JOURNAL OF VIROLOGY, May 1983, p. 490–497
0022-538X/83/050490-08$02.00/0

Vol. 46, No. 2

Emv-13 (*Akv-3*): a Noninducible Endogenous Ecotropic Provirus of AKR/J Mice

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Received 10 November 1982/Accepted 2 February 1983

All AKR/J mice carry at least three endogenous ecotropic viral loci which have been designated *Emv-11* (*Akv-1*), Emv-13 (*Akv-3*), and Emv-14 (*Akv-4*) (Jenkins et al., J. Virol. **43**:26–36, 1982.) Using two independent AKR/J-derived sets of recombinant inbred mouse strains, AKXL (AKR/J × C57L/J) and AKXD (AKR/J × DBA/2J), as well as the HP/EiTy strain (an *Emv-13*-carrying inbred strain partially related to AKR/J mice) (Taylor et al., J. Virol. **23**:106–109, 1977), we have examined the association of these endogenous viral loci with virus expression. Strains which transmit *Emv-11* or *Emv-14* or both were found to produce virus spontaneously, whereas strains that transmit *Emv-13* alone were negative for virus expression. Restriction endonuclease digestion and hybridization with an ecotropic virus-specific hybridization probe of DNAs from strains which transmit only *Emv-13* yielded enzyme cleavage patterns identical to those observed with DNAs from strains transmitting *Emv-11* or *Emv-14* or both. These findings indicate the absence of any gross rearrangement of *Emv-13* proviral sequences. Cell cultures derived from recombinant inbred strains that carry only *Emv-13* failed to express detectable infectious virus, viral proteins, or cytoplasmic ecotropic virus-specific RNA even after treatment with 5-iodo-2-deoxyuridine or 5-azacytidine, an inhibitor of DNA methylation. Our results indicate that a mechanism(s) other than methylation of *Emv-13* proviral DNA is responsible for inhibition of *Emv-13* expression.

Type C murine leukemia viruses (MuLVs) are endogenous, genetically transmitted viruses carried by a variety of inbred strains. Three major classes of MuLV have been defined on the basis of host range: ecotropic MuLVs, which infect and replicate preferentially on mouse cells; xenotropic MuLVs, which infect and replicate on cells of several heterologous species; and amphotropic MuLVs, which replicate on both mouse cells and cells of heterologous species (11). Depending on the genetic background of the host, different states of virus expression can occur, ranging from complete repression to partial expression of some viral polypeptides to production of complete infectious virus. In addition, infectious virus can be induced from cells of many inbred strains in vitro. For example, halogenated pyrimidines, such as 5-iodo-2-deoxyuridine (IdUrd), have been shown to induce both ecotropic and xenotropic MuLVs (22). Cycloheximide, an inhibitor of protein synthesis, only induces expression of xenotropic virus (1). These results suggest that ecotropic and xenotropic proviruses are subject to independent cellular regulatory mechanisms (5, 34). The low infectivity of endogenous avian leukosis virus and murine leukemia virus DNA in transfection assays suggests that virus transcription is regulated by linkage to *cis*-acting regulatory DNA sequences (8). Considerable evidence has also been presented linking various patterns of viral gene expression to differences in the extent of DNA methylation (7, 10, 14, 33).

The endogenous ecotropic MuLVs of AKR mice have been extensively studied (12, 24, 32). DNAs of all AKR/N and AKR/J mice carry at least three endogenous ecotropic proviral loci. Two of these proviruses are carried by both AKR substrains and have been designated *Akv-1* and *Akv-3* (32). The third locus has been termed *Akv-2* in AKR/N mice and *Akv-4* in AKR/J mice (32). Recently, the nomenclature of endogenous ecotropic viral loci has been changed (19). These endogenous *Akv* loci are now designated *Emv-11* (*Akv-1*), Emv-12 (Akv-2), *Emv-13* (*Akv-3*), and *Emv-14* (*Akv-4*). *Emv-11* and *Emv-13* are present at the same chromosomal sites in the genomes of both AKR/J and AKR/N mice. *Emv-12* (AKR/N) and *Emv-14* (AKR/J) are integrated at different sites (32). These substrain differences are not restricted to AKR/N and AKR/J mice. Substrain differences have been found among most AKR

