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Definitive Evidence That the Murine C-Type Virus Inducing Locus *Akv-1* Is Viral Genetic Material

(mouse leukemia virus/congenic mice/iododeoxyuridine induction/DNA·DNA hybridization)

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ABSTRACT **DNA of the AKR mouse contains a set of murine leukemia virus sequences that are not present in DNA of the NIH Swiss mouse. NIH mice partially congenic for the AKR murine-leukemia-virus-inducing locus *Akv-1* contain this set of sequences, and, in a three-point cross segregating for *Akv-1* on an NIH background, the sequences segregated with *Akv-1*. It is concluded that the *Akv-1* locus contains viral sequences.**

Many types of evidence have suggested that the genetic material of murine leukemia virus (MuLV) exists as chromosomal loci in the normal mouse genome. Early findings by Gross (1) suggesting the transmission of virus by germ cells have been confirmed by studies showing that all strains of mice have the capacity to produce MuLV or certain of its antigens (2), that in virus-positive mouse strains the viral genome is present in all cells (3–5), that the expression of virus or viral antigens is determined by classical Mendelian genes (6–9), and that sequences complementary to MuLV RNA are present in cellular DNA (10, 11). The question of whether the chromosomal loci for viral induction represent genes that are permissive for expression of MuLV genetic material located elsewhere in the cell, or are the viral genomes themselves, has not been resolved. Indirect evidence favoring the latter has come from studies of genetic segregation of naturally occurring host-range variants of MuLV (12, 13).

Two recent developments from our laboratory have provided a means to answer this question definitively. First, by mouse breeding experiments, we have mapped the chromosomal location of one of the two virus-inducing loci of the high-virus AKR strain (14), and have isolated this locus (*Akv-1*) from the rest of the AKR genome by serially backcrossing to the virus-negative NIH Swiss strain. *Akv-1* is on chromosome 7 (Linkage Group I), being about 12 map units from *Gpi-1*, and 30 units from *c*, the albino locus, with gene order centromere–*Akv-1*–*Gpi-1*–*c*.

Abbreviations: C_0t, product of DNA concentration and length of hybridization reaction, calculated as nucleic acid absorbancy at 260 nm times hours of incubation divided by 2; T_e50, temperature at which 50% of the hybrid molecules became single-stranded and eluted from the hydroxyapatite column; $NaDodSO_4$, sodium dodecyl sulfate; MuLV, murine leukemia virus; PB, phosphate buffer.

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Second, we have demonstrated that although all inbred mouse strains contain DNA sequences homologous to a portion of MuLV RNA (that is, RNA from the mouse-tropic Gross-AKR type virus found in high-virus, high-leukemic mouse strains), they differ in that DNA from strains that have the capacity to produce this virus, such as AKR, contains all of the sequences of the virus, while DNA from the NIH Swiss strain, from which mouse-tropic virus has never been recovered, lacks a portion of the viral sequences (11). We will refer to that set of viral sequences which is present in DNA of AKR and other virus-yielding mouse strains and absent in NIH DNA as AKR-specific viral DNA sequences.

If it could be shown that in segregating or congenic mice, inheritance of the *Akv-1* locus is invariably accompanied by inheritance of the AKR-specific viral DNA sequences, their identity would be proved.

MATERIALS AND METHODS

Viral [^3H]DNA Probe. Virus-specific single-stranded [^3H]-DNA was prepared from the Gross-AKR subgroup MuLV strain AKR-L1. The propagation and purification of the virus, and the synthesis and characterization of the probe have been described previously (11,15).

Mice. AKR/J (*Akv-1*$^+$, *Gpi-1*^{b}, *c*), NIH Swiss (*Akv-1*$^-$, *Gpi-1*^{b}, *c*), and C57Br/J (*Akv-1*$^-$, *Gpi-1*^{a}, *c*$^+$) mice were used. Mice carrying the *Akv-1* locus on an NIH genetic background were obtained as follows: AKR was backcrossed to C57Br for two backcross generations [in the original studies in which *Akv-1* was identified; (ref. 16)] and then crossed to NIH females for six generations. At each generation the hybrid male to be mated was selected for genotype *Akv-1*$^+$–*Gpi-1*^{a}–*c*$^+$/*Akv-1*$^-$–*Gpi-1*^{b}–*c*. A litter of nine embryos representing the fifth generation of mating with NIH was used for the study of viral sequences in a segregating generation. After the sixth generation of mating with NIH, virus-positive hybrids were brother–sister mated; of their progeny, virus-positive mice homozygous for *Gpi-1*^{a} were utilized for further inbreeding, in order to maximize attainment of homozygosity at *Akv-1*. Embryos of the third generation of brother–sister mating were used in the present study. These mice are considered to be partially congenic, carrying *Akv-1* from AKR, and *Gpi-1*^{a} and *c*$^+$ from C57Br, on a predominantly NIH genetic background.

