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Mutations in Human Immunodeficiency Virus Type 1 gp41 Affect Sensitivity to Neutralization by gp120 Antibodies

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Three closely related molecular human immunodeficiency virus type 1 (HIV-1) clones, with differential neutralization phenotypes, were generated by cloning of an *Nco*I-*Bam*HI envelope (*env*) gene fragment (HXB2R nucleotide positions 5221 to 8021) into the full-length HXB2 molecular clone of HIV-1 IIIB. These *env* gene fragments, containing the complete gp120 coding region and a major part of gp41, were obtained from three different biological clones derived from a chimpanzee-passaged HIV-1 IIIB isolate. Two of the viruses thus obtained (4.4 and 5.1) were strongly resistant to neutralization by infection-induced chimpanzee and human polyclonal antibodies and by HIV-1 IIIB V3-specific monoclonal antibodies and weakly resistant to soluble CD4 and a CD4-binding-site-specific monoclonal antibody. The third virus (6.8) was sensitive to neutralization by the same reagents. The V3 coding sequence and the gp120 amino acid residues important for the discontinuous neutralization epitope overlapping the CD4-binding site were completely conserved among the clones. However, the neutralization-resistant clones 4.4 and 5.1 differed from neutralization-sensitive clone 6.8 by two mutations in gp41. Exchange experiments confirmed that the 3′ end of clone 6.8 (nucleotides 6806 to 8021; amino acids 346 to 752) conferred a neutralization-sensitive phenotype to both of the neutralization-resistant clones 4.4 and 5.1. From our study, we conclude that mutations in the extracellular portion of gp41 may affect neutralization sensitivity to gp120 antibodies.

Experimental human immunodeficiency virus type 1 (HIV-1) infection of chimpanzees and natural HIV-1 infection of humans induce type-specific as well as group-specific neutralizing antibodies in the course of infection (3, 9, 10, 18, 25, 33). Viruses resistant to neutralization by autologous sera emerge during the course of human HIV-1 infection (1, 16, 29, 31, 32). HIV-1 variants with reduced sensitivity to neutralization by autologous but not by heterologous broadly neutralizing sera occur as early as 1 year after infection (1, 31). Despite the V3-dependent restriction of neutralizing capacity of sera during the early phase of infection, the V3 sequence of the neutralization-resistant viruses appeared to be unchanged (32). These findings in humans confirmed our earlier report on the emergence of neutralization-resistant virus variants in chimpanzees experimentally infected with HIV-1 IIIB (19). Virus recovered from a chimpanzee and passaged 32 weeks after infection had a reduced sensitivity to V3-specific experimental as well as autologous chimpanzee sera, although the amino acid sequence of the neutralizing epitope within the V3 loop was unchanged (19). Reitz and coworkers have shown that human neutralizing serum can select in vitro a mutant resistant to the selecting serum as a result of a mutation in gp41 (24, 35). Although the specificity of the antibodies in the serum driving this selection is not known, this gp41 mutation appears to indirectly affect the neutralization epitopes of the CD4-binding site of gp120 or epitopes overlapping with this region (26b). In this study, we further analyzed the genetic background of changes in neutralization phenotype due to mutations outside the primary antibody-binding site and the possible mechanism responsible for these changes in neutralization phenotype. For this purpose, we studied three molecular clones differing in neutralization sensitivity, obtained by cloning of *env* gene fragments derived from a chimpanzee-passaged HIV-1 IIIB isolate (911.32) (19).

