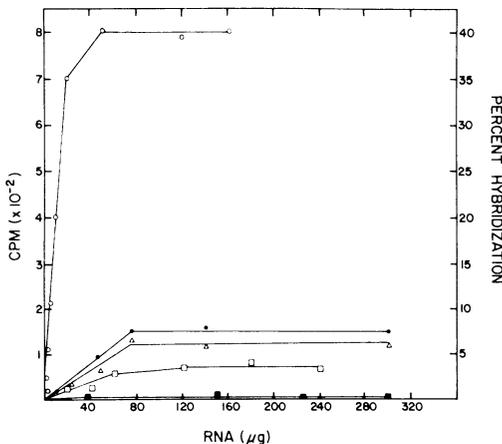


FIG. 2. Hybridization between oligo(dT)-cellulose purified RNA and ^3H -DNA viral product. The ^3H -DNA product used (2,000 acid counts/min) was preannealed to 35S viral RNA (see Materials and Methods). Symbols: \circ , dT-cellulose purified RNA from K-NRK cells; \bullet , from SVT2 cells; Δ , from R4 cells; \square , from MC5-5 cells, and from \blacksquare , SV80 cells.

Equilibrium centrifugation of RNA:DNA hybrids in Cs_2SO_4 density gradients. To confirm that the radioactivity resistant to degradation by nuclease S_1 represented the formation of an RNA:DNA hybrid, K-A31, SVT2, and BALB/c 3T3 clone A31 cytoplasmic RNA were annealed to the ^3H -DNA product prepared from 70S viral RNA as a template for AMV polymerase, and the reaction mixtures were analyzed after centrifugation in Cs_2SO_4 gradients. Figure 3A shows the profile of this ^3H -DNA product after incubation with yeast RNA. After annealing the ^3H -DNA product to A31 RNA, the amount of radioactivity in the region of buoyant density from 1.60 to 1.68 g/cm^3 (23) is increased by about 50 counts/min (Fig. 3D). This represents approximately 2% of the input ^3H -DNA radioactivity. Figure 3B depicts the results of annealing 800 μg of K-A31 cytoplasmic RNA to the DNA product; 24% of the total radioactivity recovered bands in the 1.60 to 1.68 g/cm^3 density region. After annealing 800 μg of SVT2 RNA to the DNA, 6% of the radioactivity bands in the hybrid region (Fig. 3C). These results are consistent with those obtained with S_1 nuclease (Table 2) although, as previously shown (11), the enzymatic method appears somewhat more sensitive for the detection of low concentrations of RNA:DNA hybrids.

Measurement of antigen in murine tissue culture cells. The development of a radioimmunoassay for the major internal viral protein of murine type C virus, the gs antigen (28, 35), provides a method for the detection of small quantities of this protein in murine cells. The quantitation of viral gs antigen present in several of the cell lines is shown in Table 3.

All of the virus nonproducing murine lines tested contained between 26 and 54 ng of viral gs antigen per mg of total cell protein. The levels of gs antigen in the BALB/c 3T3 cultures ranged from 18 to 54 ng per mg of protein. Repeated determinations on the same preparation did not vary by more than 20% of the mean. The five transformed nonproducer cell lines examined had comparable levels of antigenic reactivity. Transformation itself, therefore, does not result in significantly increased levels of gs reactivity compared to untransformed murine cells, as previously reported (29). Two cell lines producing murine leukemia virus, Ki-SV-(MuLV)-NRK and the spontaneous virus producer, S2CL3, contain 10^3 to 10^4 times more viral antigen per mg of protein than do cultures not releasing virus. Detectable levels of this protein could not be found in the control rat line, NRK clone 2, and in the sarcoma virus transformed rat cell line, K-NRK.

