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Molecular Characterization of an Attenuated Human Immunodeficiency Virus Type 2 Isolate

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Naturally occurring strains of human immunodeficiency virus (HIV) can vary considerably in their in vitro biological properties, and such differences may also be reflected in their in vivo pathogenesis. In an attempt to define genetic determinants of viral pathogenicity, we have molecularly cloned, sequenced, and characterized an attenuated isolate of HIV type 2 (HIV-2/ST) that differs from prototype HIV-2 strains in its inability to fuse with and kill susceptible CD4-bearing target cells. A proviral clone, termed JSP4-27, was identified to be transfection competent and to fully exhibit the noncytopathic and nonfusogenic properties of its parental isolate. Nucleotide sequence analysis of this clone revealed a genomic organization very similar to that of cytopathic HIV-2 strains and an overall nucleotide sequence homology of 88 to 90%. Amino acid sequence comparison confirmed the integrity of all major viral gene products in JSP4-27 but identified two amino acid sequence substitutions in its envelope fusion region. To investigate whether these mutations were responsible for the nonfusogenic phenotype of JSP4-27, we amplified, cloned, and sequenced the envelope fusion regions of four additional HIV-2/ST strains, two of which represented in vitro-generated, fusogenic and cytopathic variants of HIV-2/ST. The analysis showed that all HIV-2/ST strains examined, including the fusogenic variants, contained the same amino acid sequence changes. On the basis of these findings, we conclude that the attenuated phenotype of JSP4-27, and that of its parental virus, is not due to a direct alteration of the envelope fusion domain. Our results also show, for the first time, that individual replication-competent proviral clones can be representative of attenuated strains of HIV.

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) represent two distinct groups of HIV known to cause acquired immunodeficiency syndrome (AIDS) in infected individuals (3, 11, 12, 21, 40). Whereas HIV-1 is the causative agent of epidemic AIDS worldwide, HIV-2 appears to be geographically restricted to West Africa (4, 7, 13, 26, 29). Numerous isolates of HIV-1 and HIV-2 have been obtained, and their biological and molecular properties have been characterized (1, 3, 11, 22, 40, 51). Nucleotide sequence analysis shows that HIV-2 is only distantly related to HIV-1 (22) and is more closely related to two primate retroviruses, simian immunodeficiency virus (SIV) strains SIV_{MAC} and SIV_{SM}, which cause an AIDS-like disease in captive macaques (8, 14, 25). Although genetically divergent, prototype HIV-1 and HIV-2 isolates have very similar biological properties, including a propensity for rapid genetic change (17, 42, 51), a similar host cell tropism, a considerable cytopathic effect on T-cell cultures and peripheral blood mononuclear cells in vitro, and the ability to form syncytia with CD4-bearing target cells (11, 40). In fact, the majority of HIV-1 and HIV-2 strains isolated from patients with immunodeficiency disease have been shown to cause cell fusion and the formation of multinucleated giant cells in culture. This represents a hallmark of productive viral infection and accounts for the profound cytopathic effect of HIV in vitro (23, 37, 49).

In contrast to these viruses, we and others have recently isolated less pathogenic strains of HIV-1 and HIV-2 that exhibit markedly different biological properties (2, 9, 16, 32, 34, 47). These particular isolates cause little or no cell death in susceptible target cells, fail to induce cell fusion with CD4-bearing immortalized T-cell lines, exhibit a restricted host cell tropism with a preference for peripheral blood mononuclear cells or macrophages, and are often derived from asymptomatic individuals. Although their in vitro biological differences are well documented, the genetic changes responsible for their attenuated phenotype are not understood. To elucidate determinants of HIV pathogenicity, we have begun to molecularly dissect a previously reported, nonfusogenic and noncytopathic HIV-2 isolate, termed HIV-2/ST, that was obtained from a healthy Senegalese prostitute (32). Although this virus replicated to high titers in tissue culture, it infected cells at a slower rate than did cytopathic strains of HIV-1 and HIV-2 and caused little or no cell killing and fusion. This was the case despite the fact that its external envelope glycoprotein was cleaved correctly, transported to the cell surface, and shown to bind to a specific epitope on CD4, which was recognized by OKT4a but not OKT4 antibodies (32). HIV-2/ST therefore appeared to bind to the CD4 molecule analogous to other HIVs, but it failed to fuse with CD4-bearing target cells, suggesting that its infectivity was greatly retarded at the level of cell entry (32).

Since HIV isolates generally represent complex mixtures of genotypically distinct viruses and since the biological phenotype of any HIV culture depends on the sum of the properties of each genotypic variant (21a, 42), we first attempted to isolate a molecular clone that was both transfection competent and representative of the in vitro properties of its parental virus. We therefore obtained three full-length proviral clones (λJSP4-27, λJSP4-32, and λJSP4-34)

