AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 8, Number 10, 1992
Mary Ann Liebert, Inc., Publishers

Genetic and Functional Analysis of a Set of HIV-1 Envelope Genes Obtained from Biological Clones with Varying Syncytium-Inducing Capacities

ARNO C. ANDEWEG,[1] MARTIJN GROENINK,[2] PAULA LEEFLANG,[1] RUUD E.Y. de GOEDE,[2] ALBERT D.M.E. OSTERHAUS,[1] MATTHIJS TERSMETTE,[2] and MARNIX L. BOSCH[1]

ABSTRACT

To study HIV-1 envelope-mediated syncytium formation we have amplified, cloned, expressed, and sequenced individual envelope genes from a set of eight biological HIV-1 clones. These clones were obtained from two patients and display either a syncytium-inducing (SI) or nonsyncytium-inducing (NSI) phenotype. Upon expression through recombinant vaccinia virus, individual envelope gene products display heterogeneous syncytium-inducing capacities which reflect the phenotype of the parental biological HIV-1 clones in all cases. For the eight biological HIV-1 clones presented here, variation of the envelope gene alone is sufficient to explain the observed variable syncytium-inducing capacity of the respective parental viruses. In addition we determined the complete nucleotide sequence of these envelope genes. The predicted amino acid sequence revealed a considerable amount of variation located mainly in the previously denominated variable regions. In various regions of envelope genes obtained from the same patient, phenotype associated amino acid variation was found. This phenotype associated amino acid variation however, is not conserved between the two sets of envelope genes derived from different patients. Four envelope sequences derived from clones obtained from one patient showed phenotype-associated amino acid variation in the fusion domain. Sequencing of 12 additional fusion domains revealed that this same variation is found in four additional clones. However, a functional test performed on recombinant vaccinia expressing mutant envelope genes showed that this observed fusion domain variation does not contribute to the variation in syncytium-inducing capacity of the envelope gene product.

INTRODUCTION

ISOLATES OF HUMAN IMMUNODEFICIENCY VIRUS type 1 (HIV-1) differ in their in vitro biological properties such as replication rate, cytotropism, and syncytium-inducing capacity.[1–3] Based on these in vitro properties one can distinguish syncytium-inducing (SI) isolates which have a high replication rate in primary cells and are generally able to grow in continuous cell lines on the one hand, and nonsyncytium-inducing (NSI) isolates on the other hand, which have a moderate to low replication rate in primary cells and do not grow in continuous cell lines.[3]

In an earlier longitudinal study, we demonstrated that detection of SI variants in asymptomatic individuals is strongly associated with subsequent rapid decline of $CD4^+$ T-cell numbers and progression to acquired immunodeficiency syndrome (AIDS), whereas from stable asymptomatic individuals only slow replicating NSI isolates can be recovered. This suggests different roles for SI and NSI variants in in vivo pathogenesis.[4,5] It has been demonstrated that syncytium formation is mediated by the envelope glycoproteins of HIV-1 although the exact mechanism has not been clarified.[6,7] Furthermore, several studies with pairs of recombinant proviruses have directly implicated the envelope gene as containing the primary determi-

[1]Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands.
[2]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, P.O. Box 9190, 1006 AD Amsterdam, The Netherlands.

nants in controlling biological variation of HIV-1,[8–10] whereas only minimal functional variation has been found so far in the regulatory genes or long terminal repeat (LTR) of HIV-1 isolates with varying biological properties.[11–15] In the present comparative study we analyze a set of envelope genes to elucidate the genetic basis of the observed variation in syncytium-inducing capacity.

The envelope glycoproteins are synthesized as a glycosylated precursor gp160, which undergoes several processing steps including oligosaccharide modification and proteolytic cleavage into two subunits; gp41 and gp120.[16] The transmembrane glycoprotein gp41 noncovalently anchors the external glycoprotein gp120. The mature glycoprotein complex probably forms tetramers or a mixture of tetramers and dimers on the surface of the virion and infected cells.[17–19] Several determinants on the envelope glycoproteins appear to be involved in the process of membrane fusion which is necessary for the infection of target cells by HIV-1 and for HIV-1-induced syncytium formation.[6,20] In the first step HIV-1 gp120 binds directly to the CD4 receptor molecule on target cells.[21,22] Variation in CD4 binding affinities of the gp120 molecules of different HIV-1 isolates suggests direct or indirect involvement of the CD4 binding site on gp120 in determining such biological properties of the virus as the ability to induce syncytia.[23,24] As a result of this binding event the hydrophobic amino terminus of gp41 is exposed probably through a series of conformational changes.[25] Mutational analyses have shown direct involvement of this region to the ensuing membrane fusion and it is therefore denominated the fusion domain.[26–29] Mutations in the fusion domain of simian immunodeficiency virus (SIV) resulted in either reduction or enhancement of SIV envelope-induced syncytium formation depending on the hydrophobicity of the introduced amino acid which indicates that natural variation in this region of HIV-1 may contribute to determining the biological phenotype of the virus.[26] Other regions of the envelope glycoproteins (e.g., the hypervariable V3 region) appear also to be involved in the process of membrane fusion following the gp120-CD4 binding.[30] In addition, variation in the V3 region has been shown to affect the biological properties of recombinant HIV-1 proviruses especially with regard to cell tropism.[31]

Here we describe the amplification, cloning, expression and sequencing of eight complete envelope genes derived from two sets of biological clones of HIV-1 with different in vitro biological properties. This approach allows us to correlate the syncytium-forming capacities of the individual envelope genes to their respective amino acid sequences and to the syncytium inducing capacity of the parental HIV-1 clones.

MATERIALS AND METHODS

Subjects

Virus was recovered from seropositive individuals visiting the outpatient clinic of the Academic Medical Centre, Amsterdam, or participating in a large cohort study.[32]

Clonal virus isolation

To obtain clonal populations of HIV-1 directly from patient peripheral blood mononuclear cells (MNC), virus isolation was performed in 96-well microtiter plates as described previously.[33] Briefly, patient MNC (10,000/well) were cocultivated with 100,000 phytohemaglutinin-stimulated MNC from healthy seronegative blood donors. Under these conditions productive HIV-1 infection was observed in less than a third of the wells, indicating clonality according to the Poisson distribution. The thus obtained biological HIV-1 clones were then characterized as syncytium-inducing (SI) or nonsyncytium-inducing (NSI) according to previously published criteria.[3] Following this procedure, multiple clones were obtained from MNC of 30 seropositive individuals. For this study four individuals (HIVach320, HIVams16, HIVams93, and HIVach373 further referred to as, respectively, #320, #16, #93, and #373) were selected whose uncloned HIV-1 isolates, obtained as described previously,[3] had a SI phenotype. By clonal isolation both NSI and SI clones could be obtained from MNC of these individuals. From one individual (#373) in whom over time a transition from NSI to SI phenotype had been observed,[34] HIV-1 clones obtained from two time points (as indicated) were included in this study.

