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Sequence Comparisons of the Anemia- and Polycythemia-Inducing Strains of Friend Spleen Focus-Forming Virus

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A nucleotide sequence analysis carried out on the envelope gene of the anemia-inducing strain of the Friend spleen focus-forming virus (F-SFFV$_A$) reveals that its product has some unique features in common with previously described polycythemia-inducing strains of F-SFFV (F-SFFV$_P$). (i) It contains an amino terminus that is highly related to the gp70 of mink cell focus-inducing viruses, (ii) it is a fusion protein containing the amino terminus of gp70 and the carboxy terminus of p15E, and (iii) it lacks the R-peptide normally found at the carboxy end of the p15E region. Although the envelope genes of F-SFFV$_A$ and F-SFFV$_P$ are quite similar overall, they do show sequence variation, particularly at the 3′ end in the p15E-related region. These variations may contribute to previously observed differences in the response of F-SFFV$_P$- and F-SFFV$_A$-infected erythroid cells to regulatory hormone or to differences in the way the envelope glycoproteins are processed. The long terminal repeat regions of F-SFFV$_A$ and the Lilly-Steeves strain of F-SFFV$_P$ were also sequenced and compared with each other and with a previously published sequence of another F-SFFV$_P$ long terminal repeat. The sequences were found to be reasonably similar to each other but different from their ecotropic parent, Friend murine leukemia virus, as a result of a deletion of one copy of the direct tandem repeat in the enhancer regions. The observation that all SFFVs have this common change in the long terminal repeat enhancer region raises the possibility that it is required for pathogenicity.

The Friend spleen focus-forming virus (F-SFFV), a replication defective retrovirus, induces an acute leukemia in mice which is manifested by rapid accumulation of erythroid precursor cells and overproduction of erythrocytes (for a review, see reference 32). F-SFFV-induced erythroleukemia is accompanied by either a polycythemic condition or a slight anemia, depending upon the virus isolate (F-SFFV$_P$ or F-SFFV$_A$, respectively). Both cause an overall increase in erythrocyte production, but in the case of F-SFFV$_A$, the infected animals have a somewhat lower-than-normal hematocrit due to an inordinate increase in plasma volume (32, 42).

The expanded population of erythroblasts present in F-SFFV$_P$- or F-SFFV$_A$-infected mice apparently differ from each other and the population in uninfected animals in their erythropoietin requirements for proliferation and differentiation. Spleen cells from F-SFFV$_P$-infected mice are capable of forming colonies of hemoglobin-positive erythroid cells in culture without the addition of exogenous hormone, whereas F-SFFV$_A$-infected spleen cells are not (16, 19, 23). The differential effects of the anemia- and polycythemia-inducing strains have been most clearly defined in an in vitro transformation assay in which both variants induce clusters of erythroid colonies (erythroid bursts) 5 days after infection of bone marrow cells (17, 18). Whereas the F-SFFV$_P$-induced bursts contain hemoglobinized mature erythrocytes, F-SFFV$_A$-induced bursts exhibit poorly hemoglobinized precursor cells. Addition of a small amount of erythropoietin to F-SFFV$_A$ cultures, however, renders the erythroid colonies indistinguishable from those induced by F-SFFV$_P$.

At the gross molecular level, F-SFFV$_A$ and F-SFFV$_P$ appear similar. They are defective in all the structural genes of the virus, namely, *gag*, *pol*, and *env* (5, 12, 14), and the envelope genes of both encode a protein which appears, at least in part, to be required for induction of disease (24). It is a high mannose-containing glycoprotein of 52,000 to 55,000 daltons, the amino terminus of which is highly related to gp70 of recombinant mink cell focus-inducing (MCF) viruses (11, 29, 34, 44, 45). gp52-55 accumulates intracellularly in the perinuclear region of cells (35, 40), but a small percentage (10%) of the F-SFFV$_P$ gene product, unlike that of F-SFFV$_A$, can be detected on the cell surface in the form of a 65,000-dalton protein containing complex carbohydrate (33). This cell surface form has been reported (41) to contain palmitic acid, a fatty acid shown to be present on other viral membrane glycoproteins. The localization of these proteins within the cell has become a distinguishing characteristic of the F-SFFV$_P$ and F-SFFV$_A$ strains.

The purpose of the present analysis was to identify DNA sequences which could be potentially responsible for (i) differences in the ways F-SFFV$_A$- and F-SFFV$_P$-infected hematopoietic cells respond to regulatory hormones and (ii) differences in the way the glycoproteins encoded by the two variants can be processed and localized within the cell. The envelope gene of F-SFFV$_A$, in particular, was sequenced and compared to published envelope sequences of F-SFFV$_P$ (2, 9, 46) because of its demonstrated requirement for disease induction and because it encodes the glycoprotein which is differentially processed. The long terminal repeats (LTRs) of F-SFFV$_A$ and the Lilly-Steeves strain of F-SFFV$_P$ were also sequenced and compared with other LTRs since regulatory sequences contained in this region of other retroviruses may influence gene expression in specific tissues (7). Comparison of the *env* genes suggests that amino acid differences encoded at the 3′ p15E-related region of the envelope gene may account for phenotypic differences between the F-SFFV$_P$ and F-SFFV$_A$ isolates. Comparison of the LTR region, on the other hand, reveals a common

