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Presentation of Native Epitopes in the V1/V2 and V3 Regions of Human Immunodeficiency Virus Type 1 gp120 by Fusion Glycoproteins Containing Isolated gp120 Domains

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The immune response to viral glycoproteins is often directed against conformation- and/or glycosylation-dependent structures; synthetic peptides and bacterially expressed proteins are inadequate probes for the mapping of such epitopes. This report describes a retroviral vector system that presents such native epitopes on chimeric glycoproteins in which protein fragments of interest are fused to the C terminus of the N-terminal domain of the murine leukemia virus surface protein, gp70. The system was used to express two disulfide-bonded domains from gp120, the surface protein of human immunodeficiency virus type 1 (HIV-1), that include potent neutralization epitopes. The resulting fusion glycoproteins were synthesized at high levels and were efficiently transported and secreted. A fusion protein containing the HXB2 V1/V2 domain was recognized by an HIV_{IIIB}-infected patient serum as well as by 17 of 36 HIV-1 seropositive hemophiliac, homosexual male and intravenous drug user patient sera. Many of these HIV^+ human sera reacted with V1/V2 domains from several HIV-1 clones expressed in fusion glycoproteins, indicating the presence of cross-reactive antibodies against epitopes in the V1/V2 domain. Recognition of gp(1-263):$V1/V2_{HXB2}$ by the HIV_{IIIB}-infected human patient serum was largely blocked by synthetic peptides matching V1 but not V2 sequences, while recognition of this construct by a broadly cross-reactive hemophiliac patient serum was not blocked by individual V1 or V2 peptides or by mixtures of these peptides. A construct containing the V3 domain of the IIIB strain of HIV-1, gp(1-263):$V3_{HXB2}$, was recognized by sera from a human and a chimpanzee that had been infected by HIV_{IIIB} but not by sera from hemophiliac patients who had been infected with HIV-1 of MN-like V3 serotype. The reactive sera had significantly higher titers when assayed against gp(1-263):$V3_{HXB2}$ than when assayed against matching V3 peptides. Immunoprecipitation of this fusion glycoprotein by the human serum was only partially blocked by V3 peptide, indicating that this infected individual produced antibodies against epitopes in V3 that were expressed on the fusion glycoprotein but not by synthetic peptides. These data demonstrated that the chimeric glycoproteins described here effectively present native epitopes present in the V1/V2 and V3 domains of gp120 and provide efficient methods for detection of antibodies directed against native epitopes in these regions and for characterization of such epitopes.

Conformation- and glycosylation-dependent epitopes in gp120, the surface protein of human immunodeficiency virus type 1 (HIV-1), are important targets for the neutralizing response to this virus. Epitopes recognized by antibodies that potently block the binding of gp120 to its receptor, CD4, are believed to be composed of residues from several separated regions of the molecule that are brought together in the native conformation (33, 45). The importance of native protein structure to the presentation of epitopes in more localized neutralization targets on gp120 is becoming clear from recent work. A major cluster of neutralizing epitopes in the V3 domain was identified by analysis of the immune response to HIV-1 with use of synthetic peptide probes, and the immunogenicity of this region has been extensively characterized with such methods (27). It is now evident that synthetic peptide probes are inadequate for the full characterization of the anti-V3 immune response. The reactivity of natural immune sera with V3 peptides in standard solid-phase assays has been found to be a poor predictor of serum reactivity with the V3 loop in native gp120 (25). Furthermore, many anti-V3 monoclonal antibodies (MAbs) have higher affinity for native gp120 than for synthetic peptides (5), and a potently neutralizing anti-V3 MAb direct against a conformation-dependent epitope has been described (21, 22). A number of recent reports have described neutralizing MAbs directed against epitopes in the V2 region, many of which are dependent on protein glycosylation and/or conformation (3, 8, 22, 28, 41, 44, 46). Unlike the case for CD4 binding site epitopes, the global structure of gp120 appears not to be necessary for presentation of native epitopes in either the V3 or V2 cluster. Straightforward and sensitive methods for detecting immune responses to these and similar epitopes within complex immune sera would greatly facilitate study of the immune response to infection and vaccination, and molecules expressing such native structures may be more effective as immunogens than are synthetic peptides.

The N-terminal domain of the ecotropic murine leukemia virus (MuLV) surface protein, gp70, was selected as a carrier protein to express glycopeptide domains from other proteins in order to present native epitopes specified by those domains. A truncation fragment that contains the internally disulfide-bonded N-terminal region of gp70 (17) has been shown to retain ecotropic receptor binding activity (7), indicating that this is an autonomously folding globular domain. Similar fragments are generated by protease digestion at hypersensitive sites following the receptor binding domain (29, 36, 37,

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40), suggesting that this region of gp70 is exposed on the intact protein. This domain is proline rich and cysteine free and carries O-linked glycans (4), all of which are common characteristics of regions with extended conformations that serve as spacer domains in membrane proteins (11). This linker region of gp70 is hypervariable in length and sequence among ecotropic and other MuLVs (7, 13, 42) and is highly immunogenic in MuLVs (29, 40) and the closely related feline leukemia viruses (6, 30). A site within this interdomain linker of gp70 was used for fusion to disulfide-bonded domains derived from HIV-1 gp120 that contain targets of neutralizing antibodies. Fusion glycoproteins containing each of two such gp120 fragments were found to be efficiently made and secreted and to present native epitopes that are seen by natural immune sera.

MATERIALS AND METHODS

Construction of vectors. The vector for expression of heterologous glycopeptides in fusion proteins with the N-terminal domain of MuLV gp70, pLRB333, was based on Friend ecotropic MuLV clone FB29 (43), the complete sequence of which was kindly made available by John Portis. The permuted DNA clone was reconstructed in this laboratory into a colinear two-long-terminal-repeat form with minimal duplicated MuLV sequences. For historical reasons, an *Sph*I-to-*Bpu*1102I restriction fragment containing *env* as well as limited flanking sequences was replaced with the homologous fragment from the Friend ecotropic MuLV isolate clone 57 (32). To construct the truncation derivative of MuLV *env* designed to receive heterologous gene fragments, PCR with Vent polymerase (New England Biolabs) was used to generate a DNA fragment in which the sequences between the desired truncation point and a *Cla*I restriction site near the 3′ end of *env* were deleted. By incorporation in the PCR primer, an *Nhe*I site otherwise absent from the FB29/clone 57 vector was inserted in frame following the codon for amino acid 263 of gp70 followed by two stop codons and the *Cla*I site. The *Nhe*I site results in addition of Ala-Ser to the C terminus of the truncated gp70 sequence.