Construction of envelope molecular clones. A chimpanzee-passaged virus variant, obtained 32 weeks after infection with HIV-1 IIIB, was isolated from chimpanzee 911 peripheral blood mononuclear cells (19). This bulk virus isolate showed reduced sensitivity to autologous sera and V3 monoclonal antibody (MAb) 0.5β, while an identical V3 region (λBH10) was found (19). *env* gene fragments of biological clones of the bulk isolate, obtained by two rounds of plaque purification on MT-4 cells and propagated in the C8166 cell line (15), were cloned into a full-length HXB2 molecular clone (6). For this cloning procedure, DNA of the biologically cloned viruses was extracted (4) and amplified by the polymerase chain reaction. Primers used to amplify the envelope region were an extended 5′ primer, pol (A*) (5′-AAGCTNCTCTGGAAAGGTGAAG GGGCAGTA-3′; HXB2R nucleotides [nt] 4493 to 4522) (17), and a 3′ primer, nef.seq.3 (5′-CTACTTGTGATTGCTCC ATG-3′; HXB2R nt 8460 to 8479) (17). The reaction mixture (100 μl) consisted of 25 mM KCl, 50 mM Tris-HCl (pH 8.3), 0.1 mg of bovine serum albumin per ml, 2 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 10 pmol of each primer, 5% glycerol, and 2.5 U of *Taq* polymerase (a gift from Perkin-Elmer Cetus, Emeryville, Calif.). Each cycle consisted of 1 min at 94°C, 2 min at 55°C, and 4 min at 72°C. After 35 cycles, the reactions were extended for 10 min at 72°C. The amplified envelope fragments were purified and digested with *Nco*I (nt 5221) and *Bam*HI (nt 8021). The *Nco*I-*Bam*HI fragment was subsequently cloned in a full-length HXB2 molecular clone lacking the *Nco*I-*Bam*HI fragment (6). Of the three infectious molecular clones thus obtained, four chimeric

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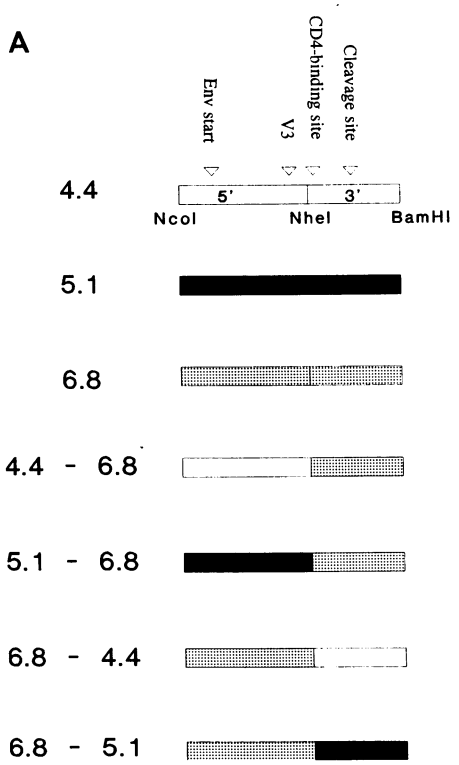

B

	Chimpanzee		Human	CD4-site		V3-site	
	36wk	87wk	1171	GP13	sCD4	178.1	5023
4.4	20	10	<10	<5	25	20	40
5.1	<20	10	<10	40	50	80	80
6.8	160	80	320	400	100	1600	800
4.4 - 6.8	640	ND	640	800	200	1600	800
5.1 - 6.8	450	ND	640	650	100	6400	3200
6.8 - 4.4	<20	ND	<10	280	50	100	200
6.8 - 5.1	40	ND	10	570	50	140	200

FIG. 1. (A) Schematic representation of the three molecular clones with different envelope regions and two-thirds of the gp41 protein and of the four exchanged chimeric molecular clones. A 4-kb fragment was obtained by the polymerase chain reaction, digested with *Nco*I and *Bam*HI, and cloned in a full-length HXB2 molecular clone. Exchange experiments were performed with *Nhe*I and *Bam*HI fragments. Depicted are the most important functional regions (*env* start region, neutralization V3 epitope, CD4-binding region, and gp120-gp41 cleavage site) and the restriction enzyme sites used (*Nco*I, *Nhe*I, and *Bam*HI). (B) Reciprocal neutralizing titers of the chimeric molecularly cloned viruses against four sets of sera. The first two sets are polyclonal antibodies; the other two sets are MAbs.

envelope molecular clones were prepared by exchange of the *Nhe*I-*Bam*HI fragment (nt 6806 to 8021; amino acids [aa] 346 to 752) (Fig. 1).

