Characterization of Endogenous and Recombinant Proviral Elements of a Highly Tumorigenic AKR Cell Line

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Received 1 February 1991/Accepted 16 May 1991

As an approach to evaluating the contribution of classes of endogenous viral sequences to leukemogenesis, a genomic library was prepared from the highly tumorigenic AKR SL12.3 cell line and screened for *env*-containing proviruses. An extensive battery of virus-derived probes and specific oligonucleotide probes were used to segregate 83 positive clones into related groups. The noncotropic endogenous retroviruses were identified as members of the polytropic, modified polytropic, or xenotropic groups. At least three unique xenotropic proviruses were detected that differed from the published xenotropic sequence within a variable region of the 5' portion of *env*. Changes among the xenotropic proviruses included relative insertions and/or deletions that maintain an open reading frame and hence the potential to encode viable envelope gene products. Several recombinant viruses were also detected. Recombination was not random and primarily involved the formation of mink cell focus-inducing class I retroviruses via recombination between polytropic elements and ecotropic virus. One other recombinant was detected which contained ecotropic virus sequences in the 5' region encoding p15 of an otherwise xenotropic provirus. An interesting observation was the finding that certain clones contained more than one provirus within the average 20-kb cloned insert. This would not be expected if integration were totally random. The de novo recombinant proviruses identified here provide a series of potential candidates to be evaluated for their contribution to the tumorigenicity of the SL12.3 cell line.

The endogenous murine retroviruses are a closely related family of type C retroviruses present as multiple copies in the mouse genome. They are normally classified by their host cell range in tissue culture, a property dictated by the gp70 protein encoded by the viral envelope gene. Ecotropic viruses can infect only mouse cells and are present as one to five copies in 75% of laboratory mouse strains (24). There is an absolute correlation between the presence of ecotropic virus sequences and the occurrence of retrovirus-induced murine leukemia. However, the presence and expression of ecotropic virus are not sufficient to cause disease (5, 7). Xenotropic viruses infect only nonmurine cells (14, 19, 30, 34, 49) and are present in all mouse strains. A third group is made up of the polytropic proviruses, which are by far the most prevalent of the endogenous retroviral elements. The polytropic proviruses have not been found as infectious viruses and are likely to be all replication defective. However, recombinant viruses containing 5' *env* sequences of the polytropic viruses can infect both murine and nonmurine cells, and it is assumed that these 5' sequences impart broadened host range properties (1, 11, 16, 17, 40, 57). The polytropic proviruses have been divided into two groups, termed polytropic and modified polytropic, based on differences in their envelope genes (15, 57, 58). Although none of the above endogenous elements have, individually, any deleterious effects on their hosts, certain recombinant viruses that arise de novo are tumorigenic in mice (7, 42, 47, 55). Evidence indicates that at least three distinct retroviral elements are involved in the production of these mink cell focus-inducing (MCF) viruses (4, 6, 20, 26, 28, 29, 41, 52,

62). In the leukemogenic class I (37) recombinants, a genetic exchange occurs in the 5' portion of the *env* gene encoding gp70 (1, 5, 6, 9, 11, 16, 22, 28, 37, 62). This usually involves the substitution of polytropic *env* sequences into an ecotropic virus background. Additionally, at least for the leukemogenic class I recombinants, a distinct recombinational event results in the substitution of xenotropic *Bxv-1* virus (19) sequences within the long terminal repeat (LTR). This latter recombination has a profound influence on the cellular tropism of the recombinant (13, 21, 23, 41, 52, 62). A second type of recombinant, termed class II (37), contains essentially the whole envelope gene of a polytropic virus on an ecotropic virus background and is nonleukemogenic. Recombination in both cases is a selective process, involving only a discrete number of proviruses from the extensive repertoire of endogenous elements. This bias toward certain sequences may be a consequence of nonrandom transcription of certain endogenous elements, making them available for selective recombination. An alternative explanation is that recombination is more general, but only a subset of random recombinants formed are amplified and propagated as infectious agents.

