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Use of TrpE/Gag Fusion Proteins To Characterize Immunoreactive Domains on the Human Immunodeficiency Virus Type 1 Core Protein

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The human immunodeficiency virus (HIV) p24 core protein is one of the most immunogenic of HIV structural proteins. Infected individuals develop high titers of antibodies against p24 early in infection, which makes anti-p24 antibodies important serological markers. However, despite the clinical importance of the anti-p24 response, no systematic study to characterize the antigenic domains on the p24 protein has been reported. We report here on the use of 12 overlapping fragments of the HIV type 1 p24 protein, synthesized in bacteria as TrpE/Gag fusion proteins, to identify at least two and possibly three antigenic domains on the p24 protein. In addition, we note that different HIV-seropositive sera exhibited different patterns of reactivity with the p24 domains presented on our fusion proteins.

The nucleocapsid of human immunodeficiency virus (HIV) is composed of multiple subunits of a protein of approximately 24,000 molecular weight called p24 (4, 20). p24 is encoded by the HIV type 1 (HIV-1) *gag* gene along with core proteins p17 and p15 as a polypeptide precursor (5, 16, 18, 21). Individuals infected with HIV mount a significant humoral response against p24. Anti-p24 antibodies, along with antibodies to the envelope proteins, are important for diagnosis of exposure to HIV. Recent studies have documented a temporal decline in the prevalence of antibodies to p24 which correlates with increasing severity of clinical symptoms (3, 10, 15, 22). It has been suggested that a loss of detectable p24 antibody, the appearance of circulating p24 antigen, and a decline in the number of CD4 cells are clinical markers which foreshadow progression to clinical acquired immune deficiency syndrome (6). Because of the importance of antibodies to p24 in serological screening of blood samples and as possible markers for disease progression, we wanted to systematically characterize the antigenic domains of the HIV-1 p24 protein. In this study we have generated twelve TrpE/Gag fusion proteins presenting overlapping regions of the p24 protein. Immunological analysis of HIV-seropositive sera using these recombinant proteins has identified at least two and possibly three antigenic domains on the p24 protein.

We constructed eight restriction enzyme deletion clones of the HIV p24 *gag* gene by using the pATH (plasmid amenable to making tryptophan hybrid) bacterial expression plasmid (Fig. 1A and Table 1) (9, 13, 24). These plasmid clones directed the synthesis of TrpE/Gag fusion proteins which contained known regions of the p24 protein. Plasmid constructions were confirmed by DNA sequencing of the 5' vector-insert junction (2, 12) and were transformed into *Escherichia coli* RR1 for recombinant protein synthesis. Plasmid-containing bacteria were grown in pATH medium and induced with β -indoleacrylic acid as described elsewhere (25). Insoluble recombinant proteins were isolated by using a modification of the procedure described by Kleid et al. (11). Bacteria were suspended in 20 ml of 50 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–2 mg of lysozyme per

ml and were left on ice for 1 h. The cells were disrupted by the addition of Nonidet P-40 and NaCl to 0.75% and 0.35 M final concentrations, respectively, and then sonicated. The insoluble material was pelleted at $9,000 \times g$ for 10 min and stored at 4°C in 10 mM Tris hydrochloride (pH 7.5).

TrpE/Gag fusion proteins were analyzed on 0.1% sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) gels and stained with Coomassie blue (Fig. 2A). Because the TrpE protein itself does not purify well in an insoluble form, total cell lysates of induced bacterial cultures carrying the parental pATH plasmid were used as controls on SDS-PAGE gels for staining (Fig. 2A and B, lanes 2; Fig. 2C, lane 1) as well as for electroblotting (Fig. 3A through C, lanes 1). In every case, the TrpE/Gag fusion proteins were synthesized at significant levels, and the amount of fusion protein relative to other bacterial proteins could be increased by the lysis and centrifugation procedure. The molecular mass of the fusion proteins relative to the 38-kilodalton (kDa) TrpE protein was generally what was predicted on the basis of addition of a known number of HIV residues. In addition, proteins from pATH 24-7, -8, -9, and -10 (Fig. 1) migrate at sizes consistent with the presence of extra residues added because of the continuation of the reading frame after the last p24 nucleotide, as determined by DNA sequencing. Therefore, the pATH 24-10 protein (Fig. 2B, lane 5) appears larger than the pATH 24-9 protein (Fig. 2B, lane 6), even though pATH 24-10 encodes fewer p24 amino acids.