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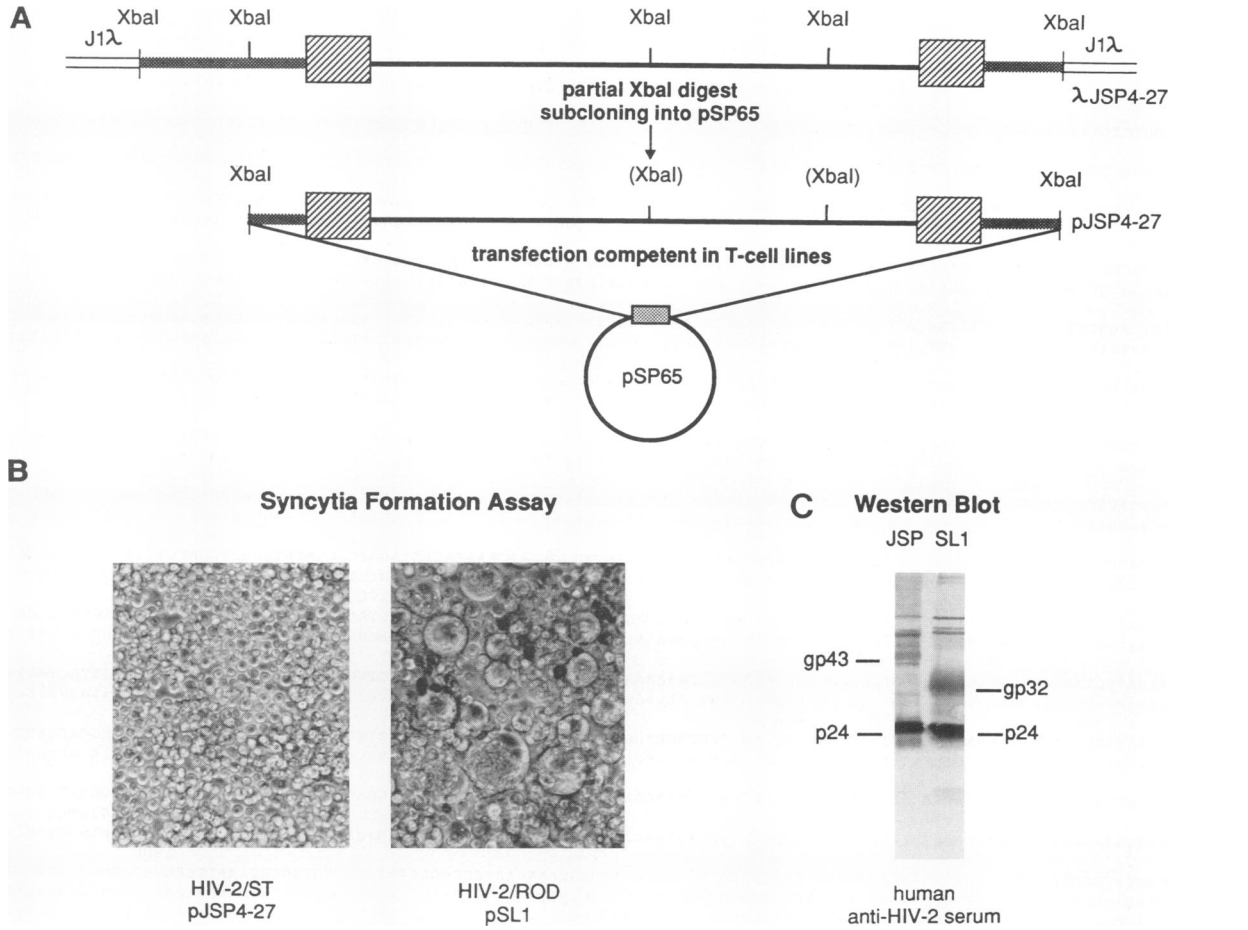

FIG. 1. (A) Construction of a replication-competent HIV-2/ST plasmid clone. λJSP4-27 (32) was partially cleaved with *XbaI* to remove bacteriophage arms (double lines) and flanking cellular sequences (hatched lines), and the resulting 14-kilobase-pair provirus-containing fragment was subsequently subcloned into pSP65. (B and C) Biological comparison of HIV-2/ST- and HIV-2/ROD-derived, genetically pure viral strains. CEMx174 cultures, productively infected (>90%) with JSP4-27 (HIV-2/ST) and SL1 (HIV-2/ROD), respectively, were examined in syncytium formation assays and by Western blot analysis. (B) No syncytium formation was observed upon cocultivation of JSP4-27-producing CEMx174 cells with uninfected CEM cells, whereas numerous and large syncytia were generated upon cocultivation of the same uninfected CEM cells with SL1-infected CEMx174 cells (identical results were obtained with H9, SupT1, and CEMx174 cells). Syncytium formation was monitored 18 h after cocultivation. (C) Western blot analysis of cell-free virions derived from these same transfection-derived cultures demonstrated differences in the sizes of the JSP and SL1 transmembrane envelope glycoproteins.

from a genomic library of a biologically cloned high-producer cell line, termed ST/B12, and subsequently transfected them into the neoplastic T-cell lines SupT1 (46) and CEMx174 (27, 44). Reverse transcriptase activity was detected in supernatants of cultures transfected with λJSP4-27 as early as 5 days posttransfection, whereas λJSP4-32- and λJSP4-34-transfected cultures revealed no signs of viral replication, indicating that these proviruses were replication defective. Immunofluorescence analysis further confirmed the presence of virus-expressing cells in λJSP4-27-transfected cultures but failed to identify virus-mediated cell fusion. Western blot (immunoblot) analysis of purified JSP4-27 virions demonstrated a protein profile similar to that of the parental HIV-2/ST virus. To facilitate subsequent transfection experiments and to allow the direct comparison of JSP4-27 with other transfection-competent HIV-2 plasmid constructs, we subcloned the proviral insert of λJSP4-27 into the plasmid vector pSP65 (Fig. 1A).

To test whether the transfection-derived JSP4-27 virions were infectious, filtered supernatants of plasmid-transfected cultures were transmitted to uninfected SupT1 and CEMx174 cells. The results showed that cell-free transmission of JSP4-27 virions was readily and reproducibly demonstrable. However, infection and spread in culture, particularly in SupT1 cells, occurred slowly and with considerable delay. These results were confirmed and extended in comparative studies with a transfection-derived, cytopathic HIV-2/ROD strain, termed SL1 (36). Whereas transfection of the SL1 provirus resulted in >90% infected SupT1 or CEMx174 cultures within 3 to 4 days posttransfection, JSP4-27-transfected cultures reached only 10% infectivity in the same time period, which indicated the same reduced ability to spread in culture that had been observed for the parental HIV-2/ST strain (32). Similarly, the transfection-derived JSP4-27 cultures did not form syncytia upon cocultivation with several CD4-bearing T-cell lines, including SupT1, CEM, H9, and CEMx174, whereas SL1-infected cultures produced numerous and large syncytia, as well as a profound cytopathic effect, with these same target cells (Fig. 1B). These data thus showed that the JSP4-27 provirus was replication competent and infectious and exhibited the same

