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Biochemical Characterization of the Amphotropic Group of Murine Leukemia Viruses

SISIR K. CHATTOPADHYAY,[1]* JANET W. HARTLEY,[2] MARILYN R. LANDER,[2] BARNETT S. KRAMER,[1] AND WALLACE P. ROWE[2]

Pediatric Oncology Branch, Division of Cancer Treatment, National Cancer Institute,[1] and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases,[2] Bethesda, Maryland 20014

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The recently described amphotropic group of murine leukemia viruses constitutes a distinct biological group, differing from the ecotropic and xenotropic groups in host range, cross interference, and serological reactivity. Viruses of this group have been detected only in wild mice from certain areas in California. By using a [^{3}H]DNA probe synthesized in an endogenous reaction from detergent-lysed amphotropic virus (strain 1504-A), it was demonstrated that the amphotropic murine leukemia viruses are distinct biochemically, in that 20% of the viral genome sequences are not shared by AKR-type ecotropic or any of three types of xenotropic murine leukemia virus tested. A subset of these amphotropic unique sequences, comprising one half of them, is present in the genome of wild mouse ecotropic viruses and in Moloney and Rauscher viruses as well. Sequences homologous to the entire genome of 1504-A amphotropic virus are present in the cellular DNA of all eight inbred mouse strains tested, as well as in wild *Mus* in Asia, in amounts varying from three to six complete viral genomes per haploid cell genome. Evidence is presented that at least 20% of the DNA sequences in both mouse- and mink-grown murine leukemia virus probes are of host-cell origin.

Naturally occurring murine leukemia viruses (MuLV's) can be separated into three major groups based on host range, interference specificity, and reactivity with neutralizing antibody. Ecotropic MuLV's, also called mouse-tropic viruses, can exogenously infect mouse cells (16), whereas xenotropic MuLV's generally cannot infect mouse cells exogenously, but can infect cells of many heterologous species (1, 2, 21-24). In contrast, amphotropic MuLV's exogenously infect mouse cells as well as cells from heterologous species. The heterologous host ranges of the xenotropic and amphotropic viruses differ in that xenotropic MuLV's can infect duck and quail cells, while amphotropic MuLV's cannot (15).

To date, amphotropic viruses have been isolated only from feral mice (*Mus musculus*) from five demes in California. The strains that we have studied derive from the La Puente trapping area (prototype strain 1504-A) and the Lake Casitas area (prototype strain 4070-A); these areas are about 40 miles apart. The strains are similar biologically and belong to the same interference group; however, antiserum to 1504-A does not neutralize 4070-A virus (15).

This report describes the use of nucleic acid hybridization techniques to study the relationship of the 1504-A virus to other MuLV's and to determine the distribution of the genome sequences in various mouse strains.

It is important to avoid confusing the amphotropic group with the MCF viruses of laboratory mice (17), which are apparent recombinants with a broad host range and which are often spoken of as having "amphotropic" host range.

MATERIALS AND METHODS

Tissue cultures. Secondary mouse embryo cultures (ME) were grown and maintained in 10% heated (56°C, 30 min) fetal calf serum (Grand Island Biological Co.) in Eagle minimum essential medium (Microbiological Associates). The SC-1 clonal line of wild mouse embryo cells (14) was grown in 10% heated fetal calf serum in McCoy 5a medium (Grand Island Biological Co.). For virus assays using this cell line, the maintenance medium was 5% heated fetal calf serum in Eagle minimum essential medium. Mink lung cells (ATCC line CCL64) (18) were maintained in 10% heated fetal calf serum in McCoy 5a medium, and assays were carried out using 10% heated fetal calf serum in the Dulbecco-Vogt modification of Eagle minimum essential medium (high glucose). Other cells used for assay and characterization of viruses have been described (15).

Viruses. The virus strains used in this study, their characterization, and the cells used for their production are summarized in Table 1. All virus pools were prepared in our laboratory except for Rauscher leukemia virus, which was obtained from the Viral Re-

TABLE 1. *Viruses and the cells in which they were propagated*

Virus strain	Characterization[a]	Grown in[b]:
AKR-L1	N-tropic ecotropic virus isolated from thymus of a leukemic AKR mouse (16, 27); XC positive	NFS (inbred NIH Swiss) METC
Moloney	NB-tropic ecotropic laboratory strain of uncertain origin (26); XC positive	NIH Swiss METC
Rauscher	NB-tropic ecotropic laboratory strain of uncertain origin (29); XC positive	BALB/c-originated cell line JLS-V9, obtained from Program Resources and Logistics, National Cancer Institute, product lot no. 0361
NZB-IU-1	Xenotropic, activated from NZB-Q cell line by IUdR treatment (15)	Mink lung cells CCL64
BALB-IU-1	Xenotropic, activated from BALB/c embryo primary cells by IUdR treatment (15)	Mink lung cells CCL64
1504-M	N-tropic ecotropic virus isolated from wild mouse embryo (no. 1504); XC positive. Virus stock used was purified by two limiting dilution passages through SC-1 cells	SC-1 cells
1504-A	Amphotropic virus isolated from same wild mouse embryo (no. 1504) as 1504-M; XC negative (15). Virus stock used was purified by two limiting dilution passages through RD cells	SC-1 cells (1504-A SC-1); CCL64 mink lung cells (1504-A mink)
4070-M	N-tropic ecotropic virus isolated from wild mouse embryo (no. 4070) cells from Lake Casitas (Calif.) (15); XC positive. Virus stock used was purified by two limiting dilution passages through SC-1 cells	SC-1 cells
4070-A	Amphotropic virus isolated from same wild mouse embryo (no. 4070) as 4070-M (15); XC negative. Virus stock used was purified by two limiting dilution passages through RD cells	SC-1 cells
Cas E no. 1-X	Xenotropic virus isolated by IUdR treatment from wild mouse embryo cells from Lake Casitas (Calif.) area	SC-1 cells
MCF(247)	Naturally occurring recombinant between ecotropic and xenotropic virus, isolated from thymus of a leukemic AKR mouse (17); N-tropic, XC negative. Virus stock used was purified by three limiting dilution passages through mink cells	SC-1 cells