To determine whether the HIV p24 determinants presented on our fusion proteins were reactive with serum antibodies, we performed Western blot (immunoblot) analysis as described elsewhere (25) by using the seven restriction enzyme-generated TrpE/Gag fusion proteins. The results (Fig. 3A and C) indicate that antibodies in an HIV-1-seropositive reference serum react with amino-terminal p24 residues present in the pATH 24-1 and pATH 24-2 proteins (Fig. 3A, lanes 2 and 3), but when the residues encoded upstream and downstream of the *Pst*I site (Fig. 1) are presented separately (pATH 24-3 and pATH 24-4), then all antibody reactivity is lost (Fig. 3A, lanes 4 and 5). Reactivity can be recovered in pATH 24-5, which maintains the integ-

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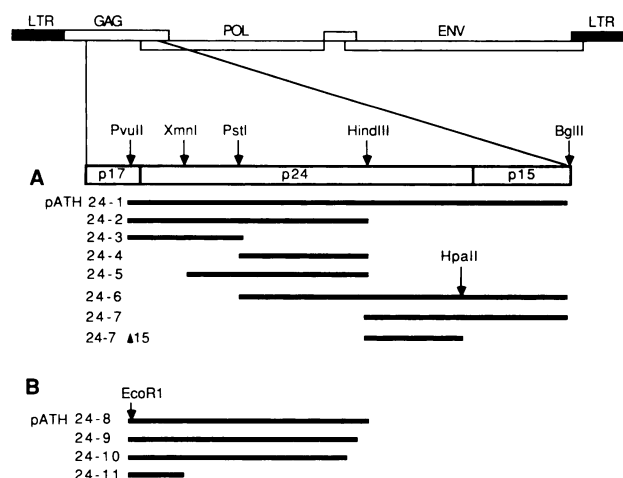


FIG. 1. Schematic representation of HIV-1 proviral genome with the *gag* DNA sequences expanded to indicate the p24 DNA segment which was cloned into the pATH expression vector. The dark lines represent HIV *gag* DNA which was digested with the indicated restriction enzymes prior to insertion into the pATH vector (A) or pATH 24-1 DNA digested with *Hind*III and subsequently digested with exonuclease III (B). Clones generated by use of existing restriction enzyme sites are designated pATH 24-1 through -7Δ15. Clones derived from pATH 24-1 by exonuclease digestion are designated pATH 24-8 through -11. The *Eco*RI site was regenerated by ligation of the p24 *Pvu*II site with the blunt-ended *Eco*RI site of the pATH vector. LTR, Long terminal repeat.

rity of the residues encoded by the *Pst*I site (Fig. 3A, lane 6). This indicates that an amino-terminal epitope exists on p24 and the upstream boundary of the epitope requires residues encoded downstream of the *Xmn*I site (nucleotide 821 [19]).

To better define the downstream boundary of the N-terminal domain, bidirectional exonuclease III deletions were performed (7) on pATH 24-1 DNA which had been digested with *Hind*III. Four clones with increasing deletions upstream from the *Hind*III site were isolated and were designated pATH 24-8, -9, -10, and -11 (Fig. 1B). The fusion proteins from these clones were analyzed by SDS-PAGE (Fig. 2B, lanes 4 through 7) and by Western blot, using our HIV reference serum (Fig. 3B, lanes 3 through 6). Removal of sequences between the *Hind*III site (nucleotide 1260) and nucleotide 1138 (pATH 24-10) did not abolish antibody

