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Nucleotide Sequence of the *env*-Specific Segment of NFS-Th-1 Xenotropic Murine Leukemia Virus

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The sequence of 863 contiguous nucleotides encompassing portions of the *pol* and *env* genes of NFS-Th-1 xenotropic proviral DNA was determined. This region of the xenotropic murine leukemia virus genome contains an *env*-specific segment that hybridizes exclusively to xenotropic and mink cell focus-forming but not to ecotropic proviral DNAs (C. E. Buckler et al., J. Virol. **41**:228–236, 1982). The unique xenotropic *env* segment contained several characteristic deletions and insertions relative to the analogous region in AKR and Moloney ecotropic murine leukemia viruses. Portions of an endogenous *env* segment cloned from a BALB/c mouse embryo gene library that had a restriction map and hybridization properties typical of xenotropic viruses (A. S. Khan et al., J. Virol. **44**:625–636, 1982) were also sequenced. The sequence of the endogenous *env* gene was very similar to the comparable region of the NFS-Th-1 xenotropic virus containing the characteristic deletions and insertions previously observed and could represent a segment of an endogenous xenotropic provirus.

The chromosomal DNA of inbred mice contains multiple copies of murine leukemia virus (MuLV)-reactive sequences (8, 27, 28). Some of these type C proviral DNAs encode infectious ecotropic or xenotropic MuLVs (24), some are expressed only in the form of viral antigens (10, 20, 29), some may contribute portions of their envelope (*env*) genes during the formation of mink cell focus-forming (MCF) MuLVs (6, 16), and others contain large deletions and may not be expressed at all (16, 22). Evaluation of the molecular organization of these different endogenous MuLV proviruses and their integration sites in cellular DNA by nucleic acid hybridization techniques has been hampered by the extensive cross-reactivity of different MuLV types. To overcome some of these problems, we constructed a recombinant plasmid (pEc_{env}), previously designated pEc-B4, that contains sequences specific for the *env* gene of ecotropic MuLVs (5). This cloned 545-base-pair (bp) *Bgl*II-*Bam*HI *env* segment maps 221 bp from the 5′ terminus of the envelope gene of the Akv MuLV and partially overlaps a 400 bp *Sma*I fragment that also specifically anneals to ecotropic proviral DNAs (7). Radiolabeled pEc_{env} DNA has been used to quantitate the number of ecotropic proviruses in different inbred mouse strains and to evaluate the stability of ecotropic proviral DNA in AKR sublines (4, 5). More recently, we described the molecular cloning of an *env*-specific DNA fragment (pX_{env}) derived from NFS-Th-1 xenotropic MuLV that hybridizes to xenotropic and MCF proviruses but not to ecotropic proviral DNA (3, 16). Restriction enzyme mapping studies and hybridization experiments indicate that the *env*-specific segments present in pEc_{env} and pX_{env} DNAs are located in analogous regions of the *env* genes from which they were derived.

Since the structure of the envelope glycoprotein plays a major role in determining the tissue tropism and host range of MuLVs (1, 9, 18), we determined the nucleotide and deduced amino acid sequences of the xenotropic *env*-specific segment and compared them with the published sequences for Moloney (Mo) and Akv MuLVs. Our analysis indicated that although portions of the xenotropic *env*-specific segment were similar to the analogous region of ecotropic proviral DNA, this part of the xenotropic *env* gene contained multiple deletions and insertions relative to the ecotropic *env* DNA that explain its unique hybridization properties and could account for the biological characteristics of xenotropic MuLVs. The nucleotide sequence of a portion of the *env* region of an endogenous BALB/c MuLV provirus was also determined. It contained all of the deletions and insertions characteristic of the NFS-Th-1 xenotropic *env* gene and therefore could represent a portion of a potentially infectious xenotropic provirus in BALB/c chromosomal DNA.

