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Molecular and Biological Characterization of the Endogenous Ecotropic Provirus of BALB/c Mice

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We have isolated two identical molecular clones of the single, endogenous ecotropic provirus of BALB/c mice. The BALB/c clones are approximately 1/10 as infectious as an exogenous proviral clone derived from AKR mice, p623. Transfection of mouse cells with each BALB/c proviral clone yielded XC-negative, N-tropic, ecotropic virus. Cotransfection of subgenomic fragments of p623 and the BALB/c provirus did not increase infectivity to the level observed for p623; however, a 292-base-pair fragment of the p623 *env* gene was found to rescue XC-plaque formation. Sequence analysis showed that the XC-negative BALB/c provirus differed from the XC-positive AKR-derived provirus at a single nucleotide at the junction of the gp70 and p15E envelope proteins. Extensive sequence analysis of the BALB/c endogenous provirus showed that it differed from the sequence of the AKR-derived provirus at approximately 0.5% of 4,500 sequenced nucleotides. In addition, the BALB/c long terminal repeat contains a single copy of the enhancer-containing sequences that are repeated twice in p623. The limited variation between the ecotropic proviruses of BALB/c mice and AKR mice suggests that few cycles of reverse transcription separate these viral genomes.

Endogenous murine leukemia viruses (MuLV) and virus-related sequences are widely dispersed throughout the mouse germ line (for a review, see reference 46). Mouse infectious or ecotropic MuLV compose one class of endogenous viral sequences that have been identified in many inbred strains of mice (5, 20, 23). The elegant genetic experiments of Rowe and co-workers first localized sequences encoding ecotropic MuLV to specific mouse chromosomes (49) and demonstrated their endogenous nature by genetic crosses of inbred mice (6). Ecotropic proviruses have now been localized to many positions within the mouse germ line; this finding suggests that viral integration events occur randomly with respect to the host genome (20, 23). Once integrated, the genomes of ecotropic viruses are stable in most inbred strains of mice (8, 20, 23). Strains sharing a common mouse progenitor have maintained the position of their endogenous ecotropic viruses during 65 years of separated inbreeding (20, 23, 24). However, new proviral integrations have been reported for some high leukemic strains, such as AKR (4, 17, 42).

The expression of endogenous ecotropic viruses has been shown to vary considerably among inbred strains (14, 33). Those strains expressing high levels of virus early in life have been shown to be at high risk for leukemia development, the AKR strain being the best-characterized example (48, 49). In contrast, low-leukemic mice produce little or no virus until relatively late in life (26, 34, 41). Halogenated pyrimidines, such as iododeoxyuridine, have been employed to induce expression of ecotropic virus from virus-negative mouse cells cultured in vitro; the frequency of induced expression of endogenous ecotropic virus has in general paralleled the results of in vivo studies (1, 14, 28).

BALB/c mice contain a single, genome-length, endogenous ecotropic provirus (20, 23) whose expression has been mapped to a segment of chromosome 5 designated *Cv-1* (22, 24). In vivo, few BALB/c mice express ecotropic virus before 6 months of age; approximately 50% of animals express moderate titers of virus by 12 months of age (34, 40, 41), with B-tropic MuLV predominating in old BALB/c mice. Several different virus isolates have been recovered from BALB/c mice, including XC-positive or XC-negative, N-tropic, nonleukemogenic isolates as well as XC-positive, B-tropic, leukemogenic viruses (19, 39). In vitro, induced cultures of BALB/c cells rarely produce XC-positive ecotropic virus (1, 13, 35). Enhanced expression, or complementation, of ecotropic virus has been observed in cells derived from progeny of BALB/c and C57 mice, and a gene tightly linked to the BALB/c provirus has been shown to be necessary for elevated virus production (13, 20, 32, 34).

Rassart et al. (45) have reported the cloning of two exogenous ecotropic viruses from a pool of virus derived from an aged BALB/c mouse. Both N- and B-tropic isolates are represented by these clones; the B-tropic isolate has been shown to be leukemogenic in susceptible mice, and a portion of the U3 region of the long terminal repeat (LTR) has been implicated in leukemogenicity (10).

To correlate the spontaneous and induced patterns of expression of ecotropic virus in BALB/c mice with the specific infectivity of its endogenous provirus, we cloned and analyzed the single, genome-length, endogenous provirus contained in the BALB/c germ line and determined the biological properties of the virus produced from transfected cells.

MATERIALS AND METHODS

Molecular cloning. BALB/cByJ mice were purchased from the Jackson Laboratory, Bar Harbor, Maine, and bred in our laboratory. BALB/c mouse embryo cells were grown in culture for 2 weeks prior to DNA extraction. Chromosomal DNA was isolated as previously described (20) and digested to completion with *Eco*RI as recommended by the supplier (New England BioLabs, Inc., Beverly, Mass.). Digested DNA was extracted with phenol-chloroform and precipitated with ethanol prior to centrifugation through a 10 to 40% neutral sucrose gradient as described by Maniatis et al. (30). Samples of each collected fraction of the gradient were examined by agarose gel electrophoresis, and those fractions

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containing DNA fragments of 21 kilobase pairs (kbp) were pooled, dialyzed, and precipitated with ethanol.

Charon 4A phage DNA (57) was prepared and ligated with T4 DNA ligase (New England BioLabs, Inc.) prior to digestion with *Eco*RI. After phenol-chloroform extraction and ethanol precipitation, phage DNA was centrifuged through a 10 to 40% neutral sucrose gradient, and fractions containing phage arms were pooled, dialyzed, and precipitated (30).

