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Nucleotide Sequence of the Envelope Gene of Friend Murine Leukemia Virus

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The envelope gene of the helper-independent, highly leukemogenic virus Friend murine leukemia virus was sequenced by using a molecular clone of a Friend murine leukemia provirus. The deduced amino acid sequences of the envelope proteins gp70 and p15^{env} were homologous to the sequences of Moloney murine leukemia virus (86%) and Akv (76%). However, a stretch of about 40 amino acid residues near the middle of gp70 was dissimilar in Friend and Moloney murine leukemia viruses and Akv. In this type-specific region the gp70s of all three viruses contained more than 30% proline residues, giving this sequence a very rigid conformation. We suggest that this rigid and highly variable region of gp70 participates in infection by recognition of cell surface receptors and, in addition, might contribute to the different oncogenic spectra of murine leukemia viruses.

The helper-independent virus Friend leukemia virus (F-MuLV) is an ecotropic retrovirus that induces erythroleukemia at a high rate in newborn mice and lymphatic leukemia with a long latency in adult mice (56). From experiments with subgenomic fragments of F-MuLV (39) and from comparative studies with several viruses of AKR mice and MCF viruses, it has been suggested that sequences within the gene for the viral envelope polypeptides or a sequence 3' distal to this gene might be involved in the genesis of leukemia (30).

The product of the envelope *env* gene of the murine leukemia viruses is co-translationally glycosylated to a precursor glycoprotein having a molecular weight of 85,000 (Pr gp85) (6, 8, 52, 59). Pr gp85 is proteolytically cleaved into gp85, which consists of the polypeptides gp70 and p15^{env}. A fraction of each of these polypeptides is disulfide linked (11, 29, 44), whereas the remaining portions of gp70 and p15^{env} are noncovalently attached to each other (51). After insertion into the plasma membrane, p15^{env} undergoes further cleavage to p12^{env} (24, 35, 41).

The envelope glycoprotein gp85 is multifunctional. gp70 forms knoblike structures on the virion surface (36), which is anchored to the viral membrane via p15^{env}. The envelope glycoproteins direct virions to cell receptors (5, 19, 28, 47) and are probably involved in penetration. In cells that are productively infected, these glycoproteins are exposed on the plasma membrane (11, 18), where progeny virus particles bud after assembly. Furthermore, envelope glycoproteins are the primary target for immunological attack in virions and virus-producing cells (18–20, 22, 27). Recently, it has been shown that immunization of mice with appropriate doses of Friend virus envelope glycoprotein gp85 completely protects mice from death caused by Friend virus-induced erythroleukemia (21).

To facilitate more detailed functional studies of the envelope protein and to characterize antigenic determinants, particularly those involved in immunoprotection, we sequenced the envelope gene of a highly leukemogenic F-MuLV strain. Furthermore, we identified potential glycosylation sites and apolar regions which probably are in contact with the viral or cellular membrane. A comparison of the F-MuLV *env* sequence with the sequences recently described for Moloney-MuLV (M-MuLV) (54) and Akv (30) showed two small regions of strong divergence flanked by large portions of homology. This allowed us to associate type-specific antigenic determinants to the variable regions and group- and interspecies-specific determinants to the homologous regions (19, 55). The position and the unusual sequence of the major variable region suggest that it functions in receptor recognition.

The distribution of stretches of uncharged amino acids in the envelope polypeptides strikingly resembles the distribution in the surface proteins of myxoviruses and paramyxoviruses (23, 58). This implies a similar anchorage of these proteins in the viral membranes and similar roles in infectivity.

MATERIALS AND METHODS

Virus and plasmid. F-MuLV clone 57 molecularly cloned in pBR322 was obtained from Allan Oliff and

Edward Scolnick. F-MuLV clone 57 is a helper-independent, NB-ecotropic, highly leukemogenic virus (39, 40) which induces rapid nonthymic leukemia after inoculation into newborn NIH Swiss mice in the absence of spleen focus-forming virus.

DNA preparation. Plasmid-containing bacteria were grown in M9 medium (4) in the presence of 50 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml and 1 mg of uridine per ml (37). Plasmid DNA was prepared by the cleared lysate procedure of Clewell and Helinski (3) and was purified through two successive cesium chloride density gradients. The DNA was then treated with 100 μg of proteinase K (E. Merck AG, Darmstadt, West Germany) per ml in the presence of 10 mM Tris, 1 mM EDTA, and 2 mg of sodium dodecyl sulfate per ml (pH 7.0) for 30 min at 37°C. Finally, the DNA was extracted three times with phenol-chloroform (1:1) and then ethanol precipitated.