Polymerase chain reaction (PCR) amplification of envelope sequences.

High-molecular weight DNA from MNC infected with clonal isolates were subjected to 35 cycles of PCR, using a set of primers either spanning the complete envelope gene or a 423 bp subregion of the envelope gene (position 7092-7514, HIV-HXB2[35]). The 423 bp region encodes the carboxy terminal part of gp120, the envelope cleavage site, and the fusion peptide located at the amino terminal end of gp41. The primers "FUS1" and "FUS2" (5′-GGAAAAGCAATGTATGCCCCTCC-3′ and 5′-CCAGACTGTGAGTTGCAACAGATGC-3′, respectively) were used to amplify this 423 bp envelope region. To amplify the complete envelope gene the primers "5000" and "7500" (5′-GACGTCCCCGGGCAGAAGACAGTGGCAATGAGAG-3′and 5′-GCATGCCCCGGGCTTTTTGACCACTTGCCACCC-3′) were used. The primer "5000" includes the first seven nucleotides of the envelope open reading frame, fixing these nucleotides for all amplified envelope genes. XmaI restriction sites (underlined) were incorporated into the primers "5000" and "7500" to facilitate cloning. PCR conditions were: 35 cycles of 1′95°C, 1′55°C, and 2.5′ 72°C with 0.7 μM primers, 2 mM Mg^{2+}, and 200 μM dNTP for the complete envelope gene and 35 cycles of 1′ 95°C, 1′ 55°C, and 2′ 72°C with 5 μM primers, 4 mM Mg^{2+} and 200 μM dNTP when amplifying the 423 bp region.

Construction of recombinant vaccinia virus

Envelope genes derived from biological clones obtained from patient #320 and #16 were cloned into the XmaI site of plasmid vector pSC11.[36] Hela cells infected with vaccinia virus (strain WR) were transfected with calcium phosphate-precipitated pSC11-*env* constructs as previously described.[36] Upon 5-bromodeoxyuridine (BudR) selection in 143 cells, recombinant virus plaques were visualized by their blue color upon overlay with 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside (XGal) due to the coexpression of the lacZ gene derived from the pSC11 vector. Independent plaques were plaque-purified three times

and the recombinant vaccinia viruses were grown to large stocks in RK13 cells. All recombinant vaccinia viruses were assayed for HIV-1 envelope expression by radioimmunoprecipitation (see below). The recombinant vaccinia virus containing the envelope genes from clones 320.2a.7 and 16.2 failed to express the expected gp160/gp120 products and these *env* genes were subsequently reamplified from the same genomic DNA. Recombinant vaccinia virus containing the *env* genes from this second PCR now expressed the expected bands and these were used for all further experiments.

Radioimmunoprecipitation

For radioimmunoprecipitation (RIPA) HeLa cells were metabolically labeled for 8 hours with [^{35}S]methionine and [^{35}S]cysteine (ICN Biomedicals) 12 hours after infection with 10 plaque-forming units (PFU) of recombinant vaccinia virus per cell. Supernatant was collected and cells were lysed 5 hours after reconstitution of the medium with unlabeled methionine and cysteine. Subsequent immunoprecipitation was performed with polyclonal sheep anti-gp120 serum (kindly provided to us by Dr. M. Page through the MRC AIDS reagent program) as previously described.[37] The precipitated proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide), according to Laemmli.[38]

CD4 binding

Secreted envelope glycoproteins were metabolically labeled as described for RIPA. Supernatant was incubated with 150 ng soluble CD4 for 3 h at 4°C and subsequently immunoprecipitated with excess monoclonal antibody to CD4 (OKT4, Ortho Diagnostic Systems Inc.). The precipitated proteins were analyzed as described for RIPA.

Syncytium-forming assay

The MNC (3×10^5) from different donors were infected with recombinant vaccinia virus or wild-type vaccinia virus (wt) at a multiplicity of infection (MOI) of 30. Infections were allowed to proceed for 48 h, at which time the cultures were assayed for syncytia. Relative sizes and numbers of syncytia found per well were scored independently on coded samples by two independent researchers.

Sequence analysis

Sequence analysis of eight complete envelope genes was performed: four were sequenced by the dideoxynucleotide chain termination method[39] using T7 polymerase (Amersham) and four others were sequenced on an Applied Biosystems Inc. automated DNA sequencer by the Central European Facility for HIV genome analysis (Georg-Speyer-Haus, Frankfurt, Germany). The amplified 423 bp fragments were analyzed by direct DNA sequencing. For direct sequencing the PCR products were purified by Centricon 30 (Amicon) filtration, denatured with NaOH and ethanol precipitated after primer annealing (FUS1 or FUS2). The precipitated DNA was then sequenced using a modified T7 polymerase according to the protocol provided by the manufacturer (U.S. Biochemical). From each isolate unambiguous DNA sequences were obtained, even in the hypervariable regions,[40] confirming the clonality of these isolates. The sequence data were analyzed using the Lasergene (DNAstar) computer programs. All sequence comparisons were performed with the gap penalty set to zero.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed on two envelope genes cloned in the filamentous phage M13 by the method described by Kunkel et al.[41] using supplies from Bio-Rad. Oligonucleotides 5′-CATAGCTCCTATCGTTC-CCACTGCTC-3′ and 5′CATAGCTCCTATCATTCCCACT-GCTC-3′ were used for mutating the fourth codon of gp41 coding region of the envelope gene of biological clones #16.1 and #16.3 from ATG to ACG and from ACG to ATG, respectively. The mutagenesis reaction changed the fourth amino acid residue of the fusion domain from methionine to threonine and reverse. The mutant envelope genes were partially resequenced for examination of the desired mutation.

RESULTS

Construction of recombinant vaccinia viruses that express env *genes derived from biological HIV-1 clones*

To examine the role and function of the envelope glycoproteins in determining the varying syncytium-inducing capacity of HIV-1, we constructed a collection of eight recombinant vaccinia viruses expressing the envelope genes of a set of biological HIV-1 clones. These biological HIV-1 clones were obtained from two patients; selected clones show similar replication rates in MNC (data not shown) but differ in their capacity to induce syncytia in MNC cultures (Table 1). The complete envelope genes of these eight clones were amplified by PCR using a set of primers introducing XmaI sites to facilitate cloning of the envelope genes downstream the P7.5 promoter of the vaccinia expression vector pSC11.[42]

