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Genetics

Complete nucleotide sequence of an infectious clone of Friend spleen focus-forming provirus: gp55 is an envelope fusion glycoprotein

[acute leukemia virus/mink cell focus-forming virus/gp70 glycoprotein/p15(E) protein/erythroleukemia]

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ABSTRACT **The Friend spleen focus-forming provirus is 6,296 base pairs (bp) in length. Compared to Moloney murine leukemia virus, it has undergone five major deletions, three substitutions, and a number of minor alterations. Otherwise, these viruses are about 90% homologous. A 16-bp palindrome is found in the region thought to be involved in packaging and dimerization of the RNA genome. Premature termination of translation of the *gag* polyprotein is attributed to a 13-bp deletion in the p12 region. A substitution of xenotropic *env* sequences was identified in the 5′ region of the *env* gene; 150 nucleotides 3′ to this substitution, a deletion of 585 bp removes the site where the normal *env* precursor protein is cleaved to form gp70 and p15(E), resulting in a fusion protein of M_r 44,725. Due to these changes, the *env* product gp55 is expected to have a substantially different conformation on the cell surface compared to either a xenotropic or ecotropic gp70 protein, and may be responsible for the rapid erythroleukemic potential of spleen focus-forming virus.**

Retroviruses that induce neoplasia can be classified with few exceptions into two groups: those that are slowly transforming and replication competent and those that have recombined with one of a number of cellular oncogenes to become rapidly transforming and replication defective (1). The oncogene can substitute within any of the viral structural genes: *gag*, which produces core proteins, *pol*, which encodes RNA-dependent DNA polymerase (reverse transcriptase), and *env*, which produces envelope glycoproteins. Each region normally produces a polyprotein that is processed by proteolytic cleavage.

Friend spleen focus-forming virus (SFFV) induces a rapid erythroleukemia in mice (2, 3). However, it neither contains a cellular oncogene nor has the ability to transform fibroblasts in culture (4). It has suffered several substitutions (5, 6) and deletions (7) compared to its helper virus, Friend murine leukemia virus (F-MuLV), and is thus replication defective. SFFV induces an unusual disease consisting of an early (1–2 weeks) proliferation and differentiation of erythroid cells (2, 8) and a late stage when transformed cells able to form tumors and permanent cell lines appear (9, 10). Which of the SFFV or F-MuLV viral genomes induces the progression of transformation has not been determined, but it is known that SFFV is responsible for the early stage (8) and is under the control of the same host factors that influence normal hemopoietic development (4, 11–13). Recombinant DNA experiments have suggested that gp55, the product of the *env* gene, may be required for the initial cellular proliferation (14). This region contains a substitution that appears to have been derived from a xenotropic retrovirus (5–7). However, mink cell focus-forming (MCF) viruses, which are also recombinants of xenotropic *env* sequences, do not induce Friend disease (15, 16).

To establish the exact nature of the SFFV rearrangements and their relationship to pathogenicity, and also to aid in the understanding of retroviral control mechanisms and evolution, we have determined the complete sequence of the Friend SFFV provirus. It was found that the 5′ region of the ecotropic F-MuLV *env* gene has been replaced by xenotropic *env* sequences, and that a deletion spanning the junction of gp70 and p15(E) results in the creation of a gp70–p15(E) fusion protein. Both of these characteristics may be important in the early phase of Friend disease.

MATERIALS AND METHODS

The 7,400-base-pair (bp) *Eco*RI insert of λgt*WES*λSFFV$_P$502 containing the infectious SFFV provirus (17) was digested with *Sau*3A, *Hpa* II, or *Taq* I. The resulting fragments were ligated into the *Bam*HI or *Acc* I site of the replicative form of the phage vector M13mp9, which was then used to transfect *Escherichia coli* strain JM103. JM103 and M13mp9 were generous gifts from J. Messing (University of Minnesota). The DNA sequences of single-stranded recombinant phages were determined by the dideoxy chain-terminating inhibitor method (18), using DNA polymerase I (Klenow fragment), deoxy- and dideoxynucleoside triphosphates from Boehringer Mannheim (Montreal), [α-^{32}P]-dATP from New England Nuclear, and a synthetic pentadecamer primer from New England BioLabs. The Maxam and Gilbert procedure (19), used to fill in gaps and verify some areas, and polyacrylamide gel electrophoresis were carried out as described (20). The sequences of all areas were determined at least once on each strand, and the sequences were determined through all restriction sites. The sequences were compiled with modifications of the FORTRAN programs DBCOMP and DBUTIL (21).

