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Fractionation of DNA Nucleotide Transcripts from Moloney Sarcoma Virus and Isolation of Sarcoma Virus-Specific Complementary DNA

ARTHUR E. FRANKEL,* RICHARD L. NEUBAUER, AND PETER J. FISCHINGER
Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20014

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Radioactive DNA complementary to nucleotide sequences in Moloney murine sarcoma virus (MSV) and Moloney leukemia virus (M-MuLV) complex was made by the endogenous reverse transcriptase reaction. These virus stocks contained a threefold excess of MSV over M-MuLV as measured by biological assay. The complementary DNA was an accurate copy of the viral RNA in that 86% of 35S viral RNA hybridized with complementary (cDNA) DNA at a 1.5 to 1 cDNA-RNA molar ratio. The complementary DNA, of a 4-6S size, was fractionated by sequential absorptions with MuLV and the feline leukemia virus pseudotype of MSV, [MSV(FeLV)] RNA. In this manner three sets of nucleotide sequences which represent different portions of the MSV viral complex were obtained: a sarcoma virus-specific fraction ($cDNA_{sarc}$) with sequences that had no homology to M-MuLV RNA but which hybridized to MSV(FeLV) RNA, a sarcoma-leukemia fraction ($cDNA_{common}$) with sequences common to MSV as well as M-MuLV viral RNA, and a $cDNA_{leuk}$ representing those nucleotide sequences found only in M-MuLV. Hybridization of MSV-MuLV viral 35S RNA with a threefold molar excess of cDNA's revealed that ~20% was hybridized with $cDNA_{sarc}$, whereas ~75% was hybridized with $cDNA_{common}$. M-MuLV 35S RNA alone did not hybridize with $cDNA_{sarc}$ but did hybridize 40 and 50% with $cDNA_{leuk}$ and $cDNA_{common}$, respectively. The $cDNA_{sarc}$ represents about 25% of the total MSV sequences, whereas the $cDNA_{common}$ represents the remainder of the MSV virus genome. Some $cDNA_{common}$ sequences were shared by two other sarcoma viruses and several distinctly different isolates of MuLV. In contrast, the MSV "sarc" sequences had little or no homology with two other murine sarcoma virus isolates.

Sarcomagenic viruses isolated from various mammalian species generally transform fibroblasts in culture and coexist with leukemia helper viruses, which furnish the virus envelope and possibily supply the reverse transcriptase enzyme (10, 11, 15, 24, 27; P. T. Peebles, B. I. Gerwin, and E. M. Scolnick, Virology, in press). The more thoroughly studied murine sarcoma viruses (MSV) were generally shown to be defective for replication but not defective for transformation. Accordingly, cells transformed only by MSV in the absence of replicating leukemia virus were isolated (1, 2). Various types of nonproducing cells of several species were characterized, some of which expressed some murine luekemia virus (MuLV) antigenic determinants and others which did not express these functions (1, 2, 13). MSV-transformed, nonproducing cells of various species stably transcribe some MuLV-specific RNA whether the cells contain MuLV antigens or not (20). Thus MuLV-specific information seems to be a genetically stable part of the MSV genome. The nucleotide sequences of MSV associated with transformation have not been clearly identified. Some strains of MSV derived after passage through rats contain as a part of their genome rat and/or rat leukemia virus sequences (22, 23). Recently, in the avian oncornavirus system the availability of nondefective transforming Rous sarcoma virus and its corresponding transformation-defective deletion variants allowed the identification of Rous sarcoma virus sequences associated with transformation (D. Stehelin et al., J. Mol. Biol., in press).

Although the nature of mammalian sarcoma viruses does not allow this approach, an alternate method was derived. The Moloney isolate of MSV (M-MSV), not known to have been passed through rats, could give rise to murine sarcoma-positive, leukemia-negative (S+L−) cells, which when infected with MuLV gave rise to virus stocks in which infectious MSV was in excess over MuLV (2, 18). This MSV

excess persisted when MSV was carried over by transspecies rescue into the cat system (16). M-MSV was then propagated for several years in cat cells as a pseudotype virus with only feline leukemia virus (FeLV) as helper [MSV(FeLV)]. It would seem reasonable to assume that only M-MSV-specific sequences were conserved in cat cells that do not contain M-MuLV sequences in uninfected cat cell DNA (A. Frankel et al., in press). Accordingly, single-stranded DNA could be made from the M-MSV-MuLV complex by the endogenous reverse transcriptase reaction, and the MuLV-specific sequences could be removed by exhaustive hybridization with MuLV RNA. The residual complementary DNA (cDNA) could be hybridized to MSV(FeLV) pseudotype RNA to verify the presence of sarcoma-specific sequences. After several sequential cycles of hybridization, we isolated sequences, termed "sarc," only associated with M-MSV. We show that the remainder of M-MSV-specific DNA shares its sequences with M-MuLV. Hybridization experiments of MSV viral 35*S* RNA with isolated sarcoma-specific cDNA showed that approximately 25% of the M-MSV genome consists of sarcoma-specific sequences, whereas the remainder is shared with M-MuLV. These M-MSV sarcoma-specific sequences were not found in Kirsten and Harvey isolates of MSV.

MATERIALS AND METHODS

Viruses. The murine sarcoma-leukemia virus mixture used to make probe was obtained from the 319 S+L− clone of MSV-transformed 3T3FL cells infected with the IC isolate of M-MuLV (2, 7). The virus had a three- to fourfold excess of focus-forming sarcoma virus to leukemia focus-inducing virus by biological assays. The viral supernatant was concentrated 2,000-fold by polyethyelene glycol precipitation (8%, wt/vol), banded through a sucrose gradient (20 to 60% sucrose in phosphate-buffered saline), pelleted through phosphate-buffered saline, and suspended in phosphate-buffered saline prior to preparing cDNA.