Ligations of mouse DNA and phage DNA were performed as suggested by Maniatis et al. (30) and packaged in vitro (56). The resulting phage library was plated on *Escherichia coli* DP50SupF, and plaques were screened by the method of Benton and Davis (2) with radiolabeled pEC-B4 (5). Two positive clones were identified, and each was purified by three rounds of plaque purification prior to amplification and DNA extraction (30).

Restriction enzyme digestion and agarose gel electrophoresis. DNA used in these studies was digested to completion with enzymes purchased from New England BioLabs, Inc., and used under conditions recommended by the supplier. Electrophoresis was performed as described (20) through 0.7 to 1.4% agarose gels (SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine), and gels were stained with ethidium bromide. Southern blots, hybridizations, and filter washes were performed essentially as previously described (20).

Transfection and cotransfection of vertebrate cells. Transfection of plasmid clones was performed with either NIH 3T3 cells or chicken embryo fibroblasts (CEF) cocultivated with mouse Sc-1 cells as cell recipients (35). Transfection of NIH 3T3 cells was performed by a modification of a procedure described by Hopkins et al. (18). Briefly, plasmid and carrier DNAs were suspended in transfection buffer and raised to 0.125 M in $CaCl_2$; DNAs were allowed to precipitate at room temperature and were then added to recipient cells pretreated with chloroquine (29). After incubation for 6 h, cells were rinsed with phosphate-buffered saline and shocked with 15% glycerol for 4 min at room temperature. Cells were then rinsed extensively and incubated overnight in Dulbecco modified Eagle medium containing 10% calf serum. Transfection of cocultivated cells was performed as previously described (35). Final DNA concentrations prior to transfection were adjusted to 20 μg/ml with sheared, calf thymus DNA as carrier (35). Cotransfection of BALB/c proviral clones and subgenomic clones of p623 was carried out as described above except that exogenous carrier DNA was omitted; the final DNA concentrations were raised to 20 μg/ml by the sole addition of subgenomic clone DNAs (37).

UV-XC assay and immunofluorescence assay. Cells were passaged at weekly intervals and scored for plaque-forming virus by the UV-XC assay (50). Immunofluorescence assays were performed as described by Hartley et al. (14). Briefly, cells were plated on microscope slide cover slips, fixed with formaldehyde, permeabilized with acetone, and stained with fluorescein-conjugated goat antibody prepared against disrupted Rauscher MuLV virions or stained in a two-step assay with a goat anti-Rauscher gp70 antiserum and fluorescein-conjugated rabbit anti-goat serum. Cells were counterstained with rhodamine, and excess antiserum was removed by extensive washes in phosphate-buffered saline. Virus-positive cells were identified by illumination with a UV light microscope.

M13 DNA sequencing. DNAs to be sequenced were cleaved with appropriate enzymes and electrophoresed through low-melt agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) gels. Fragments were identified by transillumination with long-wave UV light and eluted by heating and successive phenol extractions. DNAs were chloroform extracted, ethanol precipitated, and ligated into the appropriate M13 vector. M13mp10 and M13mp19 were used exclusively in these studies for sequence analysis and clone propagation (38). *E. coli* JM101 (13) was transformed with ligation reactions, and cells were plated in 2X YT (0.5%) top agar containing X-gal and isopropyl-β-D-thiogalactopyranoside (Bethesda Research Laboratories, Inc.). Resulting white plaques were picked and amplified in JM101, and phage were precipitated with polyethylene glycol. Single-stranded DNAs were precipitated after phenol-chloroform extraction and suspended in 10 mM Tris (pH 7.4)–0.1 mM EDTA (Sigma Chemical Co., St. Louis, Mo.). Sequencing reactions were performed essentially as described previously (51), with a 17-MER polynucleotide primer and DNA polymerase I Klenow fragment (New England BioLabs, Inc.). [α-^{35}S]dATP (650 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was used exclusively for labeling of sequencing reactions. Sequencing reactions were electrophoresed through 0.4-mm thick, 6% polyacrylamide gels containing 8.3 M urea (Bethesda Research Laboratories, Inc.); gels were fixed in 10% methanol–10% acetic acid and subsequently dried and directly exposed overnight at room temperature to Kodak XAR-5 film.

Computer-assisted sequence analysis. Sequencing data were compiled and analyzed with the assistance of the Staden sequencing programs (55) and additional sequencing programs available through the University of Wisconsin Genetics Computer Group.

RESULTS

Restriction mapping and cloning of the BALB/c endogenous ecotropic provirus. Chromosomal DNA was prepared from BALB/cByJ embryo cells and subjected to restriction enzyme digestion and agarose gel electrophoresis. After Southern blot transfer to nitrocellulose (54) and hybridization with the ecotropic virus-specific probe, pEC-B4 (5), the autoradiograph in Fig. 1A was obtained. Of the eight enzymes, seven produced a single pEC-B4 reactive fragment whose size was determined by the presence of restriction sites within the ecotropic viral genome alone (*Pst*I, *Kpn*I, and *Bam*HI) or by sites within the viral genome and flanking cellular DNA (*Xba*I, *Hin*dIII, *Sac*I, and *Pvu*II). Cleavage with these eight enzymes and another five restriction enzymes (data not shown) confirmed that a single, genome-length, ecotropic provirus was contained within the BALB/c germ line (20, 23). The ecotropic viral genome does not contain a cleavage site for *Eco*RI (20, 43); the resulting 21.4-kbp proviral fragment identified in BALB/c DNA is, therefore, bounded by enzyme recognition sequences located solely in flanking cellular DNA.