For insertion of gene fragments into this vector, DNA fragments from specific regions of HIV-1 *env* were generated by PCR. An in-frame restriction site for *Nhe*I at the 5′ end and two in-frame stop codons followed by a *Cla*I restriction site at the 3′ end were incorporated into the primers. These restriction sites were used to insert the gene fragment between the *Nhe*I and *Cla*I sites of the expression vector, generating a gene that expressed a fusion glycoprotein containing an Ala-Ser linker between the gp70 fragment and the inserted glycopeptide. In certain constructs (the V3 construct as well as the JR-FL, JR-CSF, and MN V1/V2 constructs), a *Nar*I site was inserted between the HIV-1 gene fragment and the stop codons to facilitate further modification of the fusion proteins. This resulted in a C-terminal addition of Gly-Ala to these fusion glycoproteins. To facilitate purification of the gp(1-263): $V3_{HXB2}$ fusion glycoprotein, His-8 and Gln-9 of gp70 were replaced with a sequence of six His residues. PCR overlap mutagenesis (9) was used to construct the appropriate *Sph*I-to-*Nhe*I gene fragment for insertion into the expression vector.

The V3 construct was derived from the HIV-1 HXB2d gene fragment present in an HIV-1 *env* expression vector (34), provided by Nathaniel Landau; the HXB2d V1/V2 gene fragment was also derived from this plasmid. The V1/V2 gene fragment of HIV-1 NL4-3 was obtained from pNL4-3 (1), provided by Malcolm Martin. The V1/V2 gene fragments of HIV-1 JR-FL and JR-CSF (14) were obtained from plasmid pUC112/JR-FL/3′SST and plasmid pYKJR-CSF, respectively, both provided by Irvin Chen. The V1/V2 gene fragment of HIV-1 MN was obtained from plasmid pMN-ST1, which encodes an infectious viral genome (18), provided by Marvin Reitz. The V1/V2 gene fragment of HIV-1 SF2 was obtained from plasmid pARV-Lambda7a (19), provided by Dino Dina. The numbering system used is based on that for HXB2d gp120 and begins with the first amino acid of the mature protein; the sequences are taken from the Human Retroviruses and AIDS data bank, Los Alamos National Laboratory, except for that of the MN-ST sequence, provided by Marvin Reitz.

Construction of cell lines. Cell lines expressing the truncation fragment or a fusion glycoprotein were generated by passing the retroviral vector carrying the recombinant *env* through retroviral packaging cell lines. When retroviral genomes containing a functional RNA packaging signal were transfected into a mixture of two cell lines that provide *env* proteins of different interference groups (ecotropic ψ_2 [20] or PE501 [24] and amphotropic PA317 [23]), a so-called ping-pong effect occurred, allowing the retroviral vector genomes to spread efficiently through the coculture and resulting in high-titer stocks of pseudotyped virus particles containing the vector genome. Transfection and spread of vector genomes in packaging lines was monitored with an anti-$p12^{gag}$ MAb, 10BA10, that reacts with the $p12^{gag}$ produced by the vector but not with the $p12^{gag}$ produced by the packaging lines. Supernatants from these cocultures were used to infect NIH 3T3 cells, and cell lines that express high levels of recombinant proteins were isolated.

Viral culture; immunological and biochemical procedures. Viral culture (39), indirect immunofluorescence (31), and radiolabelling and radioimmunoprecipitation (38) were performed as described elsewhere. Primary antibodies used included a hyperimmune goat anti-Rauscher MuLV gp70 serum (Microbiological Associates) and rat anti-$p12^{gag}$ MAb 10BA10 (produced in this laboratory). Sera from chimpanzee 487 infected with HIV_{IIIB} and other chimpanzees were provided by Elizabeth Muchmore of the Laboratory of Experimental Medicine and Surgery in Primates, sera from HIV-1-seropositive hemophiliac patients were provided by Margaret Hilgartner of Cornell University Medical School, sera from HIV-1-seropositive homosexual men and one intravenous drug user were provided by James Braun and Stacy Kreiswirth, and serum from an HIV_{IIIB}-infected laboratory worker, sample FF2746 from 14 November 1991 (47), was provided by William Blattner. Secondary antibodies for immunofluorescence were the appropriate rabbit anti-goat immunoglobulin (IgG) and anti-IgM or goat anti-human IgG and IgM and IgA conjugated to fluorescein isothiocyanate (Zymed).

V1/V2 domain synthetic peptides ADP 740.9 through ADP 740.17, which are each 20 amino acids long and offset by 10 amino acids and match HXB2 gp120 sequences from amino acids 82 through 181, were obtained from the MRC AIDS Reagent Project. The additional V2 domain 16-mer oligopeptides matching amino acids 135 to 149 of the HXB2d sequence (ADP 794.2) and the homologous sequences from MN (ADP 794.3) and RF (794.4), each containing an additional C-terminal Cys residue, and the analogous peptide matching the consensus sequence for this region as defined in the Los Alamos Human Retroviruses and AIDS data base (ADP 794.1) were also obtained from the MRC AIDS Reagent Project. V2 region peptides are aligned in Table 1. A linear V3 peptide corresponding to the complete sequence between the Cys residues defining the V3 loop of HXB2, TRPNNNTR KSIRIQRGPGRAFVTIGKIGNMRQAH, was provided by Seth Pincus. Peptide ADP 792.3, obtained from the MRC AIDS Reagent Project, had the same sequence for the V3 loop and included the defining Cys residues and a C-terminal Asn.

TABLE 1. V1/V2 domain sequences

Isolate or peptide (strain)	Sequence[a]
	⊢——V1——⊣ ⊢——V2——⊣
Isolate	* * * * * *
HXB2d	LKPCVKLTPLCVSLKCTDLK NDTNTNSSSGRMIMEKGEIKNCSFNISTSIRGKVQKEYAFFYKLDIIPID NDTTSYKLTSCNTSVITQACPKVS
NL4-3	---------- ---------- D---------- V--- N---R-I--------
MN-ST	----------T-N----RNTTNTN-S-AN-N-NSEGTIKG--M-------T----D-M-----LL-----VS-N --S---R-I-------I-
SF2	----------T-N----G KA-------NWKEEI ----------T----D-I---N-L-RN--VV---NASTTTNY-N-R-IH--R----------
JRCSF	----------T-N-K-V -A---T---EG- --R----------TK---D-------L-----VV--- NKNN-K-R-I--------
JRFL	----------T-N-K-V -A---TND-EGT --R----------T----DE------L-----VV--- NNN---R-I--D---------I-
Peptide	
ADP 794.1 (consensus)	--D-------L----
ADP 794.4 (RF)	R-D-T--K--L----
ADP 740.13 (HXB2)	----------
ADP 740.14 (HXB2)	----------

[a] Sequences are taken from the Human Retroviruses and AIDS data base; for HXB2d, the sequence includes amino acid residues 86 to 179 of gp120. Signals for N-linked glycosylation are underlined; positions of cysteine residues, all of which are fully conserved, are marked by asterisks.

It was obtained as the cyclic form. An MN V3 peptide, ADP 715 (RKRIHIGPGRAFYTTKN), corresponding to the tip of the V3 loop was obtained from the MRC AIDS Reagent Project.

To obtain partially purified V3 fusion glycoprotein for enzyme-linked immunosorbent assay (ELISA), supernatants from 3T3 cells expressing the His_6 form of gp(1-263):$V3_{HXB2}$ were dialyzed against phosphate-buffered saline (PBS; pH 8), NaCl was added to 0.5 M, and protein was bound to Ni^{2+}-nitrilotriacetate-Sepharose (Qiagen) in this buffer. gp(1-263):$V3_{HXB2}$ was eluted with 30 mM imidazole in PBS (pH 7.4) following a 20 mM imidazole wash. The antigen constituted only a small fraction of the Coomassie-staining protein in these preparations (data not shown) but was readily detected by direct ELISAs performed with this material.