MuLV was obtained from the supernatant of 3T3FL cells infected with the IC isolate of M-MuLV. The supernatant was concentrated by polyethylene glycol precipitation 2,000-fold and suspended in 0.15 M sodium chloride, 0.015 M Tris, 0.0015 M EDTA, pH 7.4 (NTE) for RNA extraction or banded through sucrose and suspended in phosphate-buffered saline if used to make cDNA.

The MSV(FeLV) virus complex was generated by rescue from the FG10 clone of S+L− cells into cat embryo fibroblasts with FeLV and grown in the P521 producer clone of feline CCC cells (8, 16, 17, 19). The P521 virus complex had an MSV to FeLV ratio of 3:1 by biological titrations. The virus complex was purified from supernatant fluid by banding in a continuous-flow ultracentrifuge (Electro-Nucleonics Laboratories, Inc.). The virus was pelleted and dissolved in NTE prior to RNA extraction. FeLV virus, Rickard strain (422), was obtained from Electro-Nucleonics, Inc. and dissolved in NTE for RNA extraction. Kirsten MSV-MuLV complex grown in NRK cells, Rauscher MuLV, Kirsten MuLV virus, and BALB-2 xenotropic MuLV were also obtained from Electro-Nucleonics, Inc. The FeLV pseudotype of Harvey MSV was obtained from P. Sarma and was grown in cat embryo fibroblasts, and the virus was purified identically to MSV(FeLV).

cDNA's. Radioactively labeled DNA sequences complementary to MSV-MuLV viral RNA were made by the endogenous reverse transcriptase reaction (26). The reaction mixture contained 1 ml of banded MSV-MuLV virus in phosphate-buffered saline, 1 ml of 0.5 M Tris-hydrochloride (pH 7.8), 1 ml of 5 mM $MnCl_2$, 1 ml of 0.1 M dithiothreitol, 1 ml of actinomycin D (1,000 μg/ml; Calbiochem), 0.1 ml of 2×10^{-2} M dATP, dGTP, and dCTP, 0.9 ml of 0.1 M NaF, 1 ml of 0.5 M NaCl, 1 ml of 0.15% Triton X-100, 1 ml of [^{3}H]TTP (5×10^{-5} M; 1 mCi/ml; New England Nuclear Corp.), and 1 ml of 0.1 M CMP. The final concentrations were 0.1 mg of viral protein per ml, 0.05 M Tris, 0.5 mM $MnCl_2$, 0.01 M dithiothreitol, 100 μg of actinomycin D per ml, 2×10^{-4} M bases, 9 mM NaF, 0.05 M NaCl, 0.01% Triton, 5×10^{-6} M [^{3}H]TTP, and 0.01 M CMP. The reaction mixture was vortexed and incubated for 45 min at 37 C. It was then treated with 0.7% sodium dodecyl sulfate, 0.01 M EDTA and shaken. The mixture was extracted with an equal volume of NTE-saturated phenol-chloroform-isoamyl alcohol (1:1:0.04), and the water phase was made 0.5 N NaOH and boiled for 10 min. This solution was diluted with 6 volumes of distilled water, neutralized with 2 N HCl, and concentrated on a 1-cm hydroxylapatite column. The cDNA was eluted from the column with 0.5 ml of 0.3 M phosphate buffer (PB) washes. Testing the enzymatic product prior to boiling in alkali revealed <10% double-stranded DNA by salt elutions from hydroxylapatite (6). The DNA had a specific activity of 2×10^7 counts/min per μg.

RNA. 65*S* RNA was prepared from fresh MSV-MuLV virus suspended in sterile NTE. The suspension was treated with 1% sodium dodecyl sulfate, extracted twice with equal volumes of NTE-saturated phenol, and precipitated with 2 volumes of −20 C dehydrated ethanol. Diethyloxydiformate (Eastman) (0.1%) was added prior to precipitation to inhibit nucleases (24). The RNA was left overnight at −20 C and then centrifuged at 10,000 × *g* for 30 min. The viral RNA pellet was resuspended in 0.5 ml of NTE and layered on a 10 to 30% sucrose in NTE gradient and centrifuged at 40,000 rpm for 3 h in an SW41 rotor. Optical densities at 260 nm on 0.5-ml fractions were read, and the 60–70*S* absorbance peak was pooled and precipitated with 2 volumes of ethanol in a polyallomer tube. After 6 h the high-molecular-weight RNA was pelleted and resuspended in 0.5 ml of NTE or 0.12 M PB. 30–40*S* RNA was prepared from 65*S* RNA by heating for 1 min in NTE at 100 C and centrifuging at 40,000 rpm in a 10 to 30% sucrose gradient in NTE for 5 h in an SW41

rotor. Fractions were collected, and the 30–40S absorbance peak was pooled and precipitated with 2 volumes of ethanol. After 6 h the 30–40S RNA was resuspended in 20 μl of distilled water for iodination or 400 μl of NTE for hybridization. Approximately 30 μg of high-molecular-weight RNA could be obtained from 2 liters of MSV-MuLV supernatant or 15 liters of MuLV supernatant.

For MSV(FeLV)-FeLV RNA the viral concentrate was extracted once with 1% sodium dodecyl sulfate and twice with phenol and precipitated with ethanol at −20 C. The RNA was pelleted at 10,000 × *g* for 30 min and resuspended in 0.5 ml of NTE or 0.12 M PB. High-molecular-weight viral RNA from MSV-(FeLV)-FeLV RNA was not isolated because the virus was grown in cat cells. Contaminating cat cellular RNA did not hybridize with the mouse viral sequences (Frankel et al., in press).