To clone the endogenous ecotropic genome, chromosomal DNA was cleaved with *Eco*RI and cloned with Charon 4A (57); the resulting phage were subsequently screened (2) with radiolabeled pEC-B4 (5). Of 3 × 10^5 recombinant phage plated, two independent clones, λ7D and λ8A, were identified. After purification and amplification, DNAs derived from λ7D and λ8A were cleaved with restriction enzymes, analyzed on agarose gels, and hybridized as performed previously for BALB/c genomic DNA (Fig. 1B). Comparison of the hybridization patterns obtained for the recovered clones with that seen for BALB/c genomic DNA indicated that the clones were colinear with endogenous proviral sequences (Fig. 1A and B). Cleavage of DNA from the

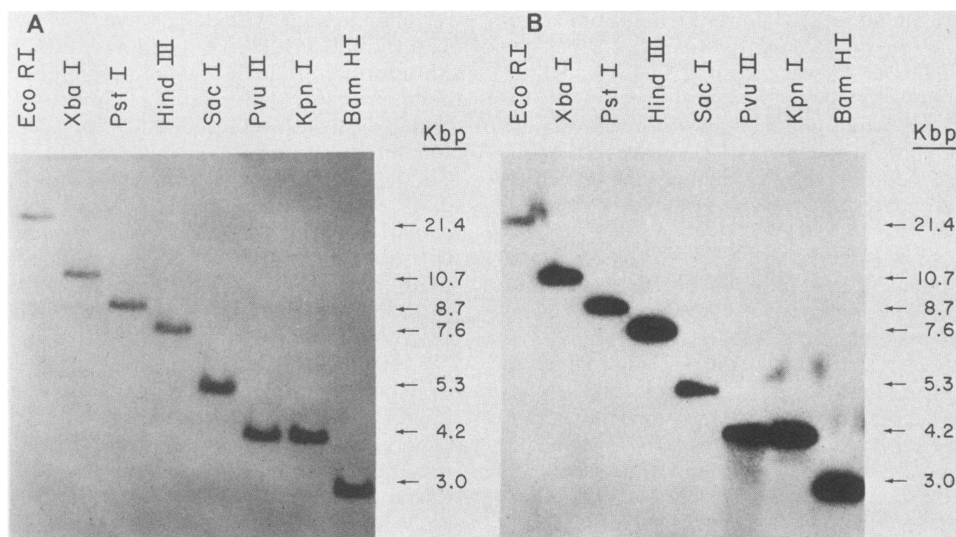

FIG. 1. Comparative Southern blot analysis of BALB/c embryo DNA and the λ7D BALB/c endogenous proviral clone. (A) BALB/c embryo DNA cleaved with the indicated restriction endonuclease, electrophoresed, transferred to nitrocellulose, and hybridized with the ecotropic virus-specific probe pEC-B4 (5). *Hin*dIII-digested lambda DNA was electrophoresed in parallel as a molecular weight marker. (B) BALB/c ecotropic proviral clone λ7D prepared as described for embryo DNA and hybridized with radiolabelled pEC-B4 (5).

recovered clones with eight additional enzymes confirmed colinearity with the endogenous provirus (data not shown). We conclude from this analysis that λ7D and λ8A are molecular clones of the endogenous ecotropic provirus of BALB/c mice and that their gross structure, as defined by restriction enzyme mapping, is indistinguishable from that of the endogenous provirus.

The 21-kbp proviral *Eco*RI fragment was subcloned from each of the recovered lambda clones into the chloramphenicol resistance gene of pBR325 (3); the resulting plasmid subclones were designated p7D and p8A. A detailed restriction map of clones of the proviral genome and approximately 6,500 base pairs of flanking cellular sequences is shown in Fig. 2. In addition to the 8.9-kbp ecotropic proviral genome, a total of 12 kbp of flanking mouse cell DNA is contained within each proviral clone.

Biological activity of clones of the endogenous BALB/c provirus. For determination of the specific infectivity of the proviral clones, various amounts of plasmid clones were transfected onto CEF (35) cocultivated with Sc-1 mouse cells. Virus-producing cultures were identified at weekly intervals by a fluorescein-tagged immunofluorescence assay (14). The proportion of virus-positive cultures was finally scored at 3 weeks posttransfection (Fig. 3). For comparison, parallel cultures of CEF plus mouse cells were transfected with a highly infectious, exogenous proviral clone derived from AKV-1, p623 (27). The resulting endpoint dilution curves show approximately one-hit titration patterns for both BALB/c- and AKR-derived proviral DNAs (Fig. 3). The specific infectivity of the BALB/c-derived proviruses was about 1/10 that observed for p623 by this method. Transfection of NIH 3T3 cells with plasmid DNA and enumeration of foci of virus-positive cells by fluorescent microscopy 10 days later established specific infectivities of 1×10^6 infectious units per pmol of p623, comparable to values reported previously (27), and 1.8×10^5 infectious units per pmol of p7D (data not shown). This sixfold difference in infectivity is comparable to that seen with CEF plus Sc-1 cells. UV-XC assays performed on cells transfected with p623 indicated that this virus was XC positive. In contrast, cultures transfected with the BALB/c-derived proviral clones failed to produce virus that induced XC syncytia.