Expression of HIV-1 envelope glycoproteins

Recombinant vaccinia virus-mediated expression of the HIV-1 envelope glycoproteins was demonstrated by RIPA. HeLa cells infected with recombinant vaccinia virus or wild-type vaccinia virus, were radiolabeled and the proteins were analyzed by immunoprecipitation followed by SDS–PAGE and autoradiography. Figure 1, panel A shows the recombinant vaccinia virus induced expression of gp160. Only in the lysates or recombinant vaccinia virus infected cells the precursor glycoprotein gp160 was precipitated by polyclonal sheep serum directed to the HIV-1 envelope protein. Under these labeling conditions the cleaved gp120 was also demonstrated in the lysates of most of the infected cell cultures (Fig. 1, panel A), and could easily be detected in the supernatants of all the recombinant vaccinia virus infected cell cultures (Fig. 1, panel B). In order to demonstrate the receptor-binding capacity of the expressed envelope proteins, gp120 present in supernatant was also coprecipitated with soluble CD4 (sCD4). Coprecipitation of gp120 by the monoclonal antibody OKT4 (directed to CD4) after incubation with sCD4, demonstrated that the eight expressed envelope proteins were all able to bind CD4 (results not

TABLE 1. SYNCYTIUM-INDUCING CAPACITY OF RECOMBINANT VACCINIA VIRUS EXPRESSED ENVELOPE GENE PRODUCTS IN RELATION TO THE PHENOTYPE OF THE PARENTAL BIOLOGICAL CLONES

Parental biological HIV-1 clones		*Syncytium-inducing capacity[c] of corresponding* env *genes expressed by rVV*
Clone #[a]	*Phenotype[b]*	
320.2a.5	SI	+++++[d]
.7	SI	+++++
.3	NSI	−
.6	NSI	−
16.1	SI	+++++
.2	SI	+++++
.3	NSI	−
.4	NSI	+

[a]The origin of each clone (and/or the corresponding recombinant vaccinia virus expressed envelope gene) is indicated as #xx.yy.z (or xx.z) where xx indicates patient code, yy the number of the particular isolate (if applicable), and z represents the clone number.

[b]Syncytium-inducing capacity of the parental biological HIV-1 clones is determined in MNC according to Tersmette et al.[3]

[c]Syncytium-inducing capacity of the recombinant vaccinia virus expressed gene products are determined in MNC.

[d]Symbols: −, no syncytia; +, <20 small syncytia per well; +++++, >200 large syncytia per well.

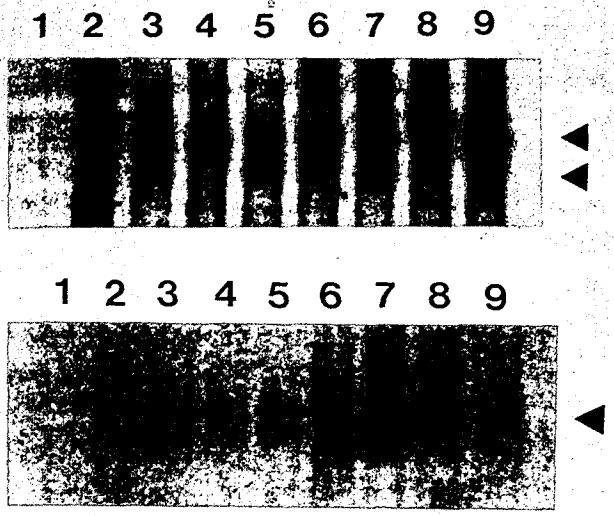

FIG. 1. Expression of HIV-1 envelope gene products by HeLa cells infected with recombinant vaccinia virus (or wt vaccinia virus). Metabolically labeled proteins from infected cell lysates (panel A) or from supernatant (culture medium) of infected cells (panel B) were immunoprecipitated with sheep polyclonal antiserum to gp120 and analyzed by SDS-PAGE. Lane 1, wt; lane 2, 320.2a.5; lane 3, 320.2a.7; lane 4, 320.2a.3; lane 5, 320.2a.6; lane 6, 16.1; lane 7, 16.2; lane 8, 16.3; lane 9, 16.4. See legend to Table 1 for description of the expressed envelope genes. Arrows indicate the precursor glycoprotein and the surface glycoprotein gp120 (panel A) or gp120 shed in the medium (panel B), respectively.

shown). This coprecipitation of gp120 was dependent on addition of soluble CD4, showing the specificity of the reaction.

Biological activity of the expressed envelope proteins

To determine the syncytium-forming capacity of the envelope proteins, human MNCs were infected with recombinant vaccinia virus or wild-type vaccinia virus. Formation of syncytia was assayed 48 h after infection. Syncytium induction by envelope genes expressed in MNCs through recombinant vaccinia viruses is shown in Figure 2. The results are summarized in Table 1. The syncytium-forming capacity of individual HIV-1 envelope genes expressed by recombinant vaccinia virus corresponds to that of the HIV-1 clones from which the envelope genes were derived. The syncytium-forming capacity of each envelope glycoprotein expressed in MNCs obtained from different donors yielded no significant differences.

Envelope gene sequence analysis

In order to define the genetic determinants of the syncytium-inducing property of HIV-1, the complete nucleotide sequence of the eight expressed envelope genes was determined. Figure 3 shows the predicted amino acid sequences of the amplified envelope genes with their origin (patient and biological clone) and the in vitro determined phenotype (SI or NSI). Comparison of these sequences revealed a substantial amount of amino acid variation located mostly in the hypervariable regions V1 through V5.[40] In these regions mutations as well as sequence length variations were observed. The overall conservation is 74.8%: 653 of the 873 predicted amino acid residues of the envelope glycoprotein are identical for all eight clones, mean amino acid homology is 88.9%. Envelope genes derived from biological clones obtained from the same patient display a higher degree of conservation: 90.2% en 95.0% for patients #320 and #16, respectively. Table 2 shows the percentage amino acid homology for all single pairs of envelope sequences. Envelope genes obtained from the same patient and displaying the same phenotype upon expression, show the highest degree of sequence homology; 95.6–99.0%.

Fusion domain variation

We have demonstrated previously that mutations in the SIV envelope fusion domain that affect the hydrophobicity of this region influence the syncytium forming capacity of the SIV envelope glycoproteins.[26] The predicted amino acid sequences of the four envelope glycoproteins obtained from patient #16 display a phenotype associated sequence variation at position number four of the fusion domain (Fig. 3). The SI envelope proteins share a methionine at this position (the fourth N-terminal amino acid residue of gp41, the fusion domain) whereas the NSI biological clone-derived envelope genes have a threonine at the same position. To examine if this mutation, which affects the hydrophobic character of the fusion domain, prevails in more HIV-1 isolates, we amplified and sequenced specifically an envelope gene fragment around the fusion domain of twelve additional biological clones. Figure 4 shows the predicted amino acid sequences with their origin (patient and isolate) and in vitro-determined phenotype of the parental biological clone (SI or NSI). This extended sequence analysis of the fusion domain