substrains analyzed (12, 24, 32). This heterogeneity in ecotropic proviral DNA copy number is thought to result from germ line infection of AKR embryos by highly viremic AKR mothers (30, 32). Germ line reinsertions have not been observed in low-viremia strains (32), supporting the theory of an infection mechanism for ecotropic virus amplification. The ecotropic proviral DNA content of AKR sublines should therefore reflect their genealogy, which has been verified experimentally (12, 24, 32). Because all AKR sublines studied carry *Emv-11*, it is assumed that this locus is the progenitor of all other *Emv* loci carried by AKR mice (12).

Genetic crosses between AKR/N mice and mice of low-virus-producing strains have demonstrated that *Emv-11* and *Emv-12* are dominant genes specifying viremia (27). These loci have been mapped to chromosomes 7 and 16, respectively (20, 28). However, *Em-13* was not detected in these crosses, suggesting that this locus is not normally expressed in AKR/N mice. This provirus could represent the third *Emv* locus, "*Akv-3*," detected by serological assays in the experiments of Ihle and Joseph (16) or the *Akv/p* locus that determines the expressin of *gs* and *G IX* viral antigens (17). The block in *Emv-13* expression may be due to DNA methylation or linkage to *cis*-acting regulatory DNA sequences, or *Emv-13* may have a structural gene defect that prevents its expression.

In the analysis of the endogenous ecotropic DNA sequences present in two AKR/J-derived sets of recombinant inbred RI mouse strains, as well as HP/EiTy (an inbred strain partially derived from AKR/J mice), three strains were identified that carry only *Emv-13* (AKXL-28, AKXD-28, HP/EiTy). The AKXL and AKXD strains were derived from crossing AKR/J with C57L/J and DBA/2J, respectively. The numerical suffix of these two independently derived strains that happen to carry *Emv-13* alone is coincidental. We have used these strains to characterize the *Emv-13* locus in more detail to determine (i) whether viral RNA or protein is spontaneously expressed by *Emv-13* in vivo and (ii) whether *Emv-13* expression is inducible with IdUrd or 5-azacytidine (5-azaCyd), an inhibitor of DNA methylation.

MATERIALS AND METHODS

Mice. Mice from RI strains AKXL-28 and AKXD-28, as well as HP/EiTy mice, were obtained from B. A. Taylor's colony at the Jackson Laboratory, Bar Harbor, Maine.

Virus induction. Procedures for the establishment of cell cultures from mouse spleen and kidney have been described previously (3). Primary and secondary cell cultures derived from strains carrying *Emv-13* were induced in subconfluent growth for 24 h with either 30 μg of IdUrd (Sigma Chemical Co.) per ml of 5-azaCyd (2 μg/ml). The cultures were then fluid changed and 24 h later were treated with mitomycin C (25 μg/ml for 30 min at 37°C) and overlaid with 2×10^5 SC-1 cells to amplify viral titers. Seven days after overlaying, and at weekly intervals thereafter for a period of 3 weeks, the cocultivated cultures were assayed for ecotropic virus expression by the XC assay (31). The highly inducible AKR/2B cell line was used as a positive control for virus induction (29). The cultures were maintained in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum and antibiotics (100 U of penicillin and 100 μg of streptomycin per ml).

Quantitating viral proteins in noninduced and induced cultures. The presence of murine gp 69/70 antigen in chemically induced cultures was determined by using a radioimmune binding assay (16). Purified viral antigens were labeled with ^{125}I by the chloramine T method (9). Cell suspensions (cell number adjusted to 10^6 cells per ml) were homogenized in 1 ml of Hanks balanced salt solution, and the cellular debris was removed by centrifugation. An equal volume of Triton X-100, 1 M KCl, and 0.1 M sodium phosphate buffer at pH 7.0 was added to the clarified supernatant fluids. The suspension was homogenized again, and 1 volume of water was added. The extract was then centrifuged at 15,000 rpm for 20 min in a Sorvall RC5-B centrifuge, and the supernatant fluids were divided into aliquots and frozen at −20°C for assays.