Cell-free supernatants from cultures transfected with the BALB/c clones were shown to contain infectious, N-tropic, ecotropic virus by infection of virus-negative, *Fv-1*-permissive mouse cells but not cells of xenogeneic origin (Table 1). Virus recovered from cells transfected with the BALB/c clones was infectious for Sc-1 ($Fv\text{-}1^{-/-}$) and NIH 3T3 ($Fv\text{-}1^{n/n}$) cells but not for BALB 3T3 ($Fv\text{-}1^{b/b}$) or mink

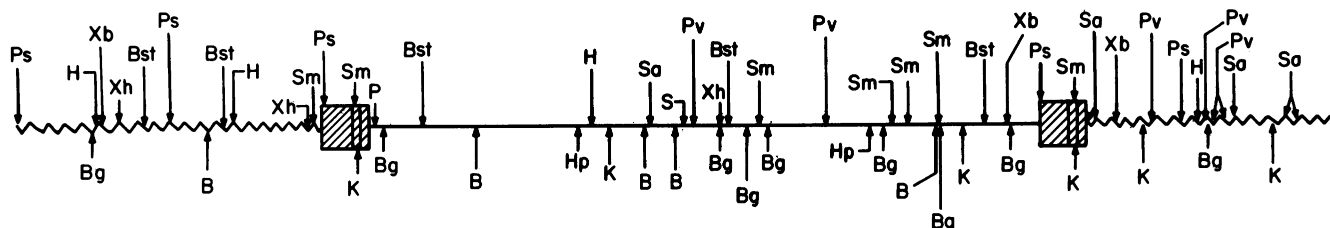

FIG. 2. Restriction endonuclease map of the BALB/c endogenous ecotropic proviral clone p7D and flanking cellular sequences. Cleavage sites are denoted as follows: B, *Bam*HI; Bg, *Bgl*II; Bst, *Bst*EII; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; P, *Pvu*I; Ps, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I.

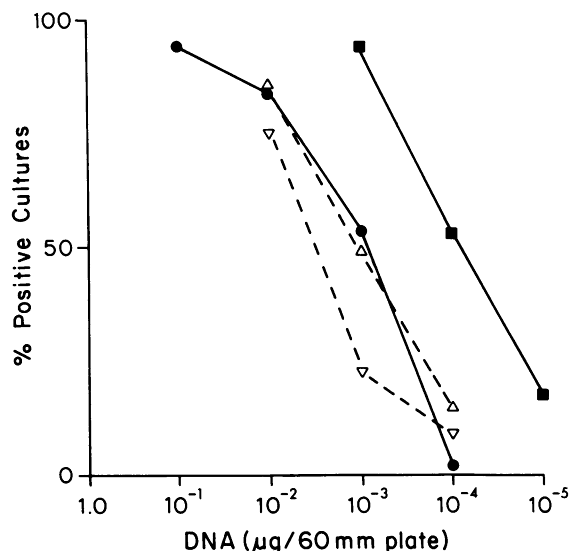

FIG. 3. Comparison of the infectivity of BALB/c proviral clone p7D and the AKV-1-derived proviral clone p623. Cultures of CEF plus Sc-1 cells were transfected with the indicated amounts of plasmid clones, and the proportion of virus-positive cultures was determined as described in Materials and Methods. Symbols: ●, BALB/c p7D provirus; ■, AKV-1-derived provirus p623; ▽, p7D plus 10 µg of p623 subgenomic clone pBaml (Fig. 4); △, p7D plus 10 µg of p623 subgenomic clone pB/S (Fig. 4). To determine infectivity values, 20 to 80 cultures were examined per titration point.

cells. Taken together, we conclude that the virus produced from cells transfected with the BALB/c proviral clones is infectious, N-tropic, ecotropic, and XC negative.

Both XC-negative BALB/c virus and XC-positive p623-derived virus were shown to cross-interfere (Table 2). Preinfection of cells with XC-positive virus produced from p623-transfected cells abolishes superinfection and focus formation by any of the pseudotyped ecotropic virus pools. Preinfection with BALB/c XC-negative virus, however, failed to block completely XC-positive virus entry and resulted in significant levels of focus formation. Continued passage of BALB/c XC-negative virus for 6 to 8 weeks posttransfection led to the recovery of XC-positive variant viruses (data not shown). XC-positive variants of the BALB/c endogenous virus blocked superinfection by p623-derived pseudotype pools to a greater extent than XC-negative BALB/c virus pools and replicated to higher titers than XC-negative virus pools (data not shown).

Cotransfection of subgenomic fragments of p623 and the BALB/c provirus rescued XC-plaque formation but not infectivity. To map the sequences responsible for lowered infectivity and XC phenotype within the BALB/c proviral clones, cotransfection experiments were performed with four cloned subgenomic fragments of p623 (Fig. 4). Cotransfection of each individual p623 subclone with the BALB/c clones did not increase the specific infectivity of p7D (Fig. 3); however, cotransfection of p7D or p8A with pBaml, an *env* gene and 3′ LTR subclone, resulted in XC-positive cultures 7 days later. To delimit the sequences contained within pBaml that were responsible for the rescue of XC-plaque formation, subclones of pBaml were constructed and individually tested for their ability to rescue plaque formation. The sequences responsible for XC-plaque rescue were detected on a 292-base-pair fragment of p623 that spanned the junction of the envelope proteins, gp70 and p15E (subgenomic clone p3′gp70-p15E). We conclude from these results that the subgenomic fragments tested are unable to rescue the infectivity of the BALB/c provirus to the level observed for p623 in cotransfection experiments and that a 292-base-pair segment of the p623 envelope gene is capable of rescuing XC-plaque formation.

TABLE 1. Host range and *Fv-1* tropism of virus produced from cells transfected with the BALB/c proviral clones

Virus pool[a]	Extent of cell infection (FFIU/ml)[b] for: Sc-1 (Fv-$1^{-/-}$)	NIH 3T3 (Fv-$1^{n/n}$)	BALB 3T3 (Fv-$1^{b/b}$)	Mink CCL4
7D	4.4×10^3	7.9×10^3	$\leq 5 \times 10^1$	$\leq 5 \times 10^1$
8A	5.5×10^3	4.4×10^3	$\leq 5 \times 10^1$	$\leq 5 \times 10^1$

[a] CEF plus Sc-1 cocultivated cells were transfected with λ7D or λ8A proviral clones, and virus pools were prepared from cell-free supernatants after two virus passages on Sc-1 cells.