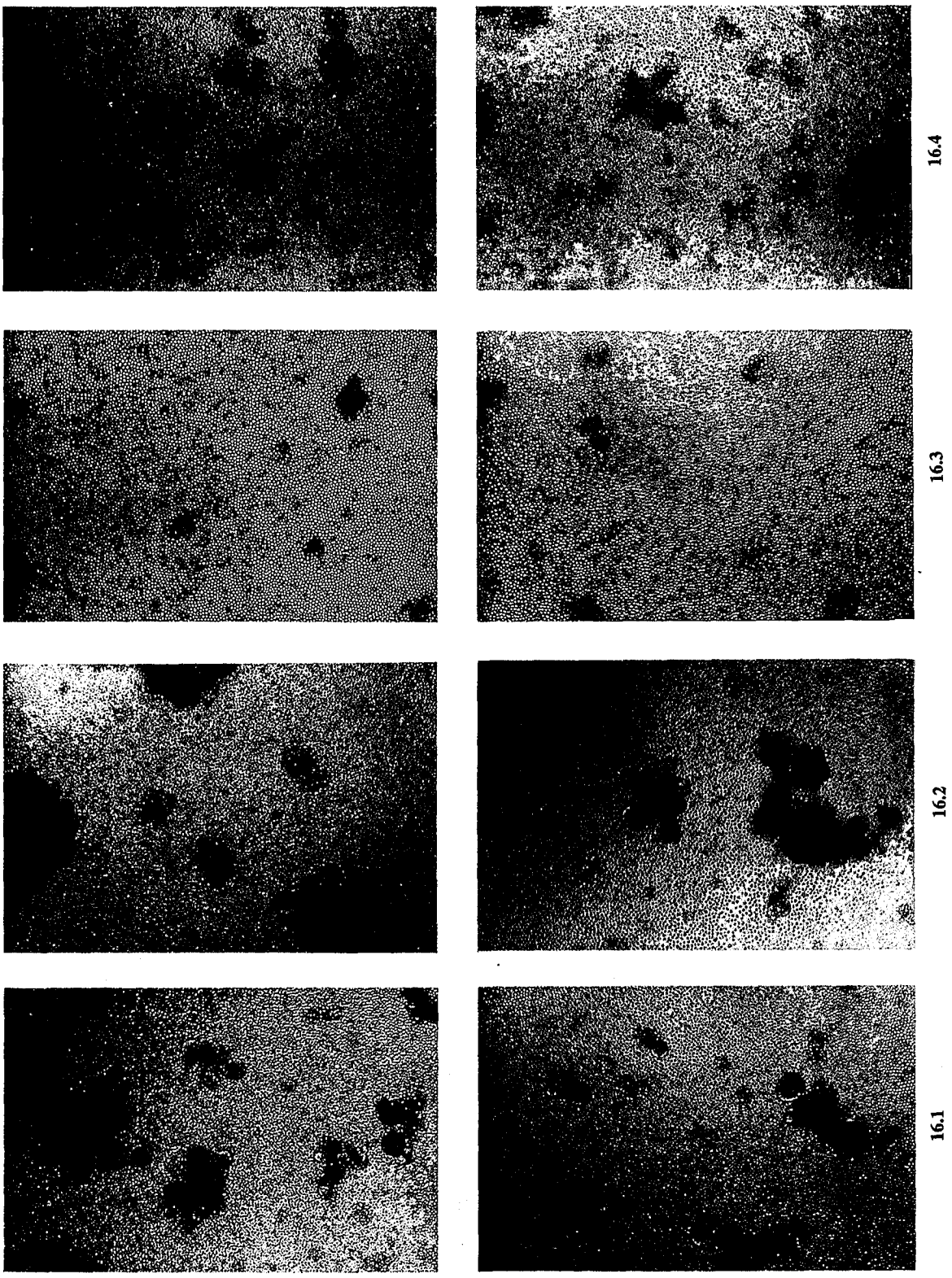

FIG. 2. Syncytia-induced in MNC upon infection with recombinant vaccinia viruses. Indicated are the biological HIV-1 clones which envelope genes are expressed by the recombinant vaccinia viruses (see legend of Table 1).

