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Characterization of a Molecularly Cloned Retroviral Sequence Associated with *Fv-4* Resistance

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The murine leukemia virus (MuLV) sequence associated with the resistance allele of the *Fv-4* gene (*Fv-4^r*) was molecularly cloned from genomic DNA of uninfected mice carrying this allele. The 5.2-kilobase cloned *EcoRI* DNA fragment (pFv4) was shown by nucleotide sequencing to contain 3.4 kilobases of a colinear MuLV-related proviral sequence which began in the C-terminal end of the *pol* region and extended through the *env* region and the 3′ long terminal repeat. Cellular sequences flanked the 3′ as well as the 5′ ends of the truncated MuLV sequence. Alignment of the N-terminal half of the pFv4 *env* sequence with ecotropic, mink cell focus-forming, and xenotropic MuLV *env* sequences established the relatedness of pFv4 and ecotropic MuLV *env* sequences. A subcloned 700-base pair segment (pFv4$_{env}$) from the 5′ *env* region of pFv4 was used as an *Fv-4*-specific probe; it hybridized specifically to the *Fv-4^r*-associated proviral sequence but not to endogenous ecotropic MuLV proviral DNA under high stringency. All *Fv-4*-resistant mice contained the same retroviral segment associated with the same flanking cellular DNA. Expression of *Fv-4^r*-specific mRNA was demonstrated in the spleens of *Fv-4^r* mice but not *Fv-4^s* mice, supporting the previously proposed resistance model based on interference.

Retroviral infections and retrovirus-induced diseases are modulated by several host genes. Some of these genes (for example, *Fv-1* and *Fv-4*) control the susceptibility of target cells to viral replication, others (such as *Sl* and *W*) control the size of the target cell population, and others (*H-2*-linked genes such as *Rfv-1* and *Rfv-2*) control immunological reactions of host to viruses or virus-infected cells (43). The *Fv-4* gene, which controls susceptibility to infection by ecotropic murine leukemia viruses (MuLVs) (18, 41, 47), was first described in a G stain of laboratory mouse (now called FRG). Subsequently, *Fv-4*-like resistance has been identified in wild Asian mice (*Fv-4w^r*) (31, 32) and in wild California mice (*Akvr-1^R*) (10). Genetic studies have shown that resistance in these different mice is due to the same genetic locus on chromosome 12 (17, 30, 32, 42).

A viral interference model has been proposed as the mechanism of *Fv-4* restriction (15). A unique cell surface antigen immunologically related to major MuLV envelope glycoprotein gp70 has been identified in normal *Fv-4^r* mice (15, 48). This glycoprotein in serological assays exhibits characteristics of ecotropic MuLV gp70s (16). More recently, an MuLV *env* sequence present in mouse chromosomal DNA was linked to *Fv-4^r* resistance; this sequence was initially identified by reactivity with an ecotropic MuLV *env* probe (22). These results are consistent with the observation that *Fv-4* resistance affects ecotropic MuLVs but not other host range types of MuLVs (47; C. A. Kozak, unpublished data). The interference model suggests that the *Fv-4* gene product (gp70) competes with ecotropic MuLV for cell surface receptors. To further describe this proviral locus and its regulation, we have molecularly cloned and characterized the endogenous MuLV *env*-related sequence associated with *Fv-4* resistance.

MATERIALS AND METHODS

Mice. BALB/c and AKR/J mice were obtained from T. Odaka, Institute of Medical Science, University of Tokyo, Tokyo Japan, who also provided frozen livers from three partially *Fv-4*-congenic mice and FRG mice. Two of the congenic mice, BALB/c-*Fv-4w^r*(Fu) and BALB/c-*Fv-4w^r*(Hz), carry the resistance allele from wild mice (*Mus musculus molossinus*) originally trapped in different areas of Japan. Both mouse strains were inbred after six backcrosses to BALB/c (32; T. Odaka, unpublished data). A third congenic mouse strain, AKR-*Fv-4^r*, carries the *Fv-4^r* gene of the FRG strain (41) on an AKR/J background and was also inbred after backcross generation 6 (T. Odaka, unpublished data).

Preparation and analysis of DNA. DNA was extracted from liver or thymus as previously described (4). Restricted DNA was fractionated by electrophoresis on 0.6% agarose gels, and cleavage products were transferred to nitrocellulose membranes for Southern blotting (40). Membranes were hybridized with ^{32}P-labeled probes in the presence of 50% formamide–20% dextran-sulfate, as previously described by Thomas (44). Hybridization was carried out for 12 to 24 h at 42°C (low stringency) or 55°C (high stringency). The membranes were washed four times, for 5 min each time, at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS); 2 times, for 15 min each time, at 55°C in 0.1× SSC–0.1% SDS; and 4 times, for 5 to 20 min per time, at 55°C in 2× SSC.

Molecular cloning of the *Fv-4^r*-associated MuLV sequence. *Eco*RI-digested BALB/c-*Fv-4w^r*(Fu) liver genomic DNA was ligated to *Eco*RI-cleaved λgtWES arms (23), packaged in vitro, and propagated in *Escherichia coli* LE392. Cloned recombinant DNA was screened as previously described (3) with a ^{32}P-labeled ecotropic *env*-specific probe, pEc-B4 (4). A recombinant lambda phage (λFv4), containing a 5.2-kilobase (kb) pEc-B4-reactive *Eco*RI fragment, was subcloned into pBR322 and was designated pFv4.