[b] FFIU, Fluorescent focus-inducing units. Test cells (2×10^5) were plated 1 day prior to infection with viral pools and assayed for the extent of infection 5 days later by fluorescence microscopy as described in Materials and Methods.

Sequence analysis localized the BALB/c proviral sequence controlling XC-plaque formation to the proteolytic processing site of the envelope precursor protein. For identification of the nucleotide differences that distinguished p623 from the BALB/c provirus within the 292-base-pair *env* gene fragment, the analogous region of the BALB/c provirus was subcloned from p7D into M13-sequencing vectors in both orientations, and each strand was sequenced by dideoxy chain termination (51). A single base difference within the 292-base-pair fragment was found to distinguish the BALB/c provirus from the sequence of p623 as reported by Herr (16) (Fig. 5). The mutation, guanine to adenine in p7D, resulted in an amino acid substitution of lysine for arginine in the carboxy-terminal amino acid of gp70, the position for proteolytic processing of the envelope protein precursor $Pr85^{env}$ to the mature viral proteins gp70 and p15E (15, 58).

LTRs of the BALB/c provirus differed from those of p623 in that they contained a single enhancer-containing sequence and several point mutations. Detailed restriction enzyme analysis

TABLE 2. Interference properties of virus derived from the BALB/c provirus

Interfering MuLV[a]	Relative FFU/ml of superinfecting pools of pseudotyped Ha-MSV[b]: AKR	BALB/c XC-negative	Amphotropic
None	1.0	1.0	1.0
AKR	0.0001	0.0006	1.3
BALB/c XC-negative	0.01	0.005	0.50
Amphotropic	0.63	0.25	0.0001
MCF-247	0.50	1.0	0.80

[a] NIH 3T3 cells were infected at high multiplicity with pools of virus derived from cells transfected with either p623 (AKR) or p7D (BALB/c XC-negative) proviral clones or with biologically cloned pools of amphotropic virus 4070A or MCF-247. Cells were challenged with pseudotype pools when judged to be confluently infected by fluorescent antibody staining as described in Materials and Methods.

[b] Pseudotyped pools of Harvey murine sarcoma virus (Ha-MSV) were produced by superinfection of a Ha-MSV nonproducer cell line with virus derived from either p623- or p7D-transfected cells or amphotropic virus 4070A. Titers for pseudotyped pools were determined for focus-forming ability on uninfected and preinfected NIH 3T3 cells. Focus-forming titers for pseudotype pools were normalized to their titer on uninfected NIH 3T3 cells (titer = 1.0). FFU, Focus-forming units.

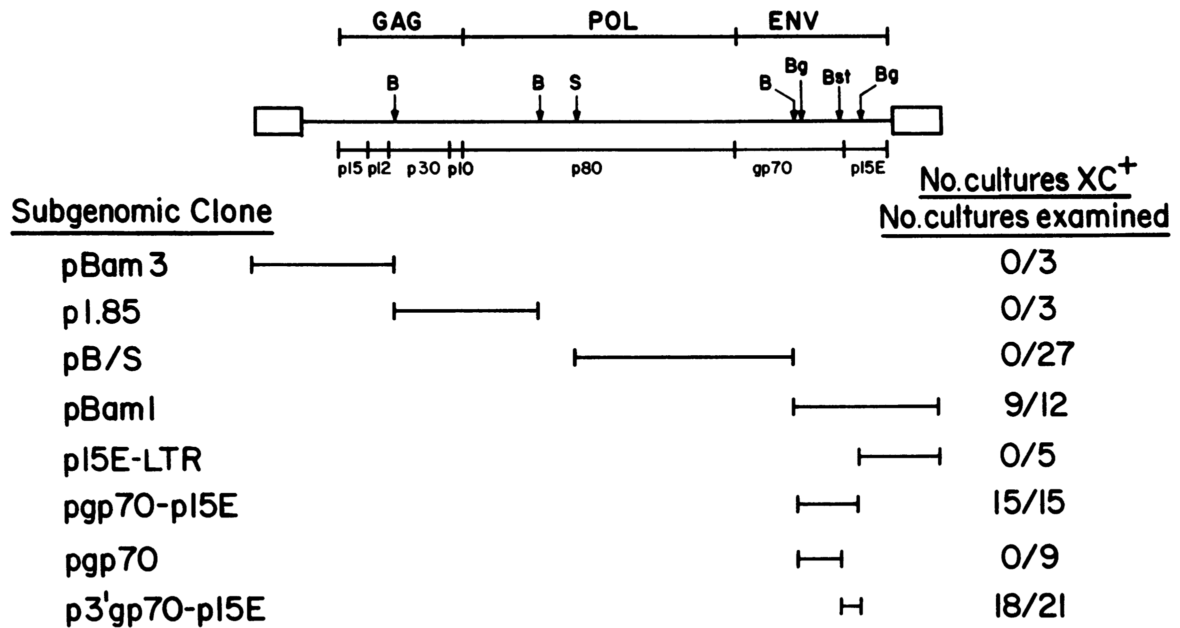

FIG. 4. Cotransfection of BALB/c proviral clone p7D with subgenomic subclones of p623. p7D DNA (0.1 μg) was cotransfected with 20 μg of the indicated subgenomic subclone of p623 onto CEF plus Sc-1 cells, and cells were assayed for the appearance of XC syncytia 7 and 14 days later. XC-positive cultures contained 50 to 1,000 syncytia per culture, whereas XC-negative cultures contained no syncytia. Indicated restriction endonuclease cleavage sites are abbreviated as described in the legend to Fig. 2.

of the p7D LTR showed that the U3 region appeared to be approximately 100-base-pairs smaller than that found for p623 (16). As the LTR provides essential cis-acting functions for the replication of the viral genome, we sequenced the endogenous BALB/c proviral LTR. A schematic comparison of the LTR regions of four ecotropic murine leukemia viruses, three derived from BALB/c and one from AKR, is shown in Fig. 6. The sequence of the endogenous BALB/c

FIG. 5. Nucleotide sequence comparison of the BALB/c (bottom row)- and AKR (top row)-derived proviruses in the region of the proviral clones that determines rescue of XC-plaque formation. Amino acids encoded by the region are indicated above and below the nucleotide sequences.