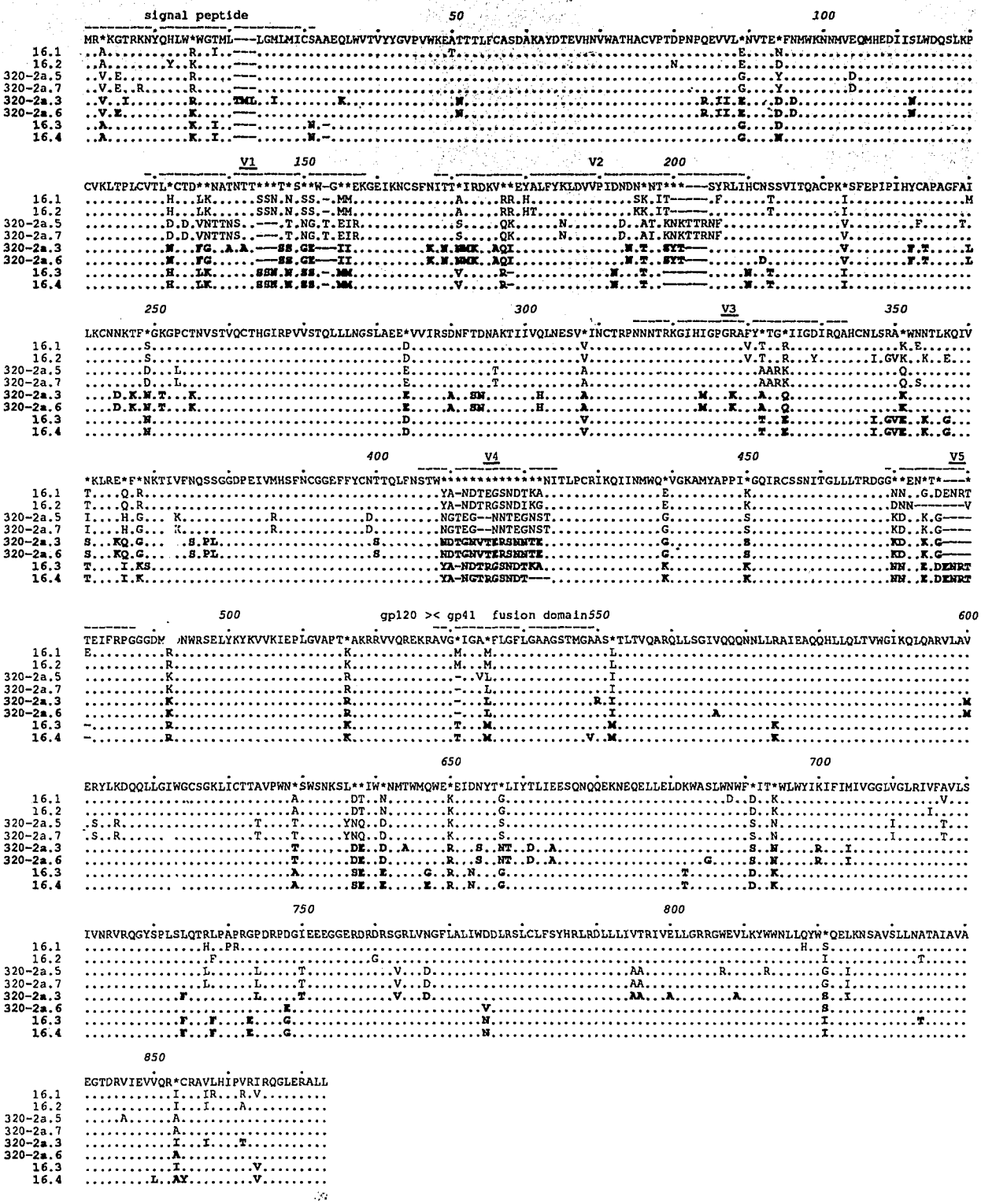

FIG. 3. Predicted amino acid sequence of eight envelope proteins. Sequences were obtained from PCR-amplified envelope genes derived from biological HIV-1 clones (codes as indicated in Table 1). The amino acid sequence is presented in the 1-letter amino acid code and alignment was performed using Clustal. In the top line a consensus sequence derived from these clones is given. Identity with the consensus sequence is indicated by (.), (−) indicates the absence of an amino acid residue at the particular position. The symbol * in the consensus sequence indicates that no consensus amino acid residue could be assigned for that position. Amino acid numbers are indicated as well as the signal peptide, the hypervariable regions V1 through V5, the gp120-gp41 cleavage site, and the fusion domain. Sequences obtained from NSI clone derived envelope genes are bold printed.

TABLE 2. SIMILARITY MATRIX OF EIGHT PREDICTED ENVELOPE AMINO ACID SEQUENCES

320.2a.5[a]	**100**[bc] [d]							
320.2a.7	**99.0**	**100**						
320.2a.3	86.1	86.7	**100**					
320.2a.6	86.1	86.6	**96.9**	**100**				
16.1	**86.6**	**87.1**	85.1	86.1	**100**			
16.2	**86.2**	**86.6**	85.0	86.2	**95.6**	**100**		
16.3	86.1	86.5	**84.8**	**86.3**	93.5	94.5	**100**	
16.4	86.5	86.9	**84.6**	**86.4**	93.2	93.9	**99.0**	**100**
	320. 2a.5	320. 2a.7	320. 2a.3	320. 2a.6	16.1	16.2	16.3	16.4

[a] Origin of envelope sequences as indicated in the legend of Table 1.

[b] Numbers represent percentage amino acid homology between each pair of sequences according to alignment of Figure 3 with gap penalty set to zero.

[c] Bold print is the observed amino acid variation occurring between HIV-1 biological clone envelope sequences displaying the same syncytium-inducing capacity.

[d] Shaded area represents observed amino acid variation between amino acid sequences obtained from the same patient.

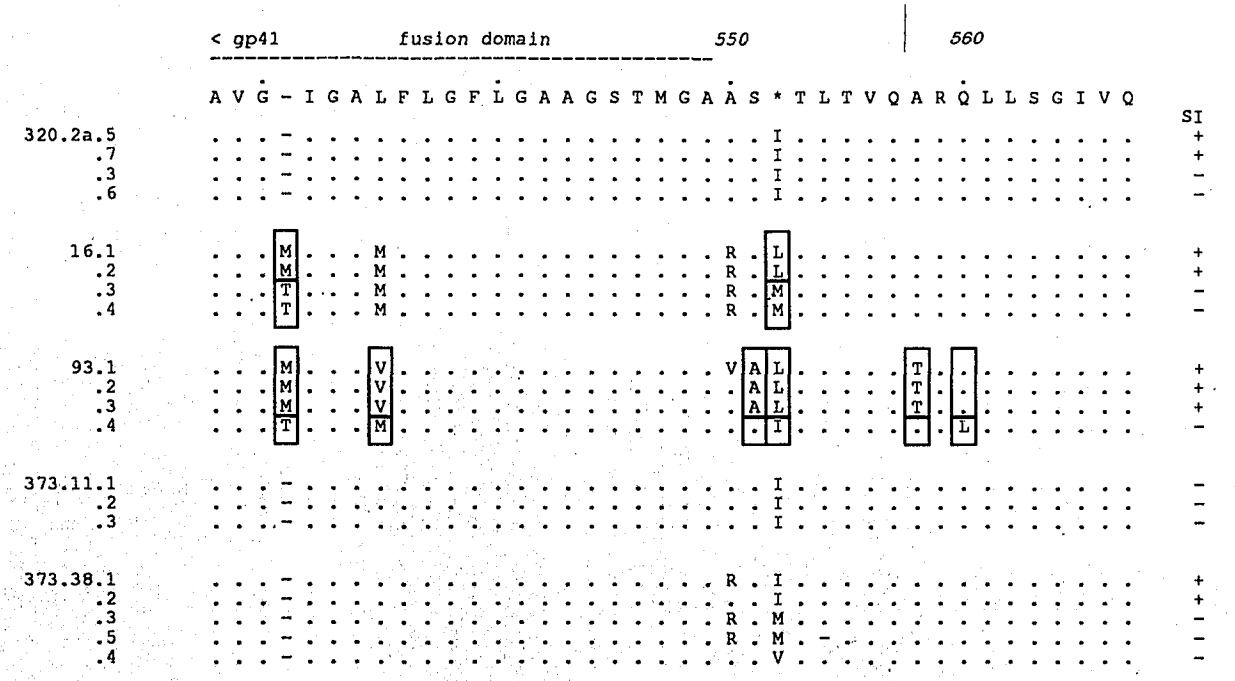

FIG. 4. Predicted amino acid sequences of the fusion domain containing N-terminal region of the transmembrane glycoprotein gp41 of 20 biological HIV-1 clones. In the top line a consensus sequence derived from these 20 clones is given. Codes indicate the biological HIV-1 clones from which the envelope sequences are obtained (see legend of Table 1). Identity with the consensus sequence is indicated by (.), (−) indicates the absence of an amino acid residue at that particular position. The symbol * in the consensus sequence indicates that no consensus amino acid residue could be assigned for that position. The phenotype of the clones is indicated: +:SI and −:NSI. Phenotype associated (SI vs. NSI) amino acid variations are boxed.