We have prepared a genomic library from the highly tumorigenic SL12.3 AKR cell line (26, 38), with the purpose of examining the diversity of both endogenous and recombinant proviruses in a transformed cell. Using the *Bam*HI-*Eco*RI 5' *env* probe from an endogenous polytropic retrovirus (32), we selected *env*-positive clones from this library. Using a battery of genomic and oligonucleotide probes, we were able to segregate these clones into related groups. As described below, we were able to define previously unreported xenotropic proviruses and also found evidence of selective recombination within this tumor. In addition, we

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Probe	Location	Fragment	Reference	Probe Class	Sequence	Reference
1.	LTR	PstI/KpnI	22	9.	Xeno GGA TGA CCC AGA ACC CGA TAT TG	48
2.	5' GAG	BglII/BamHI	22	10.	M-Poly AGT GGT CTC CAG TGG CAT CCA GGG TGC	57
3.	3' GAG	BamHI/HindIII	22	12.	Poly AGC GGT CTC CAG TGA CAT CAA GGG CGC	57
4.	5' POL	HindIII/BamHI	22	14.	Eco TTT GAC CTC CTT GTC CGA AGT AGT GTT	18
5.	3' POL	PvuII/PvuII	22	15.	Endog TCT GAC CTC GTT GTC TGA GGT GGT CCT	32
6.	5' GP 70	BamHI/EcoRI	32	16.	Eco ACT TTT AAT TTG GAC ATT ATT CTA CAG	18
7.	3' GP70	EcoRI/PstI	32	17.	Endog ACT TTC AAT TTA AGT ATT ATC TCT CAG	32
8.	p15E	PstI/PstI	33	18.	Eco CGG CAG ATT CCA CCA CCT CTC GGG CTT	18
				19.	Endog CAG CGG ACT CCA TCA TCT CCC AGG CAT	32
				JS4	M-Poly GCA GCC TCT ATA CAA CCT GGG ACG GGA G	58
				JS5	Poly GCA GCC TCT ATA GTC CCT GAG ACT GCC C	58
				JS6	Xeno ACG GTC TCT ATG GTA CCT GGG GCT CCC C	58
				JS10	Xeno ACG GTC TCT ATG GTG CCT GGG GCT CCC C	58
				JS21	Xeno ATT CTC AAA AGT CAC AAG GAA GTT TAG	59

FIG. 1. Genomic location of virus-derived probes and specific oligonucleotides. This schematic map of murine retrovirus shows the location of the virus-derived probes as well as the *gag*- and *env*-specific oligonucleotides. Probes 1 to 5 were derived from a molecular clone of MCF-247 virus (22, 25), provided by C. Holland. Probes 6 to 8 were derived from endogenous (Endog) polytropic sequences (57). JS-4, JS-5, JS-6, and JS-6/10 have been reported previously (57, 58). The sequence for xenotropic (Xeno) probe 9 was taken from O'Neill et al. (48). Modified polytropic (M-Poly) (probe 10) and polytropic (Poly) (probe 12) sequences were taken from Stoye and Coffin (57). Ecotropic (Eco) proviral sequences were taken from Herr and Gilbert (18). Restriction sites: Bg, *BglII*; B, *BamHI*; H, *HindIII*; P, *PstI*; S, *SmaI*; K, *KpnI*; Pv, *PvuII*; R, *EcoRI*.

identified clones that contained two proviruses in close proximity, which is unexpected if integration occurs randomly.

MATERIALS AND METHODS

Cell lines. The SL12.3 cell line was derived following one passage in mice and limited-dilution cloning from the lymphoma-inducing parental SL12 cell line (38). These cells have now undergone more than 50 passages in tissue culture. Cells were grown in Dulbecco minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum.

Probes. A battery of eight virus-derived probes encompassing the entire retrovirus genome was used to analyze the SL12.3 clones. The characterization of these probes and their genomic locations are detailed in Fig. 1. In addition, specific oligonucleotide probes were prepared to discriminate between closely related endogenous retroviruses by either direct or competitive hybridization. The latter probes

corresponded primarily to envelope sequences, but certain oligonucleotides were also prepared which discriminated between endogenous/xenotropic viruses and ecotropic sequences in the *gag* proteins p15 and p12 (Fig. 1).