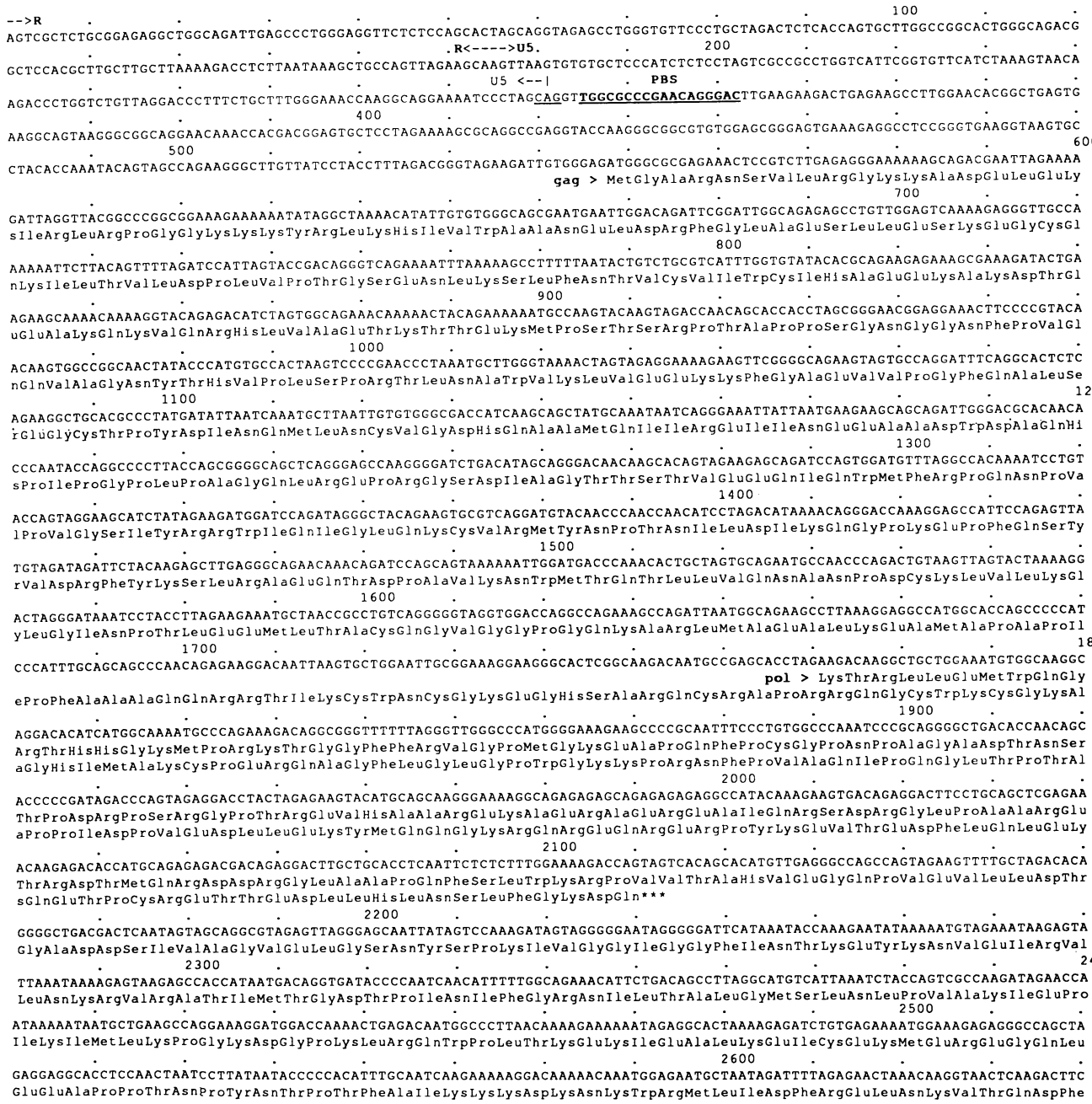

FIG. 2. Complete nucleotide sequence of the HIV-2/ST proviral genome. Shown are 9,672 bp of nucleotide sequence and the deduced amino acid sequences of the corresponding viral proteins. The sequence starts at the 5′ cap site and ends with the 3′ polyadenylation site of the viral RNA. The primer-binding site (PBS; complementary to the Lys-tRNA), polypurine tract (PPT), and short inverted repeats that flank the LTRs are underlined. Core enhancer sequences (E), Sp1-binding sites (Sp1), the TATA box (TATAA), and the polyadenylation signal (AATAAA) are shown. The U3-R and R-U5 boundaries, as well as the splice donor (SD) and splice acceptor (SA) sites, have been determined in analogy with HIV-2/ROD (22). The *vpr* open reading frame contains a premature in-frame TAA stop codon at position 5777 (***). Sequence analysis was performed by the chemical degradation method of Maxam and Gilbert (38) as well as by the dideoxynucleotide-chain termination method of Sanger et al. (45). The nucleotide sequence of JSP4-27 has been submitted to the AIDS Sequence Data Base, Los Alamos National Laboratories, as well as to the GenBank and EMBL libraries.

nonfusogenic and noncytopathic properties as previously described for the parental virus (32).

Having identified and characterized the biological features of a molecular clone of HIV-2/ST, we next sequenced its entire genome. The complete nucleotide sequence of the JSP4-27 provirus is depicted in Fig. 2. The viral genome is 9,672 base pairs (bp) long and exhibits an overall genomic organization of 5′ long terminal repeat (LTR)-*gag-pol*-central region-*env-nef*-3′ LTR, which is identical to that of other cytopathic HIV-2 and SIV_{MAC} proviruses. It contains all major open reading frames characteristic for HIV-2, including *vpx*, which is present in HIV-2 and SIV_{MAC} but not in