[a] IUdR, 5'-Iododeoxyuridine.
[b] METC, Mouse embryo tissue culture.

search Resources Branch of the National Cancer Institute.

For production of purified, unlabeled AKR and Moloney viruses, primary NIH-ME cells were treated for 45 to 60 min with 25 μg of DEAE-dextran per ml 1 day after seeding, washed once, and then infected with virus at a multiplicity of 0.1 PFU per cell. Upon confluency, the infected cells were passed to 100 or more bottles (32-ounce; ca. 0.95-liter). Collection of supernatant fluid from these secondary cells was begun when they reached confluency; fluid was collected every 4 to 6 h for several days.

The fluids were centrifuged at 9,150 × *g* for 20 min at 4°C. The virus was banded by continuous-flow zonal centrifugation in a 20 to 50% sucrose gradient in a CF-32 rotor (L3-50, Beckman-Spinco ultracentrifuge). When more than 20 liters was to be processed, initial concentration was done in a model K (Electronucleonics) centrifuge (through the kind courtesy of Raymond V. Gilden and Steve Harshman of the Frederick Cancer Research Center). The samples containing virus (measured by absorbance at 280 nm [A_{280}] and complement fixation) were pooled and pelleted at 99,000 × *g*. The pellet was resuspended in NTE buffer (0.1 M sodium chloride; 0.01 M Tris-hydrochloride, pH 7.4; 0.001 M EDTA) and stored at −70°C. Other virus pools were prepared similarly except that continuous producer cell lines were used as mentioned in Table 2.

Mice for DNA preparation. Except for wild *Mus*, from which adult tissues were processed, mouse embryos were used for obtaining DNA. The following strains were used: AKR/J, C3H/FgLW (received from E. A. Boyse), A/HeN, NZW/N, NZB/N, C57L/J, 129/J, and NIH Swiss.

Pooled embryos or tissues were washed with TNE buffer (0.05 M Tris-hydrochloride, pH 8.0; 0.1 M NaCl; 0.01 M EDTA). A cell suspension was made in a glass tissue grinder, followed by homogenization in a Sorvall Omnimixer (10,000 rpm, 2 min in an ice bath) if necessary.

Cells were lysed with 0.4% sodium dodecyl sulfate (SDS), and protein was digested by overnight incubation with Pronase (100 μg/ml; DNase-free, Calbiochem) at room temperature. Protein was removed by two extractions with TNE buffer-saturated distilled phenol. DNA was precipitated with 2 volumes of al-

cohol and dissolved in 1× SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 6.9). RNA was removed by treatment with 100 μg of pancreatic RNase (X11a, Sigma Chemical Co.) per ml and 50 U of RNase T_1 (Calbiochem, B grade) per ml for 1 h at 37°C, followed by two extractions with buffer-saturated phenol. After extensive dialysis in 0.1× SSC plus 0.4 mM EDTA, the DNAs were sheared by passing them twice through an orifice with a pressure drop of 40,000 lb/in^2 using a French pressure cell (American Instrument Co.). The sheared DNA solution was then forced through a Metricel GA-6 filter (cellulose acetate; pore diameter, 0.45 μm; Gelman Instrument Co.); such filters do not bind DNA (19). The filtered DNA solution was shaken twice with an equal volume of chloroform-isoamyl alcohol (24:1), adjusted to 0.2 M sodium acetate, precipitated with 2 volumes of alcohol, and redissolved in 0.01 M phosphate buffer (PB; equimolar concentration of NaH_2PO_4 and Na_2HPO_4; molarity refers to phosphate; pH 6.8) with 1 mM EDTA. DNAs extracted by this method had an A_{260}/A_{280} ratio of 1.85 to 1.95. DNA concentrations were determined from A_{260} (considering 21.0 A_{260} units = 1 mg of DNA).

Purification of RNA. Viral RNA was extracted from purified virus by an SDS-phenol method followed by alcohol precipitation (30) without addition of any carrier RNA. RNA was dissolved in 10 mM PB with 1 mM EDTA. When 60 to 70S RNA was isolated, the RNA was dissolved in 0.2 ml of 10 mM Tris-hydrochloride (pH 7.4) with 1 mM EDTA and layered on a preformed 5 to 20% sucrose gradient prepared in the same buffer. After 2 h of centrifugation at 182,000 × *g*, the gradient was fractionated, and the fractions containing 60 to 70S RNA were pooled, dialyzed against 10 mM PB with 1 mM EDTA, and concentrated by vacuum dialysis. RNA purified by this method had an A_{260}/A_{280} ratio of 1.98 to 2.05. RNA concentrations were determined from spectrophotometric measurement (25.0 A_{260} units = 1 mg of RNA).