DNA probes. Ecotropic MuLV gp70-related sequences were identified with the ecotropic *env*-specific pEc-B4 probe (4) derived from the DNA clone of λAKR623 (26). This probe specifically hybridized to endogenous ecotropic MuLV proviral DNAs at high stringency or to *Fv-4^r*-related

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sequences under relaxed conditions. The pFv4$_{env}$ probe, which specifically hybridized to *Fv-4*-associated MuLV sequences at high stringency, was a 0.7-kb *Bam*HI fragment, subcloned from the *env* region of clone pFv4 (see Fig. 1) and subsequently inserted into the *Bam*HI site of pBR322. Other MuLV probes used included a 1.9-kb *Bam*HI fragment derived from the *gag* region of AKV (28), a 1.4-kb *Xho*I-*Hpa*I fragment isolated from C-terminal half of the AKV *pol* region (13), a 0.9-kb *Bam*HI-*Xba*I fragment from the AKV gp70-p15(E) region (28), and a 0.6-kb *Kpn*I fragment containing long-terminal-repeat (LTR) sequences from cloned Harvey sarcoma proviral DNA (11, 28). Probes were labeled ([α-^{32}P]ATP) by the nick-translation procedure of Maniatis et al. (27).

Restriction enzymes, gel electrophoresis, nucleotide sequencing, and data analysis by computer. Restriction enzymes were purchased from New England Biolabs, Beverly, Mass., or Boehringer Mannheim Biochemicals, Indianapolis, Ind., and used as suggested by the manufacturers. Restriction fragments were separated in horizontal agarose gels (0.8 to 2.0%) and purified by the glass bead procedure (45).

T4 polynucleotide kinase and calf intestinal alkaline phosphatase were purchased from P.L. Biochemicals, Milwaukee, Wis. [γ-^{32}P]ATP (3,000 Ci/mmol) from Amersham Corp., Arlington Heights, Ill., was used for the kinase reaction. DNA was sequenced by the partial-degradation method of Maxam and Gilbert (29). The computer programs of Queen and Korn (34), Wilbur and Lipman (46), and Hopp and Woods (14) were used for translation to amino acids, identification of restriction sites, determination of sequence homology, and generation of the hydrophilicity profiles of the deduced amino acid sequences.

Cellular RNA isolation and Northern blots. Cellular RNA was isolated from cells by the guanidine thiocyanate method and was purified by ultracentrifugation onto a CsCl cushion (8, 38). RNA preparations were fractionated on 1% agarose gels in the presence of 2.2 M formaldehyde (24), transferred to nitrocellulose filters (44), and hybridized to labeled DNA probes in 50% formamide at 45°C as previously described (35). The filters were washed twice (10 min) in 2× SSC–0.1% SDS at 50°C, twice (15 min) in 0.1× SSC–0.1% SDS at 50°C, and twice (5 min) in 2× SSC at 50°C.

RESULTS

Cloning of pFv4. BALB/c and AKR/J mice contain 1 and 3 endogenous ecotropic MuLV loci (4, 6), respectively, that react with the ecotropic *env*-specific probe pEc-B4 (4). The single endogenous ecotropic provirus present in the BALB/c mouse genome is located within a 23-kb *Eco*RI fragment. *Eco*RI digestion of genomic DNA from BALB/c mice congenic for *Fv-4* generates the same 23-kb reactive fragment as well as an additional 5.2-kb fragment originally derived from the *Fv-4*^{r} parent (22). This 5.2-kb *Eco*RI fragment was cloned from the *Eco*RI-digested liver DNA of a BALB/c mouse congenic for *Fv-4* into λgtWES phages. Of the 1.3 × 10^6 recombinant phages screened, 2 contained a 5.2-kb insert that hybridized to the pEc-B4 probe. The insert present in one of these phage (λFv4A) was subsequently subcloned into the *Eco*RI site of pBR322 and designated pFv4.

Restriction mapping and alignment of pFv4 sequences. The restriction map of pFv4 is presented in Fig. 1 and compared with that of AKV623, a cloned ecotropic MuLV provirus (26). The positions of the *Pst*I, *Sma*I, and *Kpn*I sites in the LTR of AKV623 and of the single *Xba*I and *Bam*HI sites in the *env* gene at 3.7 and 2.8 map units, respectively, are identical to those present in pFv4 (Fig. 1). Identification of retroviral sequences present in pFv4 was determined by hybridizing a series of labeled MuLV probes (see above) to restricted preparations of pFv4 DNA. These experiments indicated that MuLV-related *pol*, *env*, and LTR sequences (located between map units 1.3 and 4.7 [Fig. 1]) were present in the pFv4 clone (data not shown), a result confirmed by nucleotide sequencing (see below).

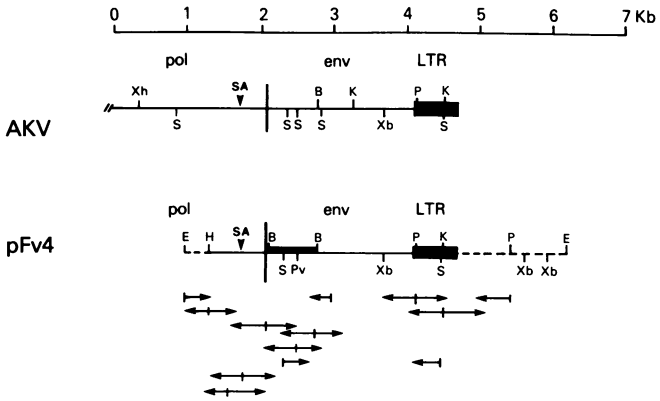

FIG. 1. Alignment of the cloned *Fv-4*^{r}-associated retroviral sequence pFv4 with the *pol*, *env*, and 3′ LTR regions of AKV proviral DNA. The 0.7-kb *Bam*HI fragment (heavy-lined areas from map positions 2.1 to 2.8) is the *env*-specific probe sequence (pFv4$_{env}$) subcloned from pFv4. Broken lines represent cellular DNA sequences. The nucleotide sequencing strategy is shown at the bottom. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I; S, *Sma*I; Xb, *Xba*I; SA, consensus splice acceptor sequence site.