The radioimmune precipitation assays were kindly done by David Johnson (Scripps Clinic) exactly as described in reference 16. The ability of the cleared suspension to inhibit the 50% binding point was determined, and the results were expressed in nanograms of murine gp 69/70 per milliliter of cell suspension.

The presence of viral antigens was also determined by the indirect fluorescence assay as described by Hilgers et al. (13). Cover slips were fixed 10 to 14 days after removal of the inducing agent, incubated with a broadly reacting antiserum prepared in goats against Tween–ether-disrupted Moloney MuLV (supplied by the Research Resources Biological Carcinogenesis Branch of The National Cancer Institute), and stained with fluorescein isothiocyanate rabbit anti-goat antiserum. The anti-Moloney MuLV serum used will react with both viral core and envelope proteins. Controls for each fluorescence test included cells that were incubated with normal goat serum and conjugated serum or with fluorescein isothiocyanate antiglobulin alone; the controls were consistently negative. NIH/3T3 cells infected with AKR-MuLV were used as a positive control.

Isolation of cellular RNA. Total cellular RNA from fresh tissues and cytoplasmic RNA from cells in culture were prepared by phenol extraction in the presence of the RNase inhibitor vanadyl adenosine (4). Briefly, vanadyl complex (Bethesda Research Laboratories) adjusted to a final concentration of 10 mM in 3 ml of cold low-salt Tris buffer (20 mM Tris-hydrochloride [pH 7.4], 10 mM NaCl, and 3 mM MgOAc) was added to the culture plates. One ml of lysing buffer (low-salt Tris buffer contining 5% sucrose [wt/wt] and 1.2% Triton X-100 [wt/wt] was added, and the cells were observed under the microscope for lysis (15 to 20 s). The supernatant fluids were removed and centrifuged at 12,000 × *g* at 4°C in a Sorvall RC5-B centrifuge. The supernatant fluid was removed, and the

TABLE 1. Spontaneous virus expression of AKR/J-derived RI mouse strains

RI strain	*Emv* loci[a]	Spontaneous virus production[b]
AKXL-24	11,13,14	+
AKXL-21	11	+
AKXL-14	14	+
AKXL-28	13	−
AKXL-29	None	−
AKXD-28	13	−
HP/EiTy	13	−

[a] The *Emv* loci present in the AKR/J-derived RI strains were assigned by restriction enzyme analysis (1, 12).

[b] Virus production was determined by the UV-XC assay. SC-1 cells were infected with mitomycin C-treated spleen cells (25 μg/ml for 30 min) or with a 10% tissue suspension. Five days later, cultures were irradiated and overlaid with XC cells. The cultures were stained and scored for virus 3 to 4 days later.

RNA was extracted with phenol followed by ethanol precipitation. Polyadenylated [poly(A)]RNA was selected by oligodeoxythymidylate cellulose (38) and analyzed by dot-blot hybridization (34) with an ecotropic virus-specific probe (DNAeco) representing a 400-base-pair fragment from the *env* gene of AKR-MuLV (6) or a probe (clone 623) representative of the total AKR-MuLV genome (DNArep) (21).

Analysis of DNA. The preparation of total cellular DNA, restriction enzyme digestion, gel electrophoresis, and transfer to nitrocellulose paper were all as previously described (19). Filters were baked, prehybridized, and hybridized at 65°C with a ^{32}P-labeled nick-translated DNAeco or DNArep probe as described previously (19, 26). Nitrocellulose filters were washed, air dried, and autoradiographed at −70°C, using Kodak XAR-5 X-ray film and Dupont Lightening-Plus intensifying screens.