Cellular RNAs were extracted by the following method. Cells were suspended in 0.05 M Tris-hydrochloride (pH 6.7)–0.3 M NaCl–0.01 M EDTA buffer containing 3.3% (vol/vol) diethyl pyrocarbonate (Eastman Kodak, Rochester, N.Y.) (31). SDS was added to a final concentration of 1%, and the mixture was incubated on ice for 10 min with occasional stirring. After 1 min of homogenization in the cold in a filled sealed container (Eberbach Corp.), RNA was extracted with an equal volume of water-saturated distilled phenol with 0.1% (wt/vol) 8-hydroxyquinoline at 60°C for 5 min with vigorous shaking. Further deproteinization was carried out by one extraction of cold water-saturated phenol as above and two extractions with equal volumes of chloroform-isoamyl alcohol (24:1 [vol/vol]). Nucleic acids were precipitated with 2 volumes of 95% ethyl alcohol. DNA was removed with 20 μg of DNase (Worthington Biochemicals) per ml followed by degradation of DNase and other remaining proteins with 15 μg of subtilisin (Nagarse, Enzyme Development Co.) per ml. RNA was precipitated by the addition of 0.1 volume of 20% potassium acetate (pH 5.4) and 2 volumes of ethyl alcohol after further removal of protein with two extractions each of phenol and chloroform-isoamyl alcohol. RNA prepared by this method had an A_{260}/A_{280} ratio of 1.9 to 2.0.

Synthesis of single-stranded, virus-specific [^{3}H]DNA. Single-stranded virus-specific [^{3}H]DNA probe was synthesized in an endogenous reverse transcriptase reaction using detergent-lysed purified SC-1-grown 1504-A virus and NIH-ME cell-grown AKR virus (3, 8, 9). The synthesized probe was purified by deproteinization with chloroform-isoamyl alcohol (24:1 [vol/vol]) and phenol, and unincorporated radioactivity was removed by Sephadex G-50 chromatography. RNA was removed by alkali treatment followed by dialysis. Briefly, a representative AKR probe synthesized by this method has a specific activity of 2 × 10^7 cpm/μg, is 98% single stranded, and is about 200 to 400 nucleotides in length. More than 90% of the probe sequences are present in the viral RNA (60 to 70S RNA from virus grown in homologous cells), and about 70% of the probe sequences are similarly represented as they are in the viral RNA (8, 9).

Hybridization of probe DNA with cellular DNAs, RNAs, and viral RNAs. Details of experimental procedures for probe DNA and cellular DNA hybridization are given in the legend to Fig. 1.

For [^{3}H]DNA probe and cellular or viral RNA hybridizations, the following procedure was used. Reactions were carried out in 1-ml sealed ampoules with 100 μl of incubation mixture containing 0.05 ng of [^{3}H]DNA probe, 0.1% SDS, 0.48 M PB, 0.5 mM EDTA, and varying amounts of cellular or viral RNA. Each incubation mixture was boiled in a sealed ampoule in a water bath for 2 min and incubated for 24 h at 65°C. Each incubation mixture was then diluted to a final concentration of 0.14 M PB and 0.4% SDS. Hybridization was assayed by hydroxyapatite (Bio-Gel HT, Bio-Rad Laboratories) chromatography. Unhybridized molecules were removed with 0.14 M PB plus 0.4% SDS at 60°C, and hybridized molecules were eluted with the same buffer at 100°C. Samples were counted after adding 12 ml of Instagel (Packard Instrument Co.) to 8 ml of aqueous solution.

Purification of amphotropic 1504-A-specific sequences. A major portion of the genomes of all (*Mus musculus*) MuLV's are homologous sequences. For better molecular characterization of these viruses, we attempted to purify the sequences of 1504-A virus that are not shared by ecotropic or xenotropic viruses of laboratory mice ("amphotropic 1504-A-specific sequences"). For the purification of amphotropic 1504-A-specific sequences, ^{3}H-labeled 1504-A probe (mouse cell grown) was hybridized with a saturating amount of AKR-L1 viral RNA (also grown in mouse cells). A total of 77.3% of the probe sequences were hybridized and included both shared viral sequences and the sequences of cellular origin. The unhybridized molecules (22.7% of the total probe counts) were separated by hydroxyapatite chromatography, purified by alkali treatment, dialyzed extensively, and concentrated by hydroxyapatite chromatography and lyophilization. These sequences were hybridized once again with a saturating amount of AKR-L1 viral RNA. At this time, only 9.6% hybridization was observed. The unhybridized molecules (20% of the total initial input counts per minute) were purified and concentrated as

mentioned and were then hybridized with the RNA from 1504-A-infected SC-1 cells. The hybridized molecules were purified through hydroxyapatite, treated with alkali to remove RNA, dialyzed, and concentrated.

RESULTS

Cellular sequences in the viral probes. In interpreting the data obtained with MuLV DNA probes, it has generally been assumed that the sequences are all of viral origin. However, there is a strong indication that there are nonviral sequences (in relation to the input virus) in the probes. We noted evidence for this previously (10) and have obtained further indications in the present studies. Table 2 shows the extent of saturation of probe sequences by cellular RNA preparations from uninfected cells and from cells infected with the same virus as used for the probe. These data provide two indications of the presence of cell sequences in the probe. First, RNA from normal mink cells reacts with significantly more of the sequences in the mink-grown xenotropic and amphotropic virus probe (20 and 45% of the probe, respectively) than in mouse-grown probes (7%). Second, RNA from cells infected with the same virus as in the probe saturated 95 to 99% of the probe, but only 60 to 80% of the probe if heterologous cells were used.