Cellular DNA for Hybridization. All DNA preparations were from embryos of 15 to 18 days' gestation.

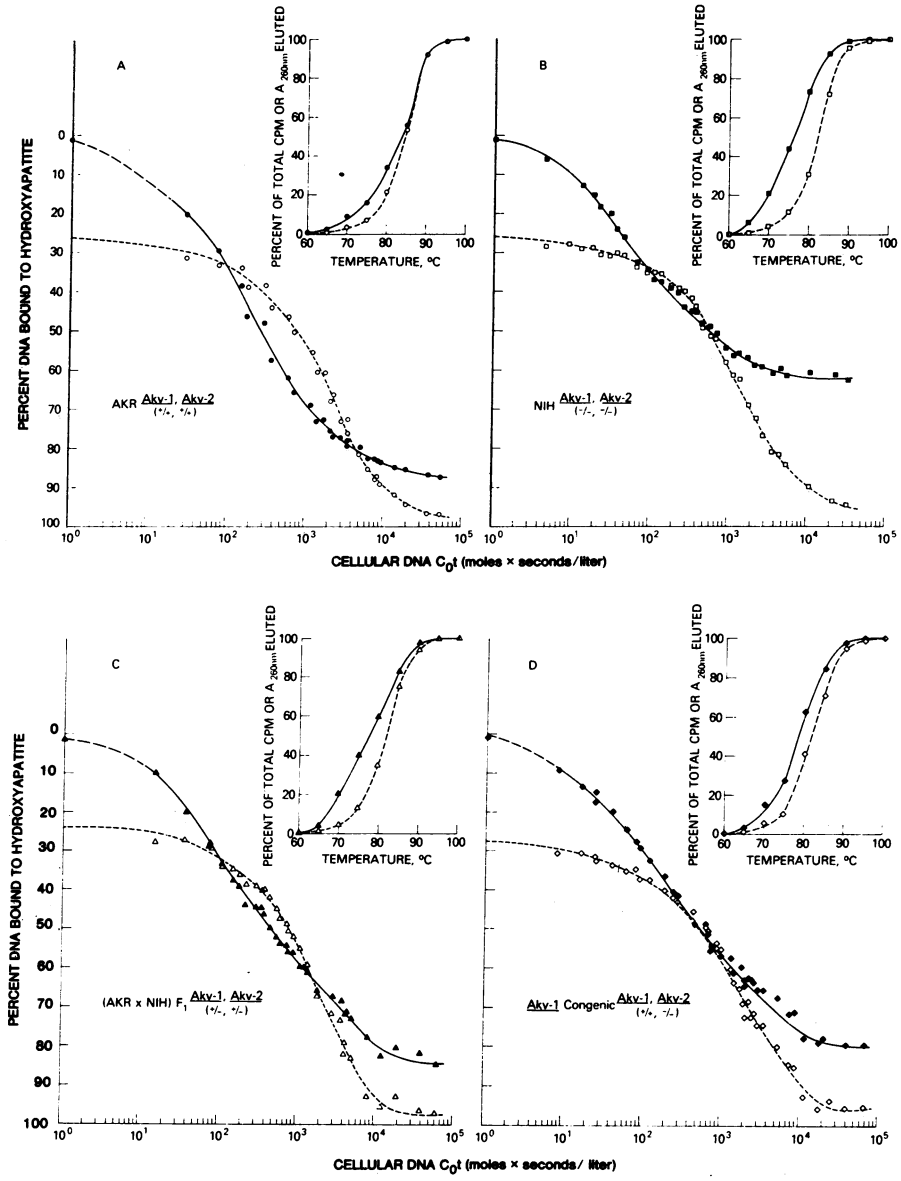

FIG. 1. Association kinetics (21) of the AKR viral [^{3}H]DNA probe with mouse embryo cellular DNAs and corresponding T_e50 analysis of the hybrids. For annealing reactions, sheared cellular DNAs (10 mg/ml) were mixed with 1 ng/ml of viral [^{3}H]DNA probe (specific activity = 2×10^7 cpm/μg) in a "Reactivial" (0.3 ml or 1.0 ml capacity). The mixtures were then denatured in 0.12 M phosphate buffer (PB) by heating at 100° for 5 min and brought up to desired salt concentrations by the addition of 4.8 M PB. All of the incubation mixtures contained 0.5 mM EDTA. Incubation mixtures with a low salt concentration (0.3 M Na^+) were incubated at 60°, whereas those with high salt concentration (1.0 M Na^+) were incubated at 65°. Samples (50 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% sodium dodecyl sulfate ($NaDodSO_4$). The extent of hybridization at each time point was assayed by hydroxyapatite chromatography. Unhybridized molecules were removed from the column with 0.14 M PB plus 0.4% $NaDodSO_4$ 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 DNA–cell DNA association), and, after addition of 12 ml of "Instagel" (Packard Instrument Co.) to 8 ml of aqueous solution, for radioactivity (to determine ^{3}H probe–cell DNA association). C_0t values represent the equivalent C_0t at 0.18 M Na^+ (22). In each panel open symbols represent the cellular DNA self-association kinetics and the closed symbols represent the association kinetics of viral [^{3}H]DNA with corresponding cellular DNA. Thermal elution profiles were obtained as follows: A 100 μl incubation mixture containing viral [^{3}H]DNA and cellular DNA at the same ratio as mentioned above, in the presence of 0.72 M Na^+, was incubated at 65° for 72–80 hr (C_0t = 4–5 × 10^4). Each incubation mixture was then brought to 0.14 M PB plus 0.4% $NaDodSO_4$ and passed over a hydroxyapatite column (60°, 0.14 M PB + 