ELISAs were performed in TiterTek Immuno-assay plates (Flow Laboratories). Antigens were adsorbed to wells for 60 min in 100 μl of carbonate buffer (pH 9.6), washed with PBS–0.05% Tween, blocked for 90 min with 2% bovine serum albumin (BSA) in PBS, and washed again with PBS–0.05% Tween. Then 100 μl of serum diluted in PBS was added for 60 min at room temperature, wells were washed with PBS–0.05% Tween, incubated for 60 min with 100 μl of alkaline phosphatase-coupled goat anti-human IgG (Zymed) diluted in 2% BSA, and washed in PBS–0.05% Tween, and 100 μl of 1-mg/ml *p*-nitrophenol phosphate in diethanolamine buffer (pH 9.8) was added. A_{405} was measured between 30 and 60 min after substrate addition. The amount of partially purified gp(1-263):$V3_{HXB2}$ used per assay was always sufficient to give at least 75% of the maximum achievable signal. Peptides were used at 100 ng per well; assays were insensitive to increased amounts of peptide. Background A_{405} reactivity in wells lacking antigen was subtracted from each of the datum points presented.

RESULTS

Synthesis and processing of fusion glycoproteins containing sequences from HIV-1 gp120. Gene constructs were prepared to express the V1/V2 and V3 domains of HXB2 gp120 in fusion glycoproteins with the N-terminal domain of MuLV gp70. The sequences and disulfide bonding patterns of the resulting chimeric proteins are shown diagrammatically in Fig. 1, based on the published secondary structures of gp70 (17) and gp120 (16). The selected fragments of gp120 corresponded to distinct disulfide-bonded domains of the protein containing hypervariable regions and neighboring conserved sequences. The V1/V2 domain was expressed as a 94-amino-acid fragment (Fig. 1A) that includes three disulfide bonds. Two of these disulfide bonds generate the V1 and V2 variable loops separated by a short stretch of conserved sequence, and the third disulfide bond generates an arm of conserved flanking sequences. This expressed sequence included six signals for N-linked glycosylation, all of which have been reported to be utilized (15, 16) and one of which (attached to Asn-156) was found to be necessary for viral growth in cell culture (15). The 46-amino-acid V3 domain fragment (Fig. 1B) contained the disulfide-bonded loop itself and five flanking amino acids N and C terminal to the defining Cys residues. It included three signals for N-linked glycosylation, one within the V3 loop and one on each flank, all of which have been reported to be utilized (15, 16) and none of which were found to be required for viral growth in cell culture (15).

The level of expression and efficiency of processing of the V1/V2 fusion glycoprotein was compared with that of gp(1-263), the N-terminal truncation product of gp70 that served as the fusion partner in these constructs, in pulse-chase experiments. The analysis of gp(1-263) was performed in cells that coexpressed a wild-type MuLV *env*, allowing comparison of the synthesis and processing of the truncation fragment with that of normal *env* proteins. A hyperimmune antiserum to gp70 was used to immunoprecipitate products containing gp70 epitopes. gp(1-263) was synthesized at a high level and was efficiently and rapidly secreted (Fig. 2A). At the end of a 30-min pulse-label, gPr80, the primary translation product of the wild-type *env*, and a 32-kDa form of gp(1-263) were seen in cell lysates. After a 1-h chase, more than 90% of gp(1-263) was found in the culture supernatant. In contrast, while the majority of gPr80 had been converted to mature gp70 at this time, only a small fraction of the gp70 had been released from the cells. This difference in the rate of secretion is probably due to the fact that the truncation fragment is a soluble protein that enters the secretion pathway and is therefore rapidly released from the cell, while gp70 is retained on cellular membranes by interaction with the viral TM protein and its release into the supernatant requires either shedding from the cell surface or incorporation into virions. Note that almost all of the gp(1-263) was processed and secreted. In contrast, only a