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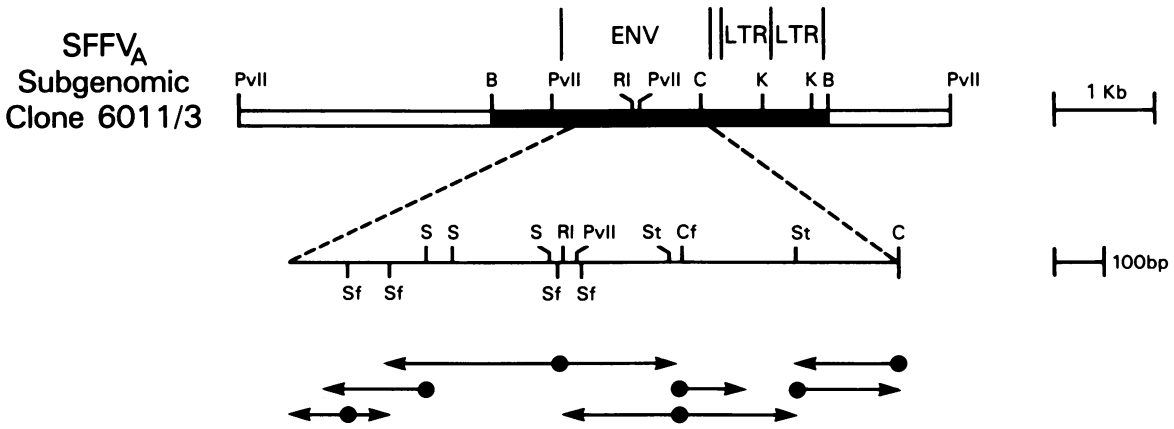

FIG. 1. F-SFFV$_A$ envelope gene sequence strategies. The technique of Maxam and Gilbert (27) was used to sequence the envelope gene of F-SFFV$_A$. A map of the subgenomic clone 6011/3 is shown; open bars represent pBR322 sequences and the solid bar represents F-SFFV$_A$ sequences. Sequencing of gel-purified DNA fragments was carried out from the restriction sites shown. B, *Bam*HI; C, *Cla*I; Cf, *Cfo*I; K, *Kpn*I; PvII, *Pvu*II; RI, *Eco*RI; S, *Sma*I; Sf, *Sfa*nI; St, *Stu*I; ENV, envelope.

feature in all SFFVs, the acquisition of which may be required for pathogenicity.

MATERIALS AND METHODS

Viral DNA. For DNA sequencing analysis, subclones 52-36 and 6011/3, derived from molecularly cloned full-length genomic clones 4-1a3 (F-SFFV$_P$, Lilly-Steeves strain) and 1310 (F-SFFV$_A$), respectively, were used (20, 25, 26). The subclones used for these studies have been shown previously to be infectious after rescue by cotransfection of fibroblasts with helper virus DNA and to have the anemic or polycythemia phenotypes of the parental genomes (20, 26). Plasmid recombinant DNA was isolated from *Escherichia coli* by using an alkaline lysis (4) and was further purified on cesium chloride gradients.

Nucleotide sequencing. Fragments of DNA for sequencing were prepared with restriction endonucleases (New England Biolabs, Inc., Beverly, Mass., and Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and purified by electrophoresis on 1 to 1.5% agarose gels or 4 to 8% polyacrylamide gels. Sequencing was performed by the method of Maxam and Gilbert (27). Fragments were labeled at the 5′ end with γ-^{32}P (New England Nuclear Corp., Boston, Mass.) and polynucleotide kinase (P-L Biochemicals, Inc., Milwaukee, Wis.) or at the 3′ end with cordycepin 5′-[α-^{32}P]triphosphate (New England Nuclear Corp.) and terminal deoxynucleotidyl-transferase (New England Nuclear Corp.) (30). The G, G+A, C+T, C, and A>C reactions were used. Sequences were visualized after electrophoresis on 20% acrylamide gels (0.4 mm by 40 cm), 12% polyacrylamide gels (0.4 mm by 80 cm), or 8% polyacrylamide gels (0.4 mm by 80 cm) and exposure of gels to Kodak XAR film at −70°C.

RESULTS AND DISCUSSION

Genetic structure of the F-SFFV$_A$ envelope gene coding region. The envelope gene of F-SFFV$_A$ was sequenced by using the strategies shown in Fig. 1. The overall genetic sequence of the F-SFFV$_A$ *env* gene is very similar to that of F-SFFV$_P$ (Fig. 2) (2, 9, 46). Three features are important in this regard and will be discussed in more detail below: (i) the amino-terminal encoding region containing endogenously derived nonecotropic sequences, (ii) the 585-base-pair (bp) deletion within the open reading frame, and (iii) premature termination of translation before the R-peptide encoding region.