RESULTS

Spontaneous virus expression in mice from AKR/J-derived RI strains. The assignment of specific *Emv* loci to each of the AKXL and AKXD RI strain members was done by restriction enzyme analysis and hybridization with a probe specific for the AKR-like ecotropic proviruses (18, 32). Two RI strain members, AKXL-28 and AKXD-28, were identified which only carried *Emv-13*. In addition, a similar analysis of DNA from HP/EiTy mice has demonstrated that this strain also only carries *Emv-13* (our unpublished data). Digestion of DNAs from these three RI strains with enzymes which monitor internal virus structure (*Pst*I, *Kpn*I, and *Bam*HI) (18, 19, 25), and hybridization with an ecotropic virus-specific probe, suggested that *Emv-13* is (i) a nondefective ecotropic provirus containing two viral long terminal repeats and (ii) similar (if not identical) in structure to the ecotropic virus spontaneously expressed by AKR mice (12, 18, 19, 32).

We have characterized the AKXL RI strains (as well as the AKXD-28 and HP/EiTy strains) for their ability to spontaneously express ecotropic virus in an attempt to correlate virus expression with the *Emv* content of each RI strain member. All AKXL strains examined, possessing *Emv-11* or *Emv-14* or both, expressed infectious virus. RI strains which transmit only *Emv-13* (AKXL-28, AKXD-28, and HP/EiTy) did not give rise to infectious virus, as determined by the XC-plaque assay (Table 1). Similar results have been observed with the AKXD RI strains (H. C. Morse, personal communication). These results suggest that *Emv-13* is a nonexpressed endogenous ecotropic viral locus.

Methylation of endogenous MuLV DNA in tissues of *Emv-13* RI strains. Several investigators have found a strong correlation between hypermethylation of endogenously acquired viral sequences and the lack of spontaneous virus expression (7, 10, 14, 33). We were interested therefore in determining whether *Emv-13* is hypermethylated, which might then account for its non-expression. To determine the extent of methylation of *Emv-13* sequences in strains which transmit only the *Emv-13* provirus, splenic DNA was isolated, digested with the restriction enzyme *Msp*I or *Hpa*II in combination with *Pst*I, and analyzed by Southern hybridization with the ecotropic virus-specific probe. The restriction enzymes *Msp*I and *Hpa*II both recognize and cleave the nucleotide sequence 5′-C-C-G-G-3′. However, when the internal cytosine is methylated, it cannot be cleaved by *Hpa*II (36). Thus, *Msp*I will determine the presence of 5′-C-C-G-G-3′ sequences, whereas *Hpa*II will define which of these sites are methylated.

*Pst*I cleaves only within the viral long terminal repeat sequences and yields a single detectable viral DNA fragment of 8.2 kilobases (kb) (25). If *Msp*I-*Hpa*II sites are absent within the viral genome, we should observe only the 8.2-kb viral fragment after *Msp*I or *Hpa*II digestion. The *Hpa*II-*Pst*I double digestion yielded the 8.2-kb viral DNA fragment (Fig. 1). The 8.2-kb fragment was not present after digestion with *Msp*I. These results indicate that the endogenous *Emv-13* provirus is hypermethylated. Identical results were observed with DNAs from AKXL-28 and HP/EiTy RI strains (data not shown). *Hpa*II-*Pst*I digestion of DNA from NIH/3T3 cells exogenously infected with AKR MuLV did not reveal the 8.2-kb fragment, thus correlating hypomethylation with virus expression.

Response of *Emv-13* strains to chemical induction. Because the *Emv-13* provirus is hypermethylated, it was of interest to determine whether chemical treatment with IdUrd or the demethylating agent 5-azaCyd would induce infectious