TABLE 1. HIV p24 nucleotide numbers in *trpE-gag* constructs^a

Clone pATH 24-	Restriction enzyme sites at termini of inserted p24 DNA	<i>gag</i> nucleotide no.
1	<i>Pvu</i> II- <i>Bgl</i> II	692-1644
2	<i>Pvu</i> II- <i>Hind</i> III	692-1260
3	<i>Pvu</i> II- <i>Pst</i> I	692-963
4	<i>Pst</i> I- <i>Hind</i> III	960-1260
5	<i>Xmn</i> I- <i>Hind</i> III	821-1260
6	<i>Pst</i> I- <i>Bgl</i> II	960-1644
7	<i>Hind</i> III- <i>Bgl</i> II	1256-1644
7Δ15	<i>Hind</i> III- <i>Hpa</i> II	1256-1400
8	<i>Pvu</i> II-X	692-1147
9	<i>Pvu</i> II-X	692-1140
10	<i>Pvu</i> II-X	692-1138
11	<i>Pvu</i> II-X	692-836

^a Nucleotide numbers are as given in reference 19. X, Endpoint generated by exonuclease III digestion. The last p24 nucleotide in each exonuclease III construct is listed in the third column.

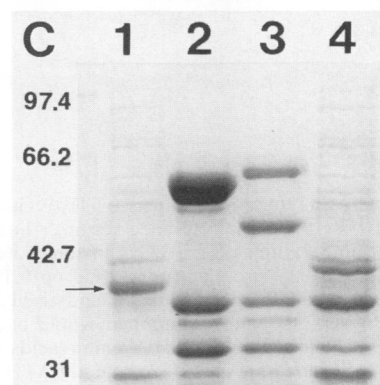
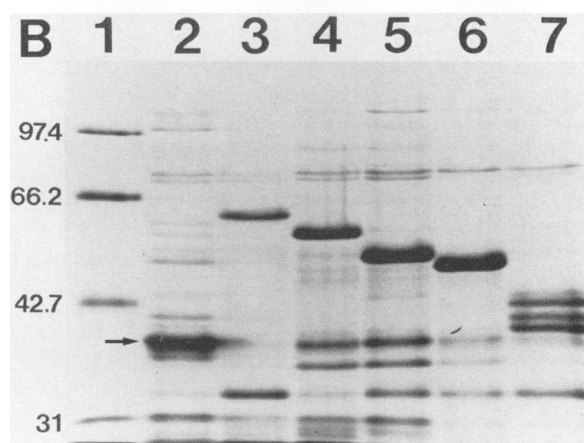
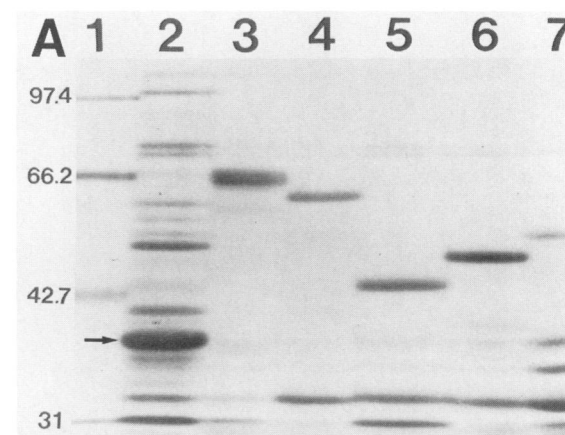


FIG. 2. Coomassie-stained SDS-PAGE gels of TrpE/Gag fusion proteins. Aliquots of fusion proteins recovered from insoluble enrichment preparations were electrophoresed on 0.1% SDS-10% PAGE gels along with whole-bacterial-cell lysate from cells synthesizing the TrpE protein without HIV amino acids (panels A and B, lanes 2; panel C, lane 1). The TrpE protein is indicated by the arrow. Molecular markers (Bio-Rad Laboratories) are shown in lane 1 of each panel, with the mass in kilodaltons of each standard given at the left. (A) Lane 3, pATH 24-1; lane 4, pATH 24-2; lane 5, pATH 24-3; lane 6, pATH 24-4; lane 7, pATH 24-5. (B) Lane 3, pATH 24-2; lane 4, pATH 24-8; lane 5, pATH 24-10; lane 6, pATH 24-9; lane 7, pATH 24-11. (C) Lane 2, pATH 24-6; lane 3, pATH 24-7; lane 4, pATH 24-7Δ15.