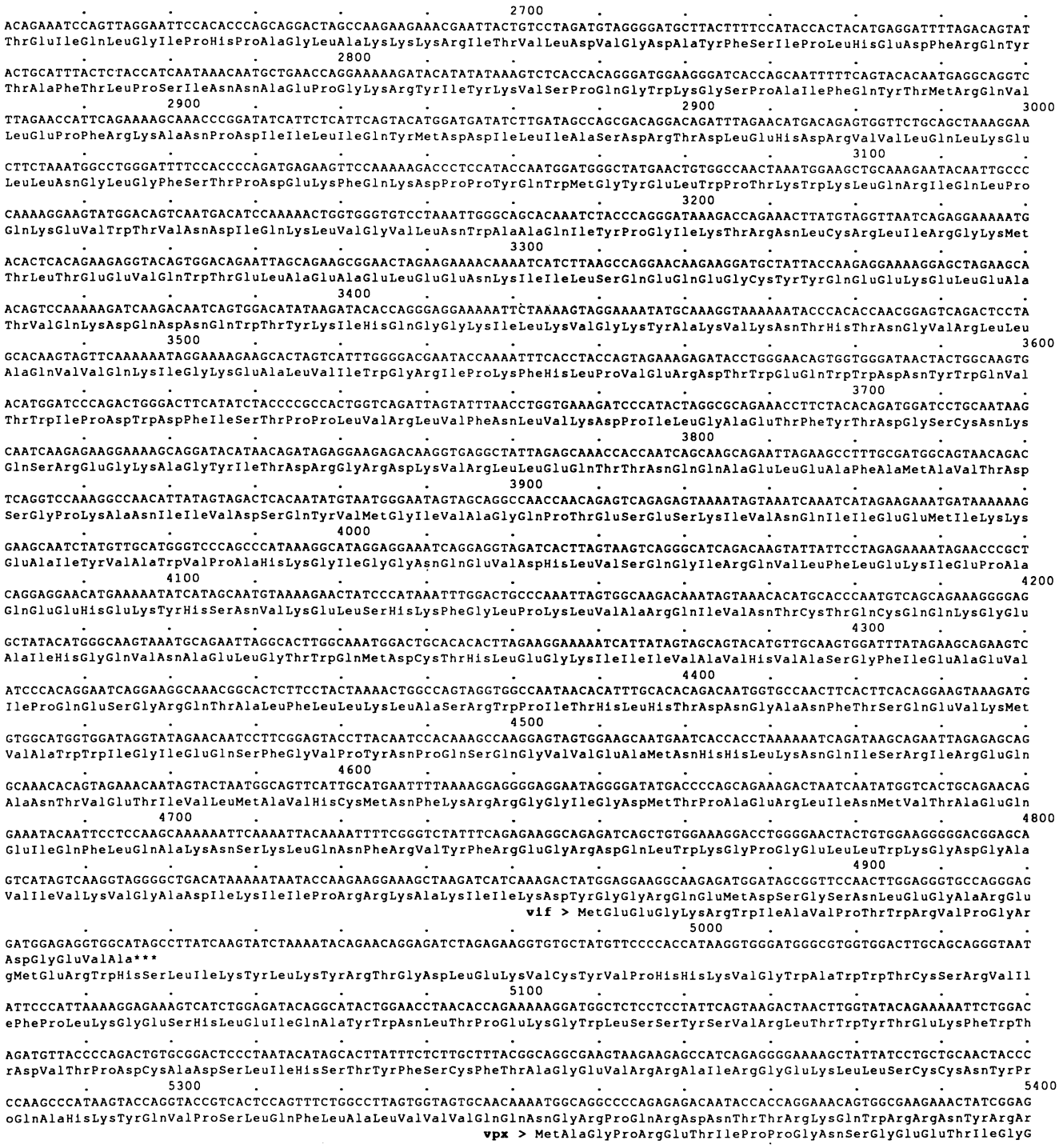

FIG. 2—*Continued.*

HIV-1 (18, 24, 30, 50), and *vpr*, which is present in HIV-1, HIV-2, and SIV_{MAC} but not in SIV_{AGM} (19). Like other HIV and SIV proviruses, HIV-2/ST is flanked by LTR sequences that are known to regulate viral gene expression. Sequence comparison with other HIV-2 LTRs showed that regulatory elements, such as the TATA box, the polyadenylation site, core enhancer sequences, Sp1-binding sites, and the *tat*-responsive region, are all present in HIV-2/ST and that their sequences are highly conserved. The HIV-2/ST LTR is of similar length, and there are no major deletions or insertions that would distinguish it from the LTRs of other cytopathic HIV-2 strains (data not shown).

Comparison of the deduced amino acid sequences of the HIV-2/ST reading frames suggested that with the exception of the *vpr* gene, they all encoded full-length and functional proteins. This open reading frame was found to contain an in-frame TAA stop codon that truncates the *vpr* protein prematurely after the first 32 amino acid residues. Since the

FIG. 2—*Continued.*

JSP4-27 provirus is fully replication competent, it can be concluded that the *vpr* gene product is not required for in vitro replication of HIV-2. This conclusion was confirmed by the biological analysis of a second *vpr*-deficient HIV-2 provirus independently constructed in our laboratory (J. C. Kappes and B. H. Hahn, unpublished data) as well as by the findings of others (15). Moreover, since *vpr*-deficient proviruses of HIV-2 are also cytopathic and fusogenic, it is unlikely that the lack of a functional *vpr* gene in HIV-2/ST is responsible for its attenuated phenotype.

Pairwise sequence alignments of JSP4-27 with other cytopathic strains of HIV-2 similarly revealed no genetic features unique to HIV-2/ST. Comparison of HIV-2/ST and HIV-2/ROD demonstrated an overall sequence divergence of 11%, which is within the expected range of genetic variability observed among geographically distant isolates of HIV-2

FIG. 2—*Continued.*

(Tables 1 and 2). Three other recently reported HIV-2 strains, HIV-2/ISY, derived from a Gambian individual with AIDS (1, 17), HIV-2/NIH$_Z$, derived from a patient with AIDS from Guinea Bissau (51), and HIV-2/GH, derived from a patient with AIDS from Ghana (28; A. Hasegawa, H. Tsujimoto, N. Maki, K. Ishikawa, T. Miura, M. Fukasawa, K. Miki, and M. Hayami, AIDS Res. Hum. Retroviruses, in press), differ from HIV-2/ROD (Cape Verde Islands [11, 22]) by 11, 12, and 12%, respectively. Among all of these strains, the Senegalese HIV-2/ST isolate was found to be most closely related to the Gambian isolate HIV-2/ISY, which shared 90% of its nucleotide sequence with HIV-2/ST.

Since infectivity, syncytium formation, and cell fusion are viral properties that are mediated by the viral *env* gene, we examined this gene in particular with respect to sequence differences unique to HIV-2/ST. Alignment of the deduced HIV-2/ST *env* sequence with those of six other cytopathic and fusogenic HIV-2 and SIV strains is shown in Fig. 3. Overall, the sizes of the various *env* sequences compared are approximately the same. In contrast to other HIV-2 and

TABLE 1. Nucleotide and amino acid sequence divergence among HIV-2 and SIV strains[a]

	Envelope Amino Acid Sequence Divergence						
	HIV-2/ST	HIV-2/ROD	HIV-2/ISY	HIV-2/NIH$_Z$	HIV-2/GH	SIV/MAC$_{142}$	SIV/SM
HIV-2/ST		19%	17%	18%	16%	28%	28%
HIV-2/ROD	11%		20%	19%	18%	28%	28%
HIV-2/ISY	10%	11%		20%	19%	30%	29%
HIV-2/NIH$_Z$	12%	12%	13%		19%	28%	28%
HIV-2/GH	11%	12%	12%	15%		29%	29%
SIV/MAC$_{142}$	23%	23%	24%	26%	24%		19%
SIV/SM	23%	22%	23%	23%	23%	15%	

Total Nucleotide Sequence Divergence

[a] The percent nucleotide sequence divergence between HIV-2/ST (JSP4-27), HIV-2/ROD (22), HIV-2/ISY (17), HIV-2/GH (Hasegawa et al., in press), SIV$_{MAC142}$ (8), and SIV$_{SM}$ (25) is shown along with the percent amino acid sequence divergence of their envelope glycoproteins. Sequences were aligned pairwise, using the Microgenie computer software (Beckman).