The first two-thirds of the F-SFFV$_A$ gp52 encoding region, including the leader peptide, is composed of sequences which are highly homologous to the gp70 of MCF viruses. We have determined the 3′ site of recombination between the putative parent Friend murine leukemia virus (F-MuLV) and endogenous MCF-like sequences to be between nucleotides 875 and 896 (as indicated under the line and between the vertical arrows in Fig. 2). This was determined by a comparison of these sequences to those of both F-MuLV and Friend MCF (F-MCF). As previously noted by Clark and Mak (10), the site of recombination between F-MuLV and endogenous *env* gene sequences in SFFVs differs from isolate to isolate. For example, the point of recombination in the Lilly-Steeves strain of SFFV$_P$ is a few bases upstream from the site indicated in Fig. 2, and the Axelrad-Steeves strain site of recombination is approximately 70 bp upstream. Interestingly, the site of recombination in F-SFFV$_A$ is identical to that present in a strain of F-SFFV$_P$ carried in Japan by Ikawa and colleagues for many years (2). The location of these different crossover regions among isolates, however, has little consequence on the envelope protein product, since at the amino acid level the strains are basically identical. The MCF-like or nonecotropic amino acid sequences terminate for all the strains at residue 278 (Fig. 3). Carboxy terminal to this, all the amino acid sequences are essentially ecotropic.

The site of the large envelope deletion, indicated in Fig. 2 between residues 982 and 983, is the position at which 585 bp of ecotropic sequences have been lost. These include part of the coding sequence for gp70 as well as p15E. The size of this deletion is exactly the same as it is in the other isolates of F-SFFV that have been sequenced (2, 9, 46). The deletion would occur in the same position as that of the other isolates if one changed a single nucleotide C at position 971 to a T (which would substitute a leucine for a proline). Thus, it is likely that all SFFV *env* genes are actually deleted in the same place and occurred only once in a common exogenous ancestral virus present in cell-free ascites extracts of Charlotte Friend (13) or in an endogenous virus picked up by recombination. Interestingly, the Rauscher SFFV (R-SFFV)

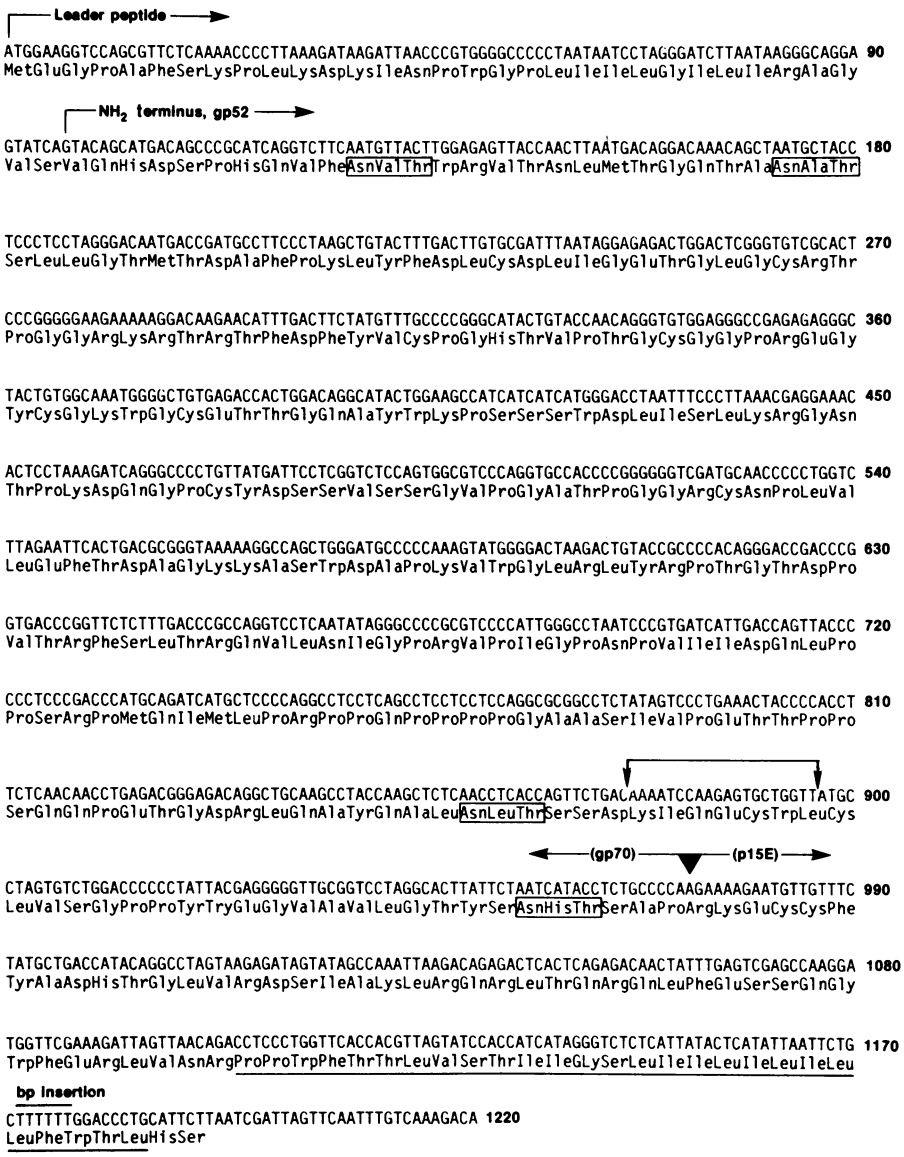

FIG. 2. Nucleotide sequence of the coding region of the F-SFFV$_A$ envelope glycoprotein, gp52-55. The deduced amino acid sequence of the protein is shown underneath the nucleotide sequence. The proposed site of recombination (see text) between F-MuLV and endogenous viral sequences is between the vertical arrows. The deletion removing 585 bp from the ecotropic portion of the envelope gene is indicated by the solid triangle. The potential glycosylation sites are indicated by boxes. The general location of the single bp insertion relative to F-MuLV is shown by a short line above the sequence. The C-terminal hydrophobic domain is indicated by a line below the sequence.

has an envelope gene deletion identical in size and position to that of the F-SFFVs (3).