(This work was performed in partial fulfilment

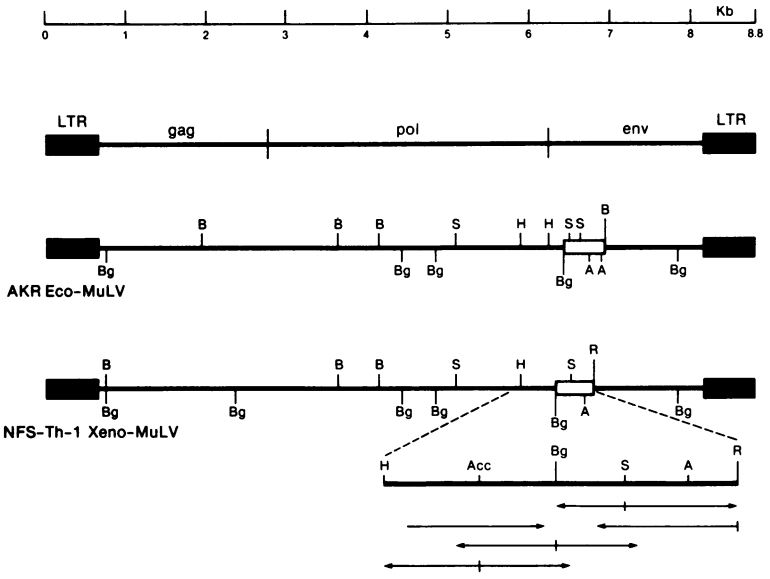

FIG. 1. Diagrammatic representation of NFS-Th-1 xenotropic and AKR ecotropic MuLV proviruses, showing the location of xenotropic and ecotropic *env*-specific segments. The construction and characterization of recombinant plasmids containing unique portions of the ecotropic and xenotropic *env* regions have been previously reported (3, 5). The strategy used for sequencing parts of the *pol* and *env* genes of NFS-Th-1 xenotropic proviral DNA is shown within dashed lines at the bottom. Abbreviations: A, *Ava*II; Acc, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hpa*I; R, *Eco*RI; S, *Sma*I.

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MATERIALS AND METHODS

Cloned retroviral DNAs used for nucleotide sequencing studies. Two recombinant plasmids that contained segments of NFS-Th-1 xenotropic proviral DNA were used for sequencing the xenotropic *env*-specific region; their construction has been previously described (3). One of the plasmids ($pXen_{5'\text{-}6.7}$) contained an 8.9-kilobase (kb) DNA insert that encompassed 6.7 kb of the 5' end of the xenotropic provirus and 2.2 kb of flanking cellular DNA. The second plasmid (pX_{env}), derived from the first, contained the 455-bp *Bgl*II-*Eco*RI *env*-specific segment of NFS-Th-1 xenotropic proviral DNA. The sequence of a portion of an endogenous MuLV *env* gene was determined from a 0.6-kb DNA segment contained within clone B-77 isolated from a BALB/c embryo gene library (16). Like other xenotropic MuLVs, the B-77 envelope segment hybridized to the xenotropic *env*-specific probe and not to the unique ecotropic *env* fragment.

DNA sequencing. DNA was sequenced by the partial degradation method of Maxam and Gilbert (19). Nucleotide sequence analyses were made with the computer program of Queen and Korn (21).

Reagents. Restriction enzymes were purchased from New England Biolabs, Beverly, Mass., Boehringer Mannheim, Indianapolis, Ind., or Bethesda Research Laboratories, Bethesda, Md., and used as described by the suppliers. T4 polynucleotide kinase and calf intestine alkaline phosphatase were obtained from PL Biochemicals, Inc., Milwaukee, Wis. [γ-^{32}P]ATP (3,000 mCi/mmol) was purchased from Amersham Corp. Arlington Heights, Ill.