It thus appears that 20 and 45% of the probe sequences are of cell origin, and analyses should take into account whether homologous or heterologous cell systems are being studied.

Relatedness of amphotropic virus to other viruses. Table 1 summarizes the characteristics of the various viruses studied, and Table 3 summarizes the data on the relatedness of amphotropic 1504-A virus to other (naturally occurring) amphotropic, ecotropic, xenotropic, and laboratory strains of MuLV. Two types of 1504-A probe preparations were studied, an unfractionated probe and an absorbed probe containing those sequences that do not cross-react with the AKR-L1 strain of ecotropic virus or with NIH mouse cell RNA. From the data obtained with the unfractionated probe, it is clear that the 1504-A virus is a unique virus, not showing full homology with any of the tested strains. More than 50% of its sequences, as represented in the [^{3}H]DNA probe, are shared with the other ecotropic, xenotropic, amphotropic, and laboratory strains of MuLV. Surprisingly, of the viruses tested, most closely related to the amphotropic 1504-A virus are the laboratory strains, Rauscher and Moloney. The other amphotropic virus tested, 4070-A, is clearly distinct from the 1504-A strain.

The absorbed probe, containing sequences specific for 1504-A virus, was obtained by subjecting the 1504-A probe to two cycles of hybridization with AKR-L1 RNA. Two preparations of absorbed probe were made; in one, AKR-L1 viral RNA was used for the absorptions, and in the other, RNA from SC-1 cells infected with AKR-L1 virus was used. The probe fraction that did not hybridize (amounting to 20% of the probe counts) was then hybridized to cellular RNA from SC-1 cells infected with 1504-A virus, and the hybridized probe molecules were then isolated and purified. The two preparations gave identical results.

The data in Table 3 confirm that these 1504-A amphotropic-specific sequences do not hybridize with AKR viral RNA or AKR virus-infected NIH mouse cellular RNA. Likewise, there is no reaction of these sequences with two xenotropic viruses of laboratory mouse origin (these two viruses show only 50% cross-hybridization

TABLE 2. *Hybridization of MuLV probes with RNA from normal cells and from cells infected with the same virus as used for making the probe*

Cellular RNA		% saturation of ^{3}H-labeled probe from[a]:			
			Amphotropic 1504-A		
Virus infection	Species of cell	Ecotropic AKR-L1 (NIH mouse cells)	SC-1 mouse cells	Mink cells	Xenotropic BALB-IU-1 (mink cells)
Same as probe	Mouse	*95*	*99*	61	ND
	Mink[b]	80	78	100	*95*
	Rat	80	ND	ND	ND
None	Mouse (NIH Swiss)	*20*	ND	ND	ND
	Mink	7	7	*45*	*20*
	Rat	10	ND	ND	ND

[a] ND, Not done. Italicized numbers represent homologous cell systems.

[b] Mink cells productively infected with the AKR-L1 ecotropic virus were obtained by Akinori Ishimoto by infection with a phenotypically mixed population followed by cloning the infected cells (A. Ishimoto, J. W. Hartley, and W. P. Rowe, manuscript in preparation).

[Chattopadhyay et al., manuscript in preparation]), with a xenotropic MuLV strain isolated from a wild mouse from the Lake Casitas trapping area, or with the MCF-247 virus (17). It is striking that half of the amphotropic-specific sequences were present in the wild mouse ecotropic viruses and in the Rauscher and Moloney viruses as well; furthermore, tests with mixtures of these RNAs showed that it is the same subset in all four viruses.

Hybridization of amphotropic virus probe with cellular DNAs of various mouse strains. The data in Fig. 1 and 2, summarized in Table 4, represent the kinetic analyses of the amphotropic probe hybridized with various normal mouse DNAs. With the exception of the DNA of wild mice from Maryland, which gave 90% saturation, the DNAs saturated more than 95% of the 1504-A probe sequences (Fig. 1). Thus, within the limits of the technique, it can be inferred that the complete genome of this virus is present in the DNA of all eight strains of laboratory mice, in the house mice from Calcutta, India, and possibly in the Maryland house mice as well. The reciprocal plots of the hybridization data (Fig. 2) show the same biphasic

TABLE 3. *Hybridization of ^{3}H-labeled 1504-A probe (SC-1 grown) and 1504-A type-specific probe with various RNAs*

Virus		Cell in which virus was grown	Type of RNA preparation	% hybridization of probe sequences with saturating amount of RNA[a]		
				Total probe		Absorbed probe, observed[b]
Group	Strain			Observed	Normalized[c]	
Amphotropic	1504-A	SC-1	Total cell	99	(100)	100
Amphotropic	1504-A	Mink	Total cell	78	(100)	100
Ecotropic	1504-M	SC-1	Total cell	55	56	51
Amphotropic	4070-A	SC-1	Total cell	77	78	80
Ecotropic	4070-M	SC-1	Total cell	59	60	40
Ecotropic	4070-M + 1504-M					50
Ecotropic	AKR-L1	NIH-ME	70S viral	75	76	0
Ecotropic	AKR-L1	NIH-ME	Total cell	71	72	0
Xenotropic	BALB-IU-1	Mink	Total cell	55	71	0
Xenotropic	NZB-IU-1	Mink	Total cell	64	82	5
Xenotropic	Cas E no. 1-X	SC-1	Total cell			5
Laboratory strain (ecotropic)	Moloney	NIH-ME	Total cell	84	85	52
Uncharacterized	Rauscher	JLS-V9	70S viral	87	88	52
	Moloney + Rauscher					63
MCF	MCF-247	SC-1	Total cell			3
			Normal mink cell	6.8	8.7	
	1504-M + Rauscher					50