Viral stock preparation for neutralization experiments. After cesium chloride gradient purification of the full-length molecular clone DNA, 5 μg of each molecular clone was electroporated into 5×10^6 SupT1 cells and maintained as described by de Jong et al. (6). On day 3, an equal amount of fresh medium, RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with 10% fetal calf serum, glutamine, and antibiotics, was added. On day 5, the supernatant fluids of the transfected cells were harvested by centrifugation at $1,000 \times g$ for 10 min and filtered through a Millipore filter (0.22-μm pore size). Supernatant fluids of HIV-1 IIIB variants and 911.32 chronically infected stocks were harvested as described previously (2). Infectious titers were determined by endpoint titration on the basis of syncytium formation, using the C8166 cell line as previously described (2, 15).

Virus neutralization assay. The cell-free virus neutralization assay was performed as described previously (2, 15). Briefly, 100 50% tissue culture infectious doses of virus stock in a volume of 40 μl was incubated in triplicate with 10 μl of twofold serial dilutions of antibodies and incubated at 37°C for 1 h. The virus-antibody mixture was then incubated with 100 μl of C8166 cells (2×10^5 cells per ml) and scored for syncytia at day 5. Neutralization titer is represented as the reciprocal of the highest serum dilution giving 99% inhibition of syncytium formation.

Sera and monoclonal antibodies. The following polyclonal and monoclonal antibodies were used to characterize the molecularly cloned viruses. Autologous plasma samples of chimpanzee 911, obtained 36 and 87 weeks after infection with HIV-1 IIIB, contain both antibodies to V3 of HIV-1 HX10 and antibodies able to block gp120-soluble CD4 (sCD4) binding and can neutralize HIV-1 HX10 and RF but not MN (data not shown); a human serum from the Dutch cohort (23), selected for high neutralizing titers against the laboratory strains HIV-1 MN, IIIB, and RF, contains HIV-1 MN V3 but not HIV-1 HX10 V3 antibodies and also contains gp120-GP13 blocking antibodies (data not shown). Human neutralizing MAb GP13, derived by Epstein-Barr virus transformation of peripheral blood mononuclear cells from an asymptomatic, HIV-1-seropositive individual, was used as a protein A-purified preparation (1 mg/ml). This human MAb binds to an epitope overlapping that of the CD4-binding site (26a). Recombinant sCD4 (1 mg/ml) was kindly provided by P. J. Maddon through the NIAID AIDS Research and Reference Reagent Program. We also used two HIV-1 IIIB V3-specific mouse MAbs, 5023 and 178.1. MAb 5023 was induced by a 15-mer synthetic BH10 V3 peptide (7), specific for RgPGRAF (aa 311 to 317; capital letters represent residues important in binding) (13), kindly provided both as ascites fluid and as a batch of purified immunoglobulin G (1 mg/ml) by P. Durda (Du Pont de Nemours and Co., North Billerica, Mass.). The recombinant gp160 (IIIB) DNA-induced MAb 178.1 (28), specific for KSiRI (aa 305 to 309) (14), was kindly provided as ascites fluid by C. Thiriart (Smith Kline, Rixensart, Belgium) and by the MRC AIDS Reagent Project.

TABLE 1. Reciprocal neutralizing titers of three uncloned HIV-1 IIIB virus isolates and the chimpanzee-passaged virus bulk isolate (911.32) against two V3-specific MAbs and a human broadly neutralizing serum

HIV-1 IIIB isolate	Titer[a]		
	V3 site MAb		1171 (human serum)
	178.1[b]	5023[b]	
HX10	520	6,400	80
HXB2	<50	570	28
HXB3	<10	1,131	10
911.32 bulk	<50	<50	<10

[a] Logarithmic mean of two or more separate experiments.
[b] Ascites fluid.

Sequencing of the *env* coding regions of the three molecular clones. Nucleotide sequence analysis of the envelope genes was performed by the Central European Sequence Facility for HIV genome analysis (H. Rübsamen, Georg-Speyer Haus, Frankfurt, Germany) by an automated sequence method. All nucleotide sequence mutations were confirmed by an automated sequence method (Applied Biosystems, Maarssen, The Netherlands), using the *Taq* dye terminator cycle kit (Applied Biosystems) and various envelope primers synthesized by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Neutralization profiles of chimpanzee-passaged virus versus HIV-1 IIIB molecular clones. Polyclonal and HIV-1 IIIB V3-specific MAbs were tested to determine the neutralization sensitivity of the three HIV-1 IIIB variants (HX10, HXB2, and HXB3) and the chimpanzee passage of HIV-1 IIIB (911.32). As shown in Table 1, the V3-binding MAbs were able to neutralize HX10 but were substantially less able to neutralize 911.32. As previously shown for the V3-specific MAb 0.5β, isolate 911.32 was also resistant to neutralization by two other V3 MAbs, 178.1 and 5023, used in this study (Table 1). This neutralization resistance could not be attributed to changes in the binding sites of these V3 MAbs (13, 14, 19), as in the core for clones HXB2 and HXB3, which are neutralization resistant by MAb 178.1 because of an S→R or S→K mutation at position 306 (14). In addition, a group-specific neutralizing human serum neutralizes the HX10 strain very efficiently but does not neutralize the 911.32 clone (Table 1). To assess more accurately the nature of the neutralization resistance of uncloned 911.32, we molecularly cloned, after biological cloning, the 911.32 virus.