A single nucleotide bp insertion in the F-MuLV-related p15E sequences of F-SFFV$_A$ causes a shift in reading frame analogous to that in F-SFFV$_P$. The area at which the insertion is found has a string of six T's compared to five T's in F-MuLV. This sequence is the same as that found in the isolate passaged for some time in the Ikawa laboratory (2).

Amino acid sequence comparison of the SFFV$_A$ *env* gene product with those of three SFFV$_P$ isolates. Figure 3 presents the gp52-55 amino acid sequences previously published by our laboratory and others for three isolates of SFFV$_P$ as well as the amino acid sequence of the SFFV$_A$ gp52. In general, the amino terminus of the envelope proteins of all SFFVs is highly conserved, particularly in the boxed-in region shown in Fig. 3. Perhaps this region is important structurally or functionally for the pathogenic function of these viruses. It is interesting to note that within this region are several cysteines, an amino acid known to be involved in maintenance of conformation.

Amino acids which are unique to SFFV$_A$ or deleted compared with all the F-SFFV$_P$s are shaded in Fig. 3. The majority of the SFFV$_A$ unique residues occur at the carboxy terminus in the ecotropic portion of the molecule. One or more of these unique amino acids probably accounts for the expression of at least some of the SFFV$_A$ phenotypic characteristics, since we have shown (unpublished data) by genetic recombination within the envelope gene itself that the 3′ end of the gene, including residues 187 and beyond, most likely determines characteristic hormone responsive-