RESULTS AND DISCUSSION

Sequence of SFFV DNA and Comparison to DNA Sequences of Ecotropic and Xenotropic Retroviruses. The sequence of SFFV DNA is shown in Fig. 1, as well as the protein-encoding regions and other features of interest. The provirus is 6,296 bp long and has two 514-bp LTRs. The DNA sequence of the related (6, 7, 20) Moloney murine leukemia virus (Mo-MuLV) (22) was compared to that of SFFV by diagonal dot matrix analysis, shown in Fig. 2. The results identify five major

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Abbreviations: SFFV, spleen focus-forming virus; F-MuLV, Friend murine leukemia virus; Mo-MuLV, Moloney murine leukemia virus; MCF virus, mink cell focus-forming virus; Mo-MuSV, Moloney murine sarcoma virus; LTR, long terminal repeat; bp, base pair(s).

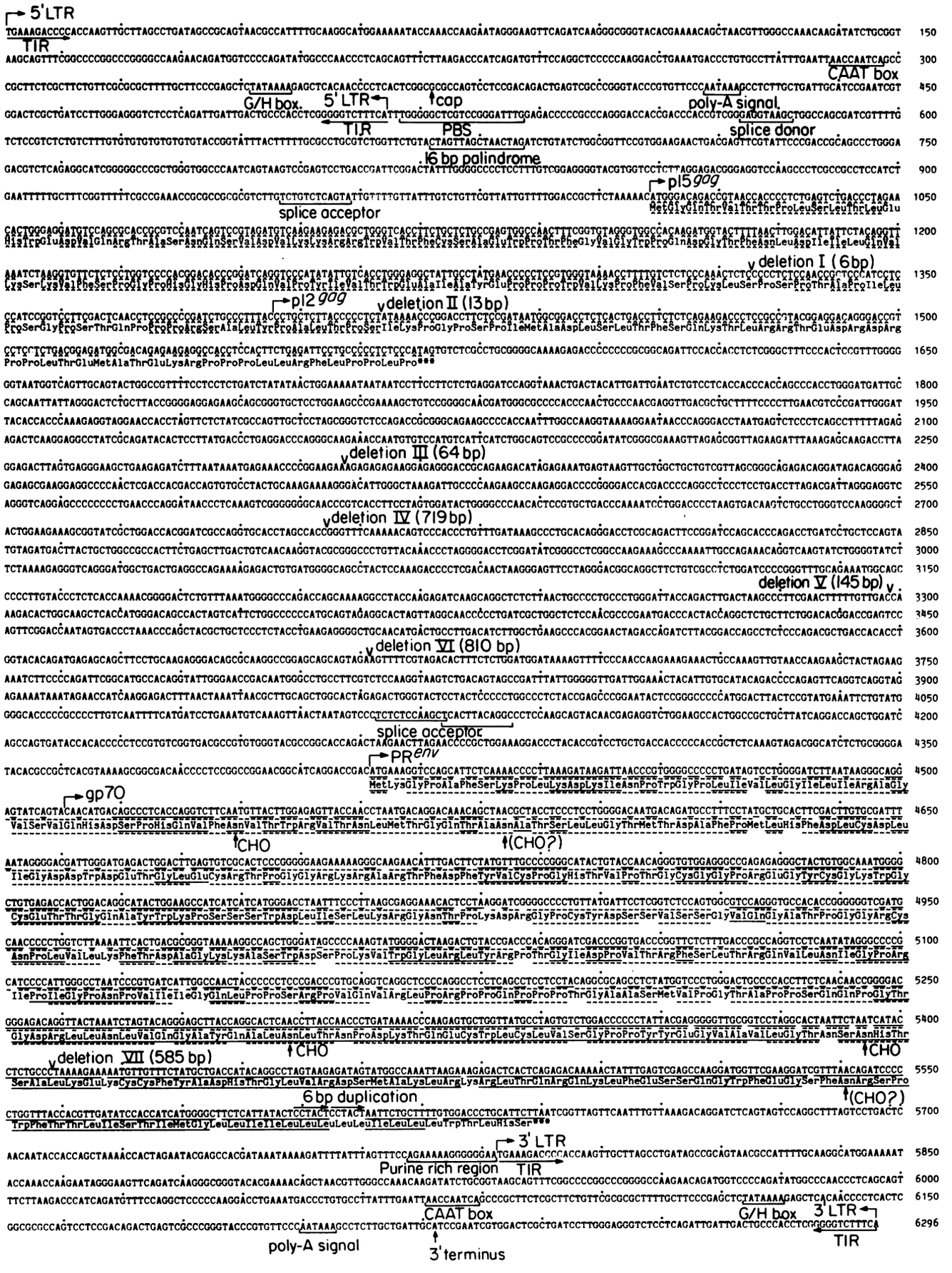

FIG. 1. (*Legend appears at the bottom of the next page.*)

