ABSTRACT Studies with a single-stranded DNA probe complementary to the RNA of mouse-tropic AKR murine leukemia virus indicate that the complete genome of the AKR-type murine leukemia virus is present in the DNA of high- and low-virus-yielding mouse strains, while DNA of non-virus-yielding strains contains only a part of the genome. Furthermore, in those strains where the genome is complete, two populations of virus-specific DNA sequences can be identified (more abundant and less abundant species) according to their rate of association with the probe. Low-virus-yielding mouse strains contain fewer copies of the less abundant species and, consequently, fewer complete viral genomes than do high-virus-yielding strains. Thus, in the ten strains tested, there is a good correlation between completeness of the genome of AKR-type murine leukemia virus in cellular DNA and the capacity of the cells to release infectious AKR-type murine leukemia virus. Moreover, the number of complete viral genomes correlates with the frequency of infectious virus production by virus-positive strains. DNA from wild Mus musculus also contained viral sequences, the sample tested showing reassociation kinetics identical to the non-virus-producing strains.

Inbred mouse strains vary markedly in their incidence of spontaneous leukemia. Mice of high leukemia strains regularly contain large amounts of mouse-tropic (AKR-type) murine leukemia virus (MLV) early in life (1). Low leukemia strains fall into two categories virologically. Some strains demonstrate AKR-type virus, but less often and later in life (low-virus-yielders). Other strains never yield AKR-type virus (non-virus-yielders) (2). All strains of mice thus far studied contain xenotropic C-type viruses, which differ markedly from the AKR-type virus in host range (being unable to exogenously infect mouse cells in tissue culture), interference specificity, and envelope antigens, but contain the same group specific (gs) antigen and RNA-directed DNA polymerase (3-6). Thus, the term "virus-yielding" in this report relates only to the AKR-type virus. Considerable evidence has accumulated indicating that in virus-yielding strains the MLV genome is present in a stable heritable form (7-9). Furthermore, it has been established that genetic material of C-type viruses is present in cellular DNA (10-16). However, since most previous studies failed to detect a difference in the viral-specific DNA of high-, low-, and non-virus-yielding strains, it has not been clear what mechanisms determine whether infectious virus is produced and the frequency with which production occurs.

We have recently reported qualitative and quantitative studies of the viral-specific nucleotide sequences present in the cellular DNA of a high-virus-yielding mouse strain (AKR) and a non-virus-yielding strain (NIH Swiss) (16). In these experiments, we used a single-stranded DNA probe made in an endogenous reaction with detergent-disrupted AKR-MLV virions. Association kinetics, as well as saturation hybridization in the presence of an excess of cellular DNA, were used in this study; it was found that the complete AKR-MLV genome is present in the AKR-DNA, whereas it is incomplete in the NIH-DNA. Scolnick et al. (17) independently observed that NIH 3T3 and a virus-negative feral mouse cell line contain a lower amount of sequences specific for Kirsten strain of murine leukemia virus in their DNA than do either BALB/c or C57BL/6, both of which are low-virus-yielding strains. In the present report, we extend our observation to other mouse strains, including low-virus-yielders and additional high- and non-virus-yielding strains. The studies indicate that of the 11 strains tested, high-virus strains contain multiple complete copies of the AKR-type MLV genome, low-virus strains possess the complete genome but contain fewer copies, and the non-virus-yielding strains lack a major portion of the viral genome.

MATERIALS AND METHODS Mouse Embryos. The laboratory mouse strains used were: high-virus-yielders: AKR/J and C3H/FgLw (received from Dr. E. A. Boyse); low-virus-yielders: BALB/cN, DBA/2N, C57BL/6J, and C3H/HeN; non-virus-yielders: NIH Swiss, C57L/J, 129/J, and NZB/N. Pregnant mice were killed by cervical dislocation and the embryos were removed aseptically and freed from the placenta and fetal membranes. AKR embryos were taken on the 14th-16th day, and C3H/FgLw on the 15th-18th day of gestation, a time when the embryos are virtually free of detectable virus (1). The other mice were obtained on the 18th-20th day of gestation. Embryos were dipped in ether, allowed to dry, and frozen at −70° until use.