Restriction analysis of DNA. Restriction endonucleases were obtained from Bethesda Research Laboratories, Bethesda, Md., and from New England Biolabs, Beverly, Mass., and were used according to the specifications of the manufacturers. DNA samples were run on agarose gels (0.6 to 1.2 g/100 ml) in 40 mM Tris–20 mM sodium acetate–1 mM EDTA (pH 7.8) or on polyacrylamide gels (7.5 g/100 ml) in 0.1 M Tris–0.1 M boric acid–2 mM EDTA (pH 8.3). Samples for electrophoresis were adjusted to 40 mg of Ficoll (Pharmacia, Uppsala, Sweden) per ml, 1 mg of bromphenol blue per ml, and 2 mg of sodium dodecyl sulfate per ml before they were loaded onto the gels. When UV light (302 nm) was to be used to visualize the electrophoresed DNA, ethidium bromide (0.5 μg/ml) was added to both the gel and the running buffer before electrophoresis. Agarose gels were run at 5 V/cm, and polyacrylamide gels were run at 10 V/cm.

DNA sequencing. DNA restriction fragments to be used for sequencing were purified on agarose or polyacrylamide gels as described above. Elution of the DNA from agarose was performed with glass powder by using the method of Vogelstein and Gillespie (57). Elution of DNA from polyacrylamide was performed as described previously (13). Restriction enzyme fragments were terminally labeled with [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs). Strand separation and sequence analysis were performed by using the method of Maxam and Gilbert (32). All parts of the genome were sequenced at least twice. Autoradiograms of sequencing gels were read independently by two workers.

RESULTS AND DISCUSSION

Localization of the envelope gene within the cloned proviral DNA. The envelope gene of a highly leukemogenic F-MuLV strain was sequenced by using F-MuLV clone 57 (40), which was kindly provided as a molecular clone in pBR322 by A. Oliff and E. Scolnick. A physical map analysis with rare cutting restriction enzymes was performed to localize the *env* gene on this DNA and to facilitate nucleotide sequencing. A comparison of the restriction sites with previously published data for related viruses (30, 54) allowed us to locate the envelope gene within the border of the left *Hin*dIII site and the *Kpn*I site (Fig. 1A).

Nucleotide sequence of the F-MuLV *env* gene. A detailed restriction enzyme analysis of the *Hin*dIII-*Kpn*I fragment was performed (Fig.