Neutralization profiles of molecular HXB2 clones chimeric for 911.32 *env* genes. A schematic representation of the envelope fragments, all cloned in a full-length HXB2 molecular clone, is shown in Fig. 1. In this way, we obtained three infectious molecular clones with different envelope regions and four infectious chimeric molecular clones with combinations of the different envelope regions. Upon transfection of these molecular clones in SupT1 or C8166 cells, the replication kinetics and the formation of syncytia for all virus clones were comparable with those for the wild-type HXB2 (data not shown). To examine neutralizing sensitivity, we tested the viral stocks of the three molecular clones (4.4, 5.1, and 6.8) against well-defined polyclonal and monoclonal antibodies (Fig. 1). Viral stock titers ranged from $10^{-3.1}$ to $10^{-2.8}$ per 40 μl. The first set of sera consisted of two autologous plasmas of chimpanzee 911. The chimpanzee plasma obtained 36 weeks after infection did not neutralize virus 5.1 but did neutralize virus 4.4 at a titer of 20 and virus 6.8 at a titer of 160. The chimpanzee plasma obtained 87 weeks after virus infection neutralized viruses 4.4 and 5.1 at reciprocal dilutions of 10 as well as virus 6.8 at a titer of 80.

The chimpanzee bulk virus isolate (911.32) is resistant to neutralization by a broadly human neutralizing serum, 1171 (Table 1). This serum neutralized virus 6.8 at a titer of 320, but again viruses 4.4 and 5.1 were neutralization resistant. To further characterize the neutralization phenotypes, we used V3- and CD4-binding-site-specific MAbs and sCD4 for confirmation. A broadly neutralizing human MAb (GP13), directed to a conserved conformation-dependent neutralization epitope that overlaps the CD4-binding site of gp120 and with neutralizing activity to both HIV-1 IIIB and MN but not RF (not shown), was used. Virus 5.1 again showed reduced neutralization sensitivity. Virus 4.4 was resistant at a titer of <5, whereas virus 6.8 was neutralization-sensitive at a titer of 400. Titers obtained with sCD4 confirmed the titers of GP13, whereas viruses 4.4 and 5.1 were less sensitive and virus 6.8 had a titer of 100. The most clear-cut difference in titers were observed with the V3-specific MAbs. Viruses 4.4 and 5.1 were neutralized only at low titers by these MAbs and were as neutralization resistant as the biological clones and the original bulk chimpanzee isolate (911) (<50; Table 1). Virus 6.8 was neutralized at a titer of 1,600 by MAb 178.1 and at a titer of 800 by MAb 5023 (different batches of MAb 5023 were used, 80 titers in Table 1 and Fig. 1 cannot be compared). These results suggest that the changes in the 2.8-kb envelope fragment of viruses 4.4 and 5.1 simultaneously affect the sensitivity for V3 antibodies as well as CD4-binding-site antibodies. Moreover, the data indicate that the 2.8-kb envelope fragment confers a neutralization phenotype to viruses 4.4 and 5.1 that is characterized by resistance to the defined set of neutralizing antibody populations present in the sera of naturally infected individuals and of experimentally infected animals.