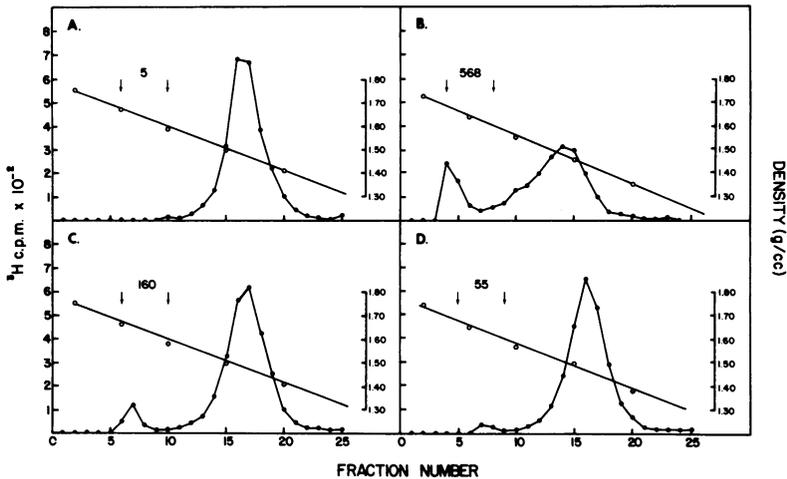


FIG. 3. Cs_2SO_4 density profile of DNA product (made from Ki-SV-(MuLV)-70S RNA and AMV polymerase) hybridized with various RNA preparations. Approximately 2,700 counts/min of the 3H -DNA product prepared from 70S RNA and AMV polymerase was present in each gradient and represented 90% of the input radioactivity. A, 3H -DNA product after hybridization with no RNA or 800 μg of yeast RNA. B, 3H -DNA product after hybridization to 800 μg of K-A31 RNA. C, Hybridization to 800 μg of SVT2 RNA. D, Hybridization to 800 μg of A31 RNA. The arrows bracket the region between densities of 1.60 to 1.68 g/cc, and the numbers above them represent the actual radioactivity present in that region.

DISCUSSION

The data presented here reveal that the virus nonproducing mouse cell lines examined contain RNA that is homologous to a portion of the murine type C viral genome. Viral RNA extracted from either the endogenous BALB virus, from the Kirsten strain of leukemia virus, or from a mixture of Kirsten leukemia and sarcoma viruses, saturates 55 to 72% of the 3H -DNA probes prepared from each of these viruses. Cytoplasmic RNA extracted from cultures producing Kirsten sarcoma and leukemia viruses hybridized to 3H -DNA product prepared from the same virions about 1% as efficiently per microgram of RNA as did 70S viral RNA extracted from these viruses. Assuming that the portion of viral RNA that is detected is representative of the entire RNA genome in the cells, it can be concluded that about 1% of the cellular RNA in these cultures is viral, in substantial agreement with previously reported values for cultures producing type C viruses (12). Assuming that a murine type C virus contains 10^7 daltons of RNA (1.7×10^{-11} μg) per viral genome (16) and that a cell contains about 10^{-5} μg of RNA, then Kirsten sarcoma and leukemia virus-infected murine cells contain Kirsten virus RNA equivalent to about 6,000 virions per cell, assuming all the cells are producing virus.

Saturating amounts of cytoplasmic RNA from Kirsten sarcoma virus transformed non-producer mouse or rat cells hybridize to 30% of

TABLE 3. Radioimmunoassay of murine type C antigenic reactivity in cell extracts

| Species | Cell line | Viral gs antigen ^a (ng/mg of cell protein) |
|--------------|------------------|---|
| BALB/c mouse | 3T3 clone A31 | 54 |
| | 3T12-3 | 40 |
| | TuT3 | 26 |
| | R4 | 55 |
| | SVT2 | 30 |
| | K-A31 | 28 |
| | S2CL3 | 70,600 |
| Rat | NRK clone 2 | <5 |
| | K-NRK | <5 |
| | Ki-SV-(MuLV)-NRK | 5,950 |

^a Based on duplicate assays of five separate cultures of the various cell lines. Cell extracts and the assay are described in Materials and Methods. Approximately 5,000 counts/min of ^{125}I -MVP3 (gs) polypeptide were incubated with a concentration of rabbit antiserum which precipitated approximately 50% of the labeled protein in a double antibody radioimmunoassay using goat anti-rabbit IgG. Doubling concentrations of the unlabeled test antigen sample were assayed for their ability to inhibit the binding of the labeled protein.

the 3H -DNA product prepared from either S2CL3 or Ki-MuLV virions and to about 40% of the DNA product prepared from Ki-SV-(MuLV) virus. These nonproducers therefore express about half of the information expressed

by virus-producing cells. The detection of virus-specific RNA in sarcoma-virus-transformed nonproducer cells with a ^3H -DNA probe prepared from Ki-MuLV has been reported previously (11), and may be due to a sequence homology shared by sarcoma and leukemia viruses.

In addition to the Kirsten sarcoma virus transformed nonproducer cells, the other transformed derivatives of BALB/c 3T3 also contain RNA which is partially homologous to 35S RNA isolated from Kirsten sarcoma and leukemia viruses. Except for MC5-5, the transformed cell lines contain an increased expression (two- to fivefold) of nucleic acid sequences which are homologous to 35S viral RNA. These transformed virus nonproducing murine cell lines thus transcribe about 9 to 21% of the information contained in the complete murine type C viral RNA. RNA from BALB/c 3T3 cells hybridized to about 2 to 8% of the various ^3H -DNA probes used; this represents about 3 to 11% of the information that can be detected in cultures producing virus. Whether the additional information that is expressed in some of the transformed cells represents transforming information, whether it is a result of the transformed state of the cells, or whether it bears no relationship to transformation remains to be determined.