Abbreviations: MLV, murine leukemia virus; AKR-DNA, 129-DNA, DBA-DNA, etc., DNA from the cells of embryos of the corresponding mouse strain; NaDodSO4, sodium dodecyl sulfate; Te50, midpoint of thermal elution profile.

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measured for and, phosphate buffer plus 0.4% [3H]DNA hybridized molecules different Instrument Co.) to 8 ml of aqueous solution, for cellular mouse measure (to hybridization at each time point was of apatite incubated Na+0.14 M NaDODSO4. The extent of hybridization at each time point was assayed by hydroxyapatite (Bio-gel HTT, Bio-Rad Laboratory) (16, 18, 22). Unhybridized molecules were removed from the column with 0.14 M phosphate buffer plus 0.4% NaDODSO4 at 60°, while the hybridized molecules were removed with the same buffer at 100°. Each fraction eluted from the hydroxyapatite column was measured for absorbance at 260 nm (to measure cell–cell DNA reassociation), and, after addition of 12 ml of “Instagel” (Packard Instrument Co.) to 8 ml of aqueous solution, for radioactivity (to measure probe–cell DNA association). Cst values represent equivalent Cst at 0.18 M Na+ (20). (a) •, Association kinetics of viral [3H]DNA probe with C3H/FlgIw mouse cellular DNA; ○, C3H/FlgIw mouse cellular DNA self-association kinetics. (b) ■, Association kinetics of [3H]DNA probe with C3H/FlgIw mouse cellular DNA; ●, C3H/FlgIw mouse cellular DNA self-association kinetics. (c) △, Association kinetics of viral [3H]DNA probe with 129/J mouse cellular DNA; □, 129/J mouse cellular DNA self-association kinetics.

Five wild Mus were caught in a home 30 miles from NIH. Liver, kidney, thymus, small intestine (opened and washed extensively with Tris-buffered saline), and spleen were pooled.

Virus. The AKR-L1 strain of MLV, originally isolated from a leukemic AKR mouse, has been serially passaged in secondary NIH Swiss mouse embryo cells. The virus purification procedure, and the purification of 70S viral [32P]RNA and unlabeled viral RNA have been described (16).

Preparation of Cellular DNA and Synthesis of Single-Stranded, Virus-Specific [3H]DNA. Cellular DNAs were prepared from embryos or tissues by a sodium dodecyl sulfate NaDODSO4–Pronease–phenol method (Chattopadhyay et al., in preparation), sheared at 40,000 lbs./inch2 in a French pressure cell (American Inst. Co.), and filtered through a GA-6 filter (Gelman Inst. Co.) (18). Single-stranded, virus-specific [3H]DNA probe was synthesized in an endogenous RNA-directed DNA polymerase reaction using detergent-lysed, purified AKR virus (19).

Hybridization of the Probe with Cellular DNAs. Initial hybridizations were carried out under conditions identical with those described (technique 1) (16). Later hybridizations have used two technical modifications and two new batches of DNA probe (technique 2). Instead of using multiple sealed ampoules for reaction mixtures as in technique 1, technique 2 uses ‘Reactivials’ (Pierce Chemical Co.). These vials permit serial sampling from a single reaction mixture without significant evaporation during long-term incubation. In technique 1, low Cst reactions were carried out with low DNA concentrations (0.2 mg/ml) and low salt (0.18 M Na+), whereas reaction mixtures for high Cst were carried out with high DNA (4.25 mg/ml) and high salt (0.72 M Na+) concentrations. In technique 2, low Cst reactions were carried out at 0.2–0.3 M Na+ and high Cst reactions at 0.8–1.0 M Na+, and all reaction mixtures contained the same concentration of cell DNA (10 mg/ml). Other technical details are described in the legend of Fig. 1. In technique 1, cellular DNA self-hybridized maximally to 90–92%, and the DNA probe hybridized maximally to 80% with AKR-DNA and 50% with NIH-DNA. In technique 2, cellular DNA self-hybridized maximally to 94–96%, and the DNA probe hybridized maximally to 88% with AKR-DNA and 69% with NIH-DNA. Cst is the product of nucleic acid absorbancy at 250 nm/ml and the hours of incubation, divided by 2. All the Cst values mentioned in this paper are the equivalent Cst at 0.12 M phosphate buffer (0.18 M Na+) (20).