0.4% $NaDodSO_4$). Single-stranded DNA was removed from the column with 0.14 M PB + 0.4% $NaDodSO_4$. The temperature of the column was then raised in a series of 5° increments, and after each increment the column was washed at the new temperature with 8 ml of 0.14 M PB + 0.4% $NaDodSO_4$. Each fraction was measured for absorbance at 260 nm (to monitor the elution of cell DNA) and after addition of 12 ml of Instagel, the radioactivity in each fraction was determined (to monitor the elution of viral [^{3}H]DNA probe). The graphs present the cumulative proportion of the DNA eluted from the column in relation to the amount bound to the hydroxyapatite at 60°, as a function of temperature. Open symbols represent the elution profile of cellular DNA self-hybrid molecules, and closed symbols represent the elution profile of viral [^{3}H]DNA–cell DNA hybrid molecules.

TABLE 1. *Segregation of viral sequences with IdU inducibility in mating segregating for Akv-1*

Strain	Embryo no.	*Akv-1*	*Gpi-1*	Color*	Genetic category†	% of viral probe–cell DNA hybridization	Cell–cell self-hybridization (%)	T_e50 of cell DNA–cell DNA hybrids (°C)	ΔT_e50‡ (°C)
AKR	1					83.1	91.6	84.5	1.3
	2					84.8	94.0		
NIH	1					67.7	95.2	83.0	7.0
	2					65.4	92.9		
	3					64.6	92.1		
(AKR × NIH)F_1						84.0	96.2	82.0	4.5
NIH Congenic for *Akv-1*						79.3	94.7	81.5	3.0
NIH × V-1 Hybrid§	2	+	ab	dark	p^+	80.2	93.8	81.0	5.0
	3	+	ab	dark	p^+	79.2	94.5	81.5	5.5
	5	+	b	white	r *Gpi-1* → *Akv-1*	81.8	95.5	81.0	5.5
	7	+	ab	white	r *c* → *Gpi-1*	78.8	93.9	81.2	5.4
	9	+	b	white	r *Gpi-1* → *Akv-1*	78.9	94.6	81.0	5.0
	1	−	ab	dark	r *Gpi-1* → *Akv-1*	67.6	94.4	81.0	9.0
	4	−	ab	dark	r *Gpi-1* → *Akv-1*	65.2	93.1	81.7	8.5
	6	−	b	white	p^-	65.6	95.6	81.5	8.0
	8	−	b	white	p^-	73.0	95.5	82.3	8.3

Each embryo was minced; one portion was trypsinized and grown in tissue culture for virus activation with 5-iododeoxyuridine (4); another portion was used for glucose-6-phosphate isomerase (*Gpi-1*) isozyme variant analysis (20), and the remainder was used for isolation of the DNA.