Total viral RNA of Kirsten sarcoma-leukemia virus, Kirsten-MuLV, Rauscher leukemia-MuLV, and Harvey sarcoma-FeLV was extracted in the same way.

High-molecular-weight RNA was iodinated by a modification of the technique of Colcher et al. (5). A 2.5-μl amount of New England Nuclear Corp. ^{125}I (1 mCi) was mixed with 3 μl of 0.3 mM $NaSO_3$, 0.3 N H_2SO_4, and 10 μl of distilled water and incubated for 5 min at room temperature. Then 2 μl of viral RNA in distilled water (1 μg) was added along with 1 μl of 1.8 M NaOAc, pH 4.5, and 1 μl of 50 mM thallium trichloride. This mixture (19 μg total) was incubated for 30 min at 65 C. Then 1.2 μl of 0.1 M $NaSO_3$ was added, and the mixture was reincubated for 10 min at 65 C. The material was then chilled and passed through a 10-cm Sephadex A-50 column to remove free iodine and, when necessary, separated from iodinated protein by a CF-11 column. ^{125}I-labeled MSV-MuLV 30–40S RNA was prepared from unlabeled 30–40S viral RNA. The prepared cDNA hybridized with 85% of the iodinated RNA tested by RNase T_1 digestion at a DNA-RNA ratio of 1:1 and 96% at a DNA-RNA molar ratio of 12:1 (see Table 1).

Fractionation of cDNA's. cDNA (0.25 to 0.75 μg or 5 to 15 million counts/min) was prepared from MSV-MuLV virus and suspended in 1 ml of 0.3 M PB. This cDNA was diluted to 0.22 M PB, and 6 to 8 μg of 70S MuLV RNA was added. This hybridization mixture was incubated at 63 C for 48 h. It was then diluted 1:1 with distilled water and loaded on a 1-cm hydroxylapatite column at 50 C. Washes of 0.14 and 0.3 M PB were done. Aliquots were precipitated with trichloroacetic acid, filtered onto 0.45-μm cellulose acetate filters, and counted. The unhybridized 0.14 M PB wash material was diluted fivefold with distilled water and concentrated on a 1-cm hydroxylapatite column. The cDNA fraction was eluted with 0.3 M PB 0.5-ml washes. Again aliquots were taken, and acid was precipitated, filtered, and counted. The hybridizing fraction was treated with 0.5 M NaOH and boiled for 10 min to remove the RNA. It was then diluted with distilled water fivefold, neutralized, and concentrated as the unhybridized fraction. With this procedure two cDNA fractions, one having MuLV-related sequences and one without MuLV sequences, were made.

Then 1 ml of each cDNA fraction in 0.3 M PB was mixed with 40 μg of MSV(FeLV)-FeLV RNA and diluted to 0.22 M PB. These were again incubated for 48 h at 63 C. The hybridization mixtures were diluted 1:1 with distilled water and loaded on 1-cm hydroxylapatite columns at 50 C. This fractionation step, carried out as before, further segregated the MuLV sequences into those common with MSV and those that were MuLV specific. Further, this step selected only those sequences not hybridizing to leukemia that can still hybridize to MSV, the "sarcoma-specific" sequences. Nonhybridizing sequences and those representing contaminating mouse cellular RNA are selected against at this fractionation and are discarded. It was found necessary to repeat the fractionation of the sarcoma-specific cDNA with MuLV RNA because the probe continued to display residual hybridization to MuLV RNA. After this second fractionation with MuLV the sarcoma-specific or $cDNA_{sarc}$ material had very little hybridization with MuLV RNA. Further fractionations with MuLV did not yield any further significant hybridization of the $cDNA_{sarc}$. We were able to obtain from 15 million counts/min of MSV-MuLV cDNA approximately 1 million counts/min of $cDNA_{common}$ – material hybridizing to leukemia and MSV(FeLV) RNA; 500,000 counts/min of $cDNA_{leuk}$ – material hybridizing to leukemia, but not sarcoma, MSV(FeLV); and 200,000 counts/min of $cDNA_{sarc}$ – material hybridizing to sarcoma but not leukemia. There was about 60% recovery of DNA at each fractionation. The losses occurred at boiling and concentrating steps and not during incubations for hybridization.

Characterization of cDNA fractions. At each stage of fractionation 300 to 1,000 counts/min of each cDNA fraction was mixed with 2 μg of 65S MSV-MuLV RNA, 0.7 μg of 70S MuLV RNA, 4 μg of MSV(FeLV) RNA, or no RNA in 0.1 ml of 0.22 M PB and incubated for 20 h at 63 C. The mixtures were then diluted into 3 ml of 0.12 M PB and loaded onto a 1-cm hydroxylapatite column at 50 C. PB washes (0.14 M and 0.3 M) were passed through the column, and acid-precipitable counts in the washes were determined. The ratio of 0.3 M PB counts/min to total counts/min represents the fraction of cDNA hybridized to RNA.

The size of the cDNA fraction was determined by centrifuging 200 to 1,000 counts/min of each cDNA fraction on a 10 to 30% (wt/vol) sucrose gradient at 34,000 rpm in an SW56 rotor for 18 h at 4 C and determining acid-precipitable counts per minute and optical densities on fractions. An *Escherichia coli* tRNA marker was centrifuged concurrently.

The purity of the DNA transcripts was ascertained by determining $C_rt_{1/2}$ for hybridization of 1,000- to 10,000-counts/min probe with 2 μg of 65S MSV-MuLV RNA in 0.1 ml of 0.22 M PB and taking aliquots at various times. The experiment was repeated with 0.8 μg of 30–40S MSV-MuLV RNA. Percentage of hybridization of the aliquots was determined by hydroxylapatite chromatography as before. C_rt curves were made as suggested by Leong and Britten (3, 4, 14). Hybrids of each probe fraction with MSV-MuLV RNA were diluted in 0.12 M PB

and loaded on hydroxylapatite columns at 50 C. These eluted at 5-C intervals with 0.12 M PB washes to obtain thermal denaturation profiles.