deletions throughout SFFV and two areas of nonhomology, which are presumably substitutions, in the *gag* and *env* regions. Because SFFV is thought to have some homology to xenotropic viruses, the *env* region of a MCF virus (23), which contains xenotropic sequences, was also compared to SFFV DNA. The *Inset* in Fig. 2 shows that the *env* region of SFFV that has poor homology to Mo-MuLV DNA has high homology to MCF virus DNA. Direct nucleotide comparison (Fig. 1) confirms the xenotropic origin of the SFFV *env* substitution. Mo-MuLV and F-MuLV (24) have equally poor homology in this substitution. The *gag* substitution shows at least 50% homology to Mo-MuLV, indicating that it is of retrovirus origin.

The sequence of SFFV DNA was compared directly to the sequences of Mo-MuLV and to F-MuLV DNAs, and two additional small deletions were found in the *gag* region. Except for deletion VI, a short repeat of three to six nucleotides can be identified at the deletion boundaries. These repeats terminate exactly at opposite ends of the deletion so that one is completely lost and the other is completely retained (Table 1). A similar situation is found in Moloney murine sarcoma virus (Mo-MuSV) (25), and in the LTR of spleen necrosis virus (26).

Repeated sequences may be important for the generation of deletions and insertions by a mechanism of homologous recombination that is unique to retroviruses, which allows noncontiguous regions of the template to be joined in the polymerase product during reverse transcription. This mechanism, known as "jumping" (26), presumably involves the complementarity between the newly synthesized product and a distant region of the template and may be mediated by breaks in the RNA molecule.

Deletion VI, similar in size and position to a deletion in Mo-MuSV (25), does not have duplication, suggesting that other factors may have been involved in its generation.

Genetic Areas of SFFV. *(i) Terminal structures.* As has been shown previously (20), this provirus is flanked by two 514-bp LTRs (27) that contain recognizable transcriptional regulatory sequences and short terminal inverted repeats (Fig. 1). A sequence complementary to the $tRNA^{Pro}$ primer for reverse transcription is found 3 bp to the 3′ side to the 5′ LTR. A purine-rich region is found just to the 5′ side of the 3′ LTR, which is thought to be involved in the initiation of second-strand synthesis during reverse transcription. Overall the SFFV and Mo-MuLV LTRs are very similar; therefore, by analogy to the Mo-MuLV transcript (22), the cap site of the SFFV RNA genome is found at position 372 and the 3′ terminus is at position 6,222.

(ii) Intron. After the region that corresponds to the transcribed RNA had been identified, two open reading frames were found that corresponded to the two known SFFV-encoded proteins (8). The first initiation triplet of the RNA molecule (position 1,009) corresponds to the Mo-MuLV *gag* initiation triplet.

Direct comparison of SFFV RNA to Mo-MuLV RNA shows that there is a substitution between this initiation codon and the 5′ LTR (position 590–670). Mo-MuSV also has a substitution in this general area (25). This region has been implicated in packaging and dimer formation of the retrovirus genomes, and has been called "E," for encapsidation (28). Despite the differences between these three related viruses, one sequence that is constant is a 16-bp palindrome starting at position 672 in SFFV