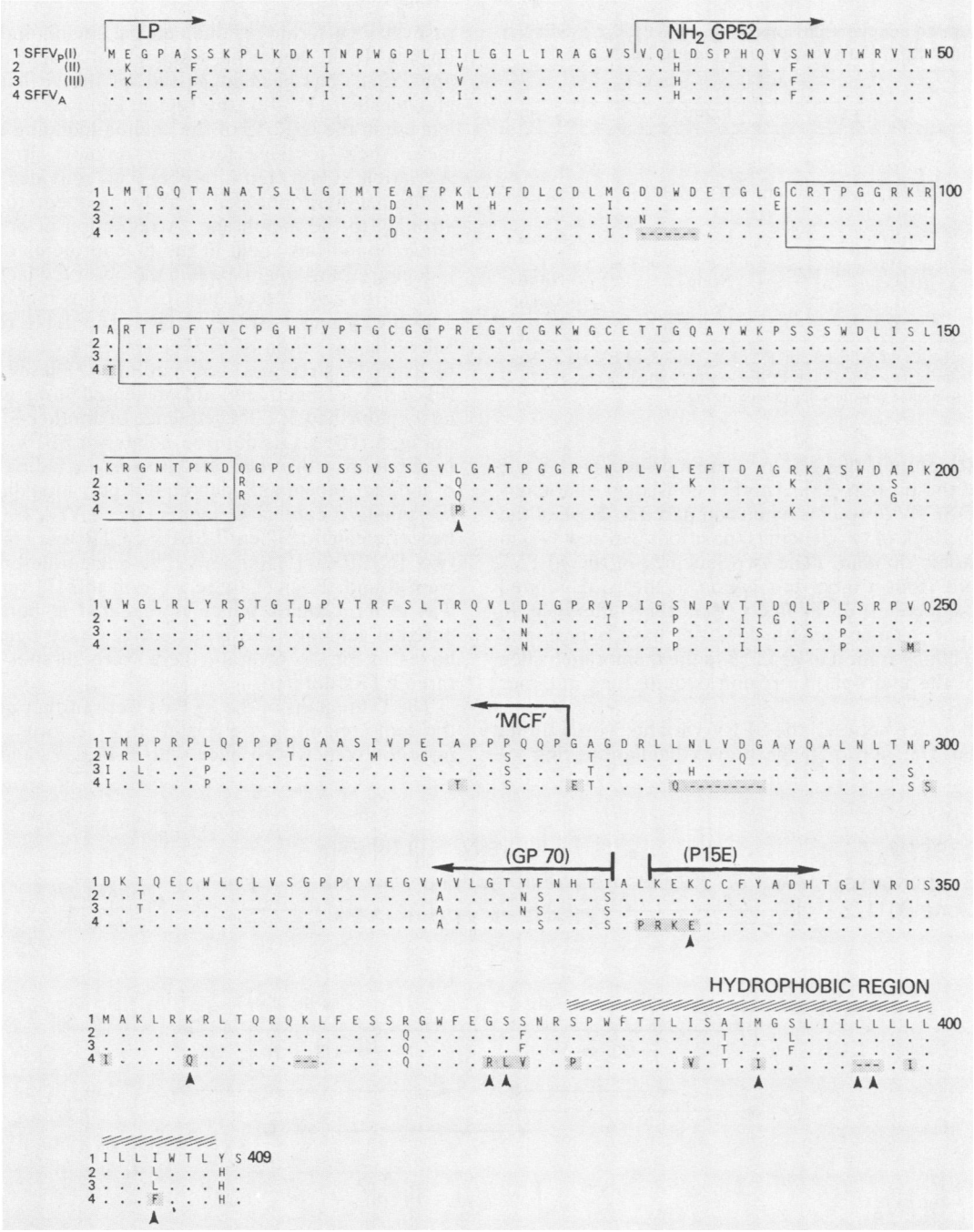

FIG. 3. Amino acid sequence comparison of gp52-55s encoded by four strains of F-SFFV. Sequences of the polycythemia-inducing viruses, $SFFV_PI$, $SFFV_PII$, and $SFFV_PIII$, have been previously published (see references 46, 9, and 2, respectively). The boxed area shows high degree of sequence homology. Shaded residues are unique to F-$SFFV_A$ compared with the three F-$SFFV_P$s. Positions with arrows are locations in which both the anemia-inducing R-SFFV, like $SFFV_A$, is different from all the F-$SFFV_P$s. LP, Leader peptide.

ness of $SFFV_A$- or $SFFV_P$-infected cells (16, 18, 19, 23) as well as the ability of the gp52-55 in infected cells to be processed to its mature form (33).

Since the R-SFFV has biological characteristics which areindistinguishable from the F-$SFFV_A$ (18) and encodes an envelope protein which fails to be transported to the cell surface (36), we compared the recently published R-SFFV envelope sequence (3) with those of F-SFFVs. Although there are a number of random sequence differences between R-SFFV and the several Friend isolates, there are several positions at which the R-SFFV sequence, like the F-$SFFV_A$ sequence, has residues that differ from all the F-$SFFV_P$s. These are indicated by arrows below the sequence in Fig. 3, and the amino acids present in those positions for all of the SFFV strains are given in Table 1. Some of the differences between the anemia and polycythemia-inducing viruses are worth noting. At position 336 there is a consistent significant charge difference; at this site the anemia-inducing viral proteins have glutamic acid residues, whereas the F-$SFFV_P$ proteins have basic lysine residues. At position 374 both of the anemia-inducing viruses are missing a glycine present in the F-$SFFV_P$s; this difference could have an important effect

TABLE 1. Amino acid comparisons among F-SFFV$_A$, F-SFFV$_P$, and R-SFFV at selected sites[a] in gp52-gp55

Amino acid position[b]	Amino acid present in:		
	F-SFFV$_P$	F-SFFV$_A$	R-SFFV
172	L, Q	P	K
336	K	E	E
356	K	Q	E
374	G	R	E
375	S	L	L
390	M	I	I
396	L	None	None
397	L	None	None
404	I, L	F	F

[a] Sites were selected at which both F-SFFV$_A$ and R-SFFV (3) residues were different from residues in all the F-SFFV$_P$s presented in Fig. 3.
[b] Amino acid sequence position designated in Fig. 3.

on protein structure if this glycine were a critical part of the backbone of the protein (28). Another consistent characteristic of the F-SFFV$_A$ proteins when compared to those of the SFFV$_P$s is the lack of two leucines (positions 396 and 397) in the hydrophobic domain. (The two leucines in the SFFV$_P$ gp52-55 result from a 6-bp duplication in the p15E-related nucleotide sequence.) It is likely that this difference in transmembrane segment length does not have a profound effect on the configuration of gp52-55 in the membrane, since the length of the hydrophobic region is quite long in either case.

A final difference between the SFFV variants worth noting is at position 375, at which the polycythemia-inducing viruses have a serine residue and the anemia-inducing viruses do not. If the serine is a site of fatty acid attachment to gp52-55, as has been suggested for the vesicular stomatitis glycoprotein (37, 38), the replacement of this residue by a leucine in the gp52-55 of the anemia-inducing viruses would cause them to have fewer or no acyl groups attached. Acylation, a late step in processing, provides proteins with additional hydrophobicity, allowing them to attach more strongly to the membrane. A reduction in or loss of covalently bound fatty acid in the glycoproteins of the anemia-inducing strains might explain the observed lack of glycoprotein on the cell surface, but this remains highly speculative.

F-SFFV$_P$ and F-SFFV$_A$ LTRs. The LTR regions of the Lilly-Steeves strain of SFFV$_P$ and SFFV$_A$ were sequenced by using the strategies shown in Fig. 4. These two sequences (PI and A, respectively) are presented along with the previously published 3′ LTR sequence of another SFFV$_P$ (PII) (8) in Fig. 5. The LTRs of three isolates of SFFV show features in common with other retroviruses, including the presence of indirect repeats at each end, a polyadenylation site, and the putative control elements, the TATA box and an enhancer element. When F-SFFV$_A$ LTR was compared to the two F-SFFV$_P$ LTRs, only five-nucleotide-bp mismatches were found. Four of these were in the U3 region, and one was in R, a region which is repeated at both ends of the parental genome. These nucleotides have been enclosed in boxes in Fig. 5. Overall, there were 28 sites at which the three LTRs differed.