To further characterize the neutralization phenotypes of these molecular viruses, we used (i) MAbs specific for the V3 region of IIIB and for the CD4-binding site and (ii) sCD4. We conclude that viruses 4.4 and 5.1 are neutralization resistant to V3 antibodies and less sensitive to gp120-CD4 blocking antibodies as well as to sCD4 and that virus 6.8 is neutralization sensitive to both gp120 antibodies and sCD4, in contrast to the bulk and biological viruses of chimpanzee 911. The neutralization sensitivity of virus 6.8 to monoclonal and polyclonal sera as well as to sCD4 (data not shown) is comparable to that of the HX10 isolate of HIV-1 IIIB (Table 1).

Sequence analysis of envelope determinants of neutralizing sensitivity. A 2,250-bp fragment (envelope start [nt 5771] to *Bam*HI [nt 8021]) of each of the molecular clones 4.4, 5.1, and 6.8, was sequenced. Alignments of the deduced amino acid sequences of the gene fragments relative to that of virus 5.1 are shown in Fig. 2. Sequence comparison of the three cloned gp120-gp41 envelope fragments shows that the gp120 antigenic sites recognized by sCD4 (22, 27) or by human MAb GP13 (influenced by amino acids at positions 88, 256, 257, 368, 370, 427, and 457 [26a]) as well as the linear epitopes in the V3 coding region or in sites that are reported to affect neutralization by polyclonal sera (RILAVERY; aa 579 to 586) (24, 35) were completely conserved among the clones. However, the neutralization-resistant variant 5.1 has one substitution in the V2 region, S-190→R, and two amino acid changes in gp41 in the C7 region, S-668→N and M-675→I, relative to the sensitive clone 6.8. The other neutralization-resistant variant, 4.4, has six amino acid substitutions in gp41, V-704→I, I-723→T, G-737→D, and N-750→D, as well as two gp41 amino acid changes the same as those in clone 5.1, S-668→N and M-675→I, relative to clone 6.8. Amino acid changes distin-