A

rVV #	parental phenotype	fusion domain amino acid sequence (first 11 residues)	syncytium inducing capacity
16.1	SI	N-Ala-Val-Gly-**Met**-Ile-Gly-Ala-Met-Phe-Leu-Gly-	+++++
16.3	NSI	N-Ala-Val-Gly-**Thr**-Ile-Gly-Ala-Met-Phe-Leu-Gly-	-
mutant 16.1	"SI"	N-Ala-Val-Gly-**Thr**-Ile-Gly-Ala-Met-Phe-Leu-Gly-	+++++
mutant 16.3	"NSI"	N-Ala-Val-Gly-**Met**-Ile-Gly-Ala-Met-Phe-Leu-Gly-	-

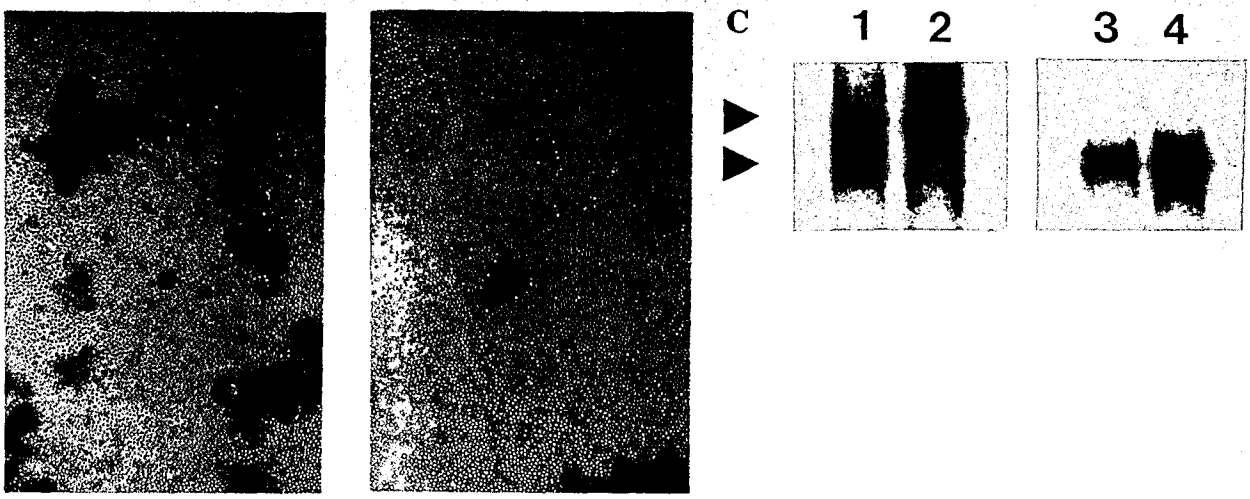

FIG. 5. *Panel A:* Syncytium-inducing capacity of parental and mutated envelope gene products. The first eleven amino acid residues at the amino terminus of gp41 are shown. Codes and symbols as indicated in table 1. *Panel B:* Syncytia induced in MNC upon infection with recombinant vaccinia virus expressing an envelope gene with a mutated fusion domain as indicated in panel A. *Panel C:* Expression of HIV-1 mutated envelope gene products by HeLa cells infected with recombinant vaccinia virus. Metabolically labeled proteins from infected cell lysates (lanes 1 and 2) or from supernatant (culture medium) of infected cells (lanes 3 and 4) were immunoprecipitated with sheep polyclonal antiserum to gp120 and analyzed by SDS-PAGE. Lane 1, mutant 16.1; lane 2, mutant 16.3; lane 3, mutant 16.1; lane 4, mutant 16.3. Arrows indicate the precursor glycoprotein and the surface glycoprotein gp120, respectively.

revealed that the in patient #16 observed phenotype associated sequence variation also occurred in patient #93 but not in patients #320 and #373 (both isolates).

Functional study of the observed phenotype-associated fusion domain variation

To assess the functional contribution of the observed phenotype associated fusion domain variation to the phenotype of HIV-1 isolates, the methionine/threonine mutation was inversely introduced in the envelope glycoproteins originally derived from biological clones #16.1 and #16.3 as shown in Figure 5 panel A. When expressed through recombinant vaccinia virus, these mutated envelope genes did not display an altered syncytium-forming capacity (Fig. 5 panels A and B). Figure 5, panel C shows that these mutated envelope glycoproteins were expressed properly. A coprecipitation with sCD4 demonstrated that these mutated gp120 molecules were still able to bind the CD4 receptor (results not shown).

DISCUSSION

In this study we determined the syncytium-inducing capacity and the nucleotide sequence of envelope genes derived from a set of phenotypically well-characterized biological HIV-1 clones obtained from two patients.

The envelope gene product of lab-adapted syncytium-inducing HIV-1 isolates like HIV-1 IIIB has been shown to induce syncytia when expressed in heterologous expression systems which demonstrates that the envelope glycoproteins of HIV-1 are by themselves sufficient to induce syncytia.[6,7] It is not known whether the lack of syncytium-inducing capacity of NSI field isolates must be attributed to functional differences between the envelope proteins of SI and NSI isolates or whether determinants located outside the envelope gene (e.g., regulatory genes, LTR, or otherwise) modulate this syncytium-inducing capacity. A limited number of studies using recombinant HIV-1 proviruses have implicated the envelope gene products as the major culprit in determining biological variation of HIV-1,[8–10] although evidence pointing to other viral genes has also been presented.[11,43]

We have expressed the complete envelope genes of eight biological clones of HIV-1 (4 SI and 4 NSI clones) to determine their SI capacity in activated human MNC. The results show that only the envelope gene products obtained from SI clones readily induce large syncytia when expressed by recombinant vaccinia virus whereas those obtained from NSI clones did not, despite the fact that they are functionally intact as demonstrated by their ability to bind sCD4. Only the NSI clone 16.4-derived gene product induced some small syncytia in MNC culture but always much less than was observed for the SI clone derived envelope genes (Table 1). This observation formally proves that envelope gene variation alone is sufficient to explain variable syncytium inducing capacities of HIV-1 isolates. For the eight HIV-1 clones presented here genetic variation outside the envelope gene need not to be invoked to explain the viral phenotypic variation.