The U3 regions of the SFFVs were further compared with the same region of the related F-MCF virus, derived from infection of newborn mice with F-MuLV, and with the U3

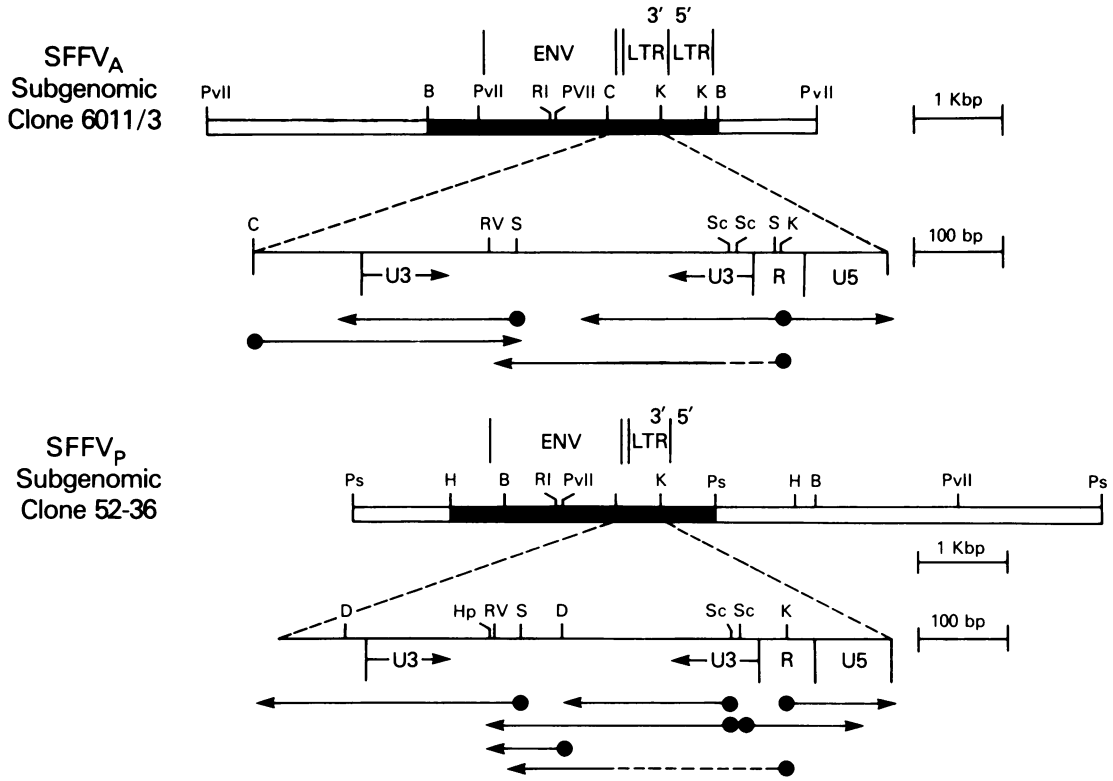

FIG. 4. LTR sequencing strategies. The technique of Maxam and Gilbert (27) was used to sequence the F-SFFV$_A$ and F-SFFV$_P$ (Lilly-Steeves strain) LTRs. Maps of subgenome clones 6011/3 and 52-36 are shown; open bars represent pBR322 sequences, and solid bars are SFFV$_A$ or F-SFFV$_P$ sequences. Sequencing of gel-purified DNA fragments was carried out from the restriction sites shown. B, *Bam*HI; C, *Cla*I; D, *Dde*I; H, *Hin*dIII; Hp, *Hph*I; K, *Kpn*I; PVII, *Pvu*II; Ps, *Pst*I; RI, *Eco*RI; RVI, *Eco*RV; S, *Sma*I; Sc, *Sac*I; ENV, envelope.