The pFv4 *env* probe and its specificity. A number of reports have shown that different classes of MuLVs contain unique sequences in the N-terminal half of the gp70 coding region, whereas the C-terminal portion of gp70 as well as p15(E) is highly conserved (13, 19, 21, 25, 33, 36, 39). Aligned sequences show distinguishing differences between ecotropic and mink cell focus-forming (MCF) or xenotropic MuLVs in the 5′ portion of gp70 (32, 35), which account for the differential specificities of the ecotropic pEc-B4 (4) and the xenotropic and MCF MuLV-reactive pX$_{env}$ (3) probes. Differences between MCF and xenotropic gp70 MuLV sequences in the 5′ half of gp70 coding regions are not extensive; however, within this segment several short runs of heterology exist (33, 36). These differences may be analogous to the short variable domains in gp85 of avian retroviruses which have recently been shown to be responsible for subgroup specificity (9).

Comparison of the *env* sequences of ecotropic MuLVs with those of pFv4 indicated that significant variability occurred within a 0.7-kb pFv4 segment defined by *Bam*HI restriction sites (positions 70 to 820 [Fig. 2]; Fig. 1). This *Bam*HI fragment was subcloned into pBR322 and was designated pFv4$_{env}$. Genomic DNA from BALB/c-*Fv-4w*^{r}(Fu) mice, containing the endogenous ecotropic BALB/c provirus as well as the *Fv-4*^{r} determinant, was digested with *Pst*I and hybridized to either the pFv4$_{env}$ or the pEc-B4 ecotropic *env* probes. Under low-stringency conditions, the pEc-B4 probe hybridized more efficiently with the endogenous ecotropic proviral DNA of BALB/c mice (the 8.3-kb fragment) than with the *Fv-4*^{r}-associated (4.2-kb fragment) retroviral sequence (Fig. 3A, lane 1). The reverse was true when the


Fv-4
Akv Eco
NZB Xeno
Akv
Xeno
BamHI
NH2-gp70
Bgl II
Sma I
Pvu I
Eco RI

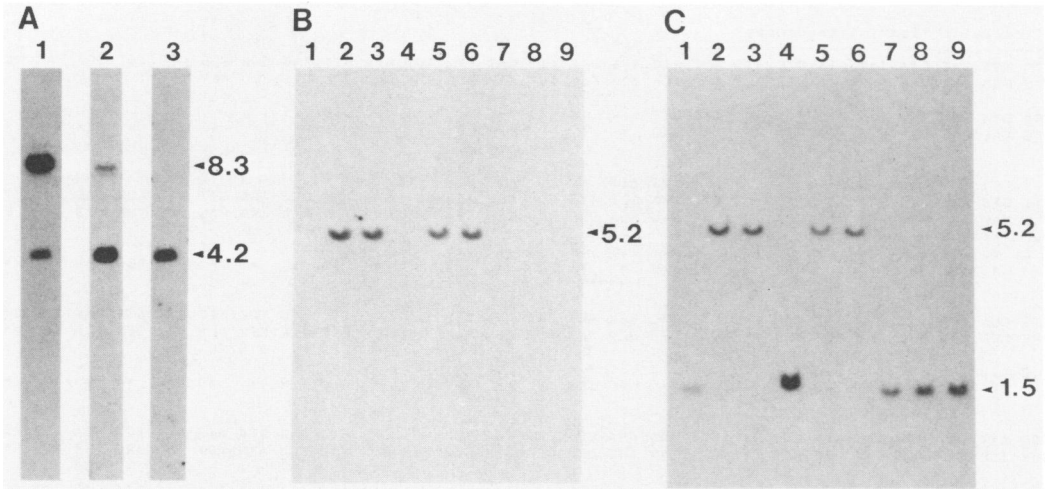

FIG. 3. Reactivity of MuLV *env* probes to *Fv-4*^{r} and *Fv-4*^{s} mouse DNAs. (A) BALB/c-*Fv-4w*^{r}(Fu) DNA was digested with *Pst*I, electrophoresed through 0.6% agarose, transferred to a nitrocellulose membrane, and hybridized to the pEc-B4 probe at 42°C (lane 1) or to the pFv4$_{env}$ probe at either 42 (lane 2) or 55°C (lane 3). The 8.3-kb fragment represents the BALB/c endogenous ecotropic provirus; the 4.2-kb fragment is the *Fv-4*^{r}-associated retroviral sequence. (B) *Eco*RI-digested genomic DNA from BALB/c (lane 1), BALB/c-*Fv-4w*^{r}(Fu) (lane 2), BALB/c-*Fv-4w*^{r}(Hz) (lane 3), AKR/J (lane 4), AKR-*Fv-4*^{r} (lane 5), FRG (*Fv-4*^{r}) (lane 6), C57BL/6 (lane 7), C3H/He (lane 8), and NFS (lane 9) strains was immobilized on filters as described above and hybridized to the ^{32}P-labeled pFv4$_{env}$ probe at 55°C. (C) The same filter as that for panel B was stripped (washed for 2 h at 68°C in 50% formamide–3× SSC to remove the first probe and rinsed for 30 min at room temperature in 2× SSC) and then rehybridized to the ^{32}P-labeled *Pst*I-*Eco*RI 3′ cellular flanking DNA probe (Fig. 1) at 55°C.