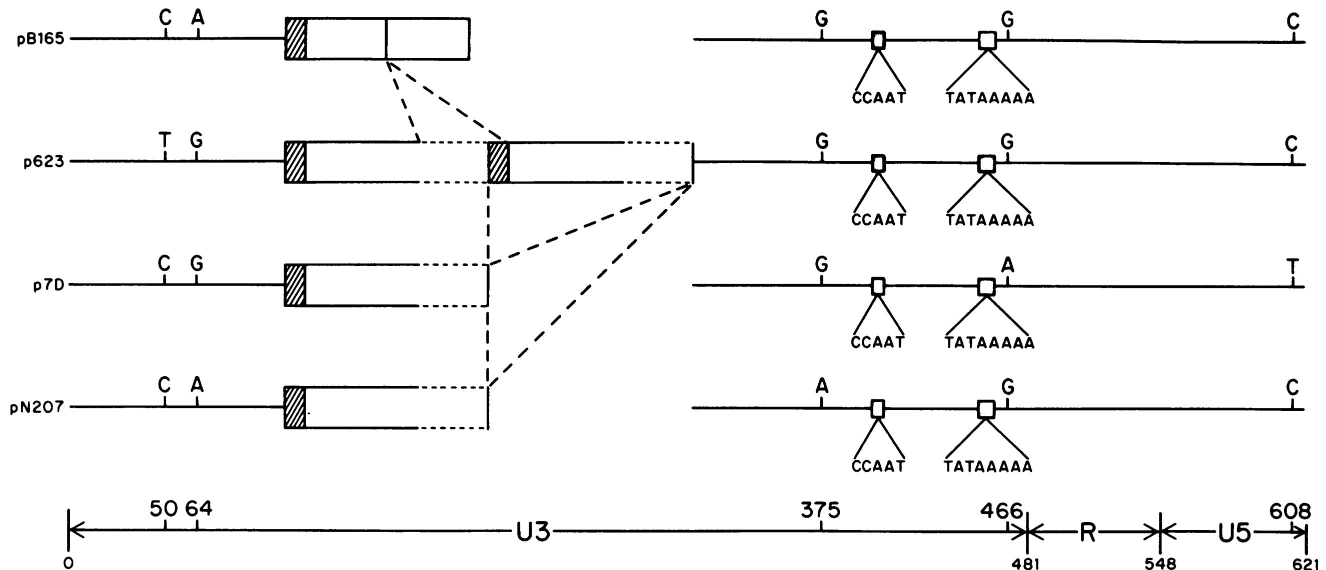

FIG. 6. Schematic sequence comparison of the LTRs of the endogenous BALB/c provirus p7D, two exogenous BALB/c-derived viruses, pB165 and pN207, and p623. Boxed regions represent sequences repeated within the p623 LTR, hatched and dotted portions of which have been deleted in the B-tropic viral LTR clone pB165. Nucleotide differences between clones are indicated, as are their positions with respect to the first nucleotide of U3. The positions of the viral CAAT and TATA boxes are illustrated, as are the sequences that compose them.

U3 region showed that a perfect duplication of 99 base pairs was present in p623 compared with the BALB/c clones, generating the tandem repeats seen in that sequence and providing for the decreased size of the BALB/c LTR. In addition, three base differences were found between p7D and p623, two in U3 (p623 positions 50 and 466; Fig. 6) and one in U5 (p623 position 608; Fig. 6). Since the 99-base-pair repeats each contain the putative viral enhancer elements (25), the endogenous BALB/c provirus contained a single enhancer element in each LTR. Other important structural features found in the LTR, CAAT and TATA boxes, and polyadenylation signals appeared indistinguishable from those found for p623. Whether these differences alter infectivity of the endogenous BALB/c proviral clones relative to p623 is currently under investigation.

Rassart et al. (45) have cloned two exogenous N- and B-tropic ecotropic viruses from a pool of virus derived from an aged BALB/c mouse (14). In Fig. 6, the sequences of the exogenous BALB/c clones pN207 and pB165 are schematically compared with those of their endogenous progenitor, represented by p7D, and p623. Both exogenous clones have single base differences relative to p7D and p623 (10), and the B-tropic isolate has deleted a portion of the tandem repeat. Comparison of the LTR sequence of the BALB/c endogenous ecotropic provirus with the LTR sequence of the endogenous ecotropic provirus of DBA mice has shown that the three nucleotide differences that distinguish the BALB/c LTR from that of p623 are shared by the DBA-derived ecotropic virus LTR (21). In addition, the DBA-derived viral LTR contains two nucleotides that are not shared by either the BALB/c- or p623-derived LTRs.