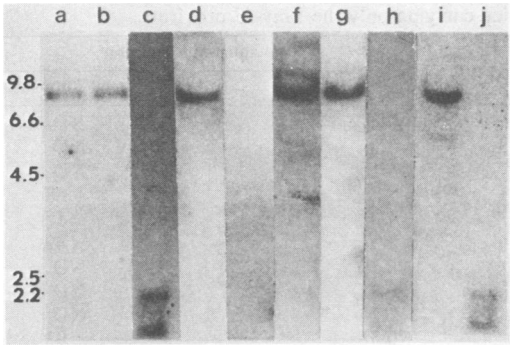

FIG. 1. Restriction enzyme analysis of DNAs from cell lines that transmit the *Emv-13* provirus. High-molecular-weight DNAs (20 μg per lane) prepared from AKXD-28, AKR/J, and AKR-MuLV-infected NIH/3T3 cell cultures were digested to completion with *Pst*I, *Hpa*II-*Pst*I, or *Msp*I-*Pst*I. The molecular weight in kilobases of the viral DNA-containing fragments, detected after electrophoresis through 0.8% agarose gels, Southern blotting, and hybridization with the ^{32}P-labeled ecotropic-specific hybridization probe (19), was calculated by using ^{32}P-labeled *Hin*dIII-digested λ DNA electrophoresed in parallel lanes of the same gels. Lanes a, d, and f contain *Pst*I digests of AKXD-28, AKR-MuLV-infected NIH/3T3, and AKR/J DNAs, respectively. Lanes b, e, and g contain *Hpa*II-*Pst*I double digests of DNAs from AKXD-28, NIH/3T3 cells exogenously infected with AKR-MuLV, and AKR/J cell cultures. Lanes c and h contain *Msp*I-*Pst*I double digests of AKXD-28 and AKR/J DNAs. Lanes i and j contain *Pst*I and *Hpa*II-*Pst*I digests, respectively, of DNA from AKXD-28 cell cultures treated with 5-azaCyd.

virus from cell cultures established from spleens and kidneys of AKXD-28, AKXL-28, and HP/EiTy mice. AKR-2B cells, a nonproducer cell line established from AKR mouse embryos (29), express endogenous ecotropic virus upon chemical induction and were used to establish optimal conditions for chemical induction. AKR-2B cells treated with IdUrd (30 μg/ml) or 5-azaCyd (2 μg/ml) produced XC-positive ecotropic virus by 7 days after induction. The levels of endogenous virus induction were similar for both inducers at the concentrations used (approximately 100 plaques per plate). However, a difference in XC plaque morphology was observed between IdUrd- and 5-azaCyd-treated cells. IdUrd-treated cells showed the large XC plaques characteristic of exogenous AKR ecotropic virus infection, whereas the 5-azaCyd-induced plaques were smaller but distinct. This difference in plaque morphology was not seen when 5-azaCyd-induced AKR-2B cells were overlaid with SC-1 cells before the XC plaque assay. This variation in plaque morphology may be due to culture conditions, to a recombination event similar to that described by Hopkins et al. (15), or to differences in the mechanism of induction by the two viral inducers. To determine the optimal chemical concentration of virus induction, we treated AKR-2B cells with several concentrations (1 to 10 μg/ml) of 5-azaCyd and overlaid the induced cells with XC cells 7 days later. At concentrations of 2 to 6 μg of 5-azaCyd per ml, the inducible XC plaque numbers (greater than 100 plaques per plate) were similar. A reduction of plaque number was observed at concentrations of 5-azaCyd above 6 μg/ml. We did not observe the cytotoxic effect or reduction in plaque number reported by Niwa and Sugahara (23) for concentrations of 5-azaCyd above 2 μg/ml (23).

In contrast to virus induction in the AKR-2B cells, spleen and kidney cultures established from the mouse strains that transmit only *Emv-13* were consistently negative for virus expression both before and after chemical treatment as determined by the XC plaque assay (Table 2) or reverse transcriptase (RT) assay (data not shown). Increased concentrations of IdUrd (50 μg/ml) or 5-azaCyd (up to 10 μg/ml) had no effect on virus expression in these cell cultures (for up to 21 days in culture).