TABLE 2. Sequence homologies among virus-specific genes of three HIV-2 proviruses

Open reading frame	% Homology					
	ST/ROD		ST/ISY		ROD/ISY	
	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
LTR	91.5		91.4		91.8	
gag	91.1	92.0	90.7	89.1	90.3	89.5
pol	91.2	91.4	91.2	91.4	91.2	91.7
vif	91.5	88.4	92.4	91.2	92.2	85.6
vpx	89.3	85.7	92.6	90.2	88.4	87.5
vpr	89.9[a]	80.0[a]	89.8[a]	84.8[a]	93.3	89.5
tat	86.7	75.4	88.7	78.5	90.5	80.8
rev	82.0	85.0	ND[b]	ND[b]	ND[b]	ND[b]
env	85.5	81.4	86.0	83.0	84.8	80.4
nef	86.1	78.9	85.8	77.7	87.0	78.5
Overall % homology	89.5		89.9		89.3	

[a] An in-frame stop codon is present in the HIV-2/ST *vpr* open reading frame.
[b] Meaningful comparison of the HIV-2/ISY *rev* gene (17) with the corresponding *rev* genes of HIV-2/ST (JSP4-27) and HIV-2/ROD (22) was not possible because of considerable length differences between their sequences. ND, Not done.

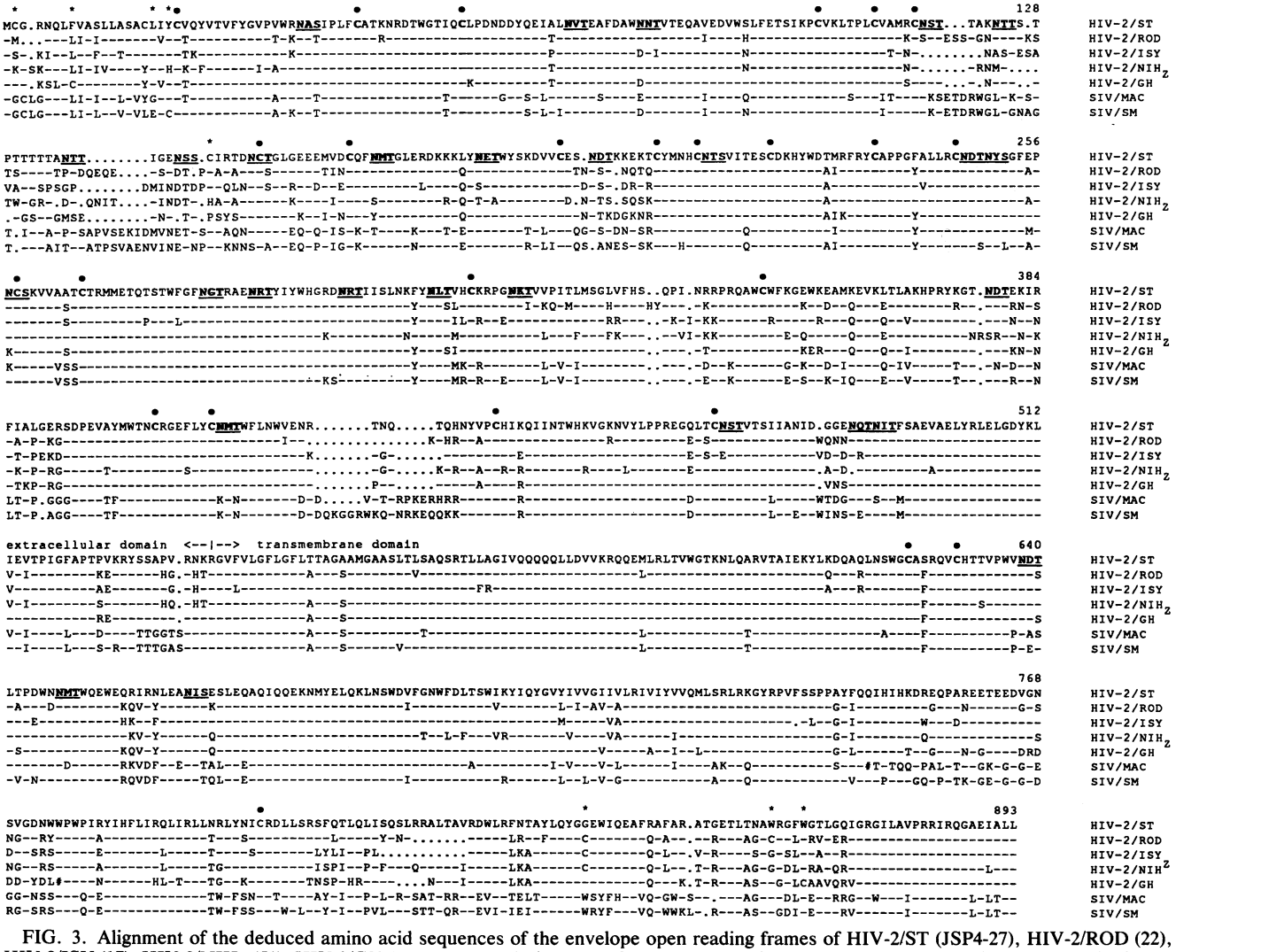

FIG. 3. Alignment of the deduced amino acid sequences of the envelope open reading frames of HIV-2/ST (JSP4-27), HIV-2/ROD (22), HIV-2/ISY (17), HIV-2/NIH$_Z$ (51), HIV-2/GH (Hasegawa et al., in press), SIV$_{MAC}$ (8), and SIV$_{SM}$ (25). The position of the presumed primary envelope precursor cleavage site, as identified for HIV-2/ROD (22), is shown. Symbols: ●, cysteine residues conserved among all seven envelope sequences; *, nonconserved cysteine residues; #, in-frame stop codons present in the sequences of SIV$_{MAC}$ and HIV-2/GH; •, gaps introduced for optimal sequence alignment. Potential N-linked glycosylation sites (NXS/T) in the HIV-2/ST envelope sequence are underlined.

SIV_{MAC} isolates, JSP4-27 contains no in-frame stop codon in its transmembrane envelope domain (Fig. 3). This is consistent with the presence of a 43-kilodalton rather than a 32-kilodalton transmembrane glycoprotein on Western blots of JSP4-27-derived virions (Fig. 1C) and is distinct from the protein profile of the HIV-2/ST parental strain, which comprises a mixture of viruses with both full-length and truncated transmembrane proteins (32). Pairwise sequence alignment shows that the HIV-2/ST *env* sequence differs from those of other HIV-2 and SIV envelopes to the same degree as they differ from each other, with amino acid sequences varying between 16 and 30% (Table 1). Of 32 cysteine residues, 25 are conserved among all viruses, which indicates a highly conserved envelope structure. In addition, HIV-2/ST contains 28 potential N-linked glycosylation sites that are arranged in a pattern similar to that of other viruses and that also include one highly conserved glycosylation site previously shown to be critical for HIV-1 infectivity (48). Finally, the HIV-2/ST *env* gene contains highly variable regions that correspond closely in distribution and size to similar hypervariable regions in the other *env* gene sequences.