Preparation of genomic library. DNA from the SL12.3 cell line was prepared as described previously (3), and a partial restriction digest was performed with the restriction enzyme *Sau3aI*. The DNA (approximately four genomic equivalents) was then ligated into the *BamHI* site of EMBL4 bacteriophage (Stratagene), packaged, and subsequently amplified in KH802 cells. The library was plated out and then screened by using a ^{32}P -labeled *Bam-EcoRI* fragment containing the 5' half of the *env* gene of an endogenous MCF-related retrovirus (32). Eighty-seven *env*-positive clones were selected and plaque-purified. Eighty-three of the clones proved to be unique, as judged by restriction analyses, and were retained for further studies. Some of these clones may represent distinct *Sau3aI* fragments containing the same integrant, and analyses with adjacent sequence probes have yet to be

performed. However, as detailed below, the majority of the proviruses identified here vary internally by at least one of the extensive list of parameters used to segregate these clones.

Southern blot hybridizations. Retrovirus-derived probes (Fig. 1) were labeled with [32 P]dCTP by previously described procedures (53) and then used in the Southern blot hybridizations. Briefly, following electrophoresis in agarose, DNA was transferred to nitrocellulose membranes as previously described by Southern (56). Hybridizations were carried out for 18 h at 37°C in 50% formamide. The membranes were washed at 37°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium *N*-laurylsarcosine–0.05% sodium PP_i, followed by a higher-stringency wash at 42°C with 0.1× SSC containing sodium *N*-laurylsarcosine and sodium PP_i.

Slot blot hybridizations. Synthetic oligonucleotides were prepared to various regions of the murine retrovirus genome (Fig. 1) and varied in length from 18 to 26 nucleotide residues. The oligonucleotides were labeled with [32 P]ATP by the T4 kinase end-labeling method (53). DNA in 1 M ammonium acetate was transferred onto nitrocellulose by using a slot blot template (Schleicher and Schuell). Oligonucleotide hybridizations with or without competition were performed as previously described (8, 44). Briefly, the membranes were prehybridized for 2 h at 37°C in 1× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum albumin)–6× SSC containing 0.05% sodium dodecyl sulfate (SDS), 100 µg of salmon sperm DNA per ml, and 0.05% sodium PP_i. The hybridization mix contained the labeled oligonucleotides plus or minus a 10-fold excess of the competitive sequence. After an initial 10-min incubation at 55°C, the temperature was lowered to 32°C over a 2-h period and the membranes were incubated for a further 16 h. The initial washes were carried out in 6× SSC–0.05% sodium PP_i at 25, 37, and then 42°C. The concentration of SSC was reduced to 1× for the final wash at 42°C. Oligonucleotides JS-4, JS-5, JS-6/10, and JS-21 were hybridized as described previously (14).

DNA sequencing. Certain viral fragments were sequenced by the chain termination method of Sanger et al. (54) following subcloning into an M13 vector.

RESULTS

Isolation of *env*-positive clones. All clones, obtained from the SL12.3 library, contained 5′ envelope sequences that hybridized with a probe derived from an endogenous polytropic virus. None of the clones hybridized with a 5′ *env* probe derived from AKR ecotropic virus (Akv [18]; data not shown), and most *env*-deleted integrants, such as those previously described (29, 51), were excluded from this analysis. Figure 1 details the battery of probes used to subdivide the selected clones into related groups. The clones were initially screened with a series of virus-derived probes which, with few exceptions, did not discriminate between the groups of integrants. They did, however, facilitate the identification of apparently complete viral genomes from those containing relatively large deletions. The clones were then further analyzed by using a series of oligonucleotide probes capable of discriminating among the closely related family of endogenous retroviral elements (Fig. 1). In addition to oligonucleotide probes used in previous studies (JS-4, JS-5, JS-6/10, and JS-21 [58, 59]), probes to 5′ regions of *env* were prepared that also discriminate among polytropic, modified polytropic, and xenotropic proviruses (Fig. 1).