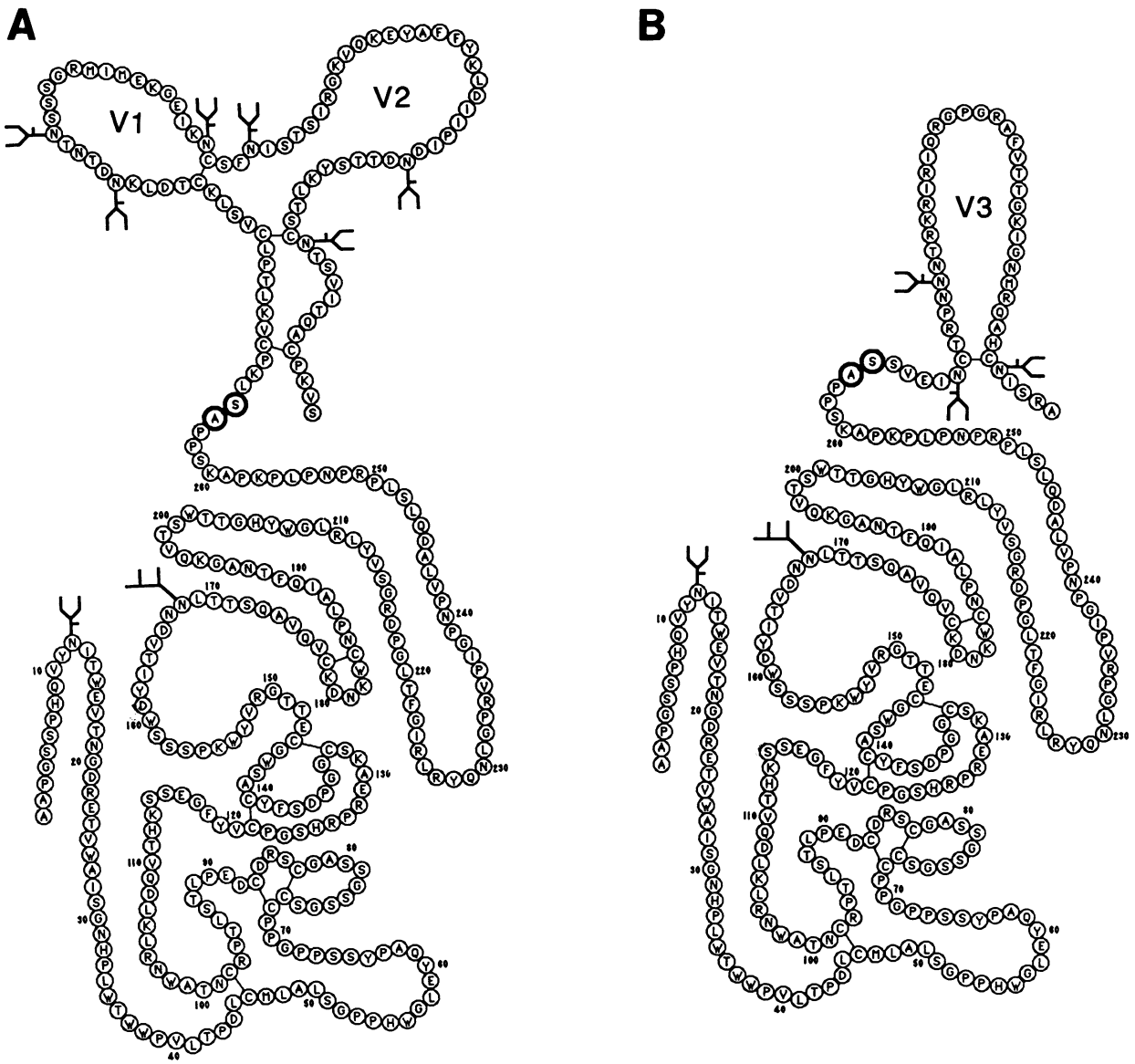

FIG. 1. Secondary structures of fusion glycoproteins. The HIV-1 sequences shown are from the HXB2d isolate, and the disulfide bonds and types of N-linked glycans shown are as postulated by Leonard et al. (16). The gp70 sequences shown are from clone 57, the disulfide bonds are as indicated by Linder et al. (17), and the types of N-linked glycans shown are as described by Kayman et al. (12). Ψ, complex N-linked glycans; Ϋ, high-mannose N-linked glycans. Amino acids encoded by the sequence of restriction sites incorporated into the constructs are highlighted. (A) gp(1-263):V1/V2$_{HXB2}$; (B) gp(1-263):V3$_{HXB2}$.

relatively small fraction of gPr80 was ever processed to gp70; most of the precursor appeared to be degraded intracellularly.

Figure 2B presents a pulse-chase analysis of the processing and secretion of the V1/V2 fusion glycoprotein. The 59-kDa primary translation product of gp(1-263):V1/V2$_{HXB2}$ was seen at the end of the 30-min pulse-label. After 1 h of chase, fusion glycoprotein started appearing in the supernatant, and by 4 h, almost all of the gp(1-263):V1/V2$_{HXB2}$ had been secreted. The secreted material was larger and more diffuse than the intracellular precursor. This was presumably due to processing of the N-linked glycans into larger complex forms in the Golgi apparatus, since enzymatic removal of N-linked glycans yielded a single well-defined species on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). The increase in apparent size for the secreted form of gp(1-263):V1/V2$_{HXB2}$, which carries eight N-linked glycans that may all be processed to complex forms, was much greater than for the secreted form of gp(1-263) (Fig. 2A), which carries only two N-linked glycans. The level of mature gp(1-263):V1/V2$_{HXB2}$ present in cell lysates was very low at all times, detectable only in film exposures longer than those shown in Fig. 2B, indicating that the fusion glycoprotein was rapidly secreted following modification of its glycans. Thus, the slower secretion seen for gp(1-263):V1/V2$_{HXB2}$ relative to gp(1-263) appeared to be due to retention of the precursor form in the endoplasmic reticulum; once transported to the Golgi apparatus, gp(1-263):V1/V2$_{HXB2}$ was rapidly secreted. This delay in transport was consistent with slower folding of the fusion

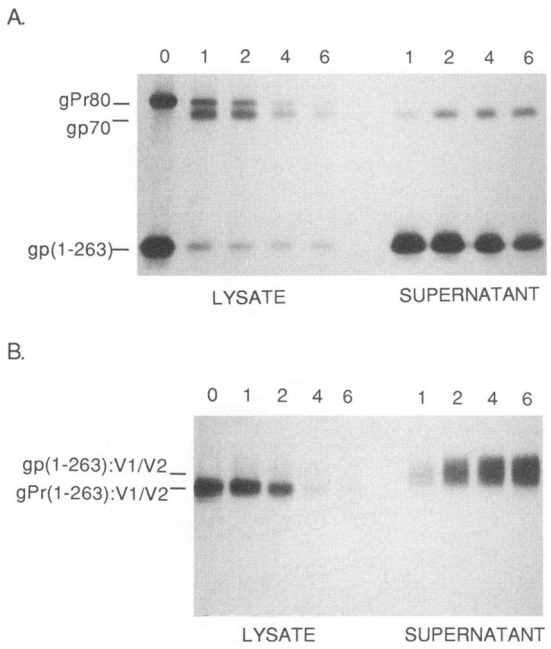

FIG. 2. Pulse-chase analysis of fusion glycoprotein processing and secretion. Cells were labelled for 30 min with [^{35}S]cysteine and chased with cold medium for the indicated times, in hours. Samples were immunoprecipitated with hyperimmune goat anti-gp70 serum and then subjected to SDS-PAGE and fluorography. NaCl and Nonidet P-40 were added to 0.5 M and 0.5%, respectively, in supernatant samples before precipitation to more closely match the buffer from which cell lysates were precipitated. Volumes of all samples were adjusted so that each lane represents the material from an equal number of cells. Cell lysate and supernatant samples are as indicated, with the time of chase in hours. (A) 3T3 cells expressing gp(1-263) and wild-type MuLV *env* proteins; (B) 3T3 cells expressing gp(1-263):V1/V2$_{HXB2}$.

glycoprotein into its globular, disulfide-bonded conformation, since such folding is widely believed to be the rate-limiting step for transport from the endoplasmic reticulum (10, 35). Processing and secretion of the less complex gp(1-263):V3$_{HXB2}$ was more rapid than that of gp(1-263):V1/V2$_{HXB2}$, more closely resembling the behavior of gp(1-263) (data not shown).

Reactivity of HIV$^+$ sera with V1/V2 fusion glycoproteins. Immune sera known to recognize HIV$_{IIIB}$ gp120 were tested for the ability to recognize gp(1-263):V1/V2$_{HXB2}$ in the absence of detergent or other denaturants. These included sera from chimpanzees experimentally infected with HIV$_{IIIB}$, from a laboratory worker accidentally infected with this virus, from a group of HIV-1-seropositive hemophiliac patients, and from a group of HIV-1-seropositive homosexual men. Since the HXB2d clone is from the HIV$_{IIIB}$ stock, the sera from the HIV$_{IIIB}$-infected human and chimpanzees were expected to include type-specific responses to this virus. These sera all recognized gp(1-263):V1/V2$_{HXB2}$, with precipitating titers in the range of 1/100 to 1/800 (data not shown). The strains of HIV-1 to which the hemophiliac patients and homosexual men had been exposed are not known but are unlikely to closely match HIV$_{IIIB}$ in the V1 and V2 regions. Nonetheless, 13 of 26 hemophiliac patient sera, four of nine homosexual male patient sera, and 0 of 1 intravenous drug user patient sera precipitated gp(1-263):V1/V2$_{HXB2}$ with titers as high as 1/200 (top panels in Fig. 3A and B and data not shown).