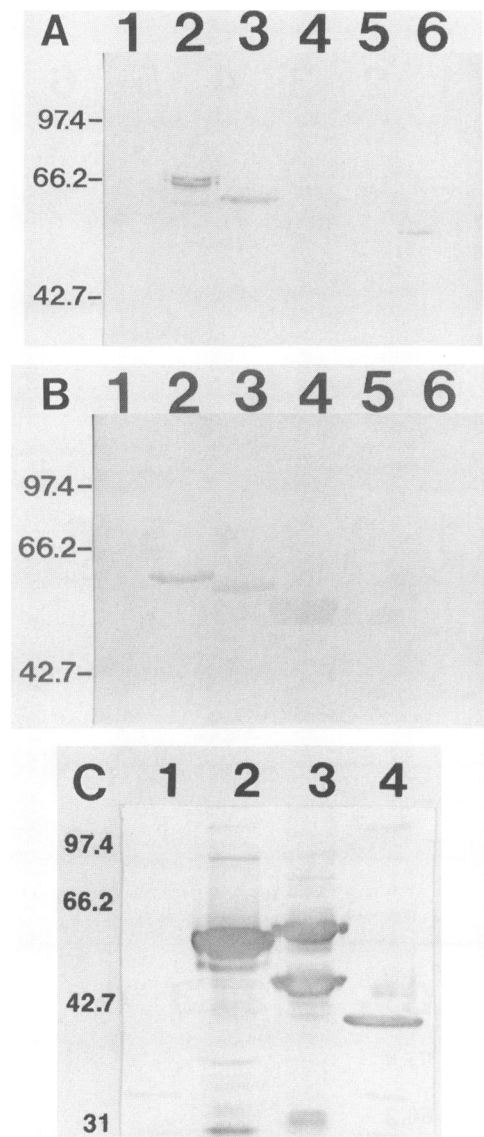


FIG. 3. Western immunoblot analysis of TrpE/Gag fusion proteins with an HIV-seropositive reference serum. Aliquots of insoluble enrichment preparations of TrpE/Gag fusion proteins identical to those used for Fig. 2 were electrophoresed on 0.1% SDS-10% PAGE gels, transferred to nitrocellulose, and used for Western immunoblot analysis. All panels contained whole bacterial lysate containing TrpE protein without any HIV amino acids in lane 1. (A) Lane 2, pATH 24-1; lane 3, pATH 24-2; lane 4, pATH 24-3; lane 5, pATH 24-4; lane 6, pATH 24-5. (B) Lane 2, pATH 24-2; lane 3, pATH 24-8; lane 4, pATH 24-10; lane 5, pATH 24-9; lane 6, pATH 24-11. (C) Lane 2, pATH 24-6; lane 3, pATH 24-7; lane 4, pATH 24-7 Δ 15. Molecular mass in kilodaltons is given at the left for markers (Bio-Rad), which were electroblotted onto the nitrocellulose filter and then Coomassie stained and destained.

reactivity (Fig. 3B, lane 4). However, removal of sequences up to nucleotide 836 (pATH 24-11) completely abolished antibody reactivity (Fig. 3B, lane 6). This analysis indicates that the amino-terminal antigenic domain is composed of, at most, 105 amino acids encoded between *gag* nucleotides 821 and 1138.

The C-terminal region of p24 also appears to contain major antibody-reactive domains. Both pATH 24-6 and -7 fusion

proteins were reactive with serum antibodies in our reference serum (Fig. 3C, lanes 2 through 4). It is not likely that this reactivity is directed toward the amino-terminal domain, because the p24 residues in pATH 24-6 are encoded beginning at the *Pst*I site and also because the residues encoded between the *Pst*I and *Hind*III sites (pATH 24-4) were not reactive when presented by themselves. Thus, it is possible that an epitope which requires amino acids encoded by an intact *Hind*III site is present or that the epitope(s) is completely contained on the region presented on the pATH 24-7 fusion protein. The reference serum used in the Western blot analysis as well as all those used in the immunodot blot analysis was characterized as lacking antibodies to p15 by Western blot when Dupont human T-cell lymphotropic virus type III-inactivated antigen strips were used (data not shown). Therefore it is likely that the reactivity observed in Fig. 3C (lanes 2 and 3) is directed toward at least one C-terminal antigenic domain on the p24 protein downstream of the *Hind*III site (Fig. 1A). To confirm this, clone pATH 25-7 Δ 15 was constructed (Fig. 1A and Table 1). Western blot analysis of this protein (Fig. 3C, lane 4) with our reference serum detected strong reactivity, indicating the presence of an extreme C-terminal domain in p24.