RESULTS AND DISCUSSION

Alignment of AKR ecotropic and NFS xenotropic proviral DNAs. Because of the extensive polynucleotide sequence homology involving large portions of the genomes of different types of MuLV, we constructed recombinant plasmids for use in hybridization experiments that contained segments specific for ecotropic and xenotropic *env* regions (3, 5). The ecotropic *env* segment consisted of the 545-bp *Bgl*II-*Bam*HI fragment, which maps 6.4 to 7.0 kb from the 5' terminus of AKR ecotropic proviral DNA (Fig. 1). The 455-bp *Bgl*II-*Eco*RI xenotropic *env*-specific segment maps to an analogous region (6.3 to 6.7 kb) of the NFS-Th-1 xenotropic provirus (3). Both of these segments have been used as hybridization probes to evaluate the molecular organization of endogenous MuLV proviral DNAs (4, 13). In view of the importance of the *env* gene in determining host range, the unique *env* segment of xenotropic proviral DNA was sequenced and compared with analogous regions of Mo and AKR proviruses.

Since we had previously ascertained that the xenotropic *env*-specific fragment did not hybridize to ecotropic proviral DNA under stringent hybridization conditions (3), it become important to establish precisely the region of heterolo-

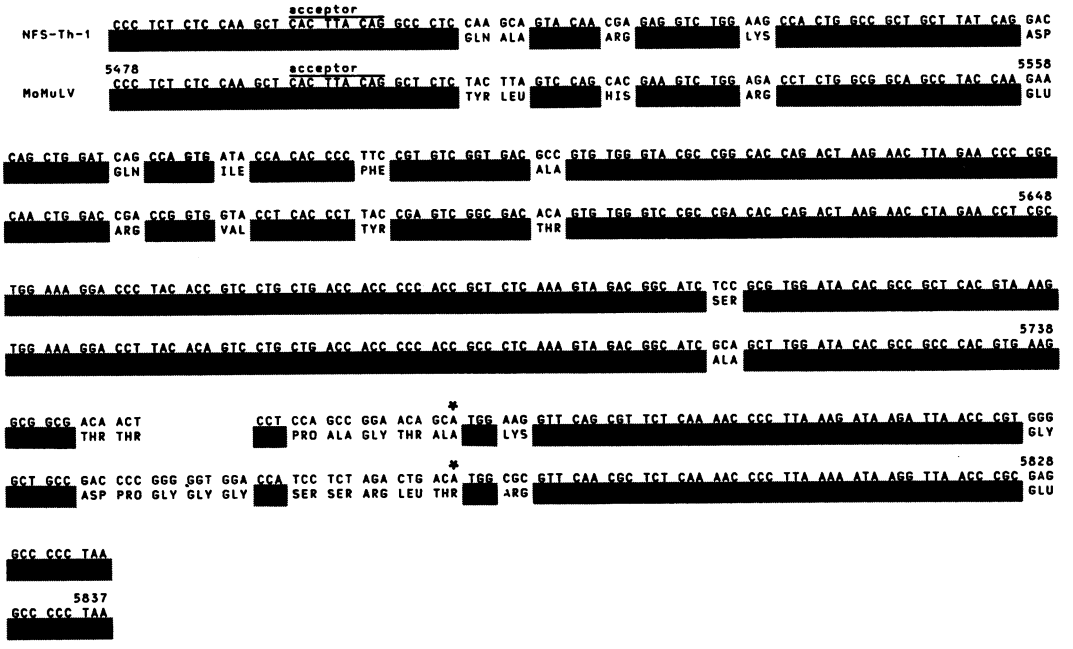
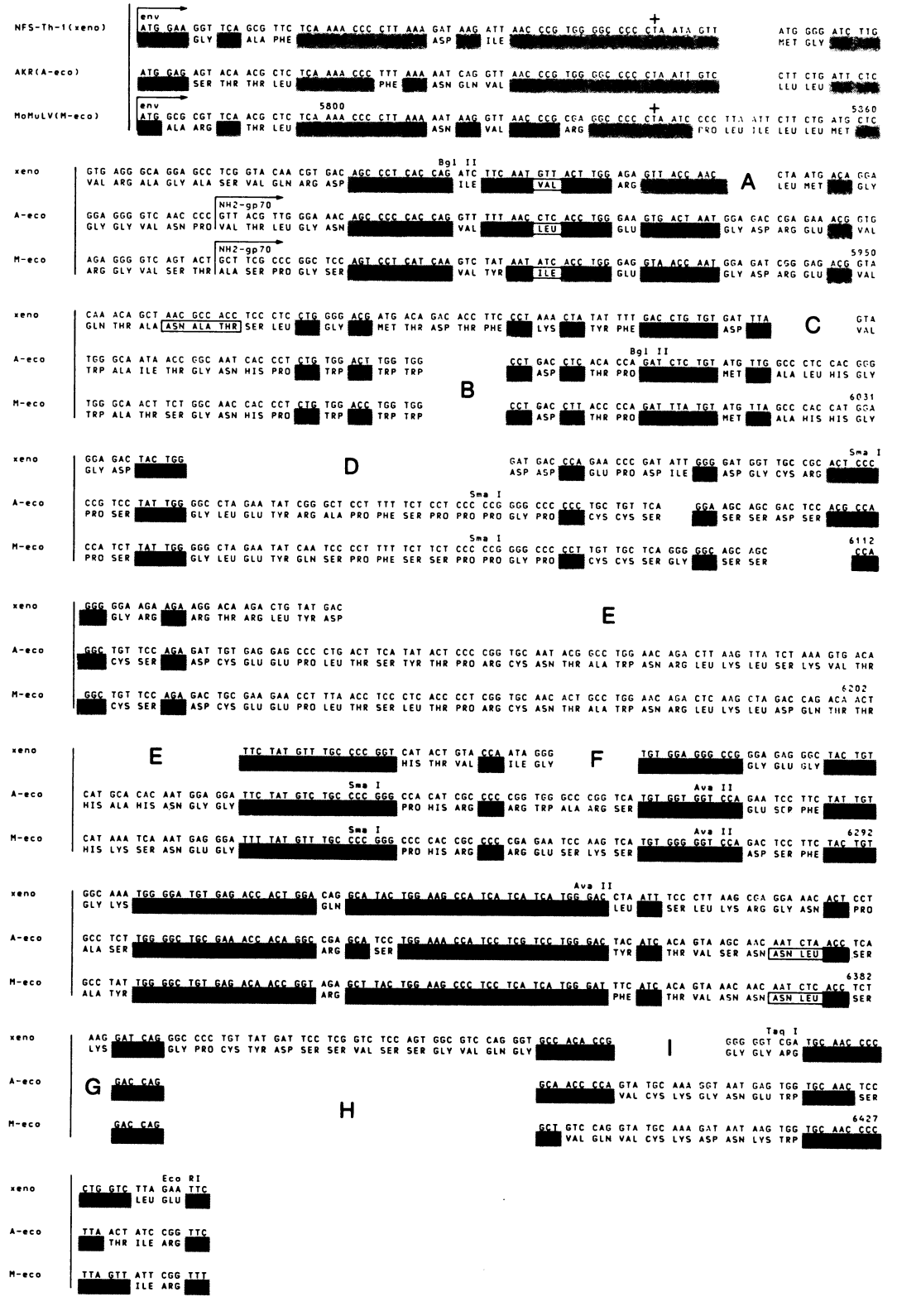