Some of the probes differed by only three or four nucleotides, and discrimination was achieved by competitive hybridization. In certain circumstances, restriction enzyme sites indicative of particular proviruses were used to aid in discrimination of the clones. On the basis of these analyses, the clones were divided into related groups, as outlined below.

Polytropic integrants. Seventeen of the retroviral clones were identified as polytropic by at least seven independent characteristics (Table 1). The LTRs contained a 192-bp insert in U3, typical of certain endogenous elements (27, 33, 43, 50, 57). This insert was apparent both by hybridization with a specific probe and by the size of the *Pst*I-*Kpn*I fragment internal to the LTR (data not shown). Polytropic integrants are somewhat underrepresented in this analysis relative to previous observations (15, 57). However, this is probably due to 3′ truncations during cloning, so that some members of this group failed to hybridize with all polytropic-specific probes. These clones were thus placed in the unclassified category (see Table 3). Six of the polytropic isolates contained relatively large deletions in *gag*, and one isolate possessed only gp70 (clone 68 [Table 1]).

Modified polytropic integrants. Fifteen clones were scored as modified polytropic, on the basis of at least nine characteristics (Table 1). Similar to polytropic integrants, the modified polytropic viruses possess large LTRs containing the 192-bp insert sequence (57). Eight of the clones had a unique *Hind*III site in the *pol* gene, previously thought to be diagnostic of the modified polytropic proviruses (57). However, the lack of this site in six other clones demonstrates a degree of polymorphism in this group. Again, as with the polytropic integrants, modified polytropic proviruses contained deletions, and it is likely that others fell into the unclassified category (see Table 3) owing to lack of 3′ sequences.

Xenotropic integrants. Initial screening for xenotropic integrants was performed by using a 5′ *env* oligonucleotide probe (Fig. 1, probe 9) corresponding to sequences of a xenotropic virus reported by O'Neill et al. (48). Four clones hybridized with this probe (Table 2). These were designated xenotropic I integrants. Two of these clones (clones 30 and 87) contained large deletions. The other two clones appeared to be complete when investigated with restriction fragment probes, but failed to hybridize with specific oligonucleotide probes to *gag* (clones 9 and 82; note that clone 82 contained two proviruses and is not listed in Table 2). Clones 9 and 82 had an extra *Kpn*I site in their LTRs. An additional 13 clones failed to hybridize with probe 9, but were judged to be xenotropic by hybridization with the more 3′ JS-6/10 probe (57, 58) (Fig. 1) and by the presence of an extra *Kpn*I site in their LTRs.

Nucleotide sequence analyses of the 5′ *env* regions of additional xenotropic isolates (Fig. 2) revealed variability at the precise location of probe 9. Changes involved the relative insertion and deletion of sequences at this site. Significantly, these changes allowed for the maintenance of an open reading frame which could encode intact *env* proteins (Fig. 2). Oligonucleotide probes specific for each of these sequences (Fig. 2, underlined sequences) were prepared and then used to screen the remaining clones. These analyses resulted in the groupings shown in Table 2, which segregated the xenotropic proviruses into more than four groups. Two clones, denoted as xenotropic by hybridization with probe JS-6/10 (note that JS-6 and JS-10 varied by only one nucleotide and were used in combination for hybridizations),

TABLE 1. Analysis of AKR SL12.3 endogenous polytropic provirus-containing clones^a

Clone	LTR ^b				gac ^c		pol ^d		gp70										p15E ^e	
	P/K (bp)	K	192	JS-21	Eco, 16	X/E, 17	+/-	B/H	M-Poly ^f		Poly ^g		Xeno ^h				Eco, 14	X/E, 15		
									JS-4	10	JS-5	12	JS-6/10	9	XEII	XEIII			XEIV	
Polytropic																				
12, 17, 18	550	-	+	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	
19	620	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	
23	550	-	+	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	
24, 60	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	
25	550	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	+	
34, 39, 56	550	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	+	
57	550	-	+	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	
66	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	
68	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	
74,77	600	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	+	
80	620	-	+	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	
Modified polytropic																				
1, 51, 63, 64	620	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	
4, 5	600	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	
8	600	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	
21, 22, 46, 50	600	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	
43, 58	600	-	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	+	
65	610	-	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	
83	660	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	

^a All the probes except for XEII to XEIV are described in Fig. 1. Probes XEII to XEIV are described in Fig. 2. The plus sign means that the clone hybridized to a particular probe or contained the particular restriction site(s).