[a] Hybridizations were carried out in 1-ml sealed ampoules with a 100-μl incubation mixture containing either 0.05 ng of total probe sequences or 0.02 ng of absorbed probe sequences, 0.1% SDS, 0.48 M phosphate buffer, 0.5 mM EDTA, and varying amounts of RNA (a maximum of 2 μg of viral RNA and 700 μg of cellular RNA from productively infected cells was used). Each incubation mixture was boiled in a water bath for 2 min and incubated at 65°C for 24 to 30 h. Hybridization was assayed by hydroxyapatite chromatography as described in the text. The results listed here refer to the percentages at saturation (plateau values).

[b] 1504-A type-specific sequences (20% of total probe counts).

[c] Normalized by cell type (mouse or mink) from which the viral RNA was obtained.

patterns as were seen previously with ecotropic virus probes (8, 9, 25). There is a steep (fast) component, followed by a less steep linear portion which extrapolates to intersect the *y* axis at approximately 1.95, the same intercept seen with the ecotropic probe. The less steep component, whose slope relative to that of the cell-cell (unique-sequence DNA) hybridization gives the number of complete copies of the viral genome, shows much variation between mouse strains. The number of copies ranges from 2 to 3 per haploid genome, in NZB and 129, to 5 to 7 in other strains. There is no correlation between the copy numbers observed with the 1504-A probe and those previously observed with the ecotropic virus probe.

The thermal stability of the cell-probe hybrid molecules (Fig. 1, insets, and Table 4) shows significant variation between strains. However, since these are complex populations of molecules, of which at least half are the group-reactive sequences which cross-react with the other MuLV genomes, the significance of these differences is not clear.

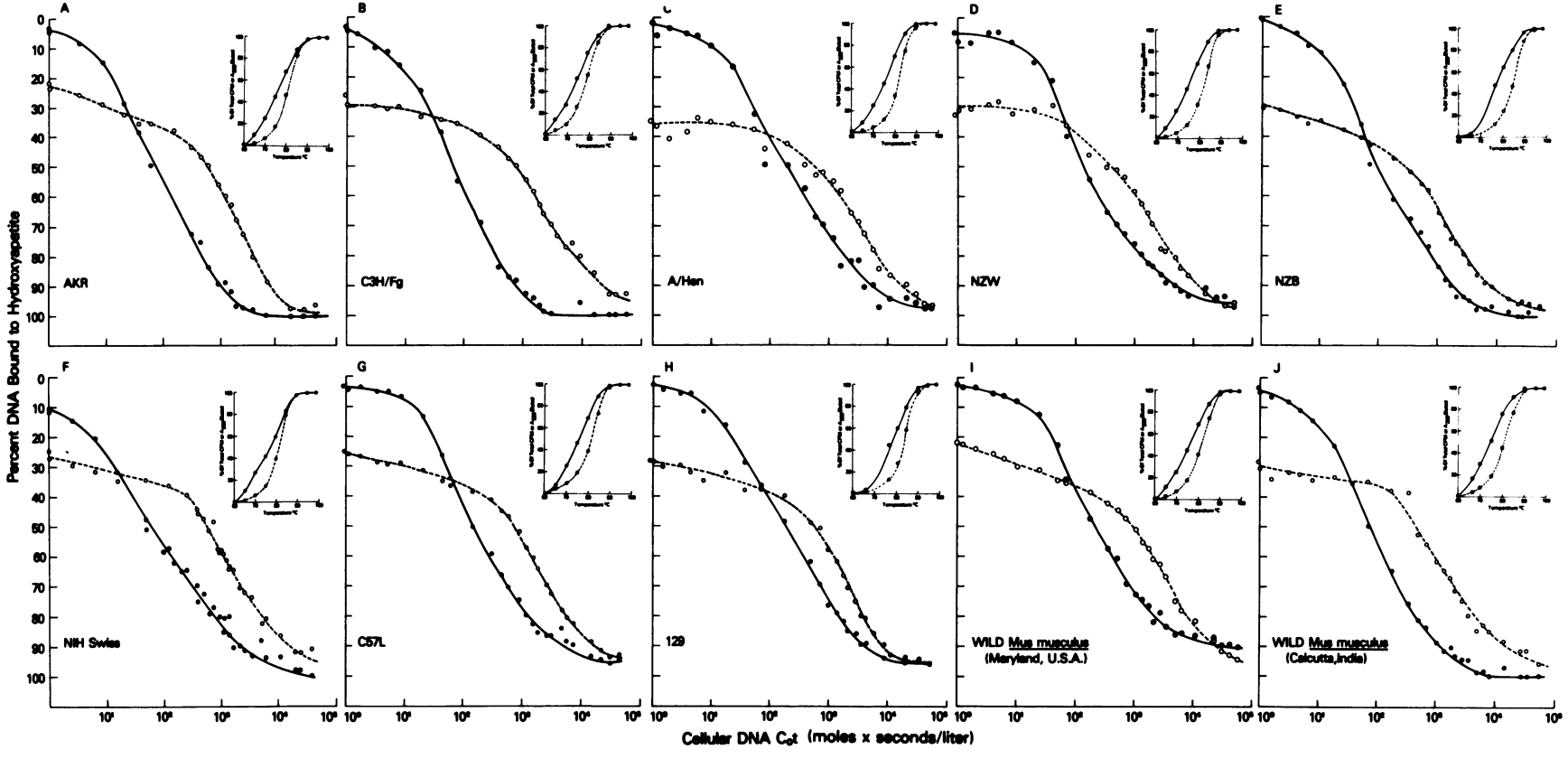
TABLE 4. *Hybridization of the ^{3}H-labeled 1504-A viral probe to the DNAs of various mouse strains*