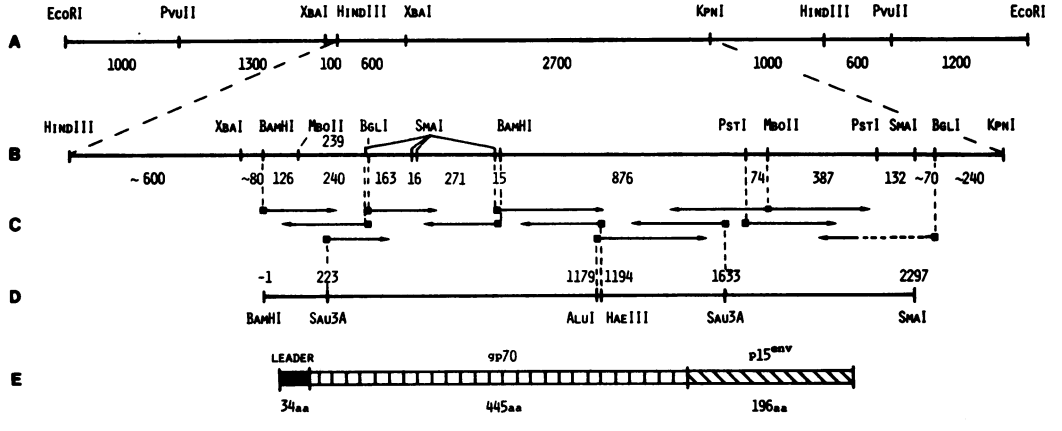

FIG. 1. Physical mapping and use of restriction fragments for sequence analysis of the *env* gene of F-MuLV. (A) Schematic representation of the F-MuLV DNA clone showing *Eco*RI, *Hin*dIII, *Kpn*I, *Pvu*II, and *Xba*I restriction endonuclease cleavage sites (40). The numbers indicate the lengths of the restriction fragments (in nucleotide pairs). (B) Cleavage sites in the *Hin*dIII-*Kpn*I fragment of the cutting enzymes used for sequencing. (C) Sequencing strategy. The lines indicate the fragments from which unambiguous sequences were obtained. The solid boxes represent 5′ ends labeled with [γ-^{32}P]ATP. (D) Selected cleavage sites of *Alu*I, *Bam*HI, *Hae*III, *Sau*3A, and *Sma*I restriction endonucleases used for sequencing. The numbers indicate the positions of the first bases of the recognition sites of the enzymes. (E) Representation of the *env* polypeptide within the boundaries of (B) and (D). The numbers of amino acid (aa) residues of various parts of the polypeptide are shown.

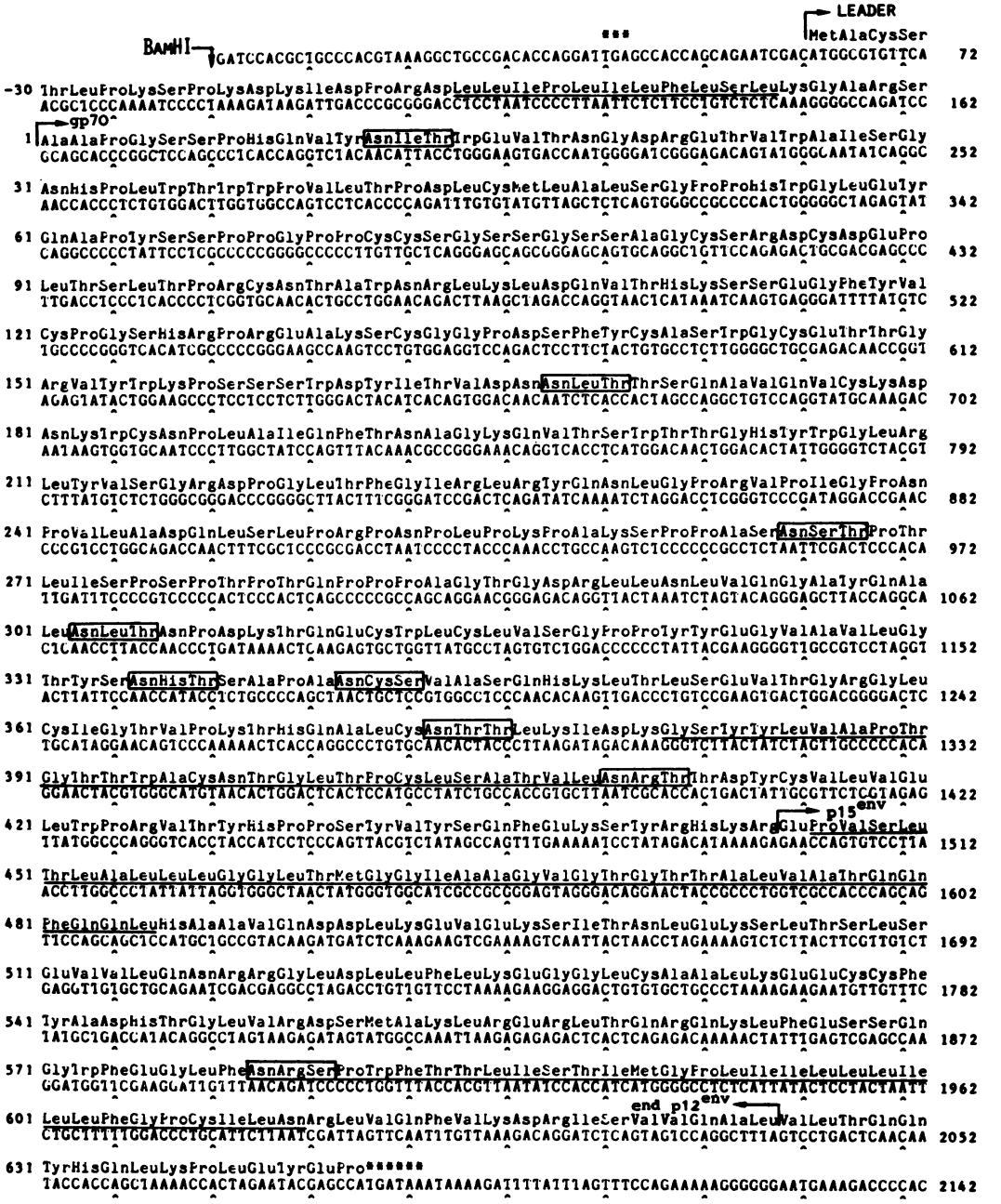

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *env* gene of F-MuLV. Potential glycosylation sites are enclosed in boxes; uncharged regions are underlined.