Although a three-dimensional structure has not been determined for any HIV or SIV envelope glycoproteins, there are certain envelope domains whose functions have been characterized by mutagenesis analysis. These include the putative CD4-binding domain (33, 35), the envelope precursor cleavage site (39), and the viral fusion sequence (5, 6, 20, 33). Since sequence changes in any one of these domains could alter the fusogenic properties of a virus (33), we analyzed the envelope sequence of HIV-2/ST for particular mutations in these areas. No changes or only conservative amino acid changes were found in an envelope domain of JSP4-27 that corresponds to the HIV-1 envelope region previously identified to be involved in CD4 binding (35). HIV-2/ST also contained an apparently functional primary envelope precursor cleavage site, with a recognition sequence (RNKR) identical to that of three other fusogenic HIV-2 or SIV strains (Fig. 3). In contrast to these isolates, however, HIV-2/ST was found to differ in 2 of 16 highly conserved amino acid residues at the N terminus of the transmembrane envelope glycoprotein which, as shown by site-directed mutagenesis, contains the viral fusion domain (5). The mutations include an alanine-to-threonine change involving amino acid residue 517 (position 12 after the cleavage site) and a serine-to-alanine change involving amino acid residue 521 (position 16 after the cleavage site). Only one other fusogenic HIV-2 strain, HIV-2/ISY, contained these same changes. However, this strain exhibited three additional mutations in this same envelope area (Fig. 3). Since the fusion domain is generally highly conserved among cytopathic HIV and SIV isolates (5), we considered the possibility that the nonfusogenic properties of HIV-2/ST resulted from these mutations.

To determine whether the observed amino acid substitutions in the HIV-2/ST envelope fusion region were likely responsible for the impaired cytopathic properties of this virus, we examined two fusogenic variants of HIV-2/ST, termed ST/24.1C and ST/24.2C (Fig. 4). Both fusogenic strains were originally derived from a biologically cloned subculture of HIV-2/ST, termed ST/24, that produced noncytopathic and nonfusogenic virions biologically indistinguishable from those of the parental HIV-2/ST isolate (32). After serial cell-free transmissions of ST/24 supernatant to uninfected SupT1 cells, large and numerous syncytia were observed on two independent occasions, which indicated the

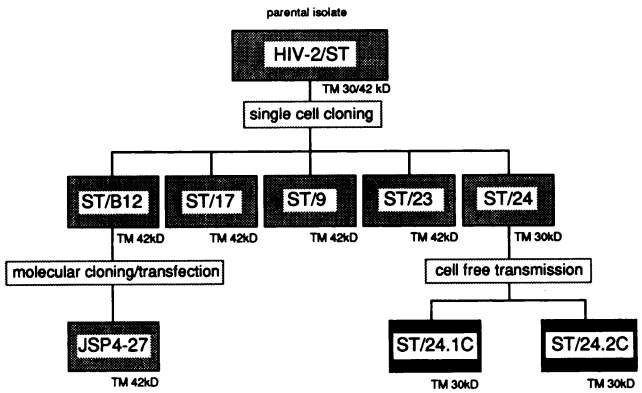

FIG. 4. Diagram of the generation of fusogenic and cytopathic variants of HIV-2/ST showing the origins of biologically distinct HIV-2/ST substrains. A total of five subcultures were established from the original HIV-2/ST isolate by limiting dilution cloning. All biologically cloned subcultures, the transfection-derived JSP4-27 cell line, and the parental bulk culture produce nonfusogenic and noncytopathic progeny virus (▩). Two fusogenic and cytopathic variants ST/24.1C and ST/24.2C were generated by repeated cell-free passage of ST/24 supernatants to uninfected SupT1 cells (■). The size of the envelope transmembrane glycoprotein (TM) for virions derived from each culture is shown. kD, Kilodaltons.

emergence of fusogenic progeny virus in the culture. Two cell lines were subsequently established (ST/24.1C and ST/24.2C) and shown to produce virions with fusogenic and cytopathic properties similar to those of prototype HIV-1 and HIV-2 isolates (J. A. Hoxie et al., manuscript in preparation). Moreover, these cell lines were confirmed to be infected with HIV-2/ST by Southern blot analysis, which revealed no changes in their *Bam*HI, *Nhe*I, *Hin*dIII, and *Pst*I cleavage patterns compared with ST/24. To identify the molecular basis for the phenotypical change in these variants and to determine whether a direct mutation of the viral fusion sequence had occurred, we amplified the envelope fusion domain of these cultures by using the polymerase chain reaction (PCR) (43).

Two oligonucleotide primers (30-mers) were designed to allow the amplification of a 544-bp envelope fragment from virus-infected cellular DNA, which included the putative precursor cleavage site as well as the envelope fusion region (Fig. 5 and 6). Both primers were synthesized according to the JSP4-27 sequence; however, sequence changes were introduced to accommodate a *Bam*HI site in the 5′ amplimer and a *Pst*I site in the 3′ amplimer (primer 1, 5′-AGAAT TGGGGGATCCTAAATTGATAGAAGT-3′; primer 2, 5′-GCTATTTAATTTCTGCAGTTCATACATGTT-3′). Total genomic DNAs of ST/24, ST/24.1C, and ST/24.2C, as well as DNA of ST/B12 as a control, were amplified by using these primers. A 100-μl sample of reaction mixture contained 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleotide triphosphates, 10 pmol of each primer, 2.5 U of *Taq* polymerase, and 1 μg of high-molecular-weight DNA. Samples were subjected to 45 amplification cycles consisting of a denaturing step at 94°C for 90 s, a primer-annealing step at 50°C for 90 s, and a primer extension step at 72°C for 135 s. Amplified envelope fragments were purified, cleaved with *Bam*HI and *Pst*I, and subsequently cloned into M13. Ten individual M13 clones per amplified DNA preparation were then isolated, and each clone was sequenced in the region, which corre-