^b The LTRs of the clones were characterized by the size, in base pairs, of an internal *PstI-KpnI* (P/K) fragment; the presence or absence of an extra *KpnI* restriction site (K); and the presence or absence of a 192-bp insert (192 [27, 32, 43, 50, 57]). Hybridization with probe JS-21 (41, 59) indicated the presence of sequences donated to MCF virus LTRs by the *Bxv-1* endogenous xenotropic virus.

^c The *gag* gene of each clone was assessed for hybridization with either an ecotropic virus-specific probe (Eco; probe 16 [Fig. 1]) or a probe that hybridized to any nonecotropic endogenous virus sequence (X/E; probe 17 [Fig. 1]).

^d The presence or absence of the *pol* region (+/-) is represented by a + or a -, respectively, as determined by hybridization with probes 4 and 5 (Fig. 1). Whether or not a clone contained a unique *BamHI-HindIII* fragment (B/H) within the *pol* region, a characteristic of certain modified polytropic elements (57, 58), is recorded as + or -.

^e The p15E regions of each clone were analyzed by using probes that hybridize specifically with ecotropic virus sequences (Eco) or that hybridize with nonecotropic endogenous proviruses (X/E).

^f Probes that hybridize to modified polytropic (M-Poly)-specific regions of *env*.

^g Probes that hybridize to polytropic (Poly)-specific regions of *env*.

^h Probes that hybridize to gp70-encoding regions of specific xenotropic proviruses, including probes JS-6/10 and 9 (Fig. 1) and XEII-IV (Fig. 2).

TABLE 2. Analysis of xenotropic provirus-containing clones from the SL12.3 cell line^a

Clone	LTR				<i>gag</i>		<i>pol</i>		gp70								P15E		
	P/K (bp)	K	192	JS-21	Eco, 16	X/E, 17	+/-	B/H	M-Poly		Poly		Xeno				Eco, 14	X/E, 15	
									JS-4	10	JS-5	12	JS-6/10	9	XEII	XEIII			XEIV
3	400	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-
6	500	+	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	+
7	500	+	-	-	-	-	+	+	-	-	-	-	+	ND ^b	ND	ND	ND	-	+
9	500	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	+
30	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-
31	500	+	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	-	+
38	500	+	-	-	-	-	+	+	-	-	-	-	+	ND	ND	ND	ND	-	-
40	500	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
44	500	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
47, 53	500	+	-	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+
67, 81	500	+	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	+
69	500	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+
84	500	+	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-
87	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-

^a All the probes with the exception of XEII to XEIV are described in Fig. 1. Probes XEII to XEIV are described in Fig. 2. For definitions of the headings in this table, see Table 1, footnotes b to h.

^b ND, not determined.

FIG. 2. Nucleotide and amino acid sequences of xenotropic virus isolates, demonstrating variability within the 5' *env* sequences. Nucleotide sequence analyses were performed by the chain termination method (54) with specific oligonucleotide primers. Underlined areas refer to the three oligonucleotide probes (probes 9, XENO II, and XENO III) used to segregate the xenotropic proviruses. The original xenotropic sequence (XENO I [48]) and polytropic and modified polytropic (57) sequences are included for comparison. Amino acid sequences are shown in the one-letter code.

failed to hybridize with probes specific for groups I to IV and as yet have not been analyzed further.