FIG. 2. Comparison of *pol* gene sequences in NFS-Th-1 xenotropic (top line) and Mo MuLV proviral (bottom line) DNAs. The sequence of 351 nucleotides extending in the 3′ direction from the *Hpa*I site located approximately 5.9 kb from the 5′ terminus of NFS-Th-1 xenotropic proviral DNA (see Fig. 1) was determined and aligned with the analogous region of Mo MuLV. Homologous deduced amino acids are shaded, and the *env* splice acceptor sequence is indicated. The asterisk marks the position of the initiator codon of the *env* precursor polypeptide, which is translated in a different reading frame (see Fig. 3). The numbers above the Mo MuLV sequence are identical to those used to Shinnick et al. (26).

gy and to determine whether the two *env* genes had any polynucleotide sequence homology. The strategy for sequencing the cloned xenotropic proviral DNA is shown in Fig. 1. Nucleotide sequencing was initiated at the *Hpa*I site (5.9 kb) located in the *pol* region of the NSF-Th-1 xenotropic MuLV provirus, which is positioned approximately 360 bp from the 5′ amino terminus of the *env* gene. As anticipated, this 3′ portion of the xenotropic MuLV *pol* gene was 80% homologous with the analogous segment of Mo MuLV (Fig. 2). Nonhomologous nucleotides frequently occurred as third-base changes, which resulted in 84% homology for the deduced amino acids. The most striking example of conservation involved the 3′ terminus of the xenotropic and ecotropic *pol* genes, in which 16 of the 17 final amino acids and the last three codons, including the TAA terminator, were identical. In this segment of Mo MuLV (and, by analogy, NFS-Th-1 xenotropic MuLV), the *pol* and *env* genes overlap one another (26). The nearly identical structure of the two proviral DNAs within the reading frame encoding the *pol* gene product aligned the two proviruses.

Sequencing of the *env*-specific segment of NFS-Th-1 xenotropic proviral DNA. The sequence of the first 573 nucleotides of the NFS-Th-1 xenotropic *env* gene could be aligned with the initial 666 nucleotides of the analogous segments of Mo

FIG. 3. Sequence of the 5′ portion of the xenotropic NFS-Th-1 *env* region and its alignment with the *env* genes of AKR and Mo ecotropic MuLVs. The sequence of 573 nucleotides extending from the 5′ terminus of the *env* gene of NFS-Th-1 xenotropic MuLV to the *Eco*RI site located at 6.7 kb was determined as described in the legend to Fig. 1 and compared with the comparable regions of two ecotropic MuLVs. The ecotropic *env*-specific segments of Akv extends from the *Bgl*II site at nucleotide 6,004 to the *Bam*HI site at 6,542; the xenotropic *env*-specific fragment is located between the *Bgl*II site at 5,901 and the *Eco*RI site at 6,442. Homologous deduced amino acids are shaded, and the 3′ terminus of the overlapping *pol* gene is indicated (+). Letters A through I indicate deletions or insertions in the xenotropic *env* sequence relative to the ecotropic *env* sequences. The amino termini of AKR and Mo MuLV gp70s and potential glycosylation sites (outlined tripeptides) in all three proviral DNAs are shown. Numbers refer to nucleotide number in the Mo MuLV genome (26).


NFS-Th-1(xeno)
AKR(A-eco)
MoMuLV(M-eco)
env
5800
5360
xeno
A-eco
M-eco
Bgl II
NH2-gp70
A
B
C
D
E
F
G
H
I
Sma I
Ava II
Taq I
Eco RI
6031
5950
6112
6202
6292
6382
6427

and Akv ecotropic MuLVs (17, 26) (Fig. 3). The difference in the size (93 nucleotides) of comparable regions between the xenotropic and ecotropic *env* genes was due to several small and two larger deletions of 36 and 78 nucleotides (Fig. 3, D and E) in the xenotropic proviral DNA. Conversely, the xenotropic *env*-specific region contained two small insertions and a larger 42-bp insertion (Fig. 3, H) relative to the two ecotropic *env* genes. The xenotropic and ecotropic *env*-specific segments shared two discrete regions of polynucleotide sequence homology. The first involved sequences encoding amino acids near the amino terminus of gp70 (corresponding to Mo MuLV nucleotides 5,891 to 5,932 [26]), in which 29 of 42 nucleotides and 11 of 14 amino acids were identical. The second occurred between Mo MuLV nucleotides 6,299 and 6,352, encompassing a stretch of 54 nucleotides, of which 48 were identical to those present in either Akv or Mo MuLV.