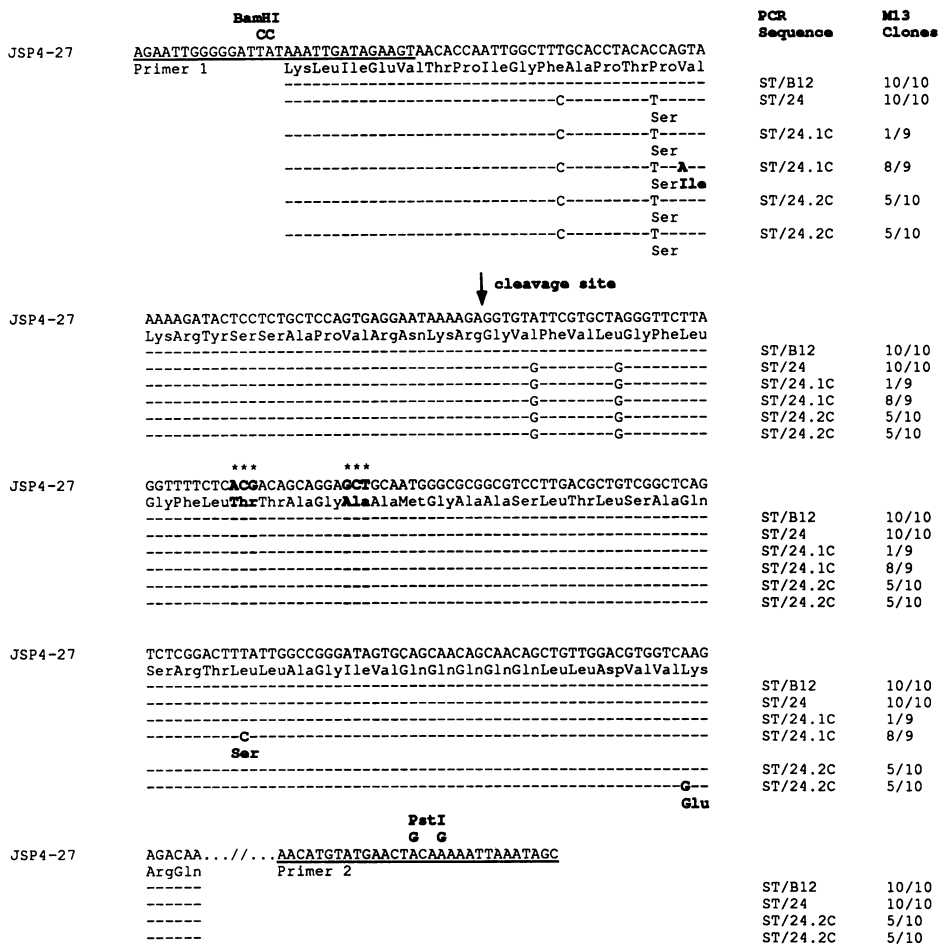

FIG. 5. Nucleotide sequence variation in the fusion domain of cytopathic and noncytopathic HIV-2/ST strains. A stretch of 230 bp of PCR-amplified envelope sequence is compared between JSP4-27 and four other HIV-2/ST strains. The boundaries of the amplified fragments are indicated. The sequences of both primers used to amplify the genomic DNA of ST/B12, ST/24, ST/24.1C, and ST/24.2C are underlined, and the base pair changes that were introduced to generate *Bam*HI and *Pst*I cloning sites are indicated. Nucleotide substitutions are shown with respect to the JSP4-27 sequence, and amino acid sequence changes unique to the cytopathic and fusogenic ST/24 strains are in boldface. Asterisks mark the threonine and alanine substitutions previously identified to distinguish JSP4-27 from most other cytopathic HIV-2 strains. The number of M13 clones analyzed per HIV-2/ST strain is listed, with frequencies referring to the proportion of clones that have identical sequences.

sponded to the N terminus of the transmembrane envelope domain. Alignment of these sequences is shown in Fig. 5.

All 10 M13 clones derived from ST/B12 contained sequences identical to that of JSP4-27, which indicated that the PCR amplification procedure was reliable and did not cause frequent misincorporations of nucleotides in this particular DNA template. Sequence comparison of amplified fragments from cell line ST/24 demonstrated no differences among the individual M13 clones but revealed four-nucleotide point mutations between these ST/24 sequences and the JSP4-27 reference sequence. In fact, all ST/24-derived strains, including the cytopathic and fusogenic ones, exhibited these same four-nucleotide sequence differences as well as the threonine and alanine substitutions previously identified in the envelope fusion region of JSP4-27. The results thus confirmed that the observed fusion sequence mutations were representative of all HIV-2/ST strains regardless of origin and biological phenotype. Interestingly, 8 of 9 M13 clones representing ST/24.1C and 5 of 10 clones representing ST/24.2C contained additional point mutations, which predicted three amino acid sequence changes with respect to the ST/24 sequence (Fig. 6). The presence of these mutations within the amplified material identifies the ST/24.1C and ST/24.2C viral strains as mixtures that comprise the parental ST/24 virus as well as additional genotypic variants. These newly generated, genotypical variants must be responsible for the phenotypical changes seen in the ST/24.1C and ST/24.2C cultures. However, the biologically significant changes appear not to occur in the envelope fusion domain of these variants.

While these studies were in progress, the biological properties of the HIV-2/ST envelope gene products were also analyzed in a eucaryotic expression system (M. J. Mulligan, P. Kumar, H. Hui, R. J. Owens, G. D. Ritter, Jr., B. H. Hahn, and R. W. Compans, submitted for publication). Vaccinia virus-expressed JSP4-27 envelope glycoproteins were compared with those of prototype HIV-1 and HIV-2 isolates. Whereas the processing, expression, and transport to the cell surface appeared to be unaltered, vaccinia virus-produced JSP4-27 envelope glycoproteins failed to form syncytia with CD4-bearing HeLa cells. Since no other HIV-2 proteins were produced in this system, these results strongly