1B). Nucleotide sequences were determined by using the method of Maxam and Gilbert (32). The sequencing strategy and the additional restriction enzyme cleavage sites used are shown in Fig. 1C and D. The sequence had one large open reading frame, which consisted of 2,043 nucleotides. The nucleotide sequence and the deduced amino acid sequence for this reading frame are shown in Fig. 2. The frame starts at nucleotide 43 and terminates at position 2,086 with two stop codons.

Besides the long open reading frame coding for the *env* polyprotein, an analysis of both DNA strands revealed several sequences which could

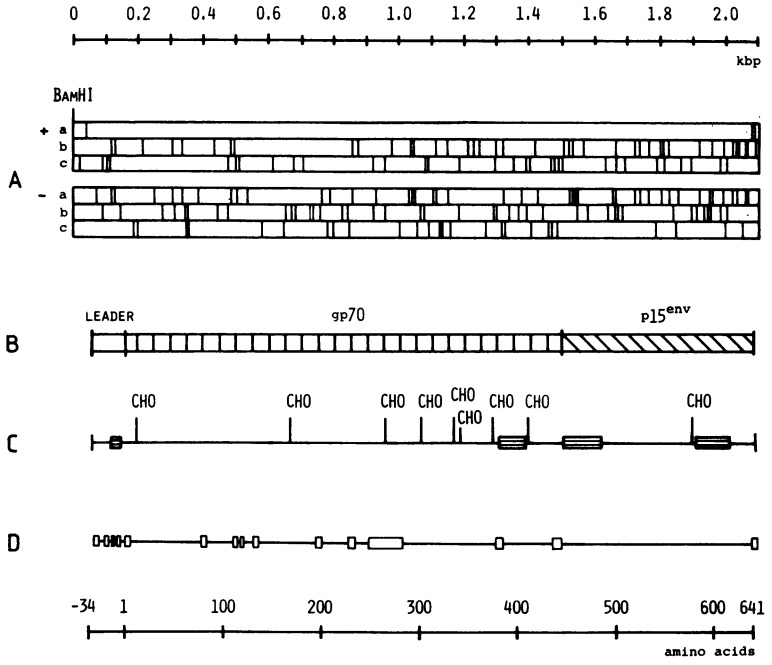

FIG. 3. Characteristics of the *env* gene and its products. (A) Distribution of termination codons in the coding (+) and noncoding (−) strands in all reading frames (a, b, and c). (B) Representation of the *env* polypeptide within the boundaries of (A). (C) Potential glycosylation sites (CHO) and large, uncharged regions (boxes) in the *env* polypeptide. (D) Positions where more than three amino acid residues vary with respect to Akv or M-MuLV. kpb, Kilobase pairs.

code for additional polypeptides up to 112 amino acids long (Fig. 3A). The longest of these was located in the coding strand between nucleotides 518 and 853. The possible significance of such a sequence remains to be elucidated.

The mRNA for the *env* gene products is spliced from a precursor RNA (12, 49). No splice acceptor sequences (53) were found within the 61 nucleotides sequenced preceding the amino terminus of the peptide leader (see below); however, this is not surprising, since in the closely related virus M-MuLV a possible splice acceptor sequence is located 154 nucleotides preceding the peptide leader (54).

Localization of the sequence coding for the envelope polyprotein within the *env* gene. The NH_2-terminal amino acids of F-MuLV gp70 have recently been identified by protein sequencing (31). From this sequence we deduced that the beginning of gp70 is the alanine residue coded for by nucleotides 163 to 165 of our sequence (Fig. 2). A methionine codon precedes this residue 102 nucleotides upstream in frame. The methionine is preceded by a stop codon at nucleotides 40 to 42. This pattern represents a leader peptide of 34 amino acids.