RESULTS

Characterization of the Single-Stranded [3H]DNA Probe. The characteristics of a probe synthesized by the method described have been discussed (16). Briefly, a representative
viral [3H]DNA probe has a specific activity of $2 \times 10^8$ cpm/µg, and is 98% single-stranded, 100% trichloroacetic acid-precipitable, and about 200–400 nucleotides in length. After hybridization with the probe (at a ratio of probe:RNA of 1.5:1), at least 69% of viral 70S RNA sequences are protected against single-strand-specific S-1 nuclease digestion (21), and a saturating amount of 70S RNA protects 87% of the DNA probe sequences.

**Hybridization of Probe with Cellular DNAs.** Tritiated single-stranded, viral-specific AKR-MLV DNA was incubated with a vast excess of unlabeled bulk cellular DNA from the various mouse strains. Association kinetics (22) of the viral DNA probe with embryo DNAs from a representative high-, low-, and non-virus-yielding strain are shown in Fig. 1. Three different kinetic patterns were obtained. The first pattern is represented by the DNA from the high-virus-yielding C3H/FgLw strain (Fig. 1a); this DNA hybridizes much faster with the viral probe than does “unique” (nonreiterated) cellular DNA with itself. At the completion of the reaction, 86% of the probe has been hybridized. The second pattern is that of the low-virus-yielding DBA strain (Fig. 1b). This DNA hybridizes initially with the viral probe at the same rate as does C3H/FgLw DNA, but the rate decreases (relative to C3H/FgLw DNA) as the hybridization proceeds, and about 81% of the viral probe is hybridized at the completion of the reaction. The hybridization of DNA from the non-virus-yielding 129 strain to the viral probe follows a third kinetic pattern (Fig. 1c). After an initial hybridization rate indistinguishable from that of the DNA from the high and low virus strains, the rate quickly slows and reaches a plateau at about 68% hybridization. This DNA is missing significantly more viral-specific sequences than is the DNA from the low-virus strain.

If one plots the reassociation kinetics (Fig. 1) as the reciprocal of the proportion of unhybridized probe (or cell DNA) (normalized to maximum hybridization equaling 100%) against the $C_0$ (Wetmur-Davidson plot) (Fig. 2) (23), more information can be gained. If all virus-specific sequences were present in the cellular DNA in equal numbers, the results would describe a single straight line with a slope proportional to the number of copies of those sequences. On the other hand, if several sets of virus-specific sequences were present, each in different proportions, several lines would be described. Using this analysis, we have previously shown that the MLV genome in AKR mouse DNA is composed of two different sets of sequences, one of which was estimated to comprise about 10 copies and the other about four copies per haploid cellular genome (16). When the hybridization data shown in Fig. 1 are transformed in this manner (Fig. 2), the DNAs from all three strains have a set of viral-specific sequences that is repeated at least seven to eight times per haploid genome. However, in the C3H/FgLw DNA, there is an additional population of sequences present in about three to four copies (Fig. 2a). The DNA from the low-virus-yielding DBA strain contains only one to two copies of this population of sequences (Fig. 2b), while the 129-DNA appears to be missing the set of less-abundant sequences altogether (Fig. 2c).

The results of hybridization of the viral probe to the DNAs of the 11 strains tested are presented in Table 1. All 11 strains fall into the three kinetic patterns described in Figs. 1 and 2, and these three patterns correlated completely with the virus-yielding state of each strain. There was a small but consistent