Sequence comparison between p623 and BALB/c endogenous provirus demonstrated that the two genomes differed at 0.5% of their sequenced nucleotides. To determine the sequence relatedness of the endogenous BALB/c provirus with the exogenous AKR-derived provirus, p623, we sequenced approximately 4,500 base pairs of p7D and compared the accumulated sequence data with the published sequence of p623 (16). We identified 22 base differences in p7D relative to the sequence of p623 (Table 3). Approximately one-third of these led to amino acid substitutions. Figure 7 provides a schematic illustration of the regions that were sequenced from p7D and illustrates that sequence variations were identified in all regions of the viral genome. The identification of only 22 base changes in 4,500 bases sequenced produced a frequency of variation of approximately 0.5%. Of the 22 differences that were identified, 21 were transitions. A

TABLE 3. Nucleotide differences noted between sequenced regions of the BALB/c endogenous ecotropic provirus and sequence of AKR-derived exogenous provirus p623

AKR position[a]	Nucleotide		Codon	Amino acid		Region affected
	AKR	BALB		AKR	BALB	
129	C	T		Noncoding		U5
333	C	T		Noncoding		Leader
647	G	A	CAG	Gln	Gln	*gag*
809	T	C	AAT	Asn	Asn	*gag*
1409	C	T	TCC	Ser	Ser	*gag*
1478	G	A	GAG	Glu	Glu	*gag*
1511	G	A	AAG	Lys	Lys	*gag*
1652	A	G	GCA	Ala	Ala	*gag*
2643	T	C	TAT	Tyr	His	*pol*
2668	T	C	GTT	Val	Ala	*pol*
2690	C	T	CTC	Leu	Leu	*pol*
2824	A	G	AAA	Lys	Arg	*pol*
4214	A	G	CGA	Arg	Arg	*pol*
4322	G	A	TTG	Leu	Leu	*pol*
5765	G	A	ACG	Thr	Thr	*pol*
5879	A	G	ACG	Thr	Ala	*env*
6214	A	C	GCA	Ala	Ala	*env*
6222	G	A	GGA	Gly	Glu	*env*
7191	G	A	AGA	Arg	Lys	*env*
7770	A	G	GAA	Glu	Gly	*env*
7875	T	C		Noncoding		U3
8291	G	A		Noncoding		U3

[a] Indicated nucleotide position reflects the numbering system previously published for p623 by Herr (16).

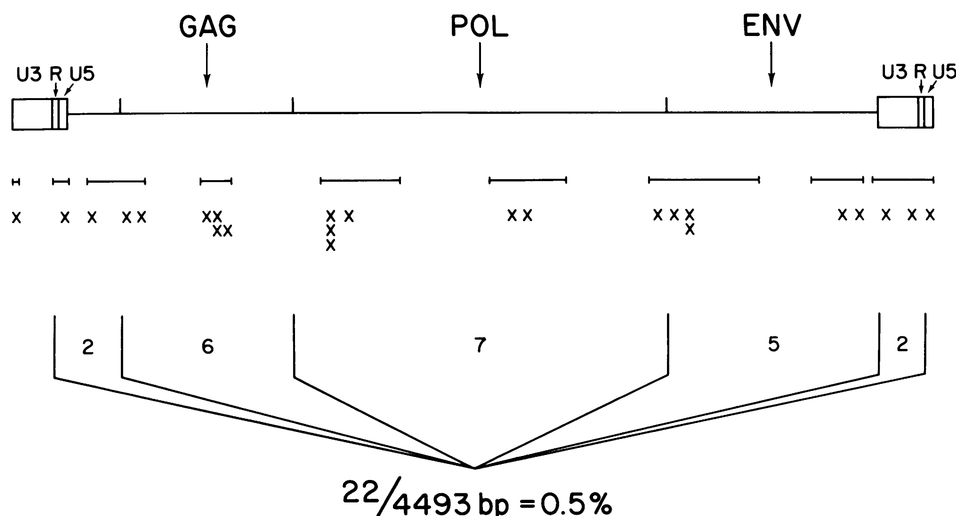

FIG. 7. Schematic illustration of the regions of the BALB/c endogenous provirus that have been sequenced and the approximate location of nucleotide differences relative to the sequence of p623. Sequenced regions are denoted by bars, and nucleotide differences are denoted by X's. Regions of the proviral genome are noted, as are the total number of nucleotide differences observed for each region.

total of four restriction endonuclease polymorphisms were identified within the BALB/c endogenous proviral genome relative to the structure of the p623 genome: (i) as has been noted previously (43), the p623 genome lacked an *Hin*dIII site within the viral *pol* gene that is characteristic of all ecotropic viruses, including the BALB/c endogenous virus; (ii) as reported above, the BALB/c LTR was 99-base-pairs smaller than the p623 LTR; (iii) the BALB/c endogenous provirus lacked an *Hpa*I site at p623 base pair position 5455, and (iv) a novel *Alu*I restriction site occurred as a result of the nucleotide difference observed in the endogenous BALB/c provirus at base pair position 1511.

DISCUSSION

The patterns of expression of ecotropic virus in inbred mice vary with each mouse strain examined. BALB/c mice express low titers of ecotropic virus late in life, and low frequencies of cells are induced to express ecotropic virus in culture. To relate this low level of ecotropic virus expression to the specific infectivity of the single, endogenous ecotropic provirus inherited by BALB/c mice, we isolated and characterized two molecular clones of the BALB/c ecotropic provirus. The specific infectivity of the clones was about 1/10 that of an exogenous proviral clone derived from AKR mice, p623, and after transfection of cells with the BALB/c provirus, XC-negative, N-tropic, ecotropic virus was recovered. The XC-negative phenotype for the BALB/c virus was correlated with an amino acid substitution at the proteolytic processing site for the envelope precursor protein $Pr85^{env}$. Extensive sequence analysis of the BALB/c provirus has shown that it differs from p623 in 0.5% of its sequenced nucleotides, and the BALB/c LTRs contain a single copy of the enhancer-containing sequences identified in the LTRs of p623.