Mouse DNA	Maximum % hybridization of probe	No. of sequence populations[a]	Approx no. of copies in each population[a]	ΔTe_{50}[b] (°C)
AKR/J	100	2	>10; 6-7	4.0
C3H/FgLW	100	2	>10; 5-6	4.5
NZW/N	98	2	>10; 3-4	7.0
A/HeN	98	2	>10; 3-4	6.0
NIH Swiss	99	2	>10; 5-6	4.0
C57L/J	96	2	>10; 3-4	5.5
129/J	96	2	>10; 2-3	5.0
NZB/N	100	2	>10; 2-3	7.5
Wild mouse (Calcutta, India)	100	2	>10; 5-6	5.5
Wild mouse (Maryland, U.S.A.)	90	2	>10; 4-5	5.0

[a] Determined from reciprocal plot (Fig. 2). The number of copies is the ratio of the slope of each line to the slope of the line described by the unique sequences of cell DNA.

[b] ΔTe_{50}, Difference between the Te_{50}'s (midpoint of thermal denaturation profile) of self-hybridized cell DNA molecules and that of probe-cell DNA hybrids.

FIG. 1. *Association kinetics of amphotropic (1504-A) viral [^{3}H]DNA probe with DNA from various strains of mice. For annealing reactions, sheared cellular DNAs (10 mg/ml) were mixed with 10^{-3} μg of viral [^{3}H]DNA probe per ml (specific activity, 2×10^7 cpm/μg) in a Reactivial (1.0-ml capacity; Pierce Chemical Co.). The mixtures were then denatured in 0.12 M PB by heating at 100°C for 5 min and brought to desired salt concentrations by the addition of 4.8 M PB. All of the incubation mixtures contained 0.4 mM EDTA. Incubation mixtures with a low salt concentration (0.18 M Na^+) were incubated at 60°C, whereas those with high salt concentration (0.72 to 0.9 M Na^+) were incubated at 65°C. Samples of 30 μl were taken at different time intervals and diluted to 3.0 ml in a final concentration of 0.14 M PB plus 0.4% SDS. The extent of hybridization at each time point was assayed by hydroxyapatite chromatography (6, 20). Unhybridized molecules were removed from the column with 0.14 M PB plus 0.4% SDS at 60°C, and the hybridized molecules were removed with the same buffer at 100°C. Each fraction eluted from the hydroxyapatite column was measured for A_{260} (to measure cell DNA-cell DNA reassociation) and, after addition of 12 ml of Instagel to 8 ml of aqueous solution, for radioactivity (to determine ^{3}H-labeled probe-cell DNA association). C_0t values represent the equivalent C_0t at 0.18 M Na^+ (4, 5). The solid line represents the hybridization kinetics of viral [^{3}H]DNA probe with cell DNA, and the broken line represents unique cellular DNA self-association kinetics. The panel insets represent the thermal elution profiles of the corresponding hybrids. A 100-μl incubation mixture containing 10 mg of sheared cellular DNA per ml, 1 ng of viral [^{3}H]DNA probe per ml, 0.12 M PB, and 0.4 mM EDTA was denatured at 100°C for 5 min. The salt concentration was then raised to 0.48 M PB and incubated at 65°C for 72 to 80 h (C_0t, 4×10^4 to 5×10^4). Each incubation mixture was then diluted to 0.14 M PB plus 0.4% SDS and passed over a hydroxyapatite column (60°C, 0.14 M PB plus 0.4% SDS). Single-stranded DNA was eluted from the column with 0.14 M PB plus 0.4% SDS, and the temperature of the column was then raised in a series of 5°C increments. At each of these temperatures, the column was washed twice with 8 ml of 0.14 M PB plus 0.4% SDS. Each fraction was measured for A_{260} to determine the percentage of self-hybridized molecules. After addition of 12 ml of Instagel, the radioactivity in each fraction was determined to assay the extent of hybridization of the viral [^{3}H]DNA probe to cellular DNA. The melting profile of the hybrids was determined by plotting the cumulative percentage of the total A_{260} units or counts per minute bound to hydroxyapatite at 60°C that were eluted at each temperature increment. Solid lines represent the melting profiles of viral probe-cell DNA hybrid, and broken lines represent melting profiles of cell DNA self-hybrid molecules. In the insets, the abscissa is temperature (°C), and the ordinate is the percentage of total counts per minute or A_{260} eluted.*


A
B
C
D
E
F
G
H
I
J
AKR
C3H/Fg
A/Hen
NZW
NZB
NIH Swiss
C57L
129
WILD Mus musculus (Maryland, U.S.A.)
WILD Mus musculus (Calcutta,India)
Percent DNA Bound to Hydroxyapatite
Cellular DNA C_0t (moles x seconds/liter)

DISCUSSION

For optimal interpretation of studies of the type reported here, it would be highly desirable to know the extent to which the [^{3}H]DNA probe is representative of the genome RNA. This was not possible in the present study because the quantity of 35S RNA obtainable from the amphotropic virus was insufficient; of the naturally occurring MuLV strains, only certain ecotropic isolates grow to sufficient titer to allow the type of biochemical studies that have been done with the high-titered laboratory strains of uncertain origin. The probe was prepared by the procedure used for the AKR-L1 probe, which has been shown to be highly representative of the AKR viral RNA (8, 9), and there is every reason to believe that probes prepared from other MuLV's would be similarly representative. Furthermore, the amphotropic probe used here resembles the AKR-L1 probe in that, in both, 60% of the counts were group-reactive MuLV sequences, 20% were specific for the class of virus, and 20% were of host-cell origin. This suggests that they have similar representativeness.