### Table 1. Summary of the hybridization results of the viral probe to the DNAs of the 11 mouse strains

<table>
<thead>
<tr>
<th>DNA from mouse strains</th>
<th>Technique 1</th>
<th></th>
<th>Technique 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max. hybridization of the probe (%)</td>
<td>No. of populations of sequences*</td>
<td>Approx. no. of copies of each population</td>
<td>Max. hybridization of the probe (%)</td>
</tr>
<tr>
<td>High-virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKR/J</td>
<td>80</td>
<td>2</td>
<td>8–10; 3–4</td>
<td>88</td>
</tr>
<tr>
<td>C3H/FgLw</td>
<td>86</td>
<td>2</td>
<td>7–8; 3–4</td>
<td>82.5</td>
</tr>
<tr>
<td>Low-virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/cJ</td>
<td>71</td>
<td>2</td>
<td>7–8; 1–2</td>
<td>81</td>
</tr>
<tr>
<td>DBA/2N</td>
<td>72</td>
<td>2</td>
<td>7–8; 1–2</td>
<td>84</td>
</tr>
<tr>
<td>C3H/HeN</td>
<td>69</td>
<td>2</td>
<td>6–7; 1–2</td>
<td>67</td>
</tr>
<tr>
<td>Non-virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIH Swiss</td>
<td>50</td>
<td>1</td>
<td>14</td>
<td>64–69</td>
</tr>
<tr>
<td>C57L/J</td>
<td>53</td>
<td>1</td>
<td>15</td>
<td>69</td>
</tr>
<tr>
<td>129/J</td>
<td>63</td>
<td>1</td>
<td>10</td>
<td>75.2</td>
</tr>
<tr>
<td>NZB/N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>63</td>
<td>1</td>
<td>7–8</td>
<td>75.2</td>
</tr>
</tbody>
</table>

* 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 unique sequences of cell DNA. In those cases where two lines are described, the estimate of the number in the more abundant population is a minimum estimate, and may be a gross underestimate.

† Range from three separate experiments.

‡ ΔTek50 is the difference between the Tek50 of self-hybridized cell DNA molecules and that of probe–cell DNA hybrids.

§ Tissue cultures were prepared from kidneys of three of the five mice, and no AKR-type MLV was detected in the culture fluids.
Fig. 3. Thermal elution profiles of the hybrids formed between cellular DNAs of high-, low-, and non-virus-yielding mouse strains and viral \[^{[\text{H}]}\]DNA probe. In each case, a 100-μl incubation mixture contained 10 μg/ml of sheared cellular DNA, 1 \times 10^{-3} μg/ml of the viral \[^{[\text{H}]}\]DNA probe, 0.12 M phosphate buffer, and 0.5 mM EDTA. After the mixtures were denatured (100° for 5 min.), they were raised to 0.48 M phosphate buffer and incubated at 65° for 72–80 hr (Cot = 4–5 \times 10^9). Each incubation mixture was then diluted to 0.14 M phosphate buffer + 0.4% NaDodSO4 and passed over a hydroxyapatite column (60°, 0.14 M phosphate buffer + 0.4% NaDodSO4). Single-stranded DNA was eluted from the column with 0.14 M phosphate buffer + 0.4% NaDodSO4, and the temperature of the column was then raised in a series of 5° increments. After each increment, the column was washed at the new temperature with 8 ml of 0.14 M phosphate buffer + 0.4% NaDodSO4. Each fraction was measured for absorbance at 260 nm to determine the percentage of self-hybridization and the Te50 of the self-hybridized molecules. After addition of 12 ml of “Instagel,” the radioactivity in each fraction was determined in order to assay the extent of hybridization of the viral \[^{[\text{H}]}\]DNA probe to cellular DNA and the elution profile of these hybrids. The melting profile of the hybrids was determined by following the percentage of the total \(A_{260}\) units or cpm that bound to the hydroxyapatite at 60° and were eluted at each temperature. (A) Hybrids formed between the \[^{[\text{H}]}\]DNA probe and high-virus-yielding C3H/FgLw DNA (●), or C3H/FgLw DNA self-hybridized molecules (○). 86% of the total input radioactivity and 93% of the total input \(A_{260}\) units were adsorbed to the column at 60°. (B) Hybrids formed between the \[^{[\text{H}]}\]DNA probe and low-virus-yielding DBA/2 DNA (▲), or DBA/2 DNA self-hybridized molecules (△). 81% of the total input radioactivity and 96% of the total input \(A_{260}\) units were adsorbed to the column at 60°. (C) Hybrids formed between the \[^{[\text{H}]}\]DNA probe and non-virus-yielding 129 DNA (■), or 129 DNA self-hybridized molecules (□). 69% of the total input radioactivity and 95% of the total input \(A_{260}\) units were adsorbed to the column at 60°.