Sequence analyses were also performed on the LTRs of certain xenotropic elements (Fig. 3). One isolate possessing a xenotropic II *env* contained an LTR with three additional *KpnI* sites, owing to the presence of three imperfect direct repeat sequences. Another isolate contained an LTR with similar repeat sequences, but only one extra *KpnI* site, owing to variation at a single base [Fig. 3, line 3, clone 55 (2)]. Tests are under way to determine whether these LTRs can act as efficient promoters of viral expression.

Recombinant viruses. Sufficient divergence has occurred between ecotropic virus and the other endogenous elements that recombinants (such as class I and II MCF viruses) can be readily discerned from endogenous elements. Oligonucleotide probes were prepared from sequences within *gag* p15 and p12 and *env* p15E (Fig. 1) that would discriminate between proviral elements containing ecotropic virus sequences and those containing polytropic, modified polytropic, or xenotropic sequences. Using these probes, we identified 12 related recombinants (Table 3). These were all class I recombinants by virtue of the presence of Akv *gag* and 3' *env* sequences and endogenous 5' *env* sequences (37). The class I integrants also were positive for hybridization with the *Bxv-1* (19) LTR probe (JS-21 in Fig. 1) (41, 59), characteristic of this group. Interestingly, all of these recombinants contained polytropic 5' gp70 sequences and none were found to contain modified polytropic or xenotropic sequences (the *Bxv-1* provirus was not represented in this study). Also, no class II (7, 37) integrants, containing Akv *gag* and endogenous *env* sequences, were detected in this library. An additional isolate was identified as recombinant, based on positive hybridization with an Akv-specific oligonucleotide probe to the p15 region of *gag* (probe 16 in Fig. 1). This clone turned out to contain two proviruses; detailed analyses are given below.

Unclassified isolates. The clones which fell into the unclassified category (Table 3) lacked *env* sequences used to discriminate among the various endogenous elements. Since the possibility existed that the sequences were not lacking, but were totally distinct from our battery of probes, we sequenced the *env* region of one isolate (clone 78) which showed positive hybridization with both the 5' *BamHI-EcoRI* probe and the full-length p15E probe 7 (Fig. 1). This analysis explained both the observed positive hybridization with the viral probes and the negative results with the synthetic oligonucleotides. The envelope gene contained a large deletion from almost precisely the beginning of gp70 to the region of p15E that is immediately 5' of the membrane-spanning domain (data not shown). The reading frame was not maintained through this deletion, so it is unlikely that this integrant produces a protein product. Although additional analyses were not performed, it is likely that similar deletions are present in other members of this group.

Double integrants. We were surprised to find that three of the approximately 20-kb clones (clones 55, 82, and 86) contained more than one retroviral element. A partial map of clone 55 is shown in Fig. 4. Clone 55 contained, on the 3' end (arbitrarily defined), an endogenous xenotropic II provirus, as judged by sequence analyses of 5' *env* and 5' LTR regions (Fig. 2 and 3, respectively). This element was truncated on the 3' end by cloning. However, it also contained a large deletion encompassing 5' packaging and p15 sequences, so that it would not encode an infectious provirus. The analysis of this clone was complicated, since hybridization with specific oligonucleotides by slot blot analyses had indicated that it contained the p15 of Akv, yet this provirus had no p15 sequences at all. Further examination revealed a second integrant approximately 5.2 kb 5' to the xenotropic II provirus. Nucleotide sequence analyses (results not shown) confirmed that this integrant possessed p15 sequences related to Akv, yet had an endogenous-xenotropic p12 and

FIG. 3. Nucleotide sequence analyses of the U3 regions of several xenotropic virus LTRs. A common *Pst*I restriction site is underlined, and boxes indicate the CAATT and TATA boxes. DR, imperfect direct repeats. The xenotropic sequence of O'Neill et al. (48) is given on the top line. Only the changes are shown for the other sequences.

xenotropic LTRs (Fig. 3, sequence 3). This provirus contained a large internal deletion encompassing *pol* and *env*. On the basis of the fact that this provirus contains both ecotropic and endogenous sequences, we have defined it as a recombinant, although whether it arose de novo or has been in the mouse genome for some time has not been determined. Tests with adjacent-sequence probes are underway to verify whether this is an integrant unique to the SL12.3 cell line. By Southern analysis with the JS-6/10 oligonucleotide probe, it was found that the 3' integrant is present in single copy in the AKR genome and corresponds to the *Xmv-27* xenotropic provirus (14) on chromosome 13 (data not shown).