To determine the positions of $p15^{env}$ and $p12^{env}$ within the precursor protein, the amino acid sequence of the F-MuLV *env* polypeptide was compared with the partial amino acid sequences determined by Oroszlan and co-workers for Rauscher MuLV and M-MuLV (41). Assuming identical processing sites in these viruses, $p15^{env}$ starts at amino acid 446 (nucleotide 1,498) and extends to amino acid 641 (nucleotide 2,085). The cleavage product of $p15^{env}$, $p12^{env}$, ends at amino acid 625 (nucleotide 2,037). To our knowledge, the exact carboxy terminus of gp70 has not been determined yet. In similar cases in which viral or nonviral membrane proteins are processed by cellular endopeptidases (10, 23), one or more of the basic amino acid residues of the new carboxy terminus of the cleavage site are excised by carboxypeptidase B. Therefore, it is possible that the carboxy terminus of gp70 is located a few amino acids to the left.

Assuming no internal splicing of the *env* mRNA, the molecular weights of the envelope polypeptides given in Table 1 could be deduced from the amino acid composition.

Location of possible glycosylation sites. In retroviruses carbohydrates are linked to proteins via the side chains of asparagine residues in sequences like Asn-X-Thr or Asn-X-Ser (14a, 34, 48). Glycosylation sites within the *env* gene

TABLE 1. Calculated molecular weights of F-MuLV *env* polypeptides

Polypeptide	No. of amino acid residues	Mol wt of the apopolypeptide
Total *env* precursor polypeptide	675	74,841
N-terminal signal peptide	34	3,741
gp70	445[a]	49,008[a]
$p15^{env}$	196	22,092
$p12^{env}$	180	20,126

[a] Since some amino acids might be cleaved off the carboxy terminus of gp70, the actual number might be slightly smaller.

products are highly conserved in the different host range classes of murine retroviruses (25, 26). Eight such sites were found in the amino acid sequence of F-MuLV gp70 (Fig. 2 and 3C); six of these sites were located in the carboxy-terminal half of gp70. In other ecotropic murine retroviruses six or seven carbohydrate chains are linked to the gp70 molecule (48). Whether all of the eight potential sites in the F-MuLV gp70 are glycosylated is not known at present. The sequence Asn-X-Thr at amino acid positions 410 to 412 is not present in either M-MuLV or Akv, but such a sequence is present in gp70 of G_{IX}^- viruses (38), where it seems to be glycosylated (7, 48).

In $p15^{env}$ the sequence Asn-Arg-Ser was found at positions 578 to 580. However, this site, which is similar to sites in Akv and M-MuLV (30, 54), seems not to be glycosylated, since only gp70 can be labeled with [^{3}H]glucosamine (33, 44).

Nonpolar regions of the *env* polypeptide. Lenz et al. (30) have proposed a model that locates gp85 and its components in the membrane, based on the positions of apolar stretches in the *env* protein of Akv. Equivalent regions of uncharged, mostly nonpolar amino acids are found in the F-MuLV *env* precursor protein (Fig. 2 and 3C). A stretch of 11 such amino acids is located in the signal peptide. A total of 28 apolar amino acids are located near the carboxy terminus of gp70. $p12^{env}$ is bounded by a stretch of 38 such amino acids at its N terminus and 30 amino acids near its carboxy terminus.

The surface glycoproteins HA_2 in myxoviruses and F_1 in paramyxoviruses carry uncharged regions at comparable positions (23, 58). The carboxy-terminal apolar stretch anchors these proteins in the viral membrane. The N-terminal apolar stretch probably interacts with a secondary receptor of the cellular membrane during infection (14, 17, 45). The striking similarity to $p12^{env}$ and the highly conserved apolar amino acids in $p12^{env}$ of other MuLVs (30, 54) suggest a similar role for $p12^{env}$.

Comparison of the nucleotide and amino acid sequences of F-MuLV, M-MuLV, and Akv. When the nucleotide and amino acid sequences of the envelope protein of F-MuLV were compared with the sequences of M-MuLV (54) and Akv (30), a few marked differences were found (Fig. 3D). Two of these differences (at amino acid positions 75 to 81 and 244 to 281) involve deletions and insertions with respect to M-MuLV and Akv (Fig. 4A and B). Three other regions with less pronounced differences are located in the peptide leader sequence (Fig. 3D). The apolar region in the F-MuLV peptide is only 11 amino acids long (as it is in M-MuLV); thus, it is much smaller than the Akv apolar region, which contains 20 residues. However, this region still contains the minimum number of amino acids necessary to traverse the membrane (10).