The difference between the total amount of the viral probe hybridized by the low-virus strains (81–84%) and by the high-virus strains (86–88%). Qualitatively, the results obtained with techniques 1 and 2 are identical; however, saturation values are higher with technique 2.

Analysis of Te50s of the Hybrids Formed Between the Probe and Cellular DNAs. The thermal melting profiles of the hybrids formed between the viral \[^{[\text{H}]}\]DNA probe and the embryo DNAs from a representative high-, low-, and non-virus-yielding strain are shown in Fig. 3. Analysis of the Te50s (midpoint of the thermal elution profile) of the hybrids formed between the \[^{[\text{H}]}\]DNA probe and DNA from various mouse strains (Table 1) revealed the following facts. (a) The hybrids formed between the DNA from all high-virus-yielding mouse strains and the \[^{[\text{H}]}\]DNA probe and DNA from various mouse strains (Table 1) revealed the following facts. (a) The hybrids formed between the DNA from all high-virus-yielding mouse strains and the \[^{[\text{H}]}\]DNA probe (Fig. 3A) are similar and have the highest Te50 (82.5–83.2°) values of all cell DNA–probe hybrids studied. (b) The Te50s of hybrids formed between the DNA from low-virus-yielding mouse strains and the \[^{[\text{H}]}\]DNA probe (Fig. 3B) show Te50s (81.5–82°) that are close to those of high-virus-yielding strains. (c) The hybrids formed between the DNA from all non-virus-yielding mouse strains and the \[^{[\text{H}]}\]DNA probe (Fig. 3C) are similar and have the lowest Te50 values (75.2–77.5°).

We tentatively interpret these Te50 patterns as indicating that the two populations of viral sequences identified in the kinetic analyses have different Te50s, the sequences common to all strains having the lower value. This could be due to the shared sequences being poorly matched to the probe or having a lower G:C content, or both.

**DNA Piece Size, Heterogeneity of Sequences in the Synthesized Probe, and the Rate of Reassociation.** Since the rate of reassociation varies with the square root of the DNA piece size (23, 24), we have determined the relative piece size of the synthesized single-stranded \[^{[\text{H}]}\]DNA probe and the sheared cellular DNA molecules by alkaline sucrose density sedimentation. The probe and cellular DNAs were cosedimented on a 5–20% alkaline sucrose gradient, and each demonstrated a single peak. The cell DNA peak sedimented slightly faster than the probe DNA peak (corresponding to an average single-stranded length for cell DNA of 400 nucleotides, and for probe DNA of 300 nucleotides). Thus, the probe \[^{[\text{H}]}\]DNA probably associated with cell DNA at a rate slightly slower than that of the cellular DNA reassociation. If so, our calculations for the copy numbers of viral sequences could be slight underestimates.

Furthermore, the association kinetics may reflect heterogeneity in the population of sequences in the synthesized probe. However, this possibility is unlikely since we performed reassociation experiments with four-fold increases and 4-fold decreases in the amount of AKR and NIH cellular DNA and obtained the identical reassociation both qualitatively and quantitatively (data not shown). Moreover, since the experiments were performed with a vast excess of cellular DNA, the rate of the association reaction was governed by the cellular DNA concentration. Heterogeneity in the probe would, therefore, not be reflected in this rate and, consequently, would not perturb our calculations for number of copies. Furthermore, when the \[^{[\text{H}]}\]DNA probe was hybridized to an excess of 70S RNA and association kinetics were measured, at least 70% of the probe reacted as if there were a single population of sequences, and the rate of reaction corresponded to the rate constant one would expect with an exact copy of 70S RNA. This experiment does not exclude the presence of a small fraction of extremely rare sequences.