Clone 86 also proved to contain two proviruses (Fig. 5). Southern analyses of this clone, followed by competitive hybridization with *env*-specific probes, confirmed the presence of two distinct *env* sequences (Fig. 5). When data from analyses of *gag* and LTR sequences are included (results not shown), the results are consistent with the interpretation that one element represents an endogenous xenotropic provirus and the other represents a recombinant provirus, by virtue of the presence of ecotropic p15E sequences in association with the polytropic 5' *env* sequences. This integrant also

hybridized with the *Bxv-1* probe, characteristic of class I recombinants. Complete mapping of clone 86 is in progress.

DISCUSSION

The SL12.3 cell line was chosen for these studies because of its highly tumorigenic nature (38, 39). Furthermore, the parental cell line and two others derived from it have been shown to have stable properties with respect to incidence, latency, and spread even after extensive in vitro culture (38, 39). It was therefore hoped that characterization of newly acquired retroviral integrants might indicate particular cellular genes that have been deregulated as a result of retroviral insertion and may therefore contribute to the tumorigenic potential of the cell. Multiple integrations of class I recombinant viruses (possibly the same one) were observed in this cell line, which could potentially alter transcription via insertional mutagenesis, resulting in transformation. Whether amplification of MCF viruses occurred before or after transformation cannot, however, be determined from this study. It is unknown whether this MCF virus arose via recombination within a progenitor of the SL12.3 cell or whether it was formed elsewhere and then infected the

TABLE 3. Analyses of recombinant unclassified provirus-containing clones from the SL12.3 cell line^a

Clone	LTR				<i>gag</i>		<i>pol</i>		gp70								p15E		
	P/K (bp)	K	192	JS-21	Eco, 16	X/E, 17	+/-	B/H	M-Poly		Poly		Xeno				Eco, 14	X/E, 15	
									JS-4	10	JS-5	12	JS-6/10	9	XEII	XEIII			XEIV
Class I recombinant-containing																			
2, 15, 79	500	-	-	+	-	-	+	-	-	-	+	+	-	-	-	-	-	+	-
48	500	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-
26, 27, 29, 33, 35, 70, 71, 76	500	-	-	+	+	-	+	-	-	-	+	+	-	-	-	-	-	+	-
Unclassified																			
10	500	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-
11	600	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
13, 62	550	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
14	500	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-
16	575	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
20	600	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41, 85	610	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
42, 61	600	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
45	580	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
49	500	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-
54	600	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
59	550	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
72	610	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
73, 75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
78	610	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

^a All the probes except for XEII to XEIV are described in the legend to Fig. 1. Probes XEII to XEIV are described in the legend to Fig. 2. For definitions of the headings in this table, see Table 1, footnotes *b* to *h*.

progenitor cell. Further work is needed to relate the amplification of this recombinant virus to transformation and/or tumorigenicity of the cell. Screening of the retroviral clones by using a battery of 15 oncogene probes failed to detect insertion into any proto-oncogenes (data not shown). However, the influence of retroviral insertion may, via enhancement or promotion, exert distal influences on cellular genes (45, 46, 61). Thus, the present report does not rule out the involvement of certain characterized oncogenes in the transformation and oncogenic potential of the SL12.3 cell line.

The finding that certain clones contained more than one provirus was surprising. Certainly, from a statistical standpoint, it would not be expected to occur with detectable frequency. We detected three such events in our analyses, all distinct from one another. Of the two that have been partially characterized (Fig. 4 and 5), it is interesting that

both appear to include a recombinant provirus integrated near an endogenous xenotropic provirus.