pFv4$_{env}$ probe was used (Fig. 3A, lane 2). At high stringency, the pFv4$_{env}$ probe reacted only with the 4.2-kb *Fv-4*^{r}-specific sequence (Fig. 3A, lane 3). Having determined that the pFv4$_{env}$ probe differentiated *Fv-4*-related *env* sequences from ecotropic MuLV *env* sequences, we then used this probe to identify *Fv-4*^{r} sequences in resistant mice. Labeled pFv4$_{env}$ DNA hybridized to a 5.2-kb fragment of *Eco*RI-digested genomic DNA from every *Fv-4*^{r} mouse tested; no reactivity with *Fv-4*^{s} (sensitive) mouse DNA was observed (Fig. 3B).

A second probe derived from pFv4 was a 700-base-pair (bp) *Pst*I-*Eco*RI fragment located in the 3′-flanking cellular DNA (between map units 5.5 and 6.2 [Fig. 1]). This probe was used to ascertain whether the *Fv-4*-related retroviral sequence was associated with the same 3′-flanking cellular DNA in all *Fv-4*^{r} mice. The 5.2-kb *Eco*RI segments in the DNA of *Fv-4*^{r} mice that hybridized to the pFv4$_{env}$ probe (Fig. 3B) also reacted with the 700-bp *Pst*I-*Eco*RI 3′-flanking, cellular-sequence probe (Fig. 3C). Both probes also hybridized to *Kpn*I, *Hin*dIII, *Pvu*II, or *Pst*I cleavage products of *Fv-4*^{r} genomic mouse DNAs as would be predicted from the map of pFv4 (Fig. 1; data not shown). Genomic DNA of *Fv-4*^{s} mice, which did not hybridize with the pFv4$_{env}$ probe (Fig. 3B), did hybridize with the 3′-flanking, cellular-sequence probe, as seen by the reactivity of a 1.5-kb *Eco*RI fragment in all five *Fv-4*^{s} mice tested (Fig. 3C). These data suggest that in all *Fv-4*^{r} mice examine, the *Fv-4*^{r}-associated retroviral sequences were inserted 5′ to the same flanking cellular sequence.

Nucleotide sequence of pFv4. Critical regions of pFv4 were sequenced to establish (i) the limits of the retrovirallike sequence and (ii) similarities or differences with known MuLV sequences. The strategy used to sequence a total of 3,900 nucleotides is shown in Fig. 1. The entire *Fv-4*^{r}-associated retroviral sequence with the exception of the midportion of *env* is shown in Fig. 2, 4, and 5. Analysis of the nucleotide sequence indicated that 3.4 kb of the 5.2-kb pFv4 insert represented MuLV-related sequences. The retroviral sequences included C-terminal *pol* nucleotides, the entire *env* gene, and 3′ LTR sequences all of which could be colinearly aligned with AKV ecotropic MuLV DNA as illustrated in Fig. 1. The nucleotides 5′ to the partial *pol* sequence and those 3′ to the LTR were not related to known MuLV proviruses and were presumed to be of cellular origin (Fig. 4 and 5).

C-terminal *pol* region. The first identifiable retroviral sequences occurred 250 bp from the 5′ *Eco*RI site in pFv4 and represented the last 850 bp of a MuLV *pol* gene (Fig. 4). This C-terminal *pol* sequence had 86% nucleotide and 92% deduced amino acid homology with AKV623 (13). The highly conserved MuLV *env* splice acceptor signal (13, 20, 33, 39), located in the *pol* region, was present in pFv4 275 nucleotides upstream from the beginning of *env* (positions 5508 through 5516 [Fig. 4]), the same position as in other MuLVs.

General features of the pFv4 *env* sequence. The nucleotide sequence of the N-terminal 1,200 bp of the *env* region (Fig. 2), as well as 255 bp of the C-terminal portion of *env* encoding a p15(E) analog (Fig. 5), was sequenced. Both segments of the pFv4 *env* region has a single open reading frame specifying amino acids which were homologous to

FIG. 2. Sequence of the amino-terminal portion of the pFv4 *env* region and its alignment with corresponding ecotropic and xenotropic MuLV sequences. The 5′ *env* sequence of pFv4 is compared with analogous sequences of ecotropic AKV623 (13, 25) and xenotropic NZB IU6 (33) MuLV sequences. Dots or asterisks, respectively, indicate nucleotide or deduced amino acid identities with pFv4. Nucleotide substitutions are shown. Gaps in sequences result from best-fit alignment and do not represent interruptions in the continuous sequence. Boxed amino acids indicate potential glycosylation sites. Recognition sites for several restriction enzymes are included for reference. Numbers shown above each group of the three aligned sequences refer only to the nucleotide position in this alignment.