Hybridization of viral RNA with cDNA fractions. The specificity of the cDNA fractions was studied by hybridizing cDNA's with high-molecular-weight, iodinated viral RNA at low and high DNA-RNA molar ratios. cDNA was dialyzed against NTE, co-precipitated in 70% ethanol with 0.1 M sodium acetate buffer and tRNA, and resuspended in 5 to 40 μl of 0.3 M PB. cDNA (10,000 to 30,000 counts/min) in 1 μl was mixed with 1 μl of 1.2 M PB and 1 μl of iodinated viral RNA containing 1,000 to 10,000 counts/min of RNA. The hybridization mixture (0.75 M Na^+) was incubated in small Eppendorf polyallomer tubes at 68 C until obtaining a C_0t of 0.3. The solution was then dissolved in 2 ml of 2× SSC and split into 2 equal volumes. One fraction was treated with RNase T_1 (200 U/ml), and both fractions were incubated for 30 min at 37 C. The nucleic acids were then precipitated with trichloroacetic acid, filtered, and counted in both a gamma counter and a liquid scintillation counter with Econoflour. The acid-precipitable gamma counts per minute after RNase treatment divided by the untreated acid-precipitable gamma counts per minute represents the percentage of hybridization. The tritium counts per minute divided by two times the gamma counts per minute represents the cDNA-RNA molar ratio (based on 2×10^7 counts/min per μg for cDNA and 10^7 counts/min per μg for iodinated RNA). Hybridizations were performed at both 10 to 12:1 and 1 to 3:1 molar ratios of cDNA-RNA.

Distribution of Moloney sarcoma and leukemia viral sequences. Each probe fraction (500 to 1,000 counts/min) was hybridized with total viral RNA extract from Kirsten MSV-MuLV, Harvey MSV(FeLV)-FeLV, Kirsten MuLV and Rauscher MuLV. The hybridizations were done in 0.22 M PB at 63 C to a final C_rt of at least 10 mol-s/liter. The extent of hybridization was analyzed by hydroxylapatite chromatography as before.

RESULTS

Fractionation scheme of MSV-MuLV cDNA. Ten cDNA preparations from MSV-MuLV were examined in a series of sequential absorptions with high-molecular-weight viral RNAs to fractionate DNA sequences. The total cDNA was a reasonably homogeneous transcript in that 70 to 86% of homologous iodinated MSV-MuLV 65S RNA was hybridized to cDNA at a 1.5:1 molar excess of probe DNA to viral 70S RNA at a C_0t of 0.24. A fractionation schema is shown in Fig. 1. The diagramed MSV-MuLV cDNA hybridized most (97%) with MSV-MuLV 65S RNA and less and approximately equally (57 to 59%) to MuLV 70S RNA and MSV(FeLV) RNA and had a 16% hybridization with no RNA present. This background was shown to be double-stranded DNA by salt elutions on hydroxylapatite (6). The cDNA was absorbed with MuLV 70S RNA to eliminate MuLV-related sequences. After fractionation with MuLV 70S RNA the fractions show a change in hybridization pattern (Fig. 1). The material hybridizing to MuLV RNA had an enriched hybridization (95%) to MuLV RNA and a somewhat lower hybridization to MSV(FeLV) RNA (78%) and 24% with no RNA. The material not hybridizing to MuLV RNA shows a reduction in both MuLV and MSV(FeLV) RNA hybridizations (55 and 52%, respectively). Further fractionation of the cDNA fractions with MSV(FeLV) RNA yields a set of sequences, $cDNA_{common}$, with ability to hybridize equally well to MuLV or MSV(FeLV) RNA – 92% for each. This fraction constitutes 0.73 of the original MSV-MuLV cDNA. Another set of sequences, $cDNA_{leuk}$, is able to hybridize with MuLV sequences (82%) but not with MSV(FeLV) sequences (7%). This $cDNA_{leuk}$ represents 0.09 of the starting material. The final fraction, $cDNA_{sarc}$, represents 0.06 of the starting material. The other 0.15 of the original cDNA is non-hybridizable material. With different preparations of $cDNA_{sarc}$ the ratio of MSV(FeLV) hybridization to MuLV hybridization ranges from 4:1 to 15:1 (as in Fig. 1). A third hybridization with MuLV RNA ultimately reduces the hybridization with MuLV to near the value of hybridization with no RNA present for all "sarc" probes. The preparation of $cDNA_{sarc}$ minimally requires both two leukemia fractionations and one MSV(FeLV) fractionation. The order of fractionation does not make any difference. The requirement for two leukemia fractionations cannot be reduced by increasing the leukemia RNA in the hybridization mixture, increasing the salt concentration of the hybridization to 0.3 M PB, lowering the incubation temperature to 50 C, or extending the time of incubation up to 72 h. The $cDNA_{leuk}$ preparation at times required repeated fractionations with MSV(FeLV) RNA to reduce hybridization to MSV(FeLV) RNA to less than 10%. A single fractionation enriches but does not lead to absolute exclusion of sequences.

It should be stressed that the terms cDNA "sarc," "leuk," and "common" are operational terms describing different sets of viral sequences. These cDNA fractions do not necessarily represent single specific genes; in the case of "common" and "leuk" these are almost certainly composed of many genes.

Properties of isolated cDNA fractions. All cDNA fractions yielded broad peaks in velocity density gradients. The unfractionated MSV-MuLV cDNA was ~4S. The $cDNA_{common}$ was 4S; the $cDNA_{leuk}$ was 3S; and the $cDNA_{sarc}$ was at times more heterogeneous but averaged 4S to 6S.