Oligo(dT)-cellulose chromatography has been employed for the purification of mammalian mRNA (7), and more recently of viral mRNA (34). This purification is based on the observation that many mammalian mRNA's contain poly(A)-rich regions (13, 18, 24) which allow the RNA to bind to dT-cellulose. The RNA of the transformed mouse cells not producing virus was purified by chromatography on dT-cellulose. Results obtained with this dT-cellulose-purified RNA show that it is enriched about 10-fold for murine type C viral specific information; the saturation values obtained were comparable to those obtained by using crude cytoplasmic RNA. Cytoplasmic RNA extracted from an SV40 transformed human cell line does not hybridize significantly to any of the ^3H -DNA probes tested, even after purification of the RNA by dT-cellulose chromatography. The lack of hybridization with poly(A) also shows that the data obtained is not the result of hybridization between oligo(dT)-rich sequences in the DNA products and poly(A) regions of mRNA.

We conclude, therefore, that there are nucleic acid sequences transcribed in murine cell lines that are homologous to murine type C viral RNA. It is not known what fraction of the cells

in a culture are transcribing this information, or, indeed, whether all the cells are transcribing the same sequences. However, information that is representative of the entire viral genome is not being detectably transcribed.

One explanation for the partial expression of the viral genome is that all of one segment of a segmented genome is transcribed, or, alternatively, that there are different initiation sites in the polycistronic message which are under independent transcriptional control. It is unlikely that the data obtained represent hybridization with cellular sequences that are present in the virus particles, since ^3H -DNA probes prepared either with purified 70S RNA as a template for AMV RNA-dependent DNA polymerase or by annealing 35S viral RNA to an endogenously prepared product still detect RNA sequences in the various mouse cells. Thus, the RNA present in the murine cells is partially homologous to that of 35S RNA isolated from Kirsten sarcoma and leukemia virions, and can therefore be considered "viral." It is possible, of course, that there are cellular RNA sequences that are an integral covalent part of the 35S viral RNA, and it could be these that are being detected in the BALB/c cells.

The unknown complexity of the DNA probes used in these experiments places a serious limitation on the quantitative interpretation of the data. It has been shown that essentially all the viral sequences present in a virus are transcribed into DNA when the single-stranded DNA is synthesized in the presence of actinomycin D (17, 19). However, the transcription is nonsymmetrical, with the majority of the DNA product representing only a small fraction (10%) of the viral genome (41). Thus, some viral sequences that are present only at a very low level in the DNA product would not be detected. Given this limitation, we can conclude that the RNA being transcribed in these various transformed cell lines consists of sequences very similar to or identical to those being transcribed by the sarcoma virus transformed nonproducer cells, since a mixture of the RNAs extracted from the various cell lines do not saturate the various ^3H -DNA probes at a level that is higher than that obtained with any one of the cell RNAs by itself.

A radioimmunoassay specific for the major murine type C virus structural protein, the gs antigen, detects low levels of antigenic reactivity in all of the murine cell lines tested. None of the transformed cell lines, regardless of their derivation, contain more of this viral gene product than does the parent line, BALB/c 3T3 clone A31. The presence of this viral protein in

murine cells could be accounted for by a spontaneous low level release of complete virus from the culture, but the failure to detect type C virus in these cells as examined by sensitive biological assays argues against this. In addition, the molecular hybridization data is strong evidence that a low level of type C virus is not being released. Since murine gs antigen is present in all the mouse cell lines examined, there has to be mRNA corresponding to this protein in the cell; the DNA probes used may or may not contain these sequences.

The recent demonstrations that virus-free clonal cell lines of hamster, mouse, rat, and cat origin can spontaneously release type C RNA virus (1, 33, 38, Livingston and Todaro, in press) and that such cell lines can be induced to secrete type C viruses after treatment with various chemical and physical agents (3, 22, 27) strongly supports the hypothesis that many or perhaps all vertebrate cells contain a latent type C viral genome as part of their complement of genes. The findings reported here provide additional evidence for the prevalence of type C viral genes in a variety of murine cell lines and show that there is a low level of synthesis of at least one virion protein (the gs antigen) in the virus-free cells. The possibility is also raised that there is an additional common transcription of viral genes in some murine cells that become transformed.

Since complete infectious type C particles can be obtained from all the BALB/c cell lines by treatment with 5-bromodeoxyuridine (Lieber and Todaro, in press), transcriptional controls must, to some extent, govern the partial expression of the endogenous type C viral genome. There may be additional controls at the translational level. Nucleic acid hybridization and immunological assays for viral proteins can be used to detect low levels of viral expression. These approaches should permit further investigation of the mechanisms which regulate the expression of the endogenous type C viral genes in vertebrate cells and whether partial viral gene expression is involved in the process of cell transformation by chemical and physical agents.

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ADDENDUM IN PROOF

We have recently found that various BALB/c cell lines contain at least two distinct classes of endoge-

nous type C viral genomes (Benveniste, Lieber, and Todaro, Proc. Nat. Acad. Sci., in press): These genomes are only partially homologous (33%) as determined by RNA:DNA hybridization and exhibit different host ranges for replication. Which of these viral genomes is transcribing the type C virus-specific information described here has not yet been resolved.

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