* Determined from eye color.

† p indicates parental; r *Gpi-1* → *Akv-1* indicates recombinant between *Gpi-1* and *Akv-1*; r *c* → *Gpi-1* indicates recombinant between *c* and *Gpi-1*.

‡ ΔT_e50 is the difference between the T_e50 of the self-hybridized cell DNA molecules and that of probe–cell DNA hybrids.

§ Mating genotype *Akv-1*$^-$–*Gpi-1*^{b}–*c* × *Akv-1*$^+$–*Gpi-1*^{a}–*c*$^+$/*Akv-1*$^-$–*Gpi-1*^{b}–*c*.

Cellular DNA from the single embryos was isolated by the urea/phosphate buffer/hydroxyapatite method (17, 18). DNA was eluted from the column with 0.48 M phosphate buffer (PB) (made up with equimolar concentrations of Na_2HPO_4 and NaH_2PO_4, pH 6.8; molarities of PB refer to concentration of phosphate), dialyzed extensively against 0.01 × SSC (1.5 mM NaCl, 0.15 mM sodium citrate) plus 0.5 mM EDTA, sheared to a piece size of 300 to 500 nucleotides per strand by twice passing through the needle valve of a French pressure cell with a pressure drop of 40,000 lb./in.^{2} (280 MPa), concentrated by vacuum dialysis to a volume of 1 ml, precipitated with 2 volumes of ethyl alcohol, and dissolved in 0.01 M PB plus 1 mM EDTA to a final concentration of 15–20 mg/ml.

Other DNAs were purified by the method described (15).

Nucleic Acid Hybridization. Kinetic studies of the association of viral [^{3}H]DNA probe and cellular DNA were performed as previously described (15, 19); details are given in the legend of Fig. 1. Saturation hybridizations of the [^{3}H]-DNA probe with cellular DNA from the individual embryos were performed as follows. Reaction mixtures of 120 μl volume in a 0.3 ml capacity "Reactivial" (Pierce Chemical Co.) containing 10 mg/ml of cellular DNAs, 1 ng/ml of [^{3}H]DNA probe (2 × 10^7 cpm/μg), 0.5 mM EDTA, and 0.12 M PB were denatured by heating at 100° for 5 min and brought up to 0.75 M Na^+ by the addition of 4.8 M PB. The incubation mixtures were then incubated at 65° for 72 hr (a C_0t of 4.3 × 10^4 mol·sec/liter). Each mixture was then diluted and assayed by hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) chromatography for hybridization or for T_e50 (elution midpoint temperature) analysis.

RESULTS AND DISCUSSION

Segregation of viral sequences with virus inducibility

When mouse cellular DNA is hybridized with a Gross-AKR type MuLV probe, virus-yielding and non-virus-yielding mouse strains differ from each other in three characteristics of the reaction (11, 15, 19): (*1*) the proportion of probe sequences hybridized to cell DNA (saturation level); (*2*) the number of classes of probe sequences represented in the cell DNA, as indicated by the biphasic or monophasic character of the reciprocal plot of the hybridization kinetics; and (*3*) thermal denaturation characteristics of the cell-probe hybrid molecules, expressed as ΔT_e50, which is the T_e50 of cell–cell hybrid molecules minus the T_e50 of the cell–probe hybrid molecules. DNA from AKR shows high saturation level (87%), two classes of sequences, and small ΔT_e50, while DNA from NIH shows lower saturation level (67%), one class of sequences, and a large ΔT_e50.

Two of these three characteristics, saturation level and ΔT_e50, can be determined with the small amount of DNA obtainable from a single mouse embryo. Also, the three linked chromosome 7 genetic markers segregating in the matings of *Akv-1* hybrid mice can be scored on mouse embryos: *Akv-1* by IdU induction of the cells grown in tissue culture, *Gpi-1* on the embryo cells before or after growth in culture, and *c* by the presence or absence of pigment in the eyes of the embryo.