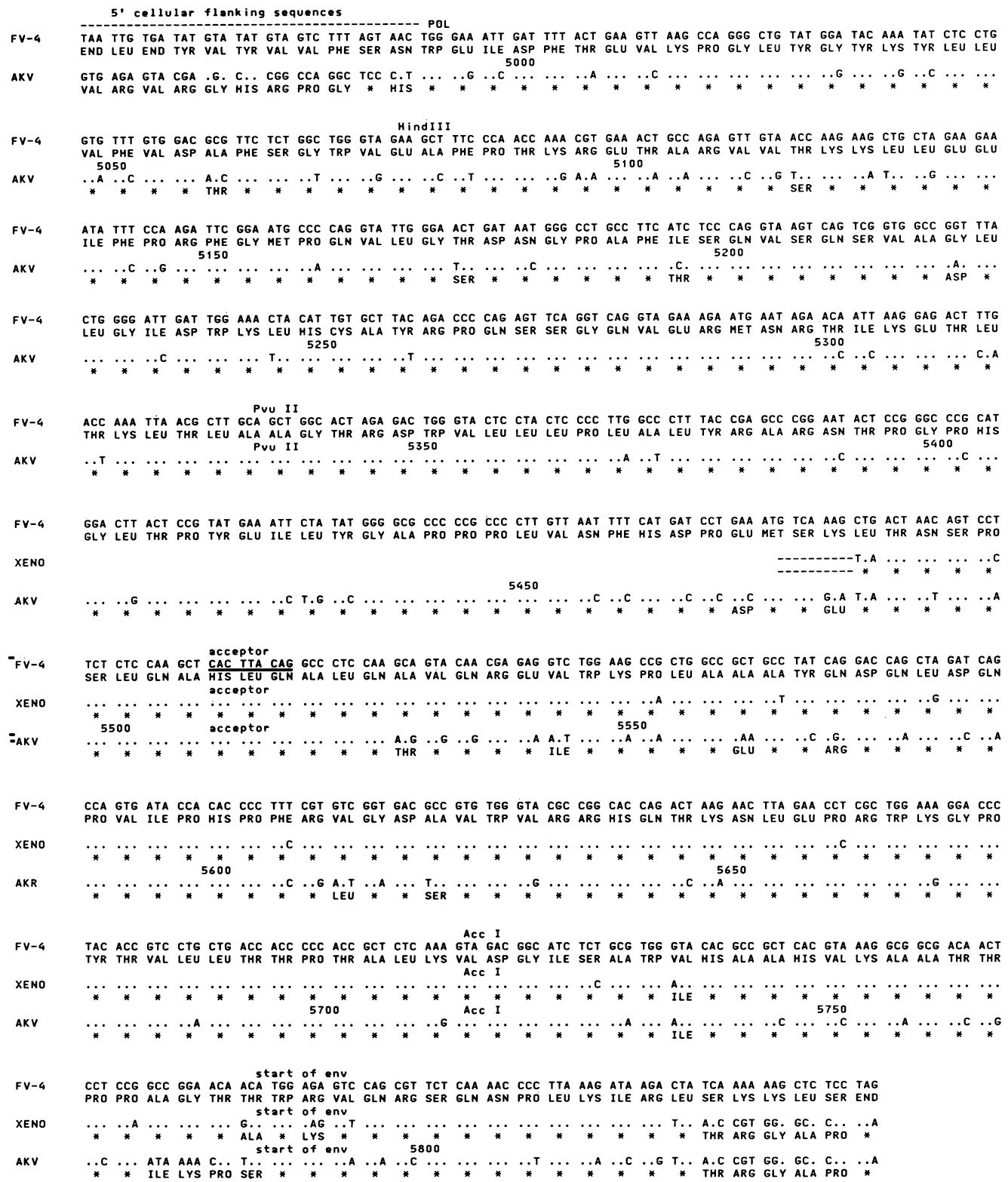

FIG. 4. Alignment of pFv4 sequences with the *pol* regions of ecotropic and xenotropic MuLV proviruses. Nucleotide and deduced amino acid retroviral sequences of pFv4 are aligned with corresponding sequences of AKV623 ecotropic (13, 25) and reported NZB IU6 xenotropic (33) MuLV sequences. Nucleotide and deduced amino acid identities are indicated by dots (.) and asterisks (*), respectively. Several restriction sites are identified for reference. The *env* splice acceptor sequence and the beginning of the overlapping *env* reading frame are indicated. AKV623 sequence numbering is by the method of Herr (13).