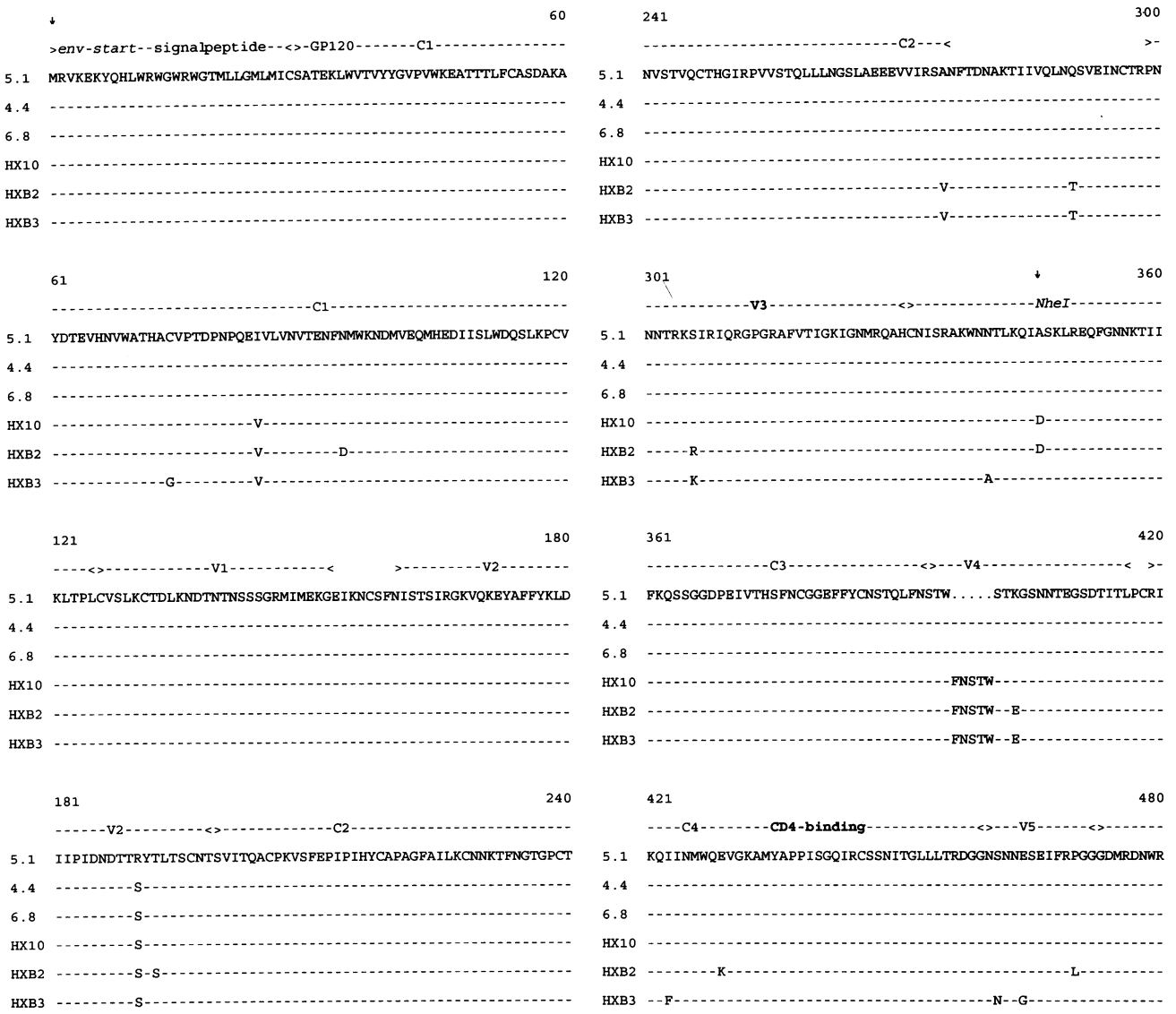

FIG. 2. Alignment of the envelope proteins and a major part of the gp41 coding regions of molecular viruses 4.4, 5.1, and 6.8 and three HIV IIIB variants, HX10, HXB2R, and HXB3, from the Los Alamos data base (17). Sequence analysis was performed by the automated sequence method as described in the text. Alignments were adjusted by eye. The predicted amino acid sequences are presented in the one-letter code. Amino acid numbers are indicated. Dashes indicate amino acid identities; points indicate gaps. The signal peptide, hypervariable region (V), constant region (C), gp120-gp41 proteolytic cleavage, *env* start, *Nhe*I, and *Bam*HI sites are indicated.

guishing these neutralization-sensitive and -resistant clones were restricted to the gp120 V2 region and the gp41 C7 region. Both the V2 region of gp120 and the second immunogenic region of gp41 (ELDKWAS; aa 662 to 668) (26, 30) are described as containing neutralization epitopes (5, 8, 12).

These results suggest that the V3 region, the CD4-binding site of gp120, and the second immunogenic region of gp41 may be domains that define the same process of entry and penetration by different means (20, 34). This process may be blocked by antibodies to continuous epitopes in each of these interactive domains or to discontinuous epitopes involving several (or all) of these interactive domains. Hwang et al. (11) and O'Brien et al. (21) have already concluded that changes in the V3 region have a direct impact on the sensitivity of a virus to sCD4 neutralization.

Exchange of gp41 and gp120 coding regions of infectious molecular clones. To further delineate the region involved in the phenotypic difference between clones 4.4, 5.1, and 6.8, exchange experiments were performed (Fig. 1). To distinguish these domains and assess their relative contribution to the neutralization phenotype as well as the possible role of the 550-bp fragment not sequenced before the envelope start site, we substituted the 3′ end (*Nhe*I-*Bam*HI; aa 346 to 752) of the *env* genes of the neutralization-resistant clones 4.4 and 5.1 for the *env* genes of the neutralization-sensitive clone 6.8 and vice versa (Fig. 1). The chimeric virus clones with the 3′ end of clone 6.8 are indeed neutralization sensitive to V3- and CD4-binding-site antibodies. The chimeric virus clones with the 3′ end of clones 4.4 and 5.1 were less neutralization sensitive than the chimeric molecular clones with the 3′ end of clone 6.8 but not as resistant as clones 4.4 and 5.1, suggesting additional contributions such as envelope structure to the