To investigate whether the absence of detectable virus expression might be associated with a lack of demethylation of *Emv-13* sequences in 5-azaCyd-treated cultures, DNAs were extracted from *Emv-13* containing cell cultures 7 days after 5-azaCyd treatment, restriction enzyme digested, Southern blotted, and hybridized with the ecotropic virus-specific probe. The 8.2-kb *Pst*I fragment characteristic of *Emv-13* was no longer resistant to digestion by *Hpa*II (Fig. 1, lanes i

TABLE 2. IdUrd and 5-azaCyd induction

Cell culture[a]	Virus production (no. of positive mice/no. treated)[b]		
	Untreated	IdUrd[c]	5-Azacytidine[d]
AKR-2B	0/10	6/6	10/10
AKXL-28	0/15	0/7	0/7
AKXD-28	0/5	0/5	0/5
HP/EiTy	0/3	0/3	0.3

[a] AKR-2B is an established cell line used here as a positive control for virus induction. Three cell cultures were established from spleens of individual RI strains and assayed for the presence of infectious virus by the XC plaque assay.

[b] The XC assay is described in detail in the text.

[c] The concentration of IdUrd used was 30 μg/ml. Cells were treated for 24 h and washed, and fresh media were added to the plates. The cultures were assayed 7 days after induction.

[d] 5-Azacytidine was used at a concentration of 2 μg/ml. Cultures were treated for 24 h and washed, fresh media were added, and the cultures were assayed for virus expression 7 days later.

TABLE 3. Viral antigen expression in mice carrying only the *Emv-13* provirus

Cell culture[a]	Treatment[b]	Viral antigen expression[c]	
		IF	gp 69/70 (ng/ml)
AKXL-28	None	0/3	ND
	IdUrd	1/4	ND
	5-azaCyd	1/5	ND
AKXD-28	None	ND	68
	IdUrd	1/4	57
	5-azaCyd	1/5	49
HP/EiTy	None	0/3	ND
	IdUrd	0/3	ND
	5-azaCyd	1/3	ND
AKR-2B	None	0/3	93
	IdUrd	3/3	4,813
	5-azaCyd	3/3	1,204

[a] The cell cultures were established from spleens or kidneys of mice from individual RI strains.
[b] The concentrations of IdUrd and 5-azacytidine used were 30 and 2 μg/ml, respectively. The cells were assayed for viral antigen expression, as described in the text, 10 days after induction.
[c] The IF results are expressed as the number of positive mice over the number tested. Spleen cultures from individual mice were established in duplicate. Gp 69/70 antigen expression was determined as described in the text, and the results are expressed in nanograms of murine gp 69/70 per milliliter of cell suspension (average of two determinations). ND, Not determined.

and j), indicating that the *Emv-13* provirus was demethylated after 5-azaCyd treatment.

Spontaneous and induced expression of viral antigens and viral RNA in mice carrying *Emv-13*. Although the various cell cultures established from the *Emv-13* strains were XC and RT negative, it was possible that viral structural proteins (p30 and gp70) were expressed in these cultures. Therefore, we assayed these cell cultures for the presence of viral proteins by indirect fluorescence (IF) assays and for gp 69/70 by radioimmunoassays. Cell cultures established from AKXD-28 mice showed gp 69/70 levels of 49 to 57 ng/ml after IdUrd or 5-azaCyd induction, which was not significantly different from the gp 69/70 levels found in uninduced AKXD-28 cells (68 ng/ml [Table 3]). In contrast, the amount of gp 69/70 in AKR-2B cells treated with IdUrd or 5-azaCyd was significantly higher (>1,204 ng/ml [Table 3]). The majority of induced AKXL-28, AKXD-28, and HP/EiTy cell cultures were also negative by IF. Occasionally, a weak positive reaction was observed in some induced cultures (Table 3). This weak positive fluorescence of presumed viral protein expression supports the earlier findings of Ikeda et al. (17), who postulated the presence of a third viral coding gene segregating in AKR/J and C57L/J crosses and the *Akv-3* locus detected by Ihle and Joseph in AKR/J and NIH crosses (16).

To assay further for viral gene expression in *Emv-13* strains, we isolated poly(A)-containing cytoplasmic RNA from control and chemically treated cells, spotted the RNA (0.5, 1, and 3 μg) onto nitrocellulose filters, and assayed for the expression of ecotropic virus-related sequences by hybridization with the ecotropic virus-specific probe (DNAeco).