Consequently, it was feasible to carry out a three-point

genetic cross involving *Akv-1*, *Gpi-1*, and *c* on individual mouse embryos, and in addition, to score for two distinct characteristics of the MuLV sequences in the cell DNA. Embryos are preferable to adult mice for such tests, since the number of MuLV sequences in the cell DNA is not complicated by the possible insertion of such sequences during the productive infection that occurs in such animals postnatally.

Table 1 shows the results of this experiment, as well as the comparable testing of individual AKR and NIH embryos, and pooled embryos of (AKR × NIH)F_1 and the partially congenic mice homozygous for *Akv-1*. In agreement with our previous findings (11, 15, 19), the DNA from AKR embryos hybridized with a greater proportion of the probe sequences than did NIH embryo DNA, and the ΔT_e50 value was much smaller with the probe–cell DNA hybrid molecules from AKR. The (AKR × NIH)F_1 DNA gave a high saturation level and an intermediate ΔT_e50, as expected. The DNA from the *Akv-1* partially congenic mice gave results similar to the F_1 hybrid, indicating that their DNA contained viral sequences not present in the NIH parental line; this finding further supports the hypothesis that the *Akv-1* locus contains these sequences.

The critical test of this was the testing of the embryos of the segregating generation. Five of the nine embryos were positive for *Akv-1*, and the DNA from all five hybridized more than 78% of the probe sequences. The four embryos negative for *Akv-1* hybridized only 65–73% of the sequences. Similarly, the five mice with *Akv-1* showed smaller ΔT_e50 values than the four mice without *Akv-1*. These correlations are all the more convincing in that five of the nine embryos were recombinants for the chromosome 7 markers, and in all cases the hybridization characteristics segregated with *Akv-1*.

These experiments show that a portion of the cell DNA hybridizable with the viral probe sequences segregated with *Akv-1;* this finding leaves little alternative but that the *Akv-1* locus contains viral sequences. It is not ruled out that the viral sequences are closely linked to *Akv-1* but are closer to the centromeric region, but this seems a highly unlikely coincidence.

Quantitation of viral sequences in *Akv-1* congenic mice

The *Akv-1* congenic mice provided a means to estimate the number of copies of the AKR virus-specific sequences that constitute this locus. As previously described (11), kinetic analyses of cell–probe DNA hybridization reactions estimate that AKR DNA contains three to four copies of the AKR virus-specific sequences per haploid genome, while NIH DNA is lacking these. Figs. 1A, 1B, 2A, and 2B show the hybridization kinetics obtained with these two strains when done with the slightly modified hybridization procedure currently employed (19), as well as the heat denaturation curves of the cell–cell and probe–cell hybrid molecules present at saturation. The AKR DNA again shows three to four copies of the AKR-specific viral sequences (the more slowly associating fraction), and a ΔT_e50 of 1.3°, indicating well-matched hybrid molecules. The NIH DNA contains only one class of viral sequences, and these are poorly matched to the viral [^{3}H]DNA, having a ΔT_e50 of 7.0°. Figs. 1C, 1D, 2C, and 2D show the same analyses of DNA from (AKR × NIH)F_1 and the *Akv-1* congenic mouse embryos. The F_1 DNA serves as a control for the quantitative estimates, since it contains half as many of the AKR-virus-specific sequences as that of the