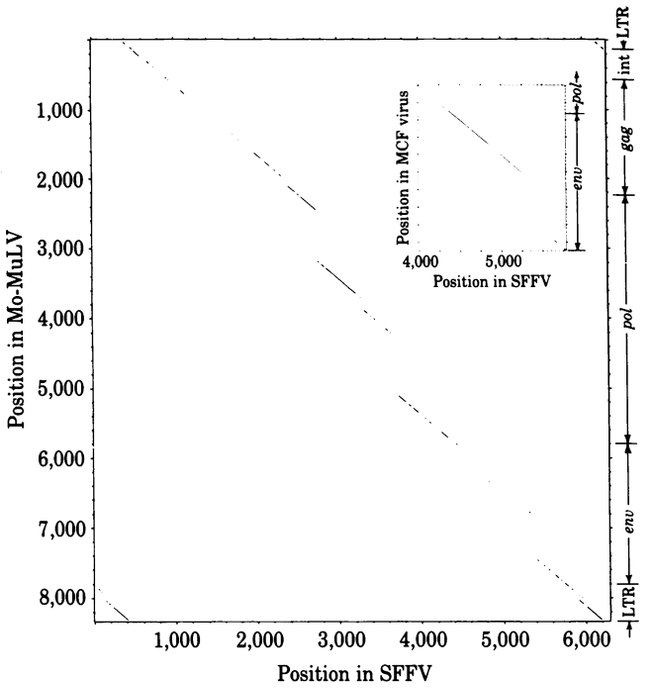

FIG. 2. Diagonal dot matrix comparison of SFFV and Mo-MuLV DNAs. Each point represents a position where there is at least 90% homology between the two viruses over 21 contiguous nucleotides. The vertical discontinuities of the diagonal line represent points of deletions in SFFV. The diagonal line fragments in the lower left and upper right corners are the result of the presence of both LTRs in the SFFV genome. int, Intron. (*Inset*) Comparison of the SFFV *env* region to the *env* region of a MCF virus, which contains xenotropic sequences.

RNA. Such a palindrome could hybridize intramolecularly to form secondary structure or intermolecularly to bind two genomes together. Its position is consistent with electron microscopic studies, which have shown that the two genomic RNA strands are held together about 300 bases from the 5′ end (29).

Unlike that in SFFV, the *gag* initiation triplet in Mo-MuLV is the fourth methionine codon in the transcript. Because the scanning model of protein translation dictates that the most 5′ AUG is used for initiation (30), a splicing event has been postulated to remove the upstream AUG triplets (22). Such an event would also remove the packaging sequences from the molecules destined to be translated rather than packaged. Consistent with this purpose is the conservation of the postulated splice sites between Mo-MuLV and SFFV.

An interesting feature within the substitution in this putative SFFV intron is a stretch of alternating thymidine residues from 597 to 633, the last 16 of which are alternating thymidines and guanosines. Such sequences have recently been found to be highly repeated in eukaryotic genomes (31) and, because they can form left-handed Z-DNA (32), they have had unusual control and recombinational functions attributed to them. It is unlikely that such a sequence could persist in a retrovirus, with such a high frequency of deletions between repeated sequences, unless it provides some function.

(iii) gag protein product. Only two viral proteins have been detected in SFFV nonproducer cells: the NH_2-terminal *gag* protein p15 and the *env*-related gene product gp55 (8). Ex-

FIG. 1 (*on preceding page*). The DNA sequence of the SFFV provirus, including the 3′ and 5′ long terminal repeats (LTRs). The amino acid sequences of the proteins produced by the *gag* and *env* regions are positioned under the nucleotide sequences. Regions of interest have been marked next to the sequence, and every 20th nucleotide has a dot over it. TIR, 11-bp terminal inverted repeat that bounds the LTRs; G/H box, Goldberg–Hogness box; cap, point of transcription initiation; PBS, primer binding site; PR^{env}, *env* precursor protein; ***, translation termination codon; CHO, point of probable polysaccharide attachment to the protein; CHO?, potential glycosylation site. The proteins and nucleotides are marked where the sequences best match those of other viruses: In *gag*, homology to Mo-MuLV is indicated by underscoring with circumflexes (^). In *env*, homology to F-MuLV is indicated by straight underlining (–), and wavy underlining (~) shows homology to xenotropic MCF virus sequences.

Table 1. Deletions and boundary repeats of the SFFV provirus

SFFV deletion		Mo-MuLV	Deletion	Boundary
No.	Position*	positions†	size, bp	repeat
I	1,325	938–943	6	G-C-C-T-C-C
II	1,413	1,032–1,044	13	C-T-A
III	2,307	1,938–2,001	64	A-G-A-G-A-G
IV	2,754	2,448–3,166	719	C-C-A-C
V	3,298	3,711–3,855	145	G-A-C
VI	3,661	4,220–5,029	810	—
VII	5,408	6,864–7,448	585	C-T-G-C-C-C

*See Fig. 1.
†From Shinnick *et al.* (22).

amination of the nucleotide sequence readily accounts for these observations.