The recognition of gp(1-263):V1/V2$_{HXB2}$ by sera from individuals exposed to random HIV isolates suggested that these sera recognized conserved epitopes presented by the fusion glycoprotein. To determine whether this was the case, fusion glycoproteins containing the V1/V2 domains from the MN, JR-CSF, JR-FL, SF2, and NL4-3 HIV-1 isolates were produced. The sequences of the V1/V2 domains are quite variable among these five isolates and HXB2d (Table 1). The six gp(1-263):V1/V2 fusion glycoproteins were immunoprecipitated with a selection of human sera (Fig. 3). The HIV$_{IIIB}$-specific laboratory worker serum reacted strongly with gp(1-263):V1/V2$_{HXB2}$ and gp(1-263):V1/V2$_{NL4-3}$, the two fusion constructs bearing sequences from HIV$_{IIIB}$ isolates, with a preference for the HXB2 construct, and reacted more weakly with gp(1-263):V1/V2$_{MN}$ (Fig. 3A, lane 9; Fig. 3B, lane 11). This serum did not react with any of the other constructs, indicating that its anti-V1/V2 response was largely type specific. Eighteen HIV$^+$ human sera from New York City hemophiliac patients and homosexual men were tested for the ability to recognize these V1/V2 fusion glycoproteins; seven precipitated between three and five of the six V1/V2 fusion glycoproteins, while the rest of these sera did not recognize any of the fusion glycoproteins at a level clearly above the background seen with HIV$^-$ human sera. gp(1-263):V1/V2$_{SF2}$ was the only construct not immunoprecipitated by any of these sera.

To determine whether these responses were directed against specific linear epitopes, selected sera were examined for reactivity with synthetic peptides matching the HIV-1 sequence expressed in gp(1-263):V1/V2$_{HXB2}$. A series of nine 20-mer oligopeptides with 10-amino-acid overlaps corresponding to residues 82 to 181 of HXB2 gp120 were tested for reactivity in solid-phase ELISAs. The HIV$_{IIIB}$-infected human serum, two highly cross-reactive hemophiliac patient sera (used in lanes 1 and 8 of Fig. 3A), and two nonreactive hemophiliac patient sera (used in lanes 3 and 7 of Fig. 3A) were examined. The HIV$_{IIIB}$-infected human serum reacted strongly only with two overlapping peptides matching V1 loop sequences, ADP 740.11 (amino acids 102 to 121) and ADP 740.12 (amino acids 112 to 131) (data not shown). On the other hand, all four of the hemophiliac patient sera tested reacted strongly and specifically with a peptide corresponding to sequences extending from the C terminus of V1 into the N-terminal region of V2, ADP 740.13 (amino acids 122 to 141), and less strongly with the next overlapping peptide, ADP 740.14 (amino acids 132 to 151); one of these sera also reacted with ADP 740.11, the amino acid 102–121 V1 region peptide (data not shown). Since the lack of correlation between reactivity of these hemophiliac patient sera with V1/V2 domain peptides by ELISA and with V1/V2 fusion glycoproteins by immunoprecipitation was surprising, the ELISA reactivities of additional sera were tested against a larger set of peptides containing sequences corresponding to this particular region from several HIV-1 strains. These assays used ADP 740.13 and ADP 740.14, and peptides matching amino acids 135 to 149 of HIV$_{IIIB}$ (ADP 794.2), the corresponding sequences of HIV$_{MN}$ (ADP 794.3) and HIV$_{RF}$ (ADP 794.4), and a consensus sequence for this region (ADP 794.1) (Table 1). All of the HIV$^+$ New York City patient sera tested reacted strongly with each of these HIV$_{IIIB}$ sequence peptides, while reactivity with the other peptides was considerably weaker or undetected (Fig. 4). These data appeared to map the peptide ELISA reactivity to amino acids 135 to 141 (IRGKVQK), the sequence common to all of the reactive peptides. Neither the universality of the recognition of HIV$_{IIIB}$ sequence peptides nor the preference for HIV$_{IIIB}$ sequence over that of HIV$_{MN}$ was seen in precipitation assays with the fusion glycoproteins. It was therefore clear that ELISA reactivity against synthetic

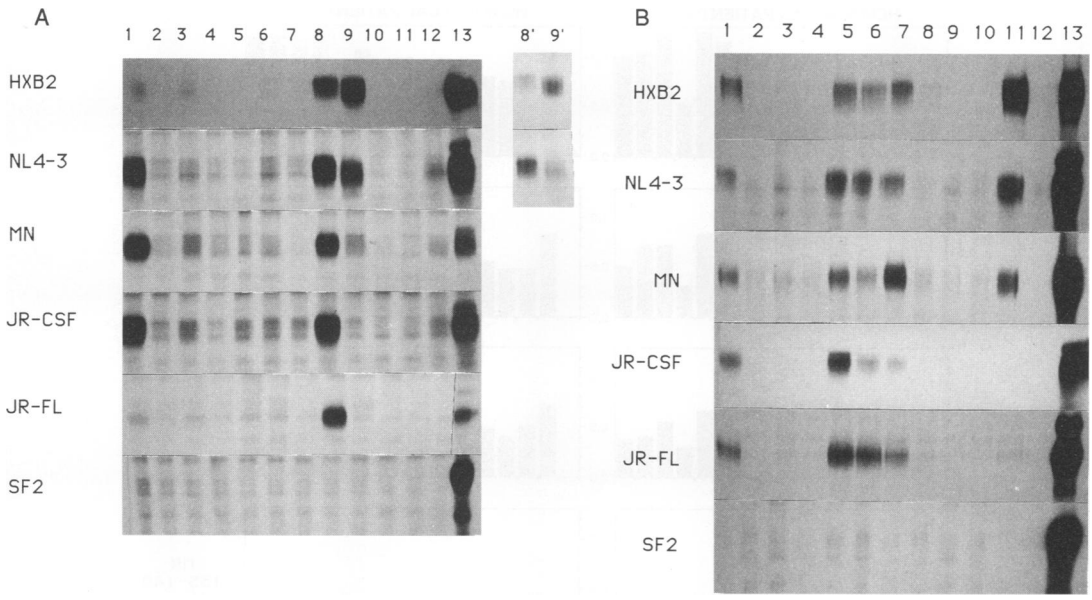

FIG. 3. Reactivity of human sera with six V1/V2 fusion glycoproteins. Culture supernatants containing [^{35}S]cysteine-labeled fusion glycoproteins were immunoprecipitated with human sera in the absence of detergent or other denaturant and then subjected to SDS-PAGE and fluorography. (A) Lanes: 1 to 8, HIV-1$^+$ hemophiliac patient sera; 9, HIV_{IIIB}-infected laboratory worker serum; 10 to 12, HIV$^-$ human serum controls; 13, hyperimmune goat anti-gp70 control; 8′ and 9′, shorter exposures of lanes 8 and 9 to visualize the relative specificity of these two sera for the HXB2 and NL4-3 constructs. Human sera were used at 1/50. (B) Lanes: 1 to 3 and 5 to 10, HIV-1$^+$ homosexual male patient sera; 4, HIV$^+$ intravenous drug user patient serum; 11, HIV_{IIIB}-infected laboratory worker serum; 12, HIV$^-$ human serum control; 13, hyperimmune anti-gp70 control. Human sera were used at 1/100.

peptides was not a predictor of activity against V1/V2 fusion glycoproteins.