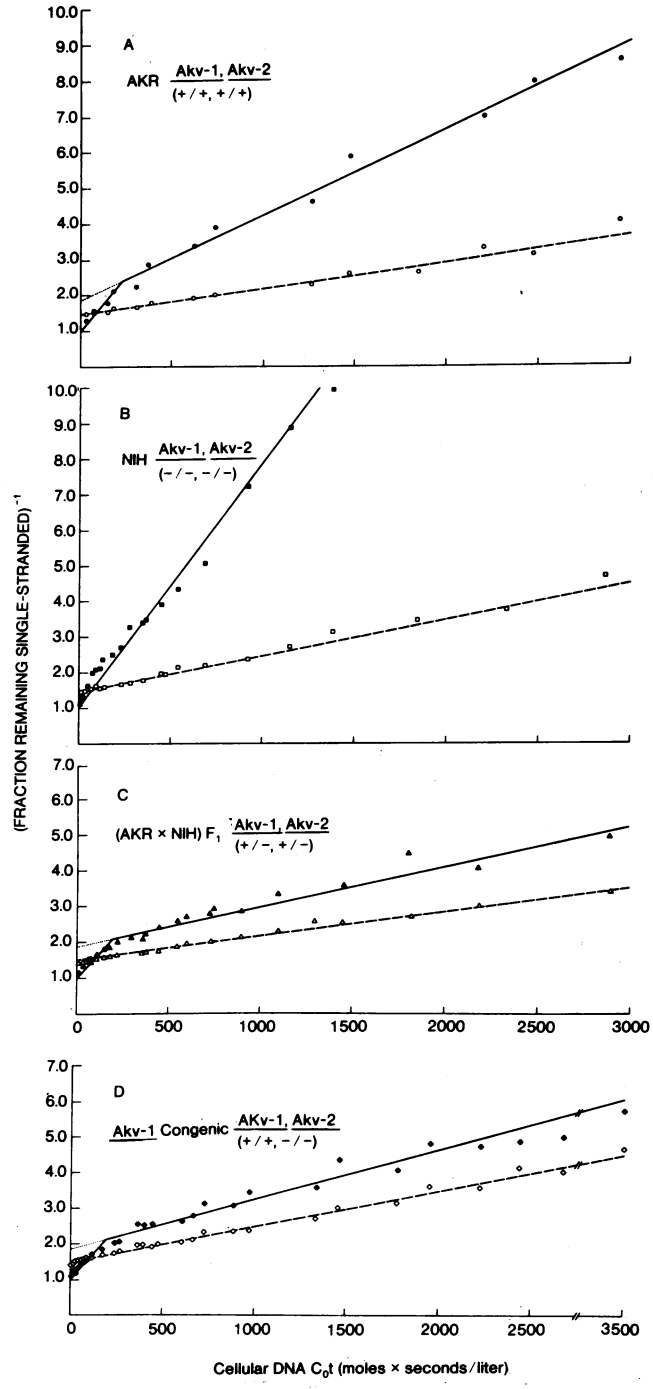

FIG. 2. Analysis of the association kinetics of [^{3}H]DNA probe with mouse embryo DNA from (A) AKR, (B) NIH Swiss, (C) (AKR × NIH)F_1, and (D) NIH Swiss congenic for *Akv-1*, and the corresponding cell DNA self-association, by the reciprocal plotting method (23). The data are from Fig. 1. The maximum observed ^{3}H-probe–cell DNA and cell DNA–cell DNA hybridizations were normalized to 100%. The symbols used here are the same as in Fig. 1. The ratios of the slope of the second component of the probe DNA–cell DNA reaction to the slope of line described by the unique sequences of cell DNA (the estimate of the number of copies of AKR-specific sequences per haploid genome) are: (A) AKR, 3.3; (B) NIH, 0; (C) (AKR × NIH)F_1, 1.6; and (D) *Akv-1* congenic, 1.45.

AKR parent; the kinetic analysis of the F_1 DNA gave data compatible with this expectation, in that there were one to

two copies of the AKR-unique viral sequences per haploid genome, and the ΔT_e50 (3.0°) was intermediate. The results with the *Akv-1*-congenic DNA were essentially identical to those with the F_1 DNA. This indicates that the *Akv-1* locus contains half of the set of AKR-virus-specific sequences present in AKR, since the homozygous *Akv-1*-congenic mice have the same number of these sequences per cell as the multiply heterozygous F_1 mice. Since AKR appears to contain more copies (3–4 per haploid genome) of the AKR-virus-specific sequences than virus-inducing loci (generally considered to be two, although three has not been ruled out), these findings with the congenic mice suggest that the *Akv-1* virus-inducing locus may contain somewhat more than one copy of these sequences. If this is the case, the presence of tandem sequences could be the reason that the locus is readily inducible.

We have reported that among virus-producing inbred mouse strains, high-virus-producers contain more copies of the AKR-specific sequences (3–4 per haploid genome) than low-virus-producers (1–2 per haploid genome) (19). The results with the F_1 and *Akv-1* partially congenic mice show that this correlation does not always hold true, since both are high-virus-producers and have the smaller number of copies. This finding suggests that the larger number of copies may be the result, rather than the cause, of the high level of virus inducibility. A highly inducible locus such as *Akv-1* results in large numbers of cells becoming infected, which is accompanied by integration of viral sequences into the cell DNA; such infection of germ cells could result in the eventual establishment of copies of the viral sequences as multiple stable chromosomal loci.

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