The xenotropic *env* gene sequence shown in Fig. 3 had a single open reading frame. The amino terminus of Mo MuLV gp70 was previously positioned at nucleotide 5,876, 100 bp downstream from the beginning of the *env* gene (26). By analogy, the amino terminus of Akv gp70 was located at the same position, although the amino acid at position 1 (valine) (17) is different from the initial amino acid (alanine) of the Mo MuLV gp70 (Fig. 3). Since the amino acid sequence of the NFS-Th-1 xenotropic MuLV gp70 has not been determined, its location within the deduced *env* precursor polypeptide can only be speculated on. If the amino terminus of the xenotropic MuLV gp70 is located at precisely the same position within the *env* gene as it is in the Akv and Mo MuLV gp70s, then the first amino acid would be serine (Fig. 3). However, this region of the xenotropic *env* gene shared little polynucleotide sequence homology with either of the two ecotropic *env* segments. A shift in the alignment of the xenotropic *env* sequence in this region by one codon in the 3′ or 5′ direction would result in a gp70 with an amino-terminal amino acid identical to Mo MuLV (alanine) or Akv MuLV (valine).

As pointed out previously, the 5′-terminal portion of the xenotropic *env* gene was highly conserved relative to the analogous segments of Mo and Akv MuLVs, particularly in the region of overlap with the *pol* gene. The direction of the shift from the *pol* to the *env* reading frame was identical. This part of the MuLV *env* gene encodes a hydrophobic leader sequence (17, 26) that is removed during the maturation of gp70 (11, 15). This portion of the NFS-Th-1 xenotropic *env* region also contains uncharged, mostly hydrophobic amino acids that are probably components of an envelope precursor. MuLV envelope glycoproteins are translated from spliced mRNAs (12, 23). A consensus 3′ acceptor sequence (25) was identified 275 nucleotides upstream from the 5′ terminus of the xenotropic *env* gene, at a location and with a nucleotide sequence identical to that present in Mo MuLV (Fig. 2).

Within the sequence shown (Fig. 3), the *env* regions of both Mo and Akv MuLVs encoded two potential glycosylation sites. One of these (between Mo MuLV nucletides 5,909 and 5,917) was also present in the xenotropic *env* gene. A second potential glycosylation site in the xenotropic *env* region with no counterpart in the ecotropic gp70 was located between nucleotides 5,960 and 5,968 (Fig. 3).

As pointed out above, the xenotropic *env* gene contains several insertions and deletions relative to the analogous segment of the ecotropic genome, which may be responsible for the unique biological properties of each MuLV type. Segment D (Fig. 3), which is missing from xenotropic MuLVs, is particularly rich in proline residues. Its absence would certainly affect the secondary structure of this portion of the xenotropic gp70 molecule. The base substitutions found in the xenotropic *env* gene that generate regions of polynucleotide heterology could have a less obvious effect on the structure of gp70 than would deletions or insertions. For example, both ecotropic and xenotropic *env* gene products have a peak of hydrophilicity (average indices of 1.02 and 1.34, respectively, calculated as described by Hopp and Woods [14]) in the region just 5′ to E (Fig. 3). Unexpectedly, an adjacent region of the xenotropic gp70 was also extremely hydrophilic (an average index of 0.94), in the area (between nucleotides 6,032 and 6,094) in which the ecotropic *env* protein had an index of −0.07. Assuming that this portion of the xenotropic envelope glycoprotein, like other strongly hydrophilic regions, is located on the surface of the gp70 molecule, this polypeptide segment could determine some of the characteristic properties of xenotropic MuLVs.

The recently published sequence of the *env* gene of BALB/c Mo MCF MuLV (2) is remarkably similar to the *env* segment of NFS-Th-1 xenotropic proviral DNA shown in Fig. 3. A comparison of both the nucleotide and derived amino acid sequences shows that homology was greater than 85%. Without exception, the MCF sequence also had the same insertions and deletions identified in the xenotropic *env* segment relative to the ecotropic MuLV *env* region (Fig. 3, A through I). In addition, the two putative glycosylation sites in the NFS-Th-1 xenotropic *env* gene were located in the same positions in the MCF sequence; the second site (nucleotides 5,960