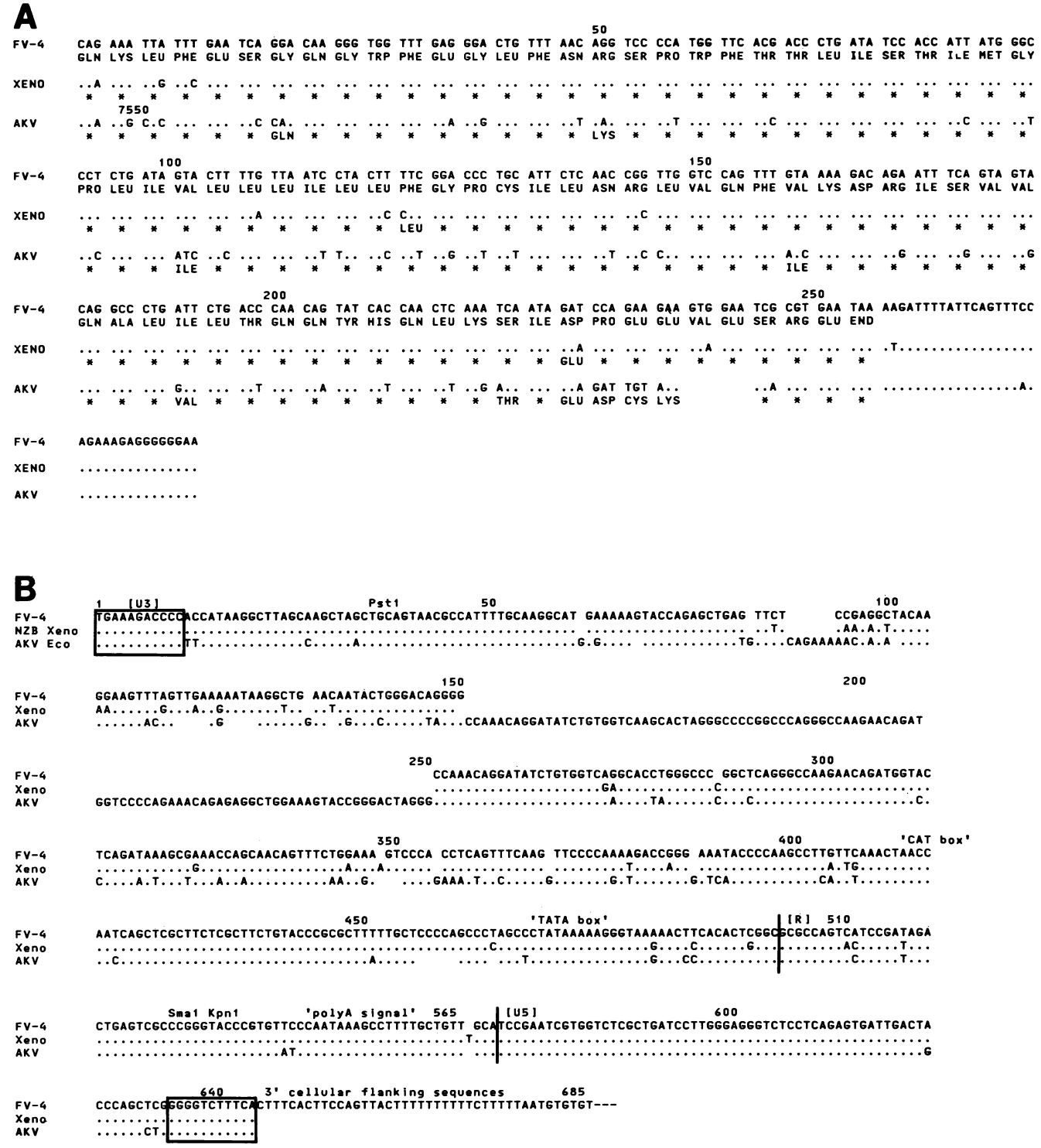

FIG. 5. Carboxy-terminal sequence of pFv4 *env* region and associated 3′ LTR and flanking cellular sequences. pFv4 retroviral sequences are aligned with corresponding AKV623 ecotropic (13, 25) and NZB IU6 xenotropic (33) MuLV sequences. Symbols for nucleotide or deduced amino acid identities are as indicated in the legend to Fig. 4. Gaps indicate alignment differences resulting from additional nucleotides in one sequence relative to the others. (A) Alignment of the carboxy-terminal p15(E) *env* region of pFv4 with AKV623 and NZB IU6 MuLV sequences. A reference nucleotide number for AKV623, calculated by the method of Herr (13), is given. (B) Alignment of pFv4-associated 3′ LTR sequence with those of AKV623 ecotropic and NZB IU6 xenotropic MuLV LTRs. The limits of U3, R, and U5 regions are indicated by vertical bars. The inverted terminal repeats are enclosed in boxes. The absence of directly repeated sequences in the U3 region of pFv4 and xenotropic LTRs is represented by the gap between positions 150 and 251. Smaller gaps indicate deletions in one aligned sequence relative to another. The numbering system used refers only to alignment positions. Characteristic LTR signal sequences and restriction sites are noted.

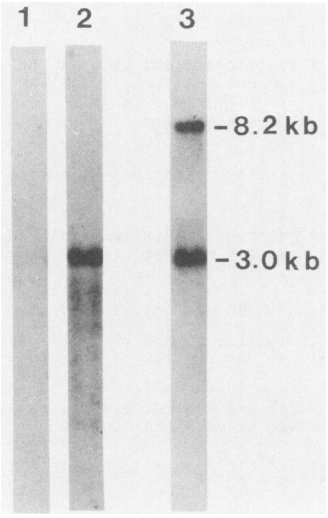

FIG. 6. Expression of *Fv-4*^{r}-associated retroviral RNA in mouse tissues. Total cell RNA (5 μg) was prepared from BALB/c spleen (lane 1), BALB/c-*Fv-4w*^{r}(Fu) spleen (lane 2), or an AKR thymic lymphoma (lane 3), electrophoresed in a 1% agarose gel containing formaldehyde, and hybridized, as described in the text, to ^{32}P-labeled pFv4$_{env}$ (lanes 1 and 2) or pEc-B4 (lane 3) DNA probe. The size markers indicate the positions of full-length and processed *env* RNAs.

ecotropic MuLV sequences. As is the case for other MuLVs, the pFv4 *env* sequence overlapped the 3′ terminus of *pol*. Alignment of the sequenced pFv4 gp70 and p15(E) regions with comparable segments of AKV623 proviral DNA indicated that the unsequenced portion of the pFv4 clone was identical in size (about 740 bp) to the corresponding AKV sequence. This suggested that a complete MuLV *env* gene was present in the pFv4 clone.