The open reading frame that starts with the initiation codon at 1,009 is largely within the *gag* substitution. Despite the poor homology between the SFFV and Mo-MuLV genomes, the deduced translation products are 78% homologous until position 1,413, where the 13-bp deletion II induces a frameshift six codons past the p15–p12 boundary, and termination occurs at 1,570. Apparently the short stretch of p12 that is properly translated is insufficient to enable anti-p12 antiserum to precipitate the abnormal protein, while still allowing the processing enzyme to recognize the correct cleavage site, since p15 of a normal size is produced (33). The translation product has a deduced M_r of 20,780.

(iv) pol *region.* The presence of three major deletions in *pol*, as well as numerous frameshifts and termination codons, makes it highly unlikely that SFFV produces its own reverse transcriptase. Analysis of SFFV nonproducer cells (33) for reverse transcriptase activity confirms this supposition (unpublished results).

(v) env *protein product.* The last open reading frame in SFFV DNA corresponds to the *env* gene in Mo-MuLV, F-MuLV, and MCF virus. The *env* precursor polyprotein is known to be produced from a subgenomic mRNA that possesses the viral 5′ end (34). Accordingly, a potential splice acceptor site has been identified in Mo-MuLV (22) which is identical to the corresponding region (4,124–4,135) in SFFV. Both viruses have a partially overlapping sequence 11 bases to the 5′ side that matches the splice acceptor consensus sequence (35) equally well.

The first ATG triplet after this sequence occurs at position 4,412 and corresponds to the presumptive initiation codon of the signal peptide of Mo-MuLV, F-MuLV, and MCF virus and is within the xenotropic *env* substitution of SFFV (Fig. 2). Direct comparison of the DNA sequences of SFFV, MCF virus, and F-MuLV (Fig. 1) shows that SFFV and MCF are almost identical in sequence until 5,260, whereas F-MuLV shows generally very poor homology throughout this region. However, between position 5,249 and the 3′ LTR, the sequence is almost identical to F-MuLV, except for the 585-bp deletion VII, which occurs at position 5,408. The reading frame is maintained between the SFFV *env* region and that of the two viruses from which it was derived, so the primary sequence of the protein produced is very similar to that of the corresponding parent, until a single base insertion occurs at 5,623, generating a termination codon at 5,639. The termination codon for the F-MuLV *env* precursor protein occurs approximately 34 codons later. This precursor protein ($PR85^{env}$) of replication-competent murine retroviruses is proteolytically cleaved (36) to form mature gp70, p15(E), plus the "R-peptide" (37). The site of cleavage between gp70 and p15(E) has been deleted from SFFV DNA and there are no other sites similar to the normal gp70-p15(E) cleavage position (Lys-Arg-Glu). The agreement between the deduced M_r of 44,725 for the primary translation product and the experimentally observed M_r of 42,000–47,000 (34, 38) for the unglycosylated protein indicates that gp55 is a fusion of the NH_2-terminal half of gp70 and the COOH-terminal half of p15(E).

There are five potential sites of N-glycosylation [Asn-X-Ser/Thr (39)] in the SFFV *env* protein sequence. Three are common to glycosylated positions in F-MuLV gp70 (40) and are likely modified. Another site within the p15(E) region is probably not glycosylated, because the identical sites in F-MuLV and Mo-MuLV are not. The status of the remaining site at position 4,883 is unknown.

The COOH-terminus of this protein is extremely hydrophobic, partly because of a 6-bp duplication between 5,605 and 5,610 and partly because of premature termination of the protein. The last 21 amino acids have the sequence Ile-Met-Gly-Leu-Leu- Ile-Ile-Leu- Leu-Leu-Leu- Leu-Ile-Leu- Leu-Leu-Trp-Thr-Leu-His-Ser. Presumably this hydrophobic stretch is embedded in the membrane and serves as an anchor for the molecule.

Origin of SFFV. The mechanism of generation of novel retroviruses is an important consideration because recombination is important in the generation of transforming viruses (1, 41). The substitution in the *env* region can be determined to end within the 12 nucleotides between 5,249 and 5,260 in SFFV. The 5′ end of this substitution cannot be identified because it occurs 5′ to the reported sequence of the F-MuLV *env* region, and it probably occurs prior to position 4,321, which corresponds to the point of recombination between Mo-MuLV and xenotropic sequences in MCF virus (23). Similarly, the boundaries of the *gag* substitution cannot be identified. It is possible that all of the virus to the 5′ side of 5,249 is xenotropic in origin, because heteroduplex analysis between SFFV and xenotropic virus DNAs shows no substitution "bubbles" (7).

The substitutions were probably generated by polymerase jumping from the Friend viral genome to a xenotropic genome during reverse transcription. The homology between F-MuLV and xenotropic sequences at the point of recombination (5,249–5,260) would enable this to occur.