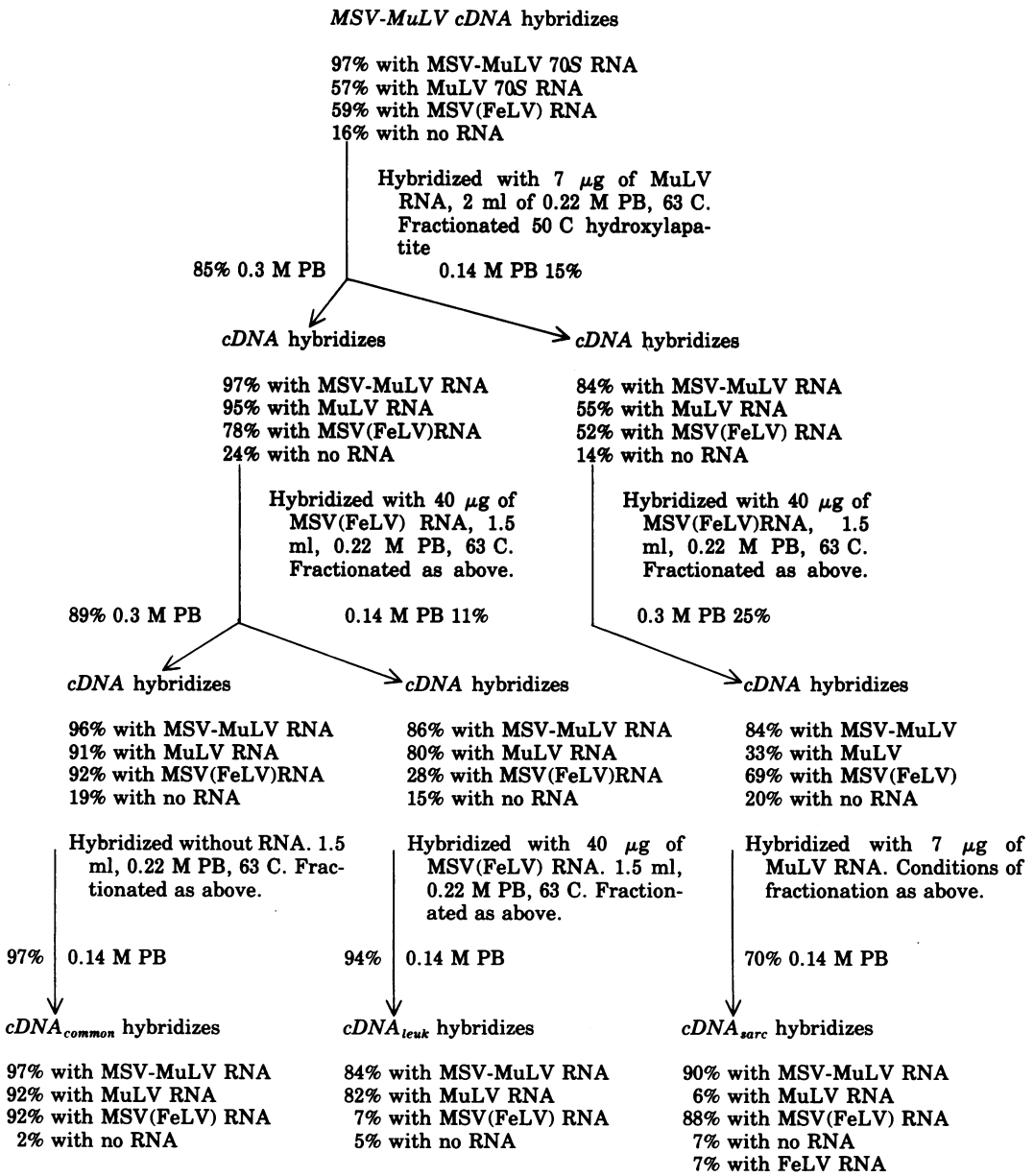

FIG. 1. *Fractionation scheme of MSV-MuLV cDNA.*

cDNA of MSV-MuLV hybridized to 65S MSV-MuLV RNA with a $C_rt_{1/2}$ of 0.05 (Fig. 2). The $cDNA_{common}$ fraction hybridized at a similar rate, with a $C_rt_{1/2}$ of 0.07. The $cDNA_{sarc}$ had a $C_rt_{1/2}$ of 0.08. The $cDNA_{leuk}$ hybridized more slowly, with a $C_rt_{1/2}$ of 0.26. The data show there are 3.25 times as many MSV-related sequences in MSV-MuLV as MuLV-related sequences. Furthermore, the complex cDNA probably does not have a major contaminant because the MuLV sequences and MSV sequences account for at least 85% of the hybridizable sequences. The observations concur with biological data which describe a three- to four-fold excess of infectious MSV to MuLV. With 35S MSV-MuLV RNA $cDNA_{sarc}$, $cDNA_{common}$, and MSV-MuLV cDNA hybridized with a $C_rt_{1/2}$ of 0.02; $cDNA_{leuk}$ hybridized with a $C_rt_{1/2}$ of 0.07. Accordingly, none of the cDNA fractions represents a minor contaminant in the 65S or 35S viral RNA.