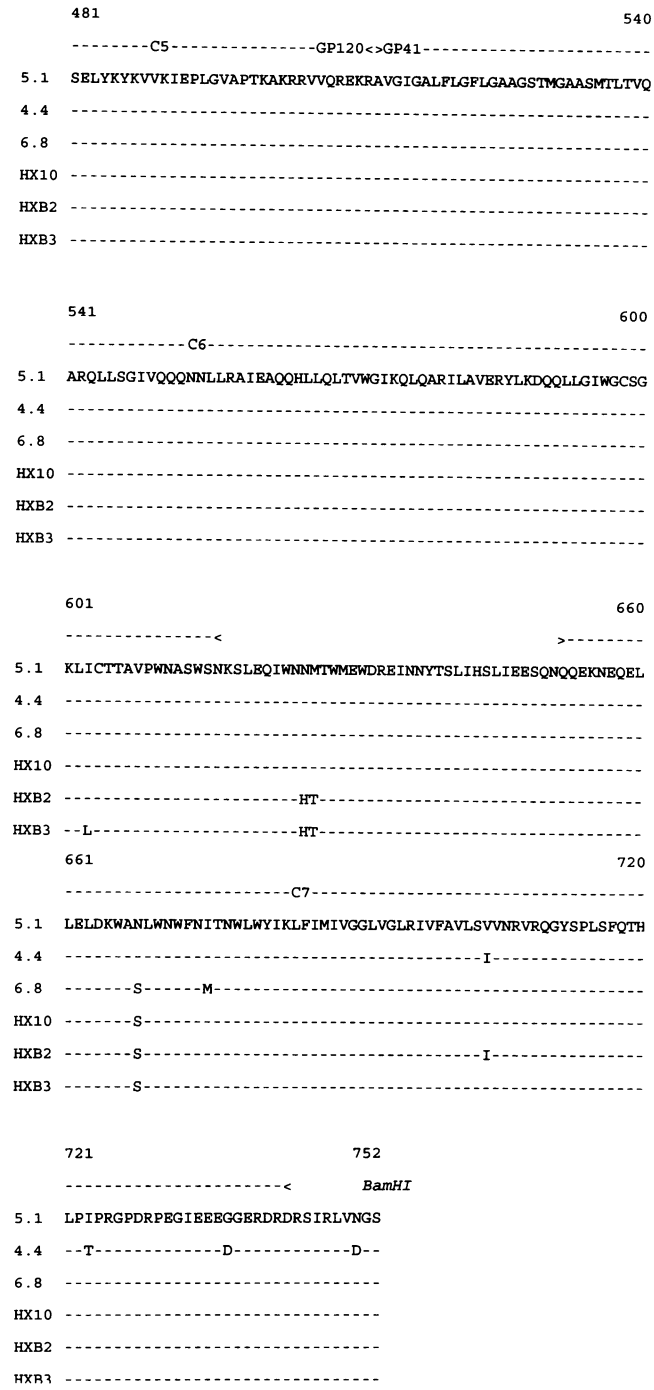

FIG. 2—*Continued.*

neutralization phenotype. Two gp41 mutations (aa 668 and 675) clearly affect the neutralization sensitivity of the viruses to V3 antibodies as well as, although to a lesser extent, the neutralizing sensitivity to gp120-sCD4 blocking antibodies and sCD4. This data set suggests again that the interaction between the gp41 region including positions 662 to 668 and V3 is stronger than with the CD4-binding site. The proposed functional interaction between the second immunogenic epitope (ELDKWAS; aa 662 to 668) of gp41 and the V3 region has not been previously described, but previous neutralization escape mutant studies have suggested that this region is not completely buried within the membrane (5).

Differences in binding of V3-specific antibodies for virion-bound gp120 may explain differences in the neutralization sensitivity of viruses. Under our assay conditions using soluble gp120, binding differences for V3 antibodies between neutralization-sensitive and neutralization-resistant viruses were not observed (data not shown).

The distinct neutralization sensitivity and the conservation of the epitopes involved suggest either that all neutralization epitopes of HIV-1 are somehow related to each other or, alternatively, that the envelope structures of these viruses are different. Third, it may be that the mutations in these clones affect the linkage between the gp120 and gp41 molecules.

The impact of the amino acid substitutions that we observed at positions 668 and 675 of the extracellular portion of gp41 on the CD4-binding site is either absent or less radical than the effect on V3 neutralization. Our results indicate that the sensitivity to neutralization by antibodies binding to gp120 can be affected not only by mutations in the gp120 coding region but also by mutations in the transmembrane protein gp41 that is associated through noncovalent interactions with gp120. These results confirm and extend previous studies by Reitz et al. (24, 35).

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