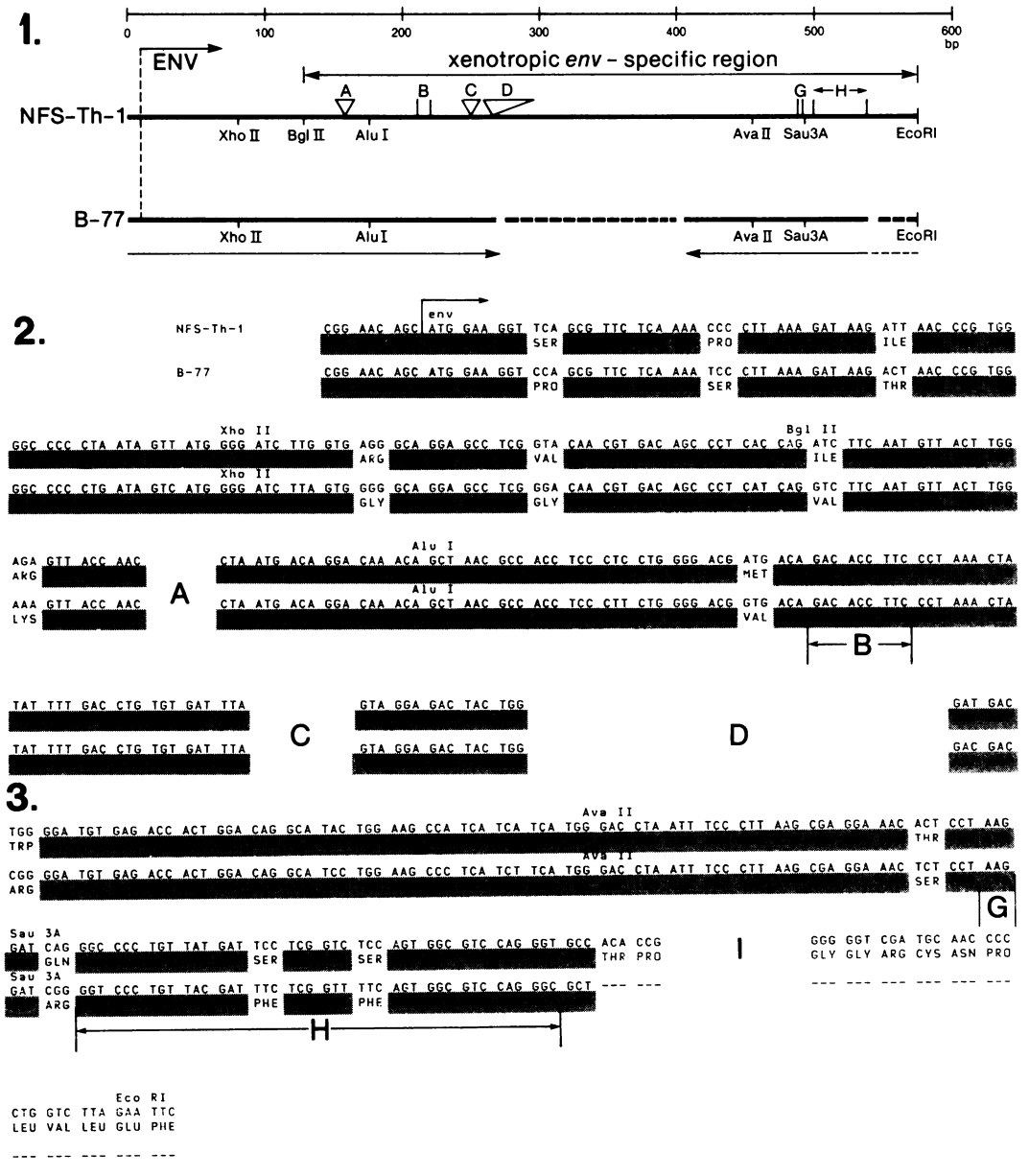


FIG. 4. Sequence of a portion of an endogenous MuLV *env* gene cloned from BALB/c mouse embryo DNA and its relationship to the *env* region of NFS-Th-1 xenotropic MuLV. (Panel 1) Diagrammatic representation of the xenotropic *env*-specific segment of NFS-Th-1 xenotropic proviral DNA and its alignment with the analogous region of clone B-77, isolated from a BALB/c embryo gene library and found to have a restriction map and hybridization properties unique to xenotropic proviruses (16). The sequencing strategy is indicated by the arrows; the regions of clone B-77 DNA not sequenced are shown by the dashed lines. (Panel 2) The sequence of 270 nucleotides of clone B-77 DNA (bottom line), extending from the *pol-env* gene junction in the 3′ direction, was aligned with the analogous segment of NFS-Th-1 xenotropic proviral DNA (top line). Identical deduced amino acids are shaded. The letters designate insertions and deletions unique to the xenotropic MuLV *env* gene relative to the ecotropic *env* segment and follow the convention described in the legend to Fig. 3. The boxed tripeptides denote potential glycosylation sites. (Panel 3) Further comparison of the nucleotide sequence of clone B-77 *env* region (bottom line) with the 3′ portion of the NFS-Th-1 xenotropic *env*-specific segment (top line) as shown in panel 1. Identical deduced amino acids are shaded. The letters indicate the insertion (H) and deletion (I) in the xenotropic MuLV *env* gene relative to the comparable regions in the ecotropic MuLV genome (see Fig. 3). Dashes indicate the nucleotide sequences of B-77 DNA that were not determined.

5,968, Fig. 3), as mentioned previously, was not coincident with any known ecotropic MuLV envelope glycosylation site.

Identification of a xenotropic *env* gene present in an endogenous MuLV provirus cloned from BALB/c mouse DNA. Khan et al. (16) cloned several endogenous MuLV proviruses from AKR and BALB/c mouse DNAs. Five of the twelve clones that contained long terminal repeat segments also had *env* regions that hybridized to the xenotropic *env*-specific probe. Restriction enzyme mapping of the five endogenous *env* segments indicated that some contained sites characteristic of MCF MuLVs (such as a *Bam*HI site at 6.2 kb associated with an *Eco*RI site at 