We did not detect a positive signal above background levels with cytoplasmic RNA from uninduced AKR-2B or AKXD-28 cell cultures (Fig. 2A). A positive signal was observed with RNA isolated 14 days after chemical induction of AKR-2B cells (Fig. 2A). However, a positive signal was not detected with RNA isolated from induced AKXD-28 cells (Fig. 2A). Results identical to those obtained with AKXD-28 cells were also observed with AKXL-28 and HP/EiTy mice (data not shown). In addition, mRNAs extracted as early as 5 days and as late as 21 days after chemical treatment were also consistently negative for ecotropic viral RNA expression. Whether viral RNA is expressed before 5 days and degraded is not known. Furthermore, we did not detect a positive hybridization signal with RNA extracted from livers and spleens of 2-month-old AKXL-28 or AKXD-28 mice (data not shown). Virus-specific RNA was detected with the DNArep probe from induced AKXL-28 and AKXD-28 cells (Fig. 2B) but not from untreated controls. However, the DNArep probe is not specific for ecotropic viral RNA and will also hybridize to xenotropic and amphotropic RNA. Therefore, we are probably inducing and detecting xenotropic viral RNA in these cultures. The possibility also exists that we are detecting ecotropic viral RNA which is defective in the region of the *env* gene recognized by the DNAeco probe. Collectively, these results indicate that viral RNA detectable by the DNAeco probe is not present in uninduced or induced cells of mice which transmit only the *Emv-13* provirus.

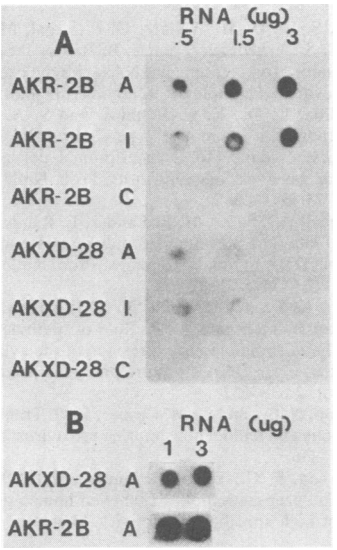

FIG. 2. Dot blot assay of RNA. Cytoplasmic mRNA was prepared from cell cultures 14 days after chemical induction by phenol extraction in the presence of the RNase inhibitor vanadyl adenosine. Polyadenylated RNA was selected by two passes through oligodeoxythymidylate cellulose (4, 39). RNA (0.5, 1.5, and 3 μg) was spotted onto nitrocellulose paper (2-μl aliquots). The dot blots were hybridized with the cloned (A) ecotropic virus-specific DNA probe or (B) probe (DNArep) representative of the total AKR-MuLV genome. The conditions for prehybridization and hybridization were exactly as described by Thomas (34). We used 10^6 cpm of denatured ^{32}P-labeled hybridization probe per ml. The probes (>2 × 10^8 cpm/μg) were prepared by nick translation (19).

DISCUSSION

DNAs of different sublines of AKR mice have been shown to be heterogeneous with respect to the number and chromosomal location of *Emv* proviruses (12, 24, 32). This polymorphism of proviral integration sites in different AKR substrains has been attributed to the sequential acquisition of proviruses by germ-line infection during inbreeding (12, 32). Using the AKR/J-derived RI strains, we have determined which *Emv* loci carried by AKR/J mice are expressed to produce infectious virus. Although closely related to one another, *Emv* proviruses carried by different inbred mouse strains have been shown to differ in their transcriptional activities. All AKR/J-derived RI strains examined that transmitted only *Emv-13* were found to be negative for infectious virus and for the production of viral gene products (as determined by XC plaque assay, IF, and radioimmunoassay), either spontaneously or after treatment with IdUrd or 5-azaCyd. In contrast, RI strains that carried *Emv-11* or *Emv-14* proviruses or both spontaneously expressed ecotropic MuLV (Table 1).