**Hybridization of the Probe Fraction Remaining Unhybridized with Non-Virus-Yielding Mouse Cell DNA.** To further examine the possibility that certain probe sequences did not hybridize to non-virus-yielding mouse cellular DNA due to lack of those sequences in the latter, we first hybridized the probe with NIH Swiss DNA and purified the fractions that did not hybridize in the following manner: NIH cell DNA (7 mg/ml) was mixed with 1 \times 10^{-3} μg/ml of the probe and boiled for 5 min at 100° (0.18 M Na+ and 0.5 mM EDTA). The salt concentration was then raised to 0.72 M Na+ (0.48 M phosphate buffer), and the mixture was incubated for 72 hr at 65° (cellular DNA Cot = 30,000). The incubation mixture was then diluted to 0.14 M phosphate buffer (plus 0.4% NaDodSO4), and unhybridized molecules were separated by hydroxyapatite chromatography. Under these conditions, 93% of the cell DNA had reassocciated and 61% of probe sequences had hybridized to cell DNA. The
unhybridized molecules were purified and concentrated by dialysis and lyophilization, and then rehybridized with fresh NIH cellular DNA or AKR cellular DNA. Only 7% of the probe rehybridized to NIH cellular DNA, but 25% associated with AKR cellular DNA. Thus, 65% of the total probe sequences associated with NIH cellular DNA, whereas 85% of the probe associated with AKR cell DNA. These saturation values are in good agreement with those determined after the probe is hybridized with NIH and AKR cellular DNA separately. Moreover, the results also confirm that NIH cell DNA lacks about 20% of the probe sequences contained in AKR cell DNA.

Since the use of the virus-specific DNA probe is one step removed from the virus itself, we have hybridized AKR and NIH cellular DNAs directly with highly labeled 70S viral [32P]RNA; the extent of the hybridization was assayed with single-strand-specific S-1 nuclease from Aspergillus oryzae (21). AKR-DNA hybridized with up to 54%, and NIH-DNA with up to 31%, of the AKR viral RNA. Therefore, it appears again that AKR-DNA contains more viral-specific material than does NIH-DNA.

**DISCUSSION**

These experiments indicate that there are two populations of virus-specific sequences in the DNA of virus-yielding strains; however, there are fewer copies of the less-abundant population in the low-virus-yielding strains than in the high-virus-yielding strains, and a small number of sequences may be missing in the low-virus-yielding strains. The non-virus-yielding strains lack the less-abundant set of virus-specific sequences altogether. The wild mouse DNA also contained viral sequences, reacting identically to the non-virus-yielding strains.

Genetic studies of crosses between virus-yielding and non-virus-yielding mouse strains have shown that the capacity to produce virus segregates in classical Mendelian patterns (2, 25, 26). From studies of the inheritance of viral genetic markers, it was inferred that the most likely explanation of these results is that the chromosomal loci detected represent viral genomes (26, 27, 5). High-virus-yielding strains (AKR, C3H/FgLw, and C58) contain two or more loci for mouse-tropic virus (2, 25, 27), while the one low-virus-yielding strain analyzed genetically, BALB/c, contains only one locus for mouse-tropic virus (5). The present findings are in full agreement with the genetic studies, and further support the hypothesis that the virus-inducing loci are viral genomes.

In contrast to the findings reported here, an earlier study failed to demonstrate any differences in the amount of virus-specific material between the DNAs of several mouse strains, including NIH and AKR (10, 11). That study used a double-stranded DNA probe from Kirsten MLV, a laboratory strain of virus. As discussed elsewhere (16), there are several theoretical advantages for the probe used here, the most important being that the single-stranded probe appears to be a much more faithful copy of the 70S viral RNA than is the double-stranded probe (28).

A small difference between the amount of the viral DNA probe that hybridizes to DNA from high- and low-virus mouse strains was consistently observed. RNA from a mouse-tropic virus isolated from a BALB/c mouse hybridizes somewhat less efficiently to the DNA probe than does RNA from AKR virus (unpublished observation). It is possible that a small number of sequences present in the high-virus strains are missing from the low-virus strains; if so, this could be another factor in determining the frequency of spontaneous virus release, together with the lower number of complete viral genomes.

Another noteworthy result of these experiments is the finding that all mouse DNAs studied contain seven or more copies of one portion of the AKR viral genome, regardless of the number of copies they contain of the remainder of the viral genome. It is probable that this “more abundant,” but incomplete, set represents the same nucleotide sequences in all mouse DNAs. It seems likely that some, or all, of these copies represent sequences shared with the xenotropic murine leukemia viruses (6), since these viruses appear to be present in all mouse strains and are known to have several proteins in common with the mouse-tropic AKR-type viruses (6, 4).

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