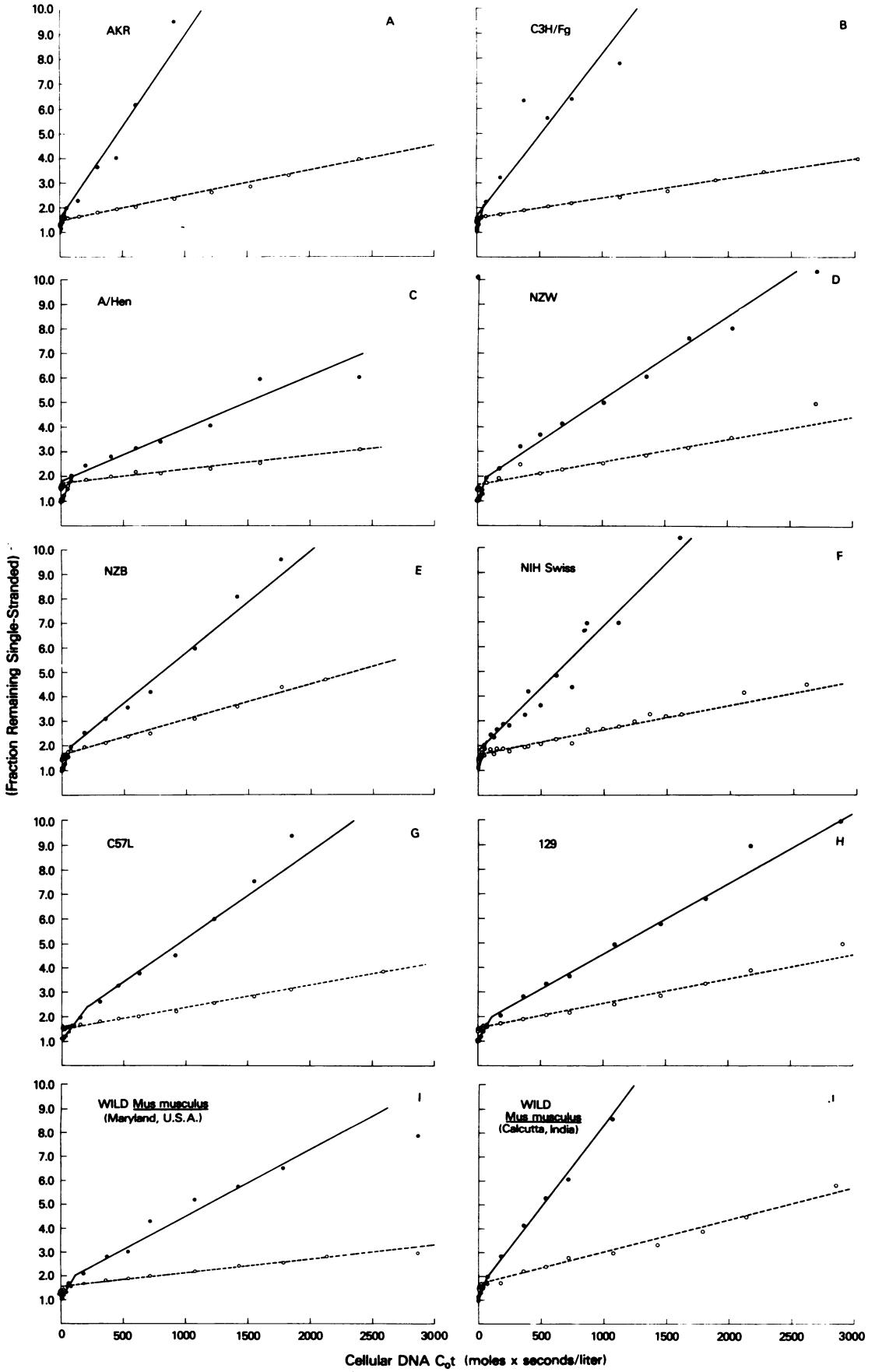
The sequence homology studies presented here confirm biological evidence that the amphotropic MuLV's are distinct from the ecotropic and xenotropic groups. This is most clear from the studies with the amphotropic-specific probe, operationally defined here as the amphotropic probe sequences that did not react with RNA from homologous species cells infected with AKR-L1 virus. These sequences, which make up one-fifth of the probe, were also absent from the three xenotropic viruses tested, including one of wild-mouse origin.