We also analyzed poly(A)-selected cytoplasmic RNA for the expression of *Emv-13*-related RNA sequences by dot blot hybridization, using an ecotropic virus-specific probe (6). We could not detect a hybridization signal above background levels with uninduced or induced RNA from *Emv-13*-bearing strains. A positive hybridization was observed with RNA from a virus expressing AKR-2B cell line and AKR/J cell cultures. These findings have been confirmed by conventional liquid hybridization analysis and by Northern blot hybridization (data not shown).

Restriction enzyme analysis of DNA isolated from the AKXL and AKXD RI strains has failed to detect an obvious deletion or gross rearrangement of *Emv-13* proviral sequences relative to the replication-competent *Emv-11* and *Emv-14* sequences (18, 32). The presence of a point mutation or a deletion, possibly within the U3 region of the LTR which contains sequences important in the regulation of viral RNA transcription (37), cannot be ruled out by these experiments. Also, *Emv-13*-specific RNA may not be detected because it is rapidly degraded within the cytoplasm. In the investigations of Baker et al. (2), it was shown that viral RNA could be detected in the nucleus but not in the cytoplasm of cell cultures established from certain chicken lines. This anomalous expression of cytoplasmic RNA may be attributed to structural features of the endogenous provirus or to an aberrant metabolism of viral RNA.

A positive reaction for viral gene expression was observed in some chemically treated cultures by both immunofluorescence assays, using an antibody prepared in goat to ether-disrupted Mo-MuLV and by dot blot hybridization with a ^{32}P-labeled probe directed against the total AKR-MuLV genome (DNArep). These same cultures were negative for viral RNA expression when the ecotropic virus-specific probe was used. However, the antiserum used in these experiments and the DNArep probe both cross-react with xenotropic MuLV. Because both IdUrd and 5-azaCyd induce the expression of xenotropic virus as well as of ecotropic virus (23), it appears that the viral RNA and proteins we detected were due to the expression of xenotropic virus. This conclusion is further supported by our finding that AKXL-28 spleen cells express xenotropic virus when cocultivated with the mink cell line (H. G. Bedigian and B. A. Taylor, unpublished data).

Selective methylation of the C-5 position of cytosine in DNA is thought to be a mechanism that influences patterns of gene expression in a number of viral and nonviral systems (10, 36,

39). Perturbations of methylation patterns in mammalian and avian cells can result in dramatic changes in gene expression and differentiation. Several investigators have recently demonstrated a positive correlation between virus expression and hypomethylation and an analogous correlation between the unexpressed viral loci and hypermethylation (7, 10, 14, 33). Our results are consistent with these findings in that the *Emv-13* provirus is hypermethylated, whereas ecotropic viral DNA present in NIH/3T3 cells exogenously infected with AKR MuLV shows a cleavage pattern characteristic of hypomethylated DNA. Double digestion of DNAs from AKXL-28, AKXD-28, or HP/EiTy cell cultures with *Msp*I-*Pst*I or *Hpa*II-*Pst*I indicated the presence of methylated sites within the *Emv-13* proviral genome. Treatment of these cell cultures with the demethylating agent 5-azaCyd resulted in the disappearance of these methylated fragments, as determined by digestion of the DNAs with *Hpa*II-*Pst*I. Yet, infectious ecotropic virus, viral RNA, and viral proteins were still not expressed in these cell cultures. From these experiments, it is not known whether the cellular DNA sequences flanking the *Emv-13* provirus remain methylated and affect *Emv-13* expression. Alternatively, there may be an internal structural defect within the *Emv-13* genome.

Our results indicate that methylation of *Emv-13* proviral DNA is not responsible for inhibition of virus transcription. It is quite possible, however, that the cellular sequences surrounding the provirus might be methylated or modified in such a way as to control the expression of integrated virus. To analyze these other possibilities in more detail, it will be necessary to molecularly clone the *Emv-13* provirus and its flanking cellular DNA sequences. The biological activity of this clone can then be studied in detail, using the DNA transfection assay to monitor biological activity.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant CA-31102 awarded by the National Cancer Institute and grant 80-05503 from the National Science Foundation (to H.G.B.); by grants MV-119 and MV-124 from the American Cancer Society, and by Public Health Service grant CA-32324 (to N.G.C. and N.A.J.).

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