Thermal denaturation of hybrids. The hy-

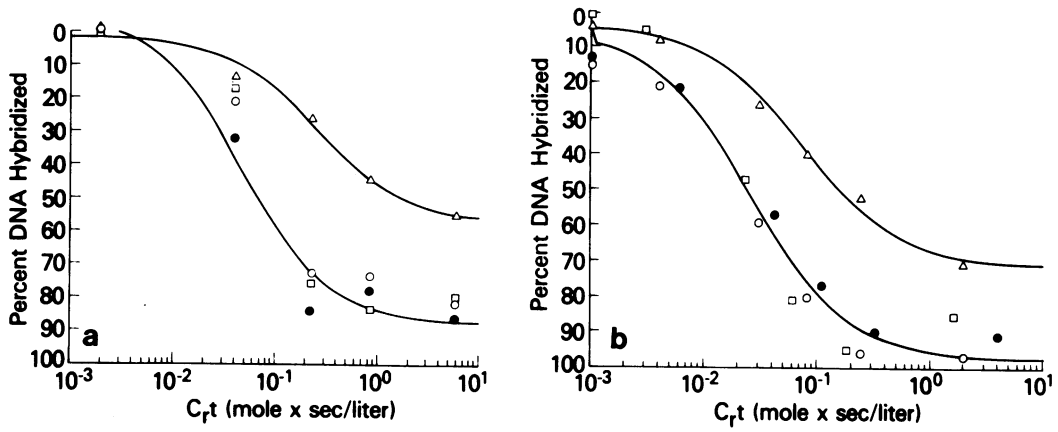

FIG. 2. *(a) Hybridization of cDNA fractions with 65S MSV-MuLV RNA. Hybridizations were performed in 0.1 ml of 0.22 M PB with 2 μg of 65S MSV-MuLV RNA and 1,000 to 10,000 counts/min of each cDNA fraction. Aliquots (0.01 ml) were taken at times varying from 2 min to 13 h. Incubation temperature was 63 C. Extent of hybridization was measured by hydroxylapatite chromatography. Symbols: ●, MSV-MuLV cDNA; ○, $cDNA_{common}$; △, $cDNA_{leuk}$; □, $cDNA_{sarc}$. (b) Hybridization of cDNA fractions with 33S MSV-MuLV RNA. Hybridizations were performed in 0.1 ml of 0.22 M PB with 0.8 μg of 30–40S MSV-MuLV RNA and 1,000 to 10,000 counts/min of each cDNA fraction. Aliquots (0.01 ml) were taken at times varying from before the addition of RNA to 23 h after the addition of viral RNA. Incubation temperature was 63 C. Extent of hybridization was measured by hydroxylapatite chromatography. Symbols are the same as in (a).*

brids between the cDNA fractions and MSV-MuLV 65S RNA had consistently high T_m's (78 to 83 C) suggesting that the selected sequences were closely matched with sequences in the high-molecular-weight viral RNA of MSV-MuLV (Fig. 3). The unfractionated cDNA hybrid with homologous RNA had a T_m of 82.5 C as did the $cDNA_{sarc}$ hybrid. $cDNA_{common}$ and $cDNA_{leuk}$ had T_m's of 77.5 C with the homologous MSV-MuLV 65S RNA.

Composition of viral RNAs based on protection of hybrids from RNase. It was of interest to determine what portions of MSV or MuLV 35S RNAs were comprised of "common," "leuk," or "sarc" sequences. Initially cDNA was also made to M-MuLV in the same way as for the MSV-MuLV complex. This MuLV cDNA hybridized 83% with 70S M-MuLV RNA, 50% with MSV (FeLV)-FeLV RNA, 24% with FeLV RNA, and 5% without RNA present. Thermal denaturation of the M-MuLV cDNA hybrid with MSV (FeLV)-FeLV was 13 C higher than with the FeLV RNA (75 and 62 C, respectively).

The MSV-MuLV and MuLV cDNA's were hybridized to their respective viral 35S RNAs, and the degree of hybridization determined by RNase T_1 digestion at relatively low molar ratios of cDNA-RNA is presented on Table 1. Large proportions of each RNA hybridized with either MSV-MuLV or MuLV cDNA. The homologous hybridization was larger, but the cDNA of MSV-M-MuLV also intrinsically hybridized more with the iodinated RNAs than the M-MuLV cDNA.

The "common" and "leuk" cDNA hybridized with portions of MSV-MuLV and MuLV RNA. The "sarc" cDNA only hybridized with MSV-MuLV RNA and not MuLV RNA. Hence the fractions have the expected sequence specificity. The MuLV genome appears to consist of approximately equal portions of "common" and "leuk" sequences, whereas the MSV genome has about 70 to 80% "common" sequences and 20 to 30% "sarc" sequences. The sum of the values of the degree of hybridization by individual probes exceeds 100%. The "leuk" sequences do not have the proven specificity of "sarc" sequences as no iodinated RNA specific for MSV was used. This is discussed below.

Distribution of Moloney sequences among murine RNA tumor viruses. $cDNA_{sarc}$ had no homology to Kirsten MSV-MuLV sequences and little (4% above background) homology to the Harvey MSV-MuLV virus complex (Table 2). There was no hybridization of "sarc" sequences with RNA from the murine ecotropic leukemia viruses or with producer cell RNA of the BALB-2 xenotropic virus.

$cDNA_{common}$ sequences hybridize to Kirsten sarcoma and Harvey sarcoma RNAs (50 to 61%) but less well to the helper virus RNA present with the Harvey sarcoma sequences–15% with FeLV RNA. Further, $cDNA_{common}$ hybridizes only slightly more with a mixture of Kirsten and Harvey sarcoma MSV RNA than to Har-