Characteristics of the N-terminal *env* region of pFv4. The N-terminal half of the MuLV *env* gene is a variable region in which characteristic differences distinguish ecotropic from MCF or xenotropic MuLVs. For example, a best-fit alignment of ecotropic and xenotropic *env* sequences generates a series of characteristic reciprocal gaps previously described by Repaske et al. (36). Xenotropic and MCF MuLVs, on the other hand, have similar patterns of insertions and deletions that clearly distinguish them from ecotropic MuLVs (33, 36). Alignment of the *env* sequence of pFv4 within the variable region fits the pattern of ecotropic and not xenotropic or MCV MuLV sequences (Fig. 2). In this region of pFv4, 73% of the nucleotides or deduced amino acids were shared with AKV. This value was comparable to the 70 to 75% conservation observed when the *env* regions of Moloney, Friend, and AKV ecotropic MuLVs were compared (13, 21, 25, 39). A similar comparison of xenotropic or MCF with ecotropic MuLV *env* sequences indicated only 30 to 40% amino acid identity. Similarly, 38% deduced amino acid homology existed between the pFv4 and MCF or xenotropic *env* sequences (19, 33, 36). Thus, by several criteria, the gp70-specific segment of pFv4 was clearly related to the ecotropic class of MuLVs. Although the N-terminal half of the gp70 coding region of the ecotropic MuLVs and the corresponding pFv4 sequences shared several structural features, the 0.7-kb *Bam*HI *env*-specific segment of the pFv4 could be used to differentiate the *Fv-4* resistance determinant from ecotropic proviral DNAs (Fig. 3).

Putative glycosylation sites are located in the same positions in both pFv4 and AKV623 with a single exception of one additional site in pFv4 at positions 1093 to 1101 (Fig. 2). Glycosylation of this additional site could account for the apparent higher molecular weight of the gp70 identified on membranes of *Fv-4*-resistant mouse cells (16).

Characteristics of the 3′ portion of p15(E). In the C-terminal portion of p15(E), pFv4 had 86% deduced amino acid sequence homology to ecotropic and MCF MuLVs and 98% homology to xenotropic MuLV (Fig. 5A). There were 50 nucleotide differences between pFv4 and AKV or MCF, whereas only 8 nucleotide differences existed between pFv4 and NZB xenotropic p15(E) sequences. At the 3′ end of the p15(E) coding region, both AKV623 and MCF247 have several amino acids deleted relative to NZB xenotropic MuLV (33) or to pFv4 sequences (Fig. 5A, positions 238 to 243).

pFv4 LTR sequence. A complete LTR containing U3, R, and U5 regions was also sequenced (Fig. 5B). Noncoding, nonretroviral sequences, presumably cellular flanking sequences, were found 3′ to the LTR (Fig. 5B). A notable feature of the flanking sequence was a 15-bp polythymidylate tract interrupted by a single C. The polyadenylate complement on the opposite strand terminated a short (132-bp) open reading frame (data not shown). With regard to the LTR, all of the characteristic landmarks were present including a 11-bp inverted repeat, a TATA box, a CCAAT box, and a polyadenylation signal. A single copy of a direct-repeat element (positions 251 to 357 [Fig. 5B]) similar to that found in NZB xenotropic (33) and MCF247 MuLVs (19) was present in the pFv4 LTR. Alignment of pFv4 LTR sequences with those of NZB xenotropic (33), MCF247 (19), and AKV ecotropic MuLVs (13, 25) indicated that the pFv4 sequence similarity with the xenotropic p15(E) region extended into the LTR. The pFv4 LTR differed from the NZB xenotropic and AKV623 ecotropic LTRs by 28 and 88 nucleotides, respectively. Furthermore, a five-nucleotide insertion characteristic of xenotropic MuLVs (33) was also identified in the pFv4 LTR (Fig. 5B, positions 457 to 461). Individual nucleotide insertions or deletions found in the xenotropic MuLV LTR sequences were conserved in the pFv4 LTR.

Analysis of mRNA expression. To ascertain whether unique species of RNA were present in *Fv-4*-resistant mice, total RNA was purified from spleens of BALB/c-*Fv-4w*^{r}(Fu) and BALB/c (*Fv-4*^{s}) mice and analyzed by Northern blot hybridization with the pFv4$_{env}$ probe. Labeled pFv4$_{env}$ DNA reacted strongly with a 3.0-kb RNA species from spleen of BALB/c-*Fv-4w*^{r}(Fu) mice (Fig. 6, lane 2), but not with RNA from a normal BALB/c (*Fv-4*^{s}) mouse (Fig. 6, lane 1). The 3.0-kb *env* message expressed in BALB/c-*Fv-4w*^{r} spleen reacted with an LTR probe but not with an MuLV *pol* probe (data not shown). RNA isolated from thymic tumors of AKR/J mice was analyzed in the same gel and hybridized with the pEc-B4 probe to show the sizes of full-length (8.2-kb) and *env* (3.0-kb) mRNAs transcribed from a typical ecotropic provirus (Fig. 6, lane 3).

DISCUSSION

We have molecularly cloned the segment of mouse genomic DNA that is associated with *Fv-4* resistance. The cloned DNA contains a truncated proviral sequence containing a portion of the *pol* gene, the entire MuLV *env* region, and the 3′ LTR; it is flanked at its termini by cellular sequences (Fig. 2, 4, and 5). Classification of the *Fv-4*^{r} *env* sequence as ecotropic was based on (i) the nucleotide and

deduced amino acid homologies of 73 and 73%, respectively, with the analogous segment of ecotropic MuLV *env* genes, (ii) the pattern (ecotropic and xenotropic) of MuLV *env* gene nucleotide insertions and deletions which correspond to ecotropic rather than xenotropic gp70 sequences (36; Fig. 2), and (iii) the conservation of the number and position of potential glycosylation sites present in ecotropic MuLV *env* regions (13, 21, 25, 39).