In addition to the phenotype we also determined the nucleotide sequence and thus the predicted amino acid sequence of the eight expressed envelope genes. The predicted protein sequences exhibited considerable amino acid variation which is mainly located in the previously denominated variable regions. Mean amino acid homology is 88.9% (range 84.6–99.0%; Table 2). Mean amino acid variation of envelope sequences obtained from the same individual was less, 90.2% and 95.0% homology for patient #320 and #16, respectively. Envelope glycoprotein sequences derived from biological clones obtained from the same individual with the same phenotype with regard to syncytium induction, exhibited the least variation (95.6%–99.0% homology; Table 2). This is a reflection of the presence of many amino acid positions displaying phenotype-associated variation between sequences with different phenotypes but obtained from the same patient. This phenotype-associated amino acid variation can be found throughout the whole sequence (e.g., in the V1 and V2 regions, in the V3 region which plays a role in the fusion process and which is the major determinant in HIV-1 neutralization, in the CD4 binding region, in the fusion domain, and in the external region of gp41). None of these variations appear to be conserved between the biological clones from the two patients (#16 and #320), at least not with regard to the exact sequences. At one position in the V3 region (amino acid 21), however, we observe consistent variation concerning charged amino acids. This change in particular could have functional consequences based on observations by others that charge density of the V3 region be important for fusion.[44,45] These and other mutations that could induce structural changes in gp120 or gp41 are of particular interest for further studies, since the lack of exact sequence conservation could mean that structure of individual regions of the envelope protein is more important than sequence, at least in determining biological properties. Alternatively, it might indicate that in different sets of envelope genes different determinants control biological variation. It is presently unclear whether in different viral populations (e.g., obtained from different patients) the biological variation of HIV-1 is controlled by the same determinants. In addition, much of the phenotype-associated amino acid variation may be the result of the presence of different viral populations distinguished by phenotypic properties other than their syncytium inducing capacity alone (e.g., cytotropism). Such shared or linked phenotypic properties will complicate the elucidation of the genetic basis of the separate phenotypic properties (e.g., the syncytium-inducing capacity in the present comparative study).

The 23 cysteine residues appeared to be highly conserved throughout these eight expressed envelope glycoproteins. Only the envelope gene obtained from clone 320.2a.5 codes for a phenylalanine at position #233 and the envelope gene of clone 16.4 codes for a tyrosine at position #854, whereas all other genes code for a cysteine at these positions (Fig. 3). The cysteine at position #854 of gp41, however, is not conserved over other known HIV-1 envelope sequences, a tyrosine at this position is frequently found.[46] In contrast to the cysteine at position #854, the cysteine at position #233 appeared to be highly conserved among HIV-1 sequences.[46] Interestingly the envelope gene product of clone 320.2a.5 which does not contain this cysteine residue does induce syncytia. Reexamination of this region by direct sequencing of a PCR-derived envelope gene fragment could not confirm the presence of this phenylalanine in the genomic DNA of MNC infected with HIV-1 clone 320.2a.5 (data not shown) which implies that it may have arisen during the envelope cloning procedure (e.g., during PCR amplification). This amino acid switch however, does not abolish CD4 binding or the syncytium-inducing capacity of the envelope glycoprotein, illustrating that the cysteine at position #233 is not crucial for these biological properties of the envelope protein.

Eight out of 20 analyzed envelope genes show amino acid variation at two positions in the fusion domain. The variation at position #531 affects the hydrophobic character of the fusion domain whereas the variation at position #535 does not (Fig. 4). Mutational studies with SIV_{mac} indicated that changing the hydrophobicity of the fusion domain could significantly modulate the fusogenic potential of the envelope protein.[26] Although the sequence analysis of the extended set of envelope genes revealed only phenotype-associated amino acid variation in some of the envelope genes, we assessed the functional contribution of this variation by inversely introducing the methionine/threonine mutation. When expressed by recombinant vaccinia virus, these mutated envelope genes do not display an altered syncytium-forming capacity, showing that this fusion domain variation does not contribute to the variation in the syncytium-inducing capacities of this subset of studied envelope genes.

The expression of chimeras of the here studied genetically highly related genes, together with site-directed mutagenesis studies, should allow the identification of the specific domains within the envelope protein that control the variable syncytium-inducing capacity of HIV-1.

ACKNOWLEDGMENTS

The authors thank C.W.H.M. Kruyssen and M.C. Eskens-Jansen for secretarial assistance, H. Vennema for help in recombinant vaccinia virus production, H. Rübsamen-Waigmann and M. Geissendorfer for sequencing service, M.A. Huynen for sequence analysis, and the Medical Research Council for providing reagents.

This work was supported by grants from the Advisory Council on Health Research (RGO no. 88-75/89039; A.C.A. and P.L.), the Netherlands Organization for Scientific Research (NWO no. 900-502-104; M.G.), and the Netherlands Foundation for Preventive Medicine (no. 28-1079).