The distribution of these 1504-A amphotropic-specific sequences among the other viruses tested was of interest in several respects. The 4070-A virus, an amphotropic virus recovered from a wild mouse in a different area of California, reacted with 80% of these sequences, further indicating that they consist, at least in part, of sequences responsible for the distinctive biological determinants (presumably *env* gene products) of the amphotropic group. A striking observation was that one portion of the amphotropic-specific sequences, amounting to half of them or about 10% of the total probe, was present in the cellular RNA of SC-1 cells infected with several unique ecotropic viruses, including Moloney virus and the ecotropic MuLV strains recovered from the same California wild mice from which the amphotropic strains were isolated. Furthermore, this subset of sequences was present in viral 70S RNA of Rauscher JLSV9 virus. While the detection of these sequences in Moloney and Rauscher virus preparations is of particular interest in view of the apparent recombinant nature of the more highly oncogenic MuLV strains (17; D. H. Troxler, D. R. Lowy, R. Howk, H. Young, and E. M. Scolnick, Proc. Natl. Acad. Sci. U.S.A., in press), this must be considered only a preliminary, suggestive observation. There are several possible explanations for the presence of these RNA sequences in these complex viral populations other than their being in the original strains.

Since no virus of the amphotropic class has been recovered from laboratory mice (though further characterization of the strains now considered to represent xenotropic isolates is called for), it was an unexpected finding that the entire set of 1504-A probe sequences is present in cell DNA of all inbred mouse strains tested. This discrepancy is in sharp contrast to the close correlation between the nucleic acid homology findings with ecotropic virus probe and the biological assays for ecotropic virus in the various inbred strains (8, 9, 25). As with the kinetic hybridization analyses with ecotropic virus, the 1504-A probe-cell DNA reaction was diphasic (or more likely, multiphasic). The more abundant sequences, present as 10 or more copies per haploid genome, represent the group-reactive sequences (9). The less abundant set probably includes, but does not consist entirely of, the amphotropic-specific sequences. The number of copies in this set shows variation between mouse strains (a range of 2–3 to 6–7 per haploid genome), as was seen with the AKR ecotropic virus (range of 0 to 3–4 per haploid genome). There was no correlation between the number of DNA copies of the two groups of virus in the various strains; for example, in the five groups in Table 4 in which the gross AKR-specific sequences are not present (NIH, C57L, 129, NZB, and the Maryland house mice), the number of amphotropic virus genomes ranges from 2–3 to 5–6.

The lack of detection of amphotropic virus in laboratory mice, despite the presence of multiple copies of the full set of genome sequences in the cellular DNA, suggests several interesting possibilities. It is conceivable that the controls of induction of provirus are qualitatively different than with ecotropic viruses, so that induction

FIG. 2. *Analyses of association kinetics of [^{3}H]DNA probe with various mouse cellular DNAs by the reciprocal plotting method (34). The data are from Fig. 1. The maximum observed [^{3}H]DNA probe-cell DNA and cell DNA-cell DNA hybridization were normalized to 100%. The symbols are the same as in Fig. 1.*


AKR
A
C3H/Fg
B
A/Hen
C
NZW
D
NZB
E
NIH Swiss
F
C57L
G
129
H
WILD Mus musculus
(Maryland, U.S.A.)
I
WILD
Mus musculus
(Calcutta, India)
J
(Fraction Remaining Single-Stranded)
Cellular DNA C_0t (moles x seconds/liter)

does not occur spontaneously or after 5'-iododeoxyuridine treatment. Alternatively, it is possible that the genome is not present in the cell DNA as complete linear proviral copies which can be transcribed into functional viral RNA genomes; for example, it can be speculated that the envelope gene of the amphotropic virus is a host gene present in several copies in the cell genome, unlinked to C-type viral sequences, and that the virus arose by recombination, as proposed in the protovirus hypothesis of Temin (32, 33). It may be relevant that in the wild mouse populations in which amphotropic virus is prevalent, its natural transmission does not appear to be by induction of genetically transmitted virus, as is the case with ecotropic virus in laboratory mice; rather, transuterine or other maternal exogenous infection of progeny appears to be the major mode of infection (12).

The evidence presented here that the viral DNA probes contain a significant amount of cell-derived sequences raises several points of theoretical and practical interest. It is possible that these sequences are genomes of endogenous oncornaviruses activated or helped by the MuLV infection (13); however, this would require postulating an endogenous mink oncornavirus, for which there is no supporting evidence. Another possibility is that the host sequences are present in viral-host recombinant molecules, perhaps containing the insertion sites; further studies of the nature of these sequences are under way. From a practical point of view, it is important to take into account what effect these host sequences might have on interpretation of cell-viral and viral-viral homology studies; the use of probes prepared with virus grown in cells of a species unrelated to those being studied for presence of viral sequences would be highly desirable. In the present study, as well as our previous reports, mouse-grown probes were used for analysis of mouse DNA. In unpublished studies, in which xenotropic virus probes grown in mink cells were tested against mouse DNA, we have observed the same kind of diphasic Wetmur-Davidson curves as in Fig. 2 and in the previous studies with ecotropic virus. Consequently, we feel reassured that the low-copy-number component is indeed viral (9–11).

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ADDENDUM IN PROOF

Since submission of this manuscript, two reports of isolations of endogenous type C RNA virus from mink lung cells [Mv1Lu (CCL64)] have been made (M. Barbacid, S. R. Tronick, and S. A. Aaronson, J. Virol. **25**:129–137, 1978; V. Klement, M. F. Dougherty, R. Roy-Burman, B. K. Pal, C. S. Shimizu, R. W. Rongey, W. Nelson-Ross, and R. J. Huebner, submitted for publication). However, it is not clear that the mink sequences that we detect in our mink-grown viral probe preparations are sequences present in these mink virus isolates.

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