The overall nucleotide sequence homologies in the envelope gene are 86% between F-MuLV and M-MuLV and 76% between F-MuLV and Akv, if the two divergent regions (Fig. 4A and B) are excluded from the calculations. The amino acid homologies in the gp85 apoprotein are 90% between F-MuLV and M-MuLV and 83% between F-MuLV and Akv (again excluding the regions of strong divergence). The similarities in the amino acid sequences of the three viruses are greatest in the $p12^{env}$ portion, where 95% of all amino acids are identical. The numbers of cysteine residues (20 in gp70 and 4 in $p12^{env}$) are the same in all three viruses. This indicates that the envelope proteins of murine retroviruses have similar conformations.

The most variable site is located between amino acid 244 and amino acid 281. About one-third of all amino acid residues in this region are proline residues (Fig. 4B), indicating complex chain conformation. In other regions of the protein, proline is not enriched (Fig. 4C). Clustering of many hydrophilic amino acids suggests (16) the presence of antigenic determinants in this region.

gp70 is a highly polymorphic protein (9, 15). Some of this polymorphism may be accounted for by the variable sites. It is surprising that all of the sites which have been described are different in all three viruses. This could reflect hot spots for somatic mutation or recombination with endogeneous *env* sequences. Such highly variable regions in a protein could evolve for two reasons. First, regions with functions having little importance are less likely to be conserved than functional domains; and second, variability of certain regions of a structural protein may allow a virus to propagate in different environments. The latter reason could explain,