6.7 kb). More revealing, however, were the unique hybridization properties of four of the five xenotropic *env*-reactive clones. Unlike typical xenotropic MuLV proviral DNA, which anneals exclusively to the pX_{env} probe and not to the ecotropic *env*-specific DNA, these four endogenous *env* segments reacted strongly with labeled pX_{env} DNA and weakly but reproducibly with the pEc_{env} probe (16). This pattern of hybridization (dual reactivity) was shown to be characteristic of the MCF *env* gene, and the results obtained with the four endogenous *env* segments suggested that they might be progenitors of infectious MCF MuLVs (16).

The fifth cloned endogenous *env* segment that was isolated from a BALB/c mouse library (associated with clone B-77) behaved like a typical xenotropic proviral DNA and hybridized only to the pX_{env} DNA probe. Since the restriction map of the B-77 *env* region was also similar to the analogous segment of the NFS-Th-1 xenotropic provirus, portions of its *env* gene were sequenced and compared with the xenotropic *env*-specific sequence. The two segments of the B-77 envelope gene analyzed were identical to comparable portions of the xenotropic *env* gene in 380 out of 408 nucleotides (93% homology) and 123 out of 136 amino acids (90% homology) (Fig. 4). All of the characteristic insertions and deletions of the NFS-Th-1 xenotropic *env* gene shown in Fig. 3, including the 42-bp insertion H, were present in the B-77 endogenous *env* segment. In addition, the B-77 *env* region, like the xenotropic *env* gene, contained a second potential glycosylation site (located between A and B in Fig. 4, panel 2) that was absent from the comparable segment of the ecotropic proviral DNA (nucleotides 5,960 to 5,968, Fig. 3). All of the deduced amino acid differences between the B-77 and the NFS-Th-1 xenotropic MuLV envelopes were single base changes. These sequencing results showed several characteristic features of xenotropic *env* genes that distinguished them from analogous segments of ecotropic MuLVs. The role of each in determining properties such as tissue tropism, host range, and binding to cell receptors awaits the construction of recombinants containing specific segments of ecotropic and xenotropic MuLV *env* genes.

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