While these data indicate that the *Fv-4*^{r} retroviral segment is closely related to ecotropic MuLVs derived from inbred mice, this segment also contained restriction enzyme cleavage sites previously recognized only in ecotropic MuLVs isolated from wild mice. For example, a *Hin*dIII site located at 1.3 maps units (Fig. 1) in pFv4 was conserved in almost all wild-mouse ecotropic MuLVs and was never found in ecotropic MuLVs derived from inbred mice (5, 7). The *Pvu*I site of pFv4 at 2.5 map units has been observed only at this position in the Castaneus-E wild-mouse ecotropic MuLV (7). Conservation of these specific restriction sites suggests that pFv4 is evolutionarily related to wild-mouse ecotropic MuLVs, which is consistent with the identification of *Fv-4*-like resistance determinants in wild Asiatic (31, 32) and California (10) mice.

Nucleotide sequencing revealed the existence of two unique and potentially significant differences between the gp70 coding sequences of pFv4 and ecotropic MuLVs. A region of amino acid heterology including a six-amino-acid insertion was present from positions 994 to 1044 (Fig. 2); in addition, a novel potential glycosylation site occurred at positions 1093 to 1101 (Fig. 2). The six-amino-acid insertion results in a peak of hydrophilicity (14) unique to *Fv-4*. This difference in an otherwise ecotropiclike gp70 sequence could be responsible for the unique antigenicity of the *Fv-4*^{r} gp70 (16). The second major differences, an additional glycosylation site, could explain the slightly higher molecular weight of the *Fv-4*^{r} gp70 (15, 16) if this site is glycosylated.

As noted above, sequence analysis of the pFv4 retroviral segment indicated that it had the structure of a truncated proviral since it was lacking approximately 5.4 kb of MuLV-related sequences. The presence of 250 bp of nonretroviral sequences that abutted the shortened MuLV *pol* region of the pFv4 insert raised the possibility that a stretch of cellular DNA had been inserted within a full-length *Fv-4* provirus, thereby displacing viral DNA sequences in the 5′ direction. In search of the missing retroviral DNA, we carried out two experiments. In one, *Fv-4*^{r} cellular DNA was cleaved with *Kpn*I, an enzyme known to cleave within the U5 portion of MuLV LTRs including the proviral DNAs of wild-mouse MuLVs (7). In this regard, the sequencing of the pFv4 segment revealed the presence of a *Kpn*I site at its usual location in the 3′ LTR. After restriction of *Fv-4*^{r} cellular DNA with *Kpn*I and transfer to a nitrocellulose membrane, a single 15-kb pFv4$_{env}$-reactive fragment was detected by Southern blot hybridization. In a second experiment, a 17-kb partial *Mbo*I cleavage product of *Fv-4*^{r} cellular DNA was molecularly cloned in a lambda phage vector by using the pFv4$_{env}$ probe to screen recombinant plaques (data not shown). The cloned DNA segment contained approximately 12 kb of cellular DNA located upstream from the *Fv-4*^{r}-associated retroviral sequences. This cellular DNA segment failed to hybridize to an MuLV LTR probe. Taken together, the results of both experiments are consistent with the absence of an LTR element within 12 kb of the 5′ terminus of the *Fv-4*-associated retroviral sequences.

The detection in *Fv-4*^{r} animals of a 3.0-kb RNA species which hybridized to the pFv4$_{env}$ probe and comigrated with the 3.0-kb *env* mRNA present in an AKR thymoma (Fig. 6) is good evidence that the *Fv-4*^{r}-associated retroviral DNA is located near a strong promoter that functions in conjunction with the pFv4 splice acceptor (Fig. 4) to generate a normal-length *env* RNA. It has previously been shown that MuLV *env* RNAs hybridize to U3- and U5-specific probes as well as with labeled MuLV *env* DNA (2). The U_5-reactive segment is transcribed from the 5′ LTR and represents retroviral RNA sequences that are spliced to *env* sequences during the processing of *env* mRNA. The 3.0-kb *Fv-4*^{r} mRNA, while hybridizing to *env*- and U3-specific probes, fails to react with an MuLV U5 LTR probe (F. Laigret and H. Ikeda, manuscript in preparation), indicating, again, that this *Fv-4*^{r} mRNA is not initiated from within a 5′ LTR.

Fv-4 may function in a way similar to that of the chicken loci *ev3*, *ev6*, and *ev9*, all of which express high levels of avian retroviral *env* glycoprotein (37) and restrict susceptibility to exogenous viruses of the same subgroup. These loci represent endogenous defective proviruses; *ev3* has a large deletion in the junction of *gag* and *pol*, *ev6* lacks a 5′ LTR and *gag* region, and *ev9* has an undetermined defect (1, 12). Since both *ev6* and *Fv-4* lack a 5′ LTR, it is quite likely that the expression of *env* gene products is under the control of a cellular promoter.

A viral interference model has been proposed for restriction of ecotropic MuLV infection in *Fv-4*^{r} mice. This concept has been supported by the detection of an *Fv-4*^{r}-associated gp70 having antigenic characteristics different from those of typical ecotropic MuLV gp70s from inbred laboratory mice. Data presented in this paper show that the sequence associated with resistance in *Fv-4*^{r} mice contained a specific *env* sequence related to an ecotropic MuLV *env* sequence. The *Fv-4*^{r}-associated *env* gene had overall characteristics which permitted its gp70 to be classified as an ecotropic type, but sufficient sequence differences existed in one region to account for a potential antigenic differences and an additional glycosylation site which could account for the larger apparent molecular weight of gp70 (see above). RNA studies showed that the *Fv-4*^{r}-associated *env* gene sequence is expressed. All of the data were compatible with *Fv-4* resistance based on the expression of an ecotropic MuLV-related gp70 which competes with the *env* glycoproteins of ecotropic MuLVs for a cell-specific receptor(s), thereby inhibiting exogenous viral infection. Additional studies on the promoter region of *Fv-4* as well as adjacent cellular sequences are in progress.

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