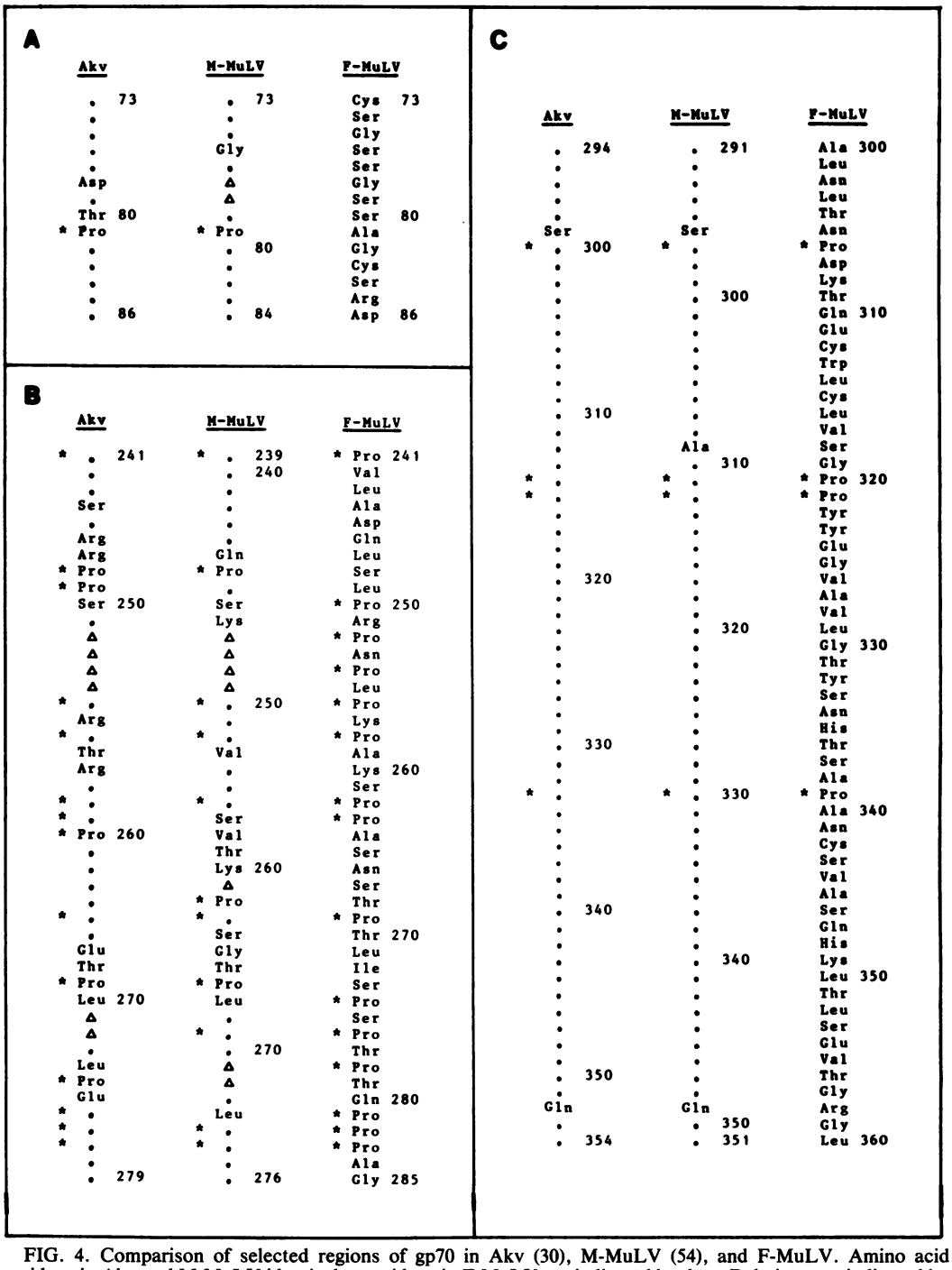

FIG. 4. Comparison of selected regions of gp70 in Akv (30), M-MuLV (54), and F-MuLV. Amino acid residues in Akv and M-MuLV identical to residues in F-MuLV are indicated by dots. Deletions are indicated by triangles. Proline residues are indicated by asterisks. The numbers indicate amino acid positions. (A and B) Regions with highest variability. (C) Region of strong homology, typical for most parts of the *env* polypeptide.

for example, the high variability restricted to certain portions of the hemagglutinin in influenza virus (42, 58). If the variable sites of gp70 function in receptor recognition, the second explanation would be favored. The unusually high proline content of the region between ami-

no acid 233 and amino acid 283 also suggests that this part of the protein is under heavy selective pressure. According to the model of Lenz et al. (30), this sequence is indeed located on the outer side of the viral membrane. The stiffness of this region, which is caused by the high proline content, might be an important requirement for interaction with a cellular receptor. In all three viruses, such a domain may have a similar function in receptor recognition.

MCF viruses acquire a polytropic host range by recombination of ecotropic and endogenous MuLV *env* genes. Moreover, unlike their parents, recombinant MCF viruses are cytopathic to mink cells and are more directly involved in lymphomagenesis (2, 50). Whether selective infection is sufficient to determine the leukemogenic potentials and spectra of nondefective MuLVs or whether additional functions (perhaps located on separate *env* gene domains or other sites of the genome) are required for transformation remains to be determined. Recent evidence suggests that F-MuLV causes leukemia via its ability to induce formation of Friend MCF virus (50).

Lenz et al. (30) have compared *env* gene sequences detectable in RNase T_1 fingerprints of leukemogenic and non-leukemogenic viruses. The only consistent difference which these authors could detect between the non-leukemogenic and leukemogenic MuLVs was a change from Arg-Leu (non-leukemogenic) to His-Met (leukemogenic) on the inner side of the membrane traversal region near the carboxy terminus of $p12^{env}$. Since the highly leukemogenic F-MuLV which we sequenced had Arg-Leu in this position (Fig. 2, positions 610 and 611), this change seems not to be related to leukemogenicity in general. Sequences in the *env* gene not detected by RNase T_1 fingerprinting or sequences in the long terminal repeat or other regions of the genome (1, 43, 46) might be responsible for the leukemogenic potentials of MuLVs. Further sequence comparisons of leukemogenic and non-leukemogenic MuLVs will certainly help characterize the regions of the genome which determine host range and transforming activity.

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ADDENDUM IN PROOF

The amino acid sequence of gp70 determined from the envelope protein of F-MuLV grown in the Eveline cell line was recently published by R. Chen (Proc. Natl. Acad. Sci. U.S.A. 79:5788–5792, 1982). Nineteen amino acid differences are to be found when this author's data are compared with our own. Since he has not separated the two isoglycoproteins of gp70 present in this virus strain, we feel that his data might in part represent a mixture of these two proteins. Some variations might also be explained by the diferent virus strains used.

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