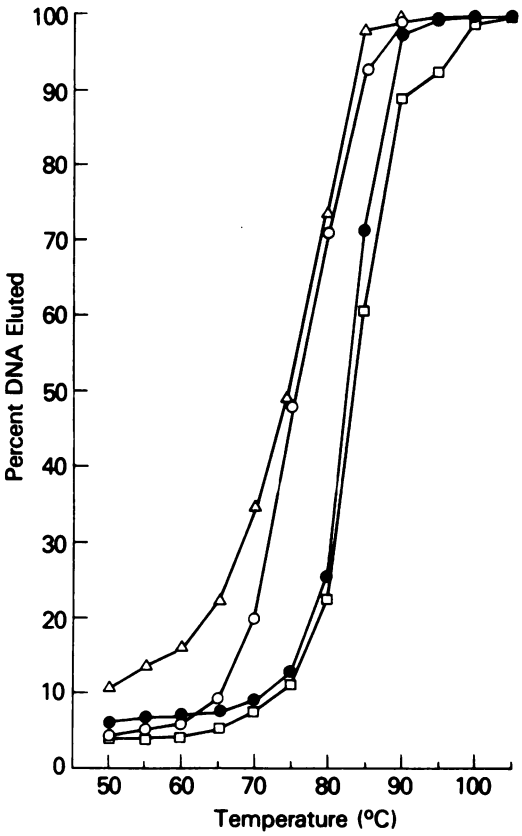

FIG. 3. *Thermal elutions of cDNA hybrids with MSV-MuLV 70S RNA. Thermal elutions were from 1-cm hydroxylapatite columns with 0.12 M PB washes at 5 C intervals. Symbols: ●, MSV-MuLV cDNA; ○, $cDNA_{common}$; △, $cDNA_{leuk}$; □, $cDNA_{sarc}$.*

vey MSV RNA alone (Table 3). This suggests that the common sequences present in Kirsten and Harvey MSVs are from the same subgroup of total Moloney common sequences. Additionally, a Kirsten MSV-MuLV cDNA, prepared similarly to the other probes, hybridized more (44%) with MSV-FeLV RNA than with FeLV RNA alone (20%). This Kirsten MSV cDNA hybridized 90% with homologous RNA with a 5% background. The hybrid of Moloney common sequences with homologous RNA had a T_m of 80 C (Fig. 4), the hybrid of Moloney common sequences with Kirsten MsV-KiMuLV RNA had a T_m of 69 C, and that with Harvey MSV RNA was 73 C. Thus, there is at least a 10% mismatch of common sequences.

DISCUSSION

Complementary DNA was transcribed from the virus complex consisting of M-MSV and M-MuLV. The cDNA was fractionated with sequential absorption into three subsets representing various portions of the viral genomes. The total cDNA was a homogeneous transcript in that essentially complete hybridization of homologous RNA was achieved at a slight molar cDNA excess.

The purity of cDNA and its fractions is sup-

TABLE 1. *Percentage of hybridization of iodinated viral RNA to cDNA fractions*[a]

cDNA representative of:	% Hybridization of viral 35S RNA	
	MSV-MuLV	MuLV
MuLV	67	68
MSV-MuLV	89	66
"common"	74.5	52
"leuk"	28	42
"sarc"	20.5	7
0	1	5

[a] Hybridization was performed as described in Materials and Methods, and the hybrids were analyzed at a C_0t of 0.3 with a cDNA-RNA molar excess of 2:4.1. In each case various cDNA concentrations were used, but a threefold excess of cDNA was sufficient to attain a maximal plateau value. RNase T_1 digestions of the hybrids were done as described in Materials and Methods.

TABLE 2. *Distribution of Moloney sarcoma and leukemia virus nucleotide sequences among murine RNA tumor viruses*

Viral RNA	Hybridization (%) to[a]:			
	MSV-MuLV cDNA	$cDNA_{sarc}$	$cDNA_{common}$	$cDNA_{leuk}$
0	11	6	2	3
M-MSV-MuLV	84	67	97	90
M-MSV-FeLV	74	69	92	7
Kirsten MSV-MuLV	62	7	50	42
Harvey MSV-FeLV	40	11	61	7
M-MuLV	86	7	92	90
Rauscher-MuLV	75	6	79	65
Kirsten-MuLV	33	3	37	40
BALB-2[b]	35	6	51	44
NZB liver[c]	NT[d]	0	26	19

[a] 500–1,000 counts/min of each cDNA fraction was hybridized in 0.22 M PB at 63 C to a final C_rt of at least 10 mol-s/liter with crude viral RNAs from each of the indicated viruses. The extent of hybridization was analyzed by hydroxylapatite chromatography.

[b] Cell RNA from A673 human cells chronically infected with BALB-2 virus from C3H/Hin-induced mouse cells used instead of viral RNA. Hybridization was taken to a final C_rt of 2,500 mol-s/liter. The RNAs from all eight viruses were tested with homologous complementary cDNA prepared in the presence of 100 μg of actinomycin D per ml and were found to hybridize to at least 70%.

[c] Cell RNA from NZB liver was used. Final C_rt of 19,000 mol-s/liter used.

[d] NT, Not tested.

TABLE 3. *Relation of Moloney common sequences with nucleotide sequences in Kirsten MSV and Harvey MSV RNAs*

Viral RNA	Hybridization to $cDNA_{common}$[a] (%)
O	2
Kirsten MSV-KiMuLV[b]	51
Harvey MSV-FeLV	61
Kirsten MSV-Kirsten MuLV + Harvey MSV-FeLV	71
Kirsten MuLV	37
FeLV	15

[a] Hybridization conditions and evaluations were the same as in Table 1.

[b] Kirsten MSV-Kirsten MuLV and Kirsten MuLV were both prepared in the same NIH 3T3 cell system.

ported by the C_rt curves with unfractionated and fractionated cDNA's. A $C_rt_{1/2}$ of 0.02 to 0.05 is near the predicted $C_rt_{1/2}$ for a mixture of two RNA tumor viruses. The similar $C_rt_{1/2}$ of the "common" and "sarc" sequences attests to their not being transcripts of minor contaminants, and the lower $C_rt_{1/2}$ of the "leuk" sequences supports the ~3:1 sarcoma excess in the mixture. The FeLV RNA hybridizations with the probe fractions were at near background level for the sarcoma-related sequences. Thus, there is no enrichment of FeLV-related sequences in the MSV(FeLV)-FeLV fractionations. Finally, the high homologous T_m's of cDNA fractions with 65S MSV-MuLV RNA favors accurate transcripts of viral sequences in the cDNA's.