Taken together, it is likely that SFFV reached its present form through a series of events (Fig. 3). The first was recombination of F-MuLV with xenotropic sequences, possibly forming a Friend MCF virus. The next stop was probably deletion of the *env* sequences to generate a helper-dependent virus that produced gp55 and was thus able to induce Friend disease. Once the helper was involved, the *gag* and *pol* structural genes could accumulate major deletions and point modifications. All of these changes were probably independent and accumulated gradually.

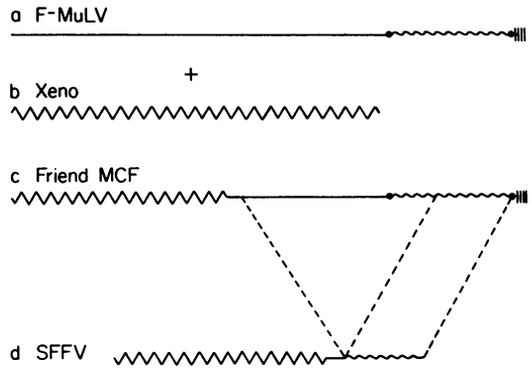

FIG. 3. Proposed mechanism of generation of SFFV from F-MuLV. (*a*) F-MuLV *env* (—, gp70; ~~, p15(E); ▮, R) recombined with (*b*) xenotropic gp70 (^^) to produce a Friend MCF virus (*c*). This underwent a major deletion and other minor changes to generate SFFV (*d*). During this process, the points of proteolytic cleavage (●) were lost.

Role of gp55 in Friend Leukemia. Comparison of three strains of SFFV showed that their only feature in common was the production of gp55 (34). This, and recombinant DNA experiments (14), suggested that gp55 might be responsible for the pathogenic effects. None of these experiments defined the origin or nature of gp55 or eliminated the possibility that other factors may be involved.

The sequence analysis of SFFV has identified two major differences between ecotropic *env* proteins and gp55. A substitution of xenotropic sequences in the region corresponding to the NH_2-terminal half of gp70 is fused to the COOH-terminal portion of p15(E) as a result of a 585-bp deletion. Which of these changes is responsible for the activity of gp55 is unknown, but the xenotropic sequences alone are not sufficient because MCF viruses are unable to induce the unique disease of SFFV (15, 16). It is likely that both events are responsible for the erythroleukemic effect, although it is not known what effect a similar deletion in an ecotropic virus would have. In addition, the SFFV LTR could affect the efficiency of viral replication in different cell types and thus play a role in the induction of the disease.

Normally, p15(E) is embedded in the membrane and anchors most of gp70 through noncovalent bonds (24). gp55 is expected to have a severely altered structure because of the NH_2-terminal substitution of xenotropic sequences, the loss of 30% of the protein, the loss of up to five glycosylation sites, and a much closer association of the gp70 portion with the membrane. As well, the premature termination and the extremely hydrophobic COOH-terminus may affect the way it is embedded in the membrane.

It seems likely that gp55 is responsible for the early proliferation of erythroid progenitor cells by inducing the cells to follow the normal hemopoietic differentiation pathway (4, 11–13, 42). This effect must be mediated at the cell surface because gp55 is a membrane protein. It might induce cellular proliferation by stimulating or interfering with a surface regulatory protein, possibly by mimicking a normal hemopoietic surface protein. This is indicated by the observation that antiserum to a SFFV-encoded antigen crossreacts with uninfected hemopoietic cells (12) and the finding that SFFV infection extends the self-renewal capacity of hemopoietic stem cells *in vivo* (42). Furthermore, the major locus that controls the susceptibility of mice to Friend disease (*Fv-2*) controls the expression of endogenous SFFV-related sequences in hemopoietic cells (4, 11) as well as the rate of proliferation of erythroid precursor cells (13).

The host loci that control normal hemopoiesis also influence the early SFFV-induced disease, suggesting that the developmental and genetic regulation of these two processes are similar. Both gp55 is clearly of viral origin, it would seem unlikely that it coincidentally resembles and mimicks some hemopoietic regulatory protein. It has been proposed that retroviruses mobilized themselves by exploiting normal cellular functions (43), so possibly the retroviral envelope proteins were originally derived from a hemopoietic cell-surface glycoprotein. Further characterizations of gp55 and its interactions within the cell membrane should lead to a better understanding of retroviral evolution and of the control mechanisms of both leukemic and normal hemopoietic differentiation.

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