The discrepancies between the peptide reactivity and recognition of V1/V2 fusion glycoproteins could mean that the two assays detect completely different sets of antibodies present in these HIV$^+$ sera or that the peptide ELISA has greater sensitivity and therefore detects reactive antibodies in a larger number of sera. This issue was investigated by determining the ability of one of the strongly cross-reactive hemophiliac patient sera to precipitate gp(1-263):$V1/V2_{HXB2}$ in the presence of a large molar excess of a mixture of the overlapping 20-mer peptides spanning amino acids 82 through 181 of the HXB2 sequence. This mixture of peptides effectively blocked precipitation by the HIV_{IIIB}-infected patient serum (Fig. 5), as did each of the V1 peptides ADP 740.11 and ADP 740.12 by themselves (data not shown), consistent with the specificity of this serum for these peptides by ELISA. However, the peptide mixture was unable to significantly inhibit precipitation by the cross-reactive hemophiliac patient serum (Fig. 5). This result suggested that the precipitating anti-V1/V2 antibodies in the cross-reactive sera were not the same as the antibodies reacting with amino acids 135 to 141 detected by ELISA. Presumably the cross-reactive anti-V1/V2 activity in these sera is directed against native epitopes that require a more accurate presentation of gp120 structure than was achieved by the synthetic peptides. The nature and significance of the anti-peptide ELISA reactivity seen with the New York City HIV$^+$ sera is not clear.

Reactivity of HIV$^+$ sera with gp(1-263):$V3_{HXB2}$. The fusion protein expressing the HXB2 V3 sequence, gp(1-263):$V3_{HXB2}$, was specifically precipitated by the sera from the HIV_{IIIB}-infected patient and chimpanzees. None of the hemophiliac patient sera precipitated this fusion glycoprotein (Fig. 6 and data not shown), consistent with peptide ELISA data that showed a strong preference of these patient sera for MN-like V3 sequences (data not shown). The reactivities of the HIV_{IIIB}-infected patient and chimpanzee sera to gp(1-263):$V3_{HXB2}$ were compared with the reactivities of these sera to two synthetic peptides corresponding to HXB2 V3 loop sequences in solid-phase ELISAs. The titers of the sera (serum dilution giving 50% maximal reaction) were determined against gp(1-263):$V3_{HXB2}$, against a synthetic linear peptide containing the complete V3 domain sequence present between the defining cysteines, and against a purportedly cyclic peptide that contained the entire V3 domain sequence, including the Cys residues that form the loop (Fig. 7). The human serum possessed a sevenfold-higher titer against the fusion glycoprotein than against either of the peptides. For the chimpanzee serum, the titer against gp(1-263):$V3_{HXB2}$ was threefold higher than against the cyclic peptide and sixfold higher than against the linear peptide. As shown in Fig. 8, the cyclic V3 peptide partially blocked immunoprecipitation of gp(1-263):$V3_{HXB2}$ by the serum from the HIV_{IIIB}-infected human patient and fully blocked immunoprecipitation by the serum from the HIV_{IIIB}-infected chimpanzee, while having no effect on immunoprecipitation by the anti-gp70 serum. Since the majority of the anti-V3 precipitating activity in these sera was blocked by the matching cyclic peptide, this peptide must have presented the epitopes seen by most of the anti-V3 antibodies in these sera. These data indicated that the anti-V3 antibodies in this chimpanzee serum had a higher affinity for gp(1-263):$V3_{HXB2}$ than for the homologous V3 peptide but contained little or no antibody against epitopes that were not presented by the cyclic V3 peptide. However, the residual precipitation by the serum from the HIV_{IIIB}-infected human patient in the presence of the cyclic V3 peptide suggested that this serum possessed anti-V3 antibodies that reacted with epitopes not effectively presented by the peptide.

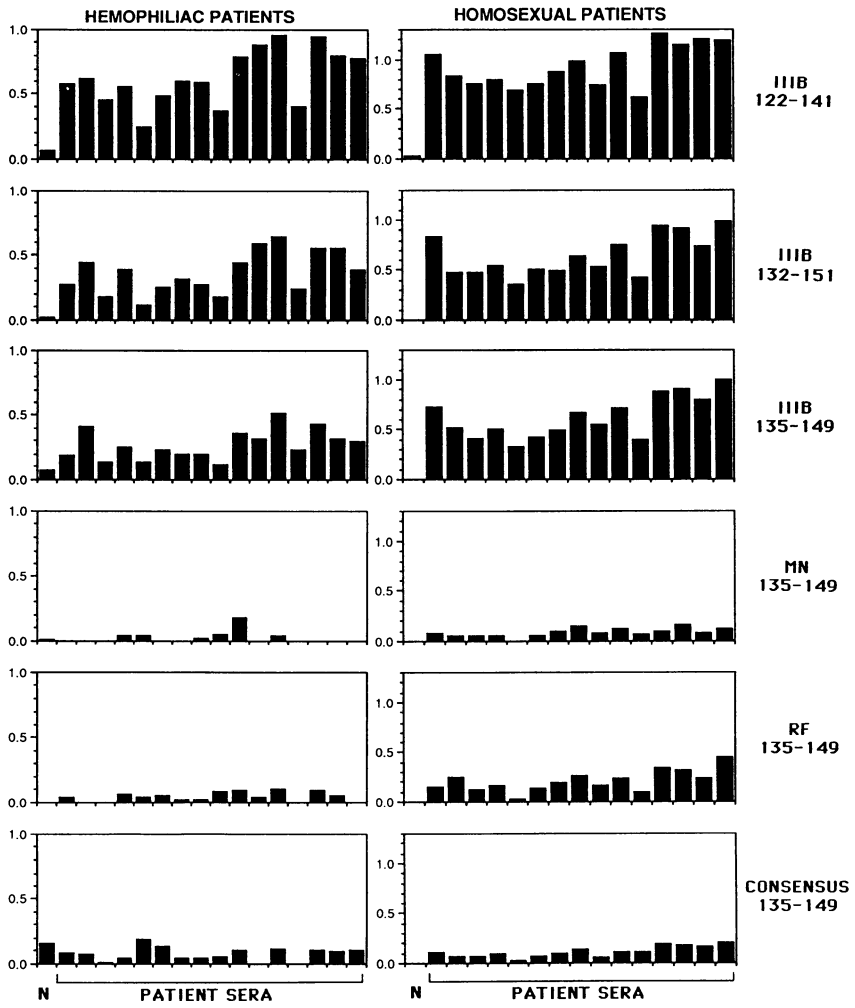

FIG. 4. Reactivity of human sera with V1/V2 region synthetic peptides by ELISA. Peptides were adsorbed to ELISA plates. Following incubation with various human sera, the relative amount of antibody bound was determined with alkaline phosphatase conjugated to goat anti-human IgG, reported as A_{405} after subtraction of background. The HIV_{IIIB} 122–141 peptide was ADP 740.13; the HIV_{IIIB} 132–151 peptide was ADP 740.14; the HIV_{IIIB} 135–149 peptide was ADP 794.2; the HIV_{MN} 135–149 peptide was ADP 794.3; the HIV_{RF} 135–149 peptide was ADP 794.4; the consensus 135–149 peptide was ADP 794.1. Column N is a normal human serum control; each of the other columns shows data for a particular patient serum; the fourth column in the homosexual patients panel shows data for the intravenous drug user patient serum.