An understanding of the composition of MSV RNA could be obtained from the hybridization experiments of iodinated viral RNA with cDNA tested by RNase digestion. Sarcoma-specific cDNA represents 20% of the total MSV-MuLV viral sequences or about 25% of the MSV sequences based on the 3:1 sarcoma excess by biological titrations and C_rt calculations performed on the same viral harvest used to make iodinated subunit RNA. The other 75% of the MSV RNA hybridizes with "common" cDNA. The Moloney leukemia viral genome consists of about equal portions of common sequences and leukemia-specific sequences. The "sarc" portion is at least as large or up to twice the size of the "sarc" portion of the Rous sarcoma genome, as reported by Stehelin et al. (in press).

The nature of sequences of the $cDNA_{sarc}$ is of interest because it may contain the sequences responsible for cell transformation. It is important to rule out that these are not spurious normal mouse DNA sequences transcribed and recognized as a part of the true MSV genome. This was hopefully avoided by the fact that $cDNA_{sarc}$ was hybridized to MSV(FeLV) RNA. Because six consecutive viral passages of MSV(FeLV) were performed in cat cells and then the infected virus-producing cat cell culture was propagated for >3 years, it is assumed that only those sequences that were the MSV genome itself were conserved.

Second, based on lack of hybridization of $cDNA_{sarc}$ with RNAs of two types of murine xenotropic viruses, $cDNA_{sarc}$ consisted of sequences other than those absent from MuLV but present in murine xenotropic oncornavirus. Third, the $cDNA_{sarc}$ partly hybridized with rat cell DNA, indicating that this M-MSV isolate is quite dissimilar from several other MSVs, which also contain information other than that of MuLV (22, 23).

This M-MSV genome gives rise in various

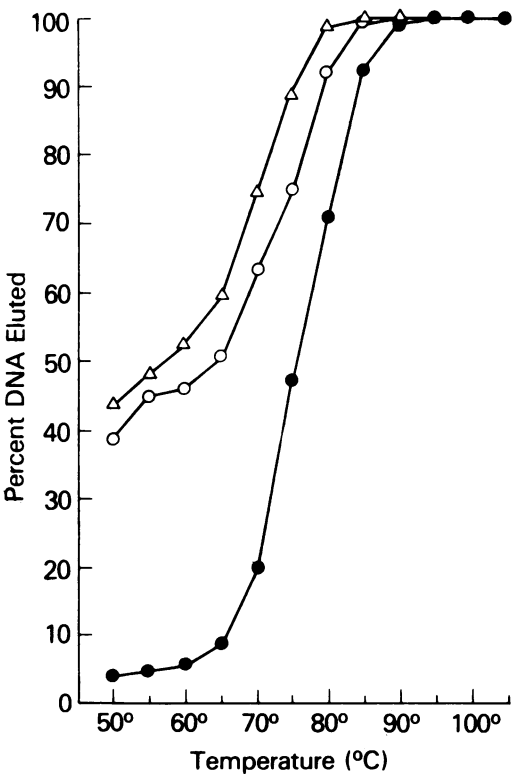

FIG. 4. *Thermal elutions of $cDNA_{common}$ hybrids with different MSV RNAs. Thermal elutions were from 1-cm hydroxylapatite columns with 0.12 M PB washes at 5 C intervals were carried out on hybrids between $cDNA_{common}$ and viral RNA of MSV-MuLV, Kirsten MSV-Kirsten MuLV, and Harvey MSV-FeLV. The hybrids were studied after reaching at C_rt of 10 mol-s/liter. Symbols: ○, M-MSV-MuLV RNA; △, Kirsten MSV-Kirsten MuLV RNA; ○, Harvey MSV-FeLV RNA.*

species to S+L− type cells, which express the antigenic gs-1 determinant normally found on the p30 polypeptide (9, 19). Actually the predominent gs-1 containing polypeptide in heterologous S+L− cells is a molecule of a p60 size which does contain, with some other determinants, the gs-1 site (17). No heterologous S+L− cell tested expresses the MuLV gp71, and recent data indicate that the detectable reverse transcriptase enzyme is only that of the helper virus (Peebles et al., in press; Fischinger and Bolognesi, unpublished data). Thus, some MuLV-specific products are a part of the S+L− type MSV genome and some other MuLV-coded information may be missing or not expressed. This is compatible with several of the other MSV genomes, which do not express MuLV proteins but do contain MuLV-specific information expressed in the RNA of transformed cells (20). Apparently the S+L− MSV genome is analogous to other hybrid oncornavirus ioslates in that it arose by the accretion of new sequences, presumably by a recombinational event (12, 20).

The lack of homology between the $cDNA_{sarc}$ of the S+L− genome and the Kirsten and Harvey MSV RNAs poses a special question. Apparently the transcription of dissimilar molecular sequences found in various MSVs can result in mouse fibroblast transformation. Even the different avian sarcoma virus genome can give rise to the same result, although avian $cDNA_{sarc}$ and mammalian transforming sarcoma viruses have no sequences in common (Stehelin et al., in press). It is important to determine whether $cDNA_{sarc}$ sequences from S+L− type MSV are represented in normal mouse DNA. Recently these sequences were indeed found in normal mouse DNA (our unpublished data). Also, one must ask whether transformation of cells by different viruses leads to expression of common sarcoma virus-associated sequences in cell RNA.

Since the submission of this manuscript, analogous data have been published which are in general agreement with the above conclusions (21).

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