REFERENCES

1. Cheng-Mayer C, Seto D, Tateno M, and Levy JA: Biologic features of HIV-1 that correlate with virulence in the host. Science 1988;24:80–82.
2. Fenyö EM, Morfeldt-Månson L, Chiodi F, Lind B, von Gegerfelt A, Albert J, Olausson E, and Åsjö B: Distinct replicative and cytopathic characteristics of Human Immunodeficiency Virus isolates. J Virol 1988;62:4414–4419.
3. Tersmette M, de Goede REY, Al BJM, Winkel IN, Gruters RA, Cuypers HT, Huisman HG, and Miedema F: Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. J Virol 1988;62:2026–2032.
4. Koot M, Vos AHV, Keet RPM, de Goede REY, Dercksen W, Terpstra FG, Coutinho RA, Miedema F, and Tersmette M: HIV-1 biological phenotype in long term infected individuals evaluated with an MT2 cocultivation assay. AIDS 1991;6:49–54.
5. Tersmette M, Lange JMA, de Goede REY, de Wolf F, Eeftink-Schattenkerk JKM, Schellekens PThA, Coutinho RA, Huisman JG, Goudsmit J, and Miedema F: Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. Lancet 1989;1:983–985.
6. Lifson JD, Feinberg MB, Reyes GR, Rabin L, Banapour B, Chakrabarti S, Moss B, Wong-Staal F, Steimer KS, and Engelman EG: Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature 1986;323:725–728.
7. Sodroski J, Goh WC, Rosen C, Campbell K, and Haseltine WA: Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. Nature 1986;322:470–477.
8. Cheng-Mayer C, Quiroga M, Tung JW, Dina D, and Levy JA: Viral determinants of human immunodeficiency virus type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. J Virol 1990;64:4390–4398.
9. Shioda T, Levy JA, and Cheng-Mayer C: Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature 1991;349:167–169.
10. York-Higgins D, Cheng-Mayer C, Bauer D, Levy JA, and Dina D: Human Immunodeficiency virus type I cellular host range, replication, and cytopathicity are linked to the envelope region of the viral genome. J Virol 1990;64,4016–4020.
11. Cheng-Mayer C, Shioda T, and Levy JA: Host range, replicative, and cytopathic properties of human immunodeficiency virus type 1 are determined by very few amino acid changes in tat and gp120. J Virol 1991;65:6931–6941.
12. Gartenhaus R, Michaels F, Hall L, Gallo RC, and Reitz MS Jr: Relative activities of HIV-1-IIIB and HIV-1-bal LTR and tat in primary monocytes and lymphocytes. AIDS Res Human Retroviruses 1991;7:681–688.
13. Meyerhans A, Cheynier R, Albert J, Seth M, Kwok S, Sninsky J, Morfeldt-Månson L, Asjö B, and Wain-Hobson S: Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. Cell 1989;58:901–910.
14. Pomerantz RJ, Feinberg MB, Andino R, and Baltimore D: The long terminal repeat is not a major determinant of the cellular tropism of human immunodeficiency virus type I. J Virol 1991;65:1041–1045.
15. Schuitemaker H, Groenink M, Meyaard L, Kootstra NA, Fouchier RAM, Gruters RA, Huisman HG, Miedema F, and Tersmette M: Early steps but not cell-type-specific signalling of the viral long terminal repeat determine HIV type I monocytetropism. 1992; submitted.
16. Kozarsky K, Penman M, Basiripour L, Haseltine W, Sodroski J, and Krieger M: Glycosylation and processing of the human immunodeficiency virus type I envelope protein. J AIDS 1989;2:163–169.
17. Doms RW, Earl PL, and Moss B: The assembly of the HIV-1 *env* glycoprotein into dimers and tetramers. Adv Exp Med Biol 1991;300:203–219.
18. Pinter A, Honnen WJ, Tilley SA, Bona C, Zaghouani H, Gorny MK, and Zolla-Pazner S: Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. J Virol 1989;63:2674–2679.
19. Schawaller M, Smith GE, Skehel JJ, and Wiley DC: Studies with crosslinking reagents on the oligomeric structure of the env glycoprotein of HIV. Virology 1989;172:367–369.
20. Stein B, Gouda S, Lifson J, Penhallow R, Bensch K, and Engleman E: pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell 1987;49:659–668.
21. Dalgleish A, Beverly P, Clapham P, Crawford D, Greaves M, and Weiss R: The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 1984;312:763–766.
22. Klatzmann D, Champagne E, Chamaret S, Gruest J, Guetard D, Hercend T, Gluckman JC, and Montagnier L: T-lymphocyte T4 molecule behave as the receptor for the human retrovirus LAV. Nature 1984;312:767–768.
23. Hoxie JA, Brass LF, Pletcher CH, Haggarty BS, and Hahn BH: Cytopathic variants of an attenuated isolate of human immunodeficiency virus type 2 exhibit increased affinity for CD4. J Virol 1991;65:5096–5101.
24. Ivey-Hoyle M, Culp JS, Chaikin MA, Hellmig BD, Matthews TJ, Sweet RW, and Rosenberg M: Envelope glycoproteins from biologically diverse isolates of immunodeficiency viruses have widely different affinities for CD4. Proc Natl Acad Sci (USA) 1991; 88:512–516.
25. Sattentau QJ and Moore JP: Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J Exp Med 1991;174:407–415.
26. Bosch ML, Earl PL, Fargnoli K, Picciafuoco S, Giombini F, Wong-Staal F, and Franchini G: Identification of the fusion peptide of primate immunodeficiency viruses. Science 1989;244:694–697.
27. Freed EO, Myers DJ, and Risser R: Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proc Natl Acad Sci (USA) 1990;87:4650–4654.
28. Gallaher WF: Detection of a fusion peptide sequence in the transmembrane protein of human immunodeficiency virus. Cell 1987;50:327–328.
29. Kowalski M, Potz J, Basiripour L, Dorfman T, Chun Goh W, Terwilliger E, Dayton A, Rosen C, Haseltine W, and Sodroski J: Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science 1987;237:1351–1355.
30. Freed EO, Myers DJ, and Risser R: Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. J Virol 1991;65:190–194.
31. Hwang SS, Boyle TJ, Lyerly HK, and Cullen BR: Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science 1991;253:71–74.
32. de Wolf F, Goudsmit J, Paul DA, Lange JMA, Hooijkaas C, Schellekens PThA, Coutinho RA, and van der Noordaa J: Risk of AIDS related complex and AIDS in homosexual men with persistent HIV antigenaemia. Br Med J 1987;295:569–572.
33. Groenink M, Fouchier RAM, de Goede REY, de Wolf F, Gruters RA, Cuypers HTM, Huisman HG, and Tersmette M: Phenotypic heterogeneity in a panel of infectious molecular human immunodeficiency virus type 1 clones derived from a single individual. J Virol 1991;65:1968–1975.
34. Tersmette M, Gruters RA, de Wolf F, de Goede REY, Lange JMA, Schellekens PThA, Goudsmit J, Huisman HG, and Miedema F: Evidence for a role of virulent human immunodeficiency virus

(HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. J Virol 1989; 63:2118–2125.
35. Ratner L, Fisher A, Jagodzinski LL, Mitsuya H, Liou RS, Gallo RC, and Wong-Staal F: Complete nucleotide sequences of functional clones of the AIDS virus. AIDS Res Human Retroviruses 1987;3:57–69.
36. Chakrabarti S, Brechling KB, and Moss B: Vaccinia virus expression vector: coexpression of B-galactosidase provides visual screening of recombinant virus plaques. Mol Cell Biol 1985; 5:3403–3409.
37. Mulligan MJ, Kumar P, Hui H, Owens RJ, Douglas Ritter Jr G, Hahn BH and Compans RW: The *env* protein of an infectious noncytopathic HIV-2 is deficient in syncytium formation. AIDS Res Human Retroviruses 1990;6:707–720.
38. Laemmli EK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
39. Sanchez-Pescador R and Urdea MS: Use of purified synthetic deoxynucleotide primers for rapid deoxynucleotide chain termination sequencing. DNA 1984;3:339–343.
40. Modrow S, Hahn BH, Shaw GM, Gallo RC, Wong-Staal F, and Wolf H: Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. J Virol 1987;61:570–578.
41. Kunkel TA, Roberts JD, and Zakour RA: Rapid and efficient site-specific mutagenesis without phenotypic selection. Meth Enzymol 1987;154:367–382.
42. Cochran MA, Puckett C, and Moss B: Mutagenesis of the promoter region for a vaccinia virus gene: Evidence for tandem early and late regulatory signals. J Virol 1985;54:30–37.
43. Spire B, Hirsch I, Neuveut C, Sire J, and Chermann JC: The env gene variability is not directly related to the high cytopathogenicity of an HIV-1 variant. Virology 1990;177:756–758.
44. Callahan LN, Phelan M, Mallinson M, and Norcoss MA: Dextran sulfate blocks antibody binding to the principal neutralizing domain of human immunodeficiency virus type 1 without interfering with gp120-CD4 interactions. J Virol 1991;65:1543–1550.
45. Fouchier RAM, Groenink M, Kootstra NA, Tersmette M, Huisman HG, Miedema F, and Schuitemaker H: Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. J Virol 1992;66:3183–3187.
46. Meyers G, Berzofsky JA, Korber B, Smith RF, and Pavlakis GN (eds): *Human Retroviruses and AIDS, A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences*. Los Alamos National Laboratory, Los Alamos, NM 1991.

Address reprint requests to:
Marnix L. Bosch
Laboratory of Immunobiology
National Institute of Public Health and
Environmental Protection
P.O. Box 1
3720 BA Bilthoven, the Netherlands