DISCUSSION

This report describes a new vector system for the efficient production of glycosylated protein fragments in fusion glycoproteins. PCR-generated gene fragments are inserted in frame

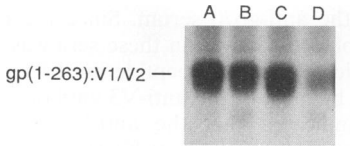

FIG. 5. Peptide inhibition of serum reactivity with gp(1-263):V1/$V2_{HXB2}$. [^{35}S]cysteine-labeled gp(1-263):V1/$V2_{HXB2}$ was immunoprecipitated with the cross-reactive human serum used in lane 8 of Fig. 3A (lanes A and B) and the HIV_{IIIB} type-specific human serum (lanes C and D) in the absence of detergent and then subjected to SDS-PAGE and fluorography. In lanes B and D, synthetic peptides spanning HIV-1 gp120 amino acids 82 to 181 (ADP 740.9 through ADP 740.17) were present at 30 μg/ml each. Sera were used at 1/50.

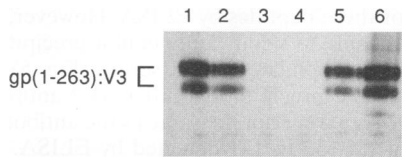

FIG. 6. Precipitation of gp(1-263):$V3_{HXB2}$. [^{35}S]cysteine-labeled gp(1-263):$V3_{HXB2}$ (lanes 1 to 4 and 6) or gp(1-263):$V3_{HXB2}$ containing the His_6 affinity tag (lane 5) was immunoprecipitated with human and chimpanzee sera in the absence of detergent or other denaturants and then subjected to SDS-PAGE and fluorography. The upper band of the doublet is the fully glycosylated gp(1-263):$V3_{HXB2}$, and the lower band is a partially glycosylated form. The following sera were used: lane 1, HIV_{IIIB}-infected human patient serum; lane 2, HIV_{IIIB}-infected chimpanzee serum; lane 3, hemophiliac patient serum shown in lane 8 of Fig. 3A; lane 4, normal human serum; lane 5, HIV_{IIIB}-infected chimpanzee serum; lane 6, hyperimmune goat anti-gp70 serum. The goat antiserum was used at a dilution of 1/100; the other antisera were used at 1:50.

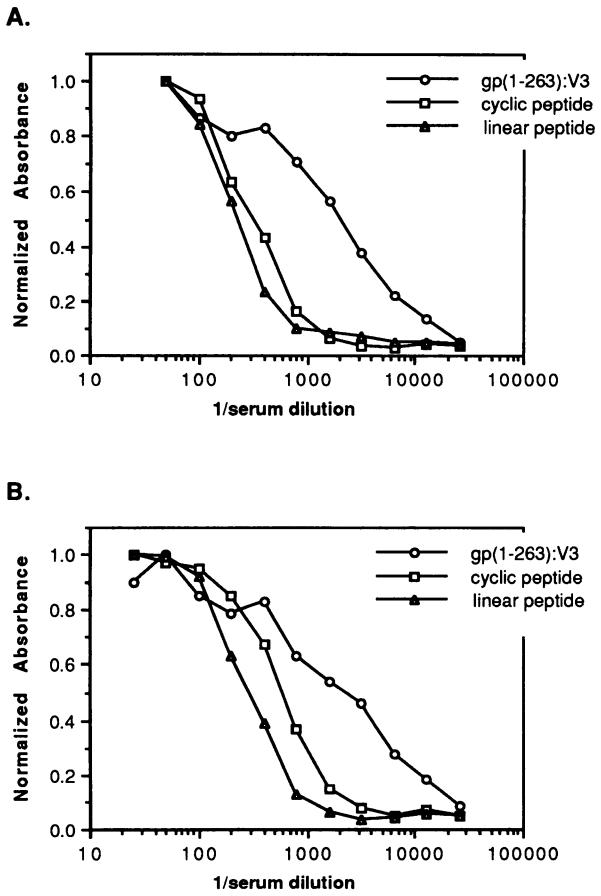

FIG. 7. ELISA titers of type-specific sera on gp(1-263):$V3_{HXB2}$ and synthetic V3 peptides. Partially purified gp(1-263):$V3_{HXB2}$ or the full-length linear or cyclic synthetic HXB2 V3 peptides were adsorbed to ELISA plates. The relative amounts of antibody bound following incubation with a series of twofold dilutions of the HIV_{IIIB}-infected human (A) and chimpanzee 487 (B) sera were determined with alkaline phosphatase conjugated to goat anti-human IgG, reported as normalized A_{405} after subtraction of background.

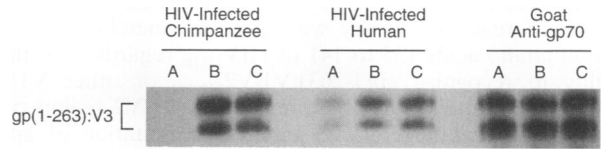

FIG. 8. Peptide inhibition of serum reactivity with gp(1-263):$V3_{HXB2}$. [^{35}S]cysteine-labeled gp(1-263):$V3_{HXB2}$ was immunoprecipitated with the HIV_{IIIB}-infected human or chimpanzee 487 serum or the goat anti-gp70 serum in the absence of detergent and then subjected to SDS-PAGE and fluorography. In lanes A, the cyclic HXB2 V3 peptide ADP 792.3 was present at 15 μg/ml; in lanes B, a short MN V3 peptide ADP 715 was present at 15 μg/ml; in lanes C, there was no peptide present. Sera were used at 1/100.

into a truncated MuLV *env* containing the N-terminal receptor-binding domain of gp70, and the chimeric gene is expressed from a retroviral vector. The fusion glycoproteins examined were glycosylated and efficiently secreted. These fusion glycoproteins retained binding activity for the ecotropic MuLV receptor (data not shown), suggesting that the carrier fragment folds autonomously in these constructs and thus is unlikely to seriously perturb other internally structured heterologous protein fragments fused to it. The expression system should also be useful for expressing O-glycosylated protein fragments, since a slightly longer truncation fragment of gp70 that includes O-glycan attachment sites was O glycosylated (data not shown). The recombinant vectors can be constructed rapidly, as can stable cell lines secreting high levels of the fusion glycoproteins.