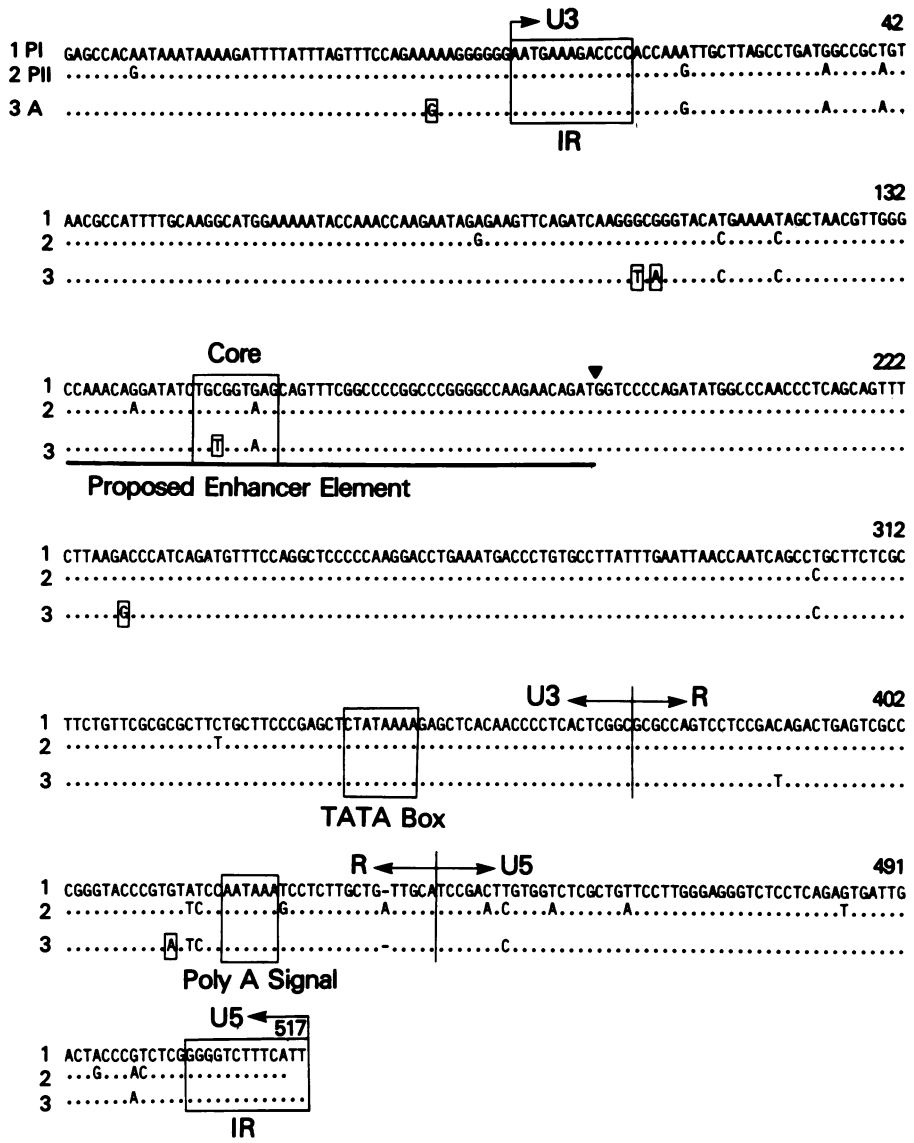

FIG. 5. Nucleotide sequence of three F-SFFV LTRs. PI, F-SFFV$_P$ (Lilly-Steeves strain); PII F-SFFV$_P$ (8); A, F-SFFV$_A$; inverted triangle, the proposed enhancer element is analagous to the enhancer element region defined by Laimins et al. (22) for Moloney sarcoma virus and represents one copy of the direct repeat found in the F-MuLV LTR (21). The core enhancer sequence is analagous to that in Moloney sarcoma virus as defined by Weiher et al. (43).

region of the putative parent of these viruses, F-MuLV (Fig. 6). An obvious difference in this region between the pathogenic envelope gene recombinant viruses (F-SFFV and F-MCF virus) and F-MuLV is the lack in the former viruses of a copy of the direct tandem repeat in the region analogous to the enhancer region of Moloney murine sarcoma virus (22). R-SFFV also has this identical deletion (3), and another F-MCF virus sequenced recently by Adachi et al. (1) has a partial loss of the same sequence. The fact that this sequence loss is a common characteristic and represents the lack of a specific set of sequences suggests that this LTR alteration may be a requirement for generation of pathogenic viruses from F-MuLV and Rauscher MuLV. This enhancer property may be required for disease induction by both SFFV and recombinant F-MCF virus, but the support for making this contention is stronger in the case of SFFV. We believe that any unique and conserved properties of the SFFV genome can be considered as potentially important for disease induction. First, the genetic information contained in SFFV, unlike that in F-MCF or F-MuLV, serves a limited function in virus replication, its primary role being disease induction. Recent sequence analyses of several F-SFFV isolates (2, 9, 46) shows that long stretches of sequences are not conserved in F-SFFV; the defective viruses have varied extensively from their original parental virus, F-MuLV, and from each other through recombination, insertion, and deletion (10). Perhaps this reflects the loss of many replicative functions which are performed by the helper virus during infection with SFFV. On the other hand, certain features of SFFV do not vary from isolate to isolate in the envelope region and LTR, suggesting that they may be crucial for disease induction. The second reason we feel that the SFFV sequence can tell us a great deal about the genetic requirements for erythroleukemia is that the leukemia induced by this virus is