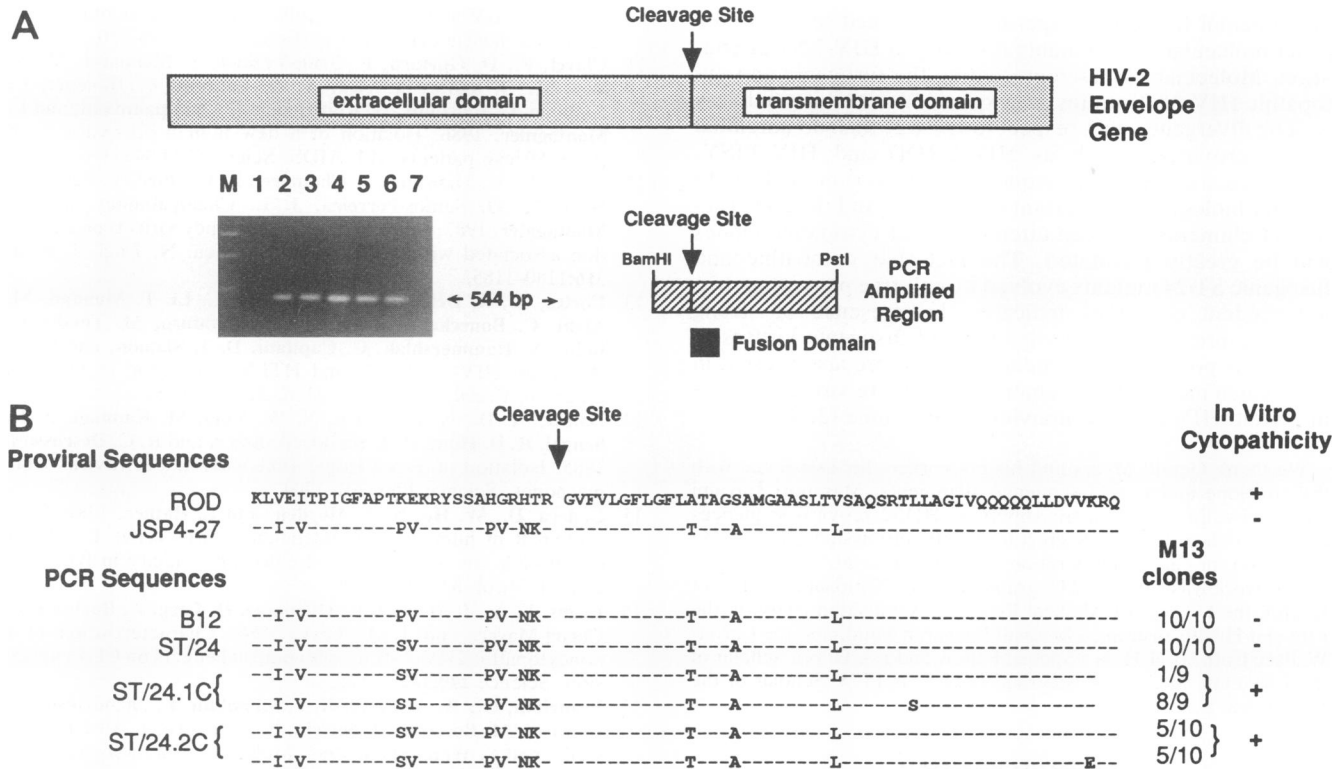

FIG. 6. Amino acid sequence variation in the fusion domain of cytopathic and noncytopathic HIV-2/ST strains. (A) Relative locations of the PCR-amplified envelope fragments in the context of the entire HIV-2 envelope open reading frame. Amplification products: Lanes: 1 and 7, uninfected peripheral blood lymphocyte DNA (negative control); 2, ST/B12; 3, ST/24; 4, ST/24.1C; 5, ST/24.2C; 6, SupT1/LK001 (HIV-2/ST-infected positive control cell line). (B) Alignment of the deduced amino acid sequences of the amplified fusion regions to the corresponding sequences of HIV-2/ROD and JSP4-27. The number of M13 clones analyzed per HIV-2/ST strain is listed, with frequencies referring to the proportion of clones that have identical sequences.

suggested that the JSP4-27 envelope glycoproteins were primarily responsible for the nonfusogenic and noncytopathic phenotype of this strain (Mulligan et al., submitted). On the basis of these results as well as the sequence data, we conclude that the nonfusogenic and attenuated properties of HIV-2/ST are indeed a function of its envelope gene products, although the causative genetic defect appears not to involve mutations within the envelope fusion domain.

There are several mechanisms other than a direct alteration of the fusion sequence that could result in the biological changes observed in HIV-2/ST. These include mutations that affect envelope-CD4 interactions, mutations that cause differences in envelope glycosylation, mutations that require additional cell surface molecules to facilitate virus-cell fusion, and mutations that reduce the stability of envelope glycoprotein complexes on the cell surface. In fact, several naturally occurring as well as genetically engineered immunodeficiency viruses are altered in their fusogenic or cytopathic properties because of one of these mechanisms. For example, possible differences in the binding affinity of HIV and SIV envelope glycoproteins to the CD4 receptor have been suggested by the finding that 25-fold more soluble CD4 is necessary to block infectivity of prototype HIV-2 compared with HIV-1 isolates (10). It is possible that the HIV-2/ST envelope glycoprotein binds the CD4 molecule with an even lower affinity, which would be expected to influence subsequent steps of viral entry, including membrane fusion and penetration. Another mechanism known to cause attenuation of virulence in naturally occurring retroviruses involves differences in posttranslational modifications of envelope glycoproteins. Poss and co-workers showed that the pathogenic determinants of an immunodeficiency-causing feline leukemia virus were dependent on the processing of particular envelope oligosaccharides (41). Since HIV-2/ST differs in number and distribution of its potential N-linked envelope glycosylation sites from other cytopathic HIV-2 strains and since size differences between the exterior envelope glycoproteins of fusogenic and nonfusogenic HIV-2/ST strains have been observed (J. A. Hoxie, personal communication), a biologically significant change in the sugar composition of the HIV-2/ST envelope cannot be excluded. Finally, a requirement of accessory molecules for virus-cell fusion represents still another potential mechanism to influence retroviral cytopathicity. Studies involving SIV_{MAC} recently revealed that this virus has a restricted host cell range that comprises only a subset of $CD4^+$ T-cell lines (27, 31). Although highly infectious and cytopathic for HUT78 and H9 cells, SIV_{MAC} does not fuse with CD4-bearing SupT1 cells. Moreover, SIV_{MAC} infects SupT1 cells only with considerable delay. It is therefore conceivable that SIV_{MAC} requires a surface molecule(s) in addition to CD4 to establish a productive infection in certain human T-cell lines. Since its infection kinetics and lack of cytopathic effect in SupT1 cells very much resemble those of HIV-2/ST, it is not unreasonable to speculate that HIV-2/ST similarly requires an additional cell surface molecule(s) for efficient cell fusion or penetration.

The availability of cytopathic variants of HIV-2/ST will be

instrumental for future experiments designed to define the exact molecular determinants involved in HIV-2/ST attenuation. Molecular clones representing the fusogenic and cytopathic HIV-2/ST strains are expected to exhibit much less genetic divergence with respect to JSP4-27 than do unrelated HIV-2 proviruses such as HIV-2/ROD and HIV-2/ISY. Therefore, a comparative sequence analysis is more likely to identify biologically important differences, and the construction of chimeras between attenuated and cytopathic clones will be greatly facilitated. The fact that cytopathic and fusogenic ST/24 mutants evolved by cell-free passage on two independent occasions indicates the presence of strong selective pressures for cytopathic and fusogenic viruses in vitro. It is possible that similar pressures are also present in vivo which may favor the emergence of more virulent strains in certain HIV-infected individuals over time (2, 9, 47).

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