The majority of the clones contained relatively large deletions in various regions of the genome. In addition, it is likely that many proviruses that appeared complete by Southern analyses would contain small deletions, yielding them noninfectious. Deletion of an LTR may well have occurred during the cloning procedure, as described for one of the integrants of clone 55 (Fig. 4). However, it is likely that the internal deletions were already present within the viral genome. In addition, one-fourth of the clones did not hybridize with any class-specific *gp70* oligonucleotides (Table 3). Sequencing of one of these clones revealed a deletion of a large portion of the *gp70* and p15E *env* proteins. It is not known whether this species is transcriptionally active or whether any of the proviruses containing deletions

FIG. 4. Schematic map of clone 55, which contains two proviruses within 15.5 kb. The heavy line represents retrovirus DNA, and the thin line represents either flanking sequence or the vector. Deletions within the retroviral genome are represented by dotted lines. Approximate scale, in kilobases, is shown beneath the map.

FIG. 5. Southern blot analyses of the double integrated clone 86. Panels A and B represent duplicate blots probed as detailed below. (A) Southern blot analysis with ^{32}P -labeled probe (probe 12 in Fig. 1) hybridized under competitive conditions in the presence of unlabeled probe 10 (Fig. 1; see Materials and Methods). (B) Southern blot analysis with ^{32}P -labeled probe 10, hybridized under competitive conditions in the presence of unlabeled probe 12. DNAs were digested as follows: lanes 1, *Bam*HI-*Bgl*II; lane 2, *Bam*HI-*Hind*III; lane 3, *Bam*HI-*Pst*I; lane 4, *Bam*HI-*Pvu*II. Note that in each case, distinct bands hybridized with the two probes. These analyses, in conjunction with all other analyses (detailed in Results), indicate that the two proviruses represent a class I MCF recombinant virus integrated near an endogenous xenotropic element.

encode functional gene products. However, it has been noted that certain viral transcripts that arise from deleted proviruses can encode truncated *gag* proteins (51). It would be of value to establish whether expression of deleted proviruses has a detrimental effect unrelated to expression of complete infectious viruses that are also present. The finding that a virus which causes a severe immunodeficiency syndrome in mice is replication defective by virtue of a large deletion encompassing *pol* and *env* (the Du5H clone [2]) suggests that transcription of certain deleted proviral elements might have a profound influence on the state of the cell, without being infectious agents.

The approach taken in this study, i.e., to develop an extensive and representative collection of proviral elements from a highly tumorigenic line, was undertaken with the view that the main mode of transformation involves insertional mutagenesis, with resultant deregulation of endogenous genetic elements. However, recent findings in the Friend virus system offer an additional possibility, i.e., the direct involvement of viral gene products in transformation. It has long been recognized that the truncated glycoprotein, gp52, plays a role in the pathogenesis of Friend spleen focus-forming virus (36). Li et al. have demonstrated that the recombinant Friend spleen focus-forming virus gp52 glycoprotein can immortalize erythropoietin-dependent cells by interacting directly with the erythropoietin receptor (35). It could be envisioned that thymic leukemia might thus result from the uncontrolled or inappropriate interaction of viral glycoprotein(s) with the interleukin-2 receptor or another, yet to be described, cytokine receptor. The long-standing observation that gp70s are expressed as differentiation antigens in normal mice, independent of virus expression (10, 12, 31, 60), presents a scenario whereby deregulated expression of viral *env* genes could contribute to transformation, independent of an insertional mutagenesis mechanism.

Regardless of the ultimate mechanism of transformation, the repertoire of proviruses described here will serve as the basis for further examination of the involvement of retroviral sequences in transformation and tumorigenicity.

ACKNOWLEDGMENTS

We thank C. Holland and N. Hopkins for cloned MCF-247 virus used as probes in this study. We also thank C. MacLeod and K. Finley for providing the SL12.3 cell line and for helpful discussions. The technical assistance of K. Lovelace is gratefully acknowledged.

This study was supported in part by grants RO1 CA25533 (J.E.), RO1 CA25803 (J.E.), R35 CA44385 (J.M.C.), and RO1 CA33730 (M.C.W.) from the National Cancer Institute. W.N.F. is a Special Fellow of the Leukemia Society of America.

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