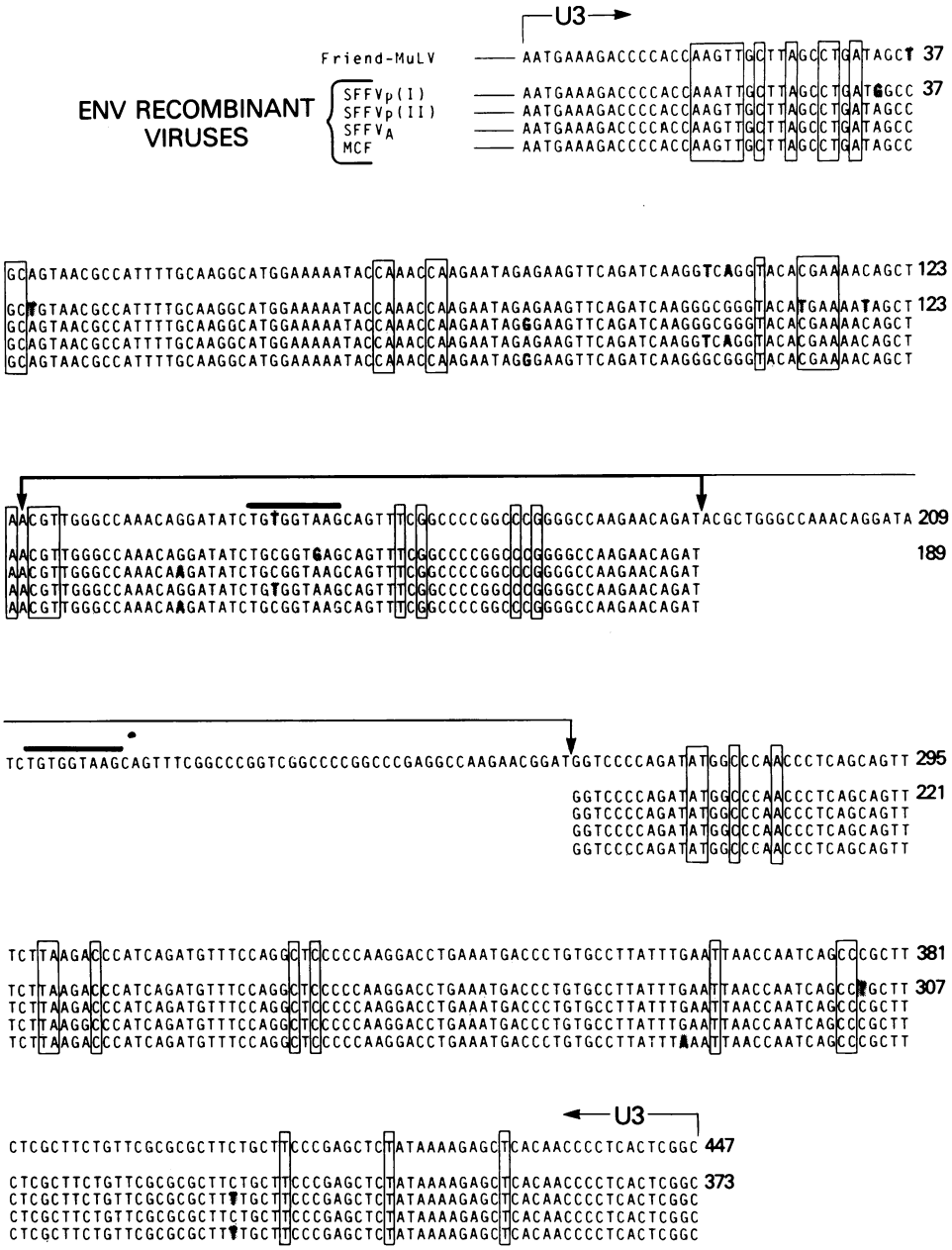

FIG. 6. Comparison of U3 regions of envelope (ENV) recombinant viruses with the U3 region of F-MuLV. The F-MuLV, F-MCF, and F-SFFV$_P$II were published previously (8, 21). The proposed enhancer region in F-MuLV is composed of a sequence which is repeated (direct tandem repeat). The location of this region is designated by lines above the sequence; arrows show the beginning and end of the repeated sequence. The heavy bar designates the enhancer core sequence. Shaded nucleotides are those which differ from the nucleotides found in the majority of viruses in this figure at the indicated position. The sequences which are enclosed in boxes are those which are not found in Moloney MuLV or Moloney MCF virus (6, 39).

so acute it allows little time in animals for genetic alteration and selection of more pathogenic viruses. The effect of this virus is seen as early as 3 days in vivo (16, 19, 23), and the proliferative response of hematopoietic cells can be assessed directly in a target cell transformation assay which demonstrates one-hit kinetics within 5 days after cells are exposed to the virus (15). Therefore, it appears with SFFV that the input virus is the one responsible for disease. This is in contrast to the erythroid disease induced by F-MuLV or F-MCF (31) in newborns, in which the induction period is longer and the roles of the input virus and recombinant viruses that may arise during infection are not clear.

It is interesting to speculate whether the alteration in the LTR of SFFVs came as a result of a recombinational event with other viral sequences or a deletion event. We believe that the sequence data suggests the latter. If the U3 region of the LTRs of SFFV had undergone a recombinational event with another viral sequence, then one would expect to see specific sequences present in all the SFFV LTRs that are not present in the F-MuLV LTR. This is not the case; sequence variations between the LTRs shown in Fig. 2 appear to have no pattern and are fairly random. There is only one nucleotide, at position 37, in the F-MuLV LTR which is changed from a T to a C in F-MCF virus and SFFV. The LTRs of the

SFFVs and F-MCF virus have retained almost all of the nucleotides that are unique to F-MuLV when compared to Moloney MuLV or Moloney MCF virus (nucleotides enclosed in boxes in Fig. 6), suggesting that the former LTRs are derived from F-MuLV.

We raise the possibility that this alteration in the LTR plays a role in regulating the expression of the virus in a particular cell type, since we have observed (unpublished observation) that spleen cells from mice with erythroid disease and immortalized erythroid precursor cells from diseased animals express much higher levels of recombinant virus envelope proteins than F-MuLV viral envelope proteins.

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