Fusion glycoproteins expressing disulfide-bonded domains of gp120 were found to present epitopes that are not presented by synthetic peptides. The fusion glycoprotein containing the V3 region of HIV_{IIIB} gp120 was immunoprecipitated by sera from a human patient and a chimpanzee that had been infected with HIV_{IIIB}. The titers of these type-specific sera were significantly higher on gp(1-263):$V3_{HXB2}$ than on homologous V3 peptides; for the laboratory worker serum, the difference was about sevenfold. Furthermore, although immunoprecipitation of gp(1-263):$V3_{HXB2}$ by the chimpanzee serum was fully blocked by excess cyclic V3 peptide, immunoprecipitation by the human serum was only partially blocked by this peptide. These data indicated that most anti-V3 antibodies present in these immune sera had higher affinity for the fusion glycoprotein than for V3 peptides and that this human serum recognized epitopes that were presented by the V3 fusion glycoprotein but not by V3 peptides. These data are consistent with reports showing that a serum sample from this patient has a 10-fold higher titer on native gp120 than on denatured protein (26) and that a large fraction of its total gp120 reactivity is with V3 epitopes (25). Little or no reactivity with this construct was found in a screen of approximately 30 HIV^+ hemophiliac patient sera of MN-like V3 serotype as determined with V3 peptides (data not shown). On the other hand, many of these sera did react with a fusion glycoprotein expressing the MN-like V3 loop from JR-CSF (data not shown). These results indicated that V3 fusion glycoproteins detected a largely type-specific response to V3 epitopes, as do V3 peptides, and suggested that reactivity with V3 fusion glycoproteins can be used to assay the type specificity and magnitude of anti-V3 responses.

The gp(1-263):$V1/V2_{HXB2}$ fusion glycoprotein was recognized by the HIV_{IIIB}-infected human and chimpanzee sera. In addition, 17 of 36 HIV^+ sera from hemophiliac, homosexual male, and intravenous drug user patients recognized this fusion glycoprotein. Since the hemophiliac patients were exposed to viruses with an MN-like V3 domain, the anti-V1/V2 reactivities of these sera were likely to be cross-reactive responses. This expectation was confirmed by the finding that hemophiliac patient and homosexual male sera that were reactive with gp(1-263):$V1/V2_{HXB2}$ also precipitated fusion glycoproteins bearing V1/V2 sequences from other HIV-1 isolates. As many as five of the six distinct V1/V2 domains examined were recognized by many of these sera. The only sequence not recognized by any of these sera was that of SF2, suggesting that SF2 gp120 represented a distinct V1/V2 serotype.

The serum of the HIV_{IIIB}-infected human reacted with V1 region peptides that matched HIV_{IIIB} amino acids 112 to 121, and immunoprecipitation of gp(1-263):$V1/V2_{HXB2}$ by this serum was largely blocked by these peptides. The anti-V1/V2 activity in this serum therefore reacts primarily with a linear epitope(s) in V1. Similar data mapping the V1/V2 region reactivity of this patient to amino acids 110 to 120 were recently reported (28). The significance of results from experiments using synthetic peptides to characterize the other

human sera is unclear. All of the New York City patient sera examined reacted strongly with peptides matching the V2 region amino acids 135 to 141 of HIV_{IIIB}, regardless of their ability to recognize gp(1-263):$V1/V2_{HXB2}$ or other V1/V2 fusion glycoproteins, yet these and other V1/V2 region peptides failed to interfere with immunoprecipitation of gp(1-263):$V1/V2_{HXB2}$ by a highly cross-reactive serum. The inability of the synthetic peptides to interfere with the cross-reactive response strongly suggested that the epitope(s) involved required native protein structures presented by gp(1-263):$V1/V2_{HXB2}$ but not by synthetic peptides.

The lack of correlation between reactivity with V1/V2 fusion glycoproteins and the V2 region peptides raises questions of the relevance of serum ELISA reactivity with these peptides. V2 peptide reactivity was specific in that this reactivity was seen with peptides matching the HIV_{IIIB} sequence but not the HIV_{MN} or consensus sequence and that most normal human sera did not react strongly with the HIV_{IIIB} V2 peptides. The IIIB 135–141 sequence is highly positively charged (containing two Lys, an Arg, and a Gln), but this is partially compensated for by a Gly-to-Asp shift in the non-IIIB sequences. One possibility is that the reactivity of these sera with the IIIB peptide is due to a nonspecific charge interaction. The low level of reactivity seen for the homosexual male sera with the HIV_{RF} sequence peptide might then be due to the presence of two nearby positively charged residues absent from the other sequences. The prevalence of this reactivity in HIV^+ but not normal sera might result from the elevated levels of immunoglobulin commonly found in HIV-infected patients (48). However, ELISA analysis of sera from HIV^+ patients in London with these peptides found reactivity with the amino acid 135–149 consensus sequence peptide with 21% of the sera examined, and the positive sera exhibited a preference for the MN sequence over that of IIIB (22). A second study recently reported reactivity of sera from HIV^+ patients from New York but not London with a peptide corresponding to HXB2 amino acids 121 to 140 (28). These geographical preferences suggest the possibility of strain-specific effects but are difficult to rationalize with a nonspecific charge interaction hypothesis. Furthermore, the fact that a number of potent neutralizing rat MAbs were shown to recognize the HXB2 132–141 V2 peptide (22) indicates that this region contains functional epitopes. The discrepancies between ELISA and immunoprecipitation data may reflect detection of low-affinity antibodies in solid-phase formats (25). Taken together, these data indicate that caution is called for in the interpretation of ELISA results with peptides containing the N-terminal sequence of V2.

Although peptide reactivity data did not allow localization of the epitope(s) seen by the cross-reactive sera within the sequences expressed by the V1/V2 fusion glycoproteins, comparison of the expressed sequences that were and were not recognized suggests that the critical region is likely to be within V2. The flanking conserved sequences are retained in the SF2 construct that is not recognized by any of these sera, and the MN sequence that is recognized is much more divergent in V1 than is the SF2 sequence. The SF2 sequence is the most divergent sequence in the relatively conserved N-terminal region of V2 and in the highly variable C-terminal region of V2, both of which have been shown to be important for the epitopes of neutralizing MAbs (22, 25, 44). The lack of recognition of the SF2 construct by these sera indicates that these reactivities represent epitope-specific complementarity determining region-mediated recognition by the antibodies rather than the V_H3-mediated binding of antibodies that has recently been described for SF2 gp120 (2).

The nature of the native cross-reactive epitopes in the V1/V2 domain recognized by the human sera described in this study is of interest. Analyses of gp(1-263):$V1/V2_{HXB2}$ with appropriate MAbs demonstrated that this fusion glycoprotein presents both conformation- and glycan-dependent epitopes (22, 41, 47a). One or both of these classes of epitopes are likely to be the basis of the preference of immune sera for fusion glycoprotein over synthetic peptide. The fact that many MAbs recognizing epitopes in V2 neutralize viral infectivity (3, 8, 22, 25, 46, 46a) suggests that the anti-V1/V2 antibodies in these sera may also have neutralizing activity. Experiments to characterize the V1/V2 epitopes seen by the cross-reactive human sera and to analyze the functional activities of human antibodies against these epitopes are in progress.

The work described here demonstrated expression of epitopes by both the V1/V2 and V3 fusion glycoproteins that were not presented by synthetic peptides. Fusion glycoproteins expressing native epitopes from specific regions of gp120 can be used to monitor antibodies that have not previously been resolved in analyses of human sera; whether any such humoral responses correlate with protection against infection by HIV-1 or progression to AIDS needs to be determined. If so, these fusion glycoproteins would provide an important tool for the evaluation of HIV vaccine trials and could themselves prove to be effective immunogens for inducing such antibodies.

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