Although expression of ecotropic virus in BALB/c mice and cultures of their induced cells occurs at low frequencies, the specific infectivity of the endogenous provirus inherited by BALB/c mice approximates that of an exogenous proviral clone derived from a high-virus, high-leukemic mouse strain, AKR. This disparity may reflect the restriction imposed on in vivo virus replication by the BALB/c *Fv-1* gene and the XC-negative phenotype of the virus induced in vitro from the endogenous provirus. An additional means by which the production of ecotropic virus may be regulated has been provided by an analysis of the integration site of the BALB/c provirus. The BALB/c proviral integration event occurred such that the ecotropic virus is integrated, in opposite transcriptional orientation, within the LTR of a VL30 virus-related element (J. M. Horowitz and R. Risser; manuscript in preparation). The expression of a chimeric message from the VL30 LTR, containing VL30 and anti-sense BALB/c ecotropic virus sequences, could result in diminished production of viral RNA and proteins from the ecotropic provirus. Experiments in progress should establish whether expression of the BALB/c provirus is regulated in cis by the presence of VL30 sequences.

The infectivity of the BALB/c proviral clones was not increased to the level observed for the AKR-derived provirus by cotransfection with subgenomic clones of p623. There are several possible explanations for this result: (i) the BALB/c proviral clones may contain multiple sequence differences that are not contained on any one subgenomic clone of p623; (ii) a 480-base-pair segment of the viral *pol* gene was not tested in our cotransfection experiments; (iii) sequences flanking the BALB/c provirus may limit virus expression; and (iv) the efficiency of cotransfection precludes the observation of a tenfold increase in specific infectivity. We are currently addressing these possibilities by performing in vitro restriction fragment exchanges between p623 and the BALB/c proviral clones.

XC-plaque formation traditionally has been used to monitor the production of ecotropic viruses. Our results suggest that the formation of XC plaques can be diminished by minor perturbations in the structure of the proteins conferring ecotropic host range. The substitution of a lysine residue for the carboxy-terminal arginine of gp70 renders the virus produced from the BALB/c proviral clones XC-negative. We are currently investigating whether this amino acid substitution leads to aberrant processing of the envelope protein precursor. Continued passage of the BALB/c XC-negative virus leads to the recovery of XC-positive variants. Restriction digests and hybridization analysis of unintegrated viral DNA preparations from cells infected with BALB/c XC-positive variant viruses suggest that minor sequence alter-

ations are responsible for conversion to the XC-positive virus phenotype. The positions of such alterations have yet to be mapped.

Several other XC-negative ecotropic viruses have been previously reported; two isolates were recovered from BALB/c or related mice (9, 19, 36, 44, 50, 52, 53). Induction of cells derived from BALB/c-related strains, such as C3H, CBA, and A, have also resulted in low frequencies of XC-positive, ecotropic virus-producing cells, consistent with their inheritance of the ecotropic provirus of BALB/c mice (14). Southern analysis of DNAs derived from low-leukemic mice has shown that strains such as SEC, SEA, HRS, and RF have also inherited an ecotropic provirus allelic to that of BALB/c mice (20, 31) and hence represent strains from which new isolates of XC-negative ecotropic virus may be derived. The determinants of several XC-negative Moloney-MuLV isolates have been mapped; all have been shown to reside within the *gag-pol* region of the viral genome (9, 36, 52, 53). All but one of these isolates are reported to produce low levels of reverse transcriptase activity and to be poorly infectious (36). Taken together, these results indicate that the determinants of XC phenotype are not confined to one region of the viral genome and that the determinants of XC-negative viruses may vary with each isolate analyzed.

Enhanced virus expression, or complementation, in hybrid (BALB/c × C57) mouse cells may be due to phenotypic conversion of the BALB/c XC-negative ecotropic virus to an XC-positive virus. Rescue of XC-plaque formation in cells of F1 animals could be facilitated by envelope protein complementation or recombination of viral sequences contributed by the ecotropic genomes of BALB/c and C57 mice. The ecotropic viruses of (BALB/c × C57) F1 animals may thus produce XC-positive virus by providing functions for plaque formation that are incompetent when either virus is expressed alone. A complete understanding of the mechanism of complementation for the BALB/c and C57 endogenous ecotropic viruses requires that the restrictions on the production of XC-positive virus from C57 cells be determined. The molecular cloning of the ecotropic provirus of C57 mice should provide insight into this question.

Restriction enzyme and oligonucleotide analyses of many ecotropic viruses have shown them to be highly related (20, 23, 43, 47), and our results indicate that the overall sequence homology between the AKR- and BALB/c-derived proviruses is quite striking. This conservation of sequence has occurred despite the independent inbreeding of AKR mice 20 years after the establishment of the BALB/c inbred strain and the acquisition of their endogenous ecotropic proviruses by different integration events. Although we cannot rule out the role of selection in the integration and maintenance of such conserved viral genomes, we suggest that few cycles of reverse transcription separate the AKR- and BALB/c-derived proviruses. The remarkable sequence conservation noted between the LTRs of the BALB/c- and DBA-derived ecotropic proviruses is also consistent with a common origin for endogenous ecotropic proviruses. We propose that the endogenous ecotropic viruses of inbred mice are the result of rare germ line infection events prior to inbreeding of mouse strains and their subsequent dispersal about the mouse genome by reintegration events similar to those observed in contemporary viremic strains such as AKR (4, 17, 42). Our sequence analysis is consistent with evidence of common ancestry of inbred mouse strains provided by examination of restriction polymorphisms in mouse mitochondrial DNA (11) and a recent report of common strain ancestry for AKR and BALB/c mice on the basis of an extensive compilation of isoenzyme markers (12). Studies on the inheritance of endogenous mouse mammary tumor virus proviruses by inbred mice provided evidence for their introduction into the mouse germ line prior to strain segregation by multiple, rare, independent infection events (7). Our results extend these observations to another family of endogenous mouse viruses and suggest a single virus ancestor for the ecotropic proviruses of inbred mice.

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