We next wanted to determine whether the putative p24 domains detected by our reference serum are also detected by serum antibodies from other HIV-infected individuals. We therefore screened our TrpE/Gag fusion proteins by using 14 additional sera previously characterized as containing antibodies to the HIV-1 gp41 envelope protein by our immunodot blot assay (25) and, in the case of pATH 24-7 Δ 15, by Western blot. One of the advantages of the immunodot blot is that many different antigens may be tested for antibody reactivity simultaneously with only minimal amounts of patient sera. As a negative control for antibodies against the TrpE or other bacterial proteins, the pATH 24-11 protein was included on each antigen strip. This protein does not contain the p24 epitopes described here and appears negative on Western blot (Fig. 3B, lane 6). The patterns of reactivity to our TrpE/Gag fusion proteins can be divided into four groups based on the immunodot blot results: (i) those that do not have detectable anti-p24 antibodies, (ii) those that have antibodies which recognize the C terminus only, (iii) those which recognize the N terminus only, and (iv) those which recognize both N and C termini. The first group contained three sera which did not contain detectable anti-p24 antibodies. Two of these sera were confirmed p24 antigen positive by Abbott human T-cell lymphotropic virus type III antigen enzyme immunoassay. Therefore, these two sera which were anti-envelope positive, anti-p24 negative, and p24 antigen positive were similar to serum samples from individuals exhibiting clinical symptoms of acquired immune deficiency syndrome (1, 17). A second group of four p24 antigen-negative sera contained antibodies reactive only with the C-terminal domain(s) present on pATH 24-1, -6, and -7. A third group was defined by a single serum which recognized only the amino-terminal (*Pst*I) domain. A fourth group of six p24 antigen-negative sera contained antibodies reactive with both the amino-terminal domain (pATH 24-1, -2, and -5) and the C-terminal domain(s) (pATH 24-1, -6, and -7). Three sera from individuals not infected with HIV were tested by immunodot blot and did not contain detectable antibodies to any TrpE/Gag fusion proteins. No *E. coli*-specific antibodies were detected on the control dots, which indicates that the reactivity observed was directed against the HIV domains present on the fusion proteins and not

against the TrpE protein itself or the contaminating bacterial proteins.

Of the 10 sera that recognized the C-terminal domain(s) presented by pATH 27-6 and -7, 7 have higher titers of antibodies specific for the pATH 24-6 protein than of antibodies for the pATH 24-7 protein, as judged from the intensity of the dot blot results with equivalent amounts of immobilized antigen. This implies that these seven sera contain antibodies reactive against a domain which is composed of residues encoded by sequences which flank the *Hind*III site as well as antibodies to an extreme C-terminal domain formed by residues encoded downstream of the *Hind*III site. Western blot analysis of these seven sera with pATH 24-7 Δ 15 confirmed that all of these sera also contain antibodies to the extreme C-terminal region of p24. Overall, 11 of the 12 sera which contain anti-p24 antibodies (including our reference serum [Fig. 3]) were reactive with the pATH 24-7 Δ 15 protein, which implies that this region may encode an immunodominant domain. Only one serum was found to be reactive with the N-terminal p24 domain exclusively.

It has been reported previously that sera characterized by Western blot as lacking antibodies to p15 were reactive with p24 domains present on a protein analogous to our pATH 24-6 protein (14). Our results are consistent with this finding and show that an immunodominant domain exists at the extreme C terminus of p24 as presented on the pATH 24-7 Δ 15 protein. In addition, a second C-terminal domain, detected by a majority of the sera tested, requires amino acids encoded by the *Hind*III site. A plot of the hydrophobicity of the p24 protein (not shown) is consistent with these results, as it predicts that the regions encoded around the *Pst*I and *Hind*III sites and the C terminus of p24 are strongly hydrophilic and thus may be located on the external surface of the native p24 protein.

Although others have expressed the HIV-1 *gag* gene in *E. coli* (8, 23), our panel of TrpE/Gag fusion proteins is designed to present discrete portions of the intact protein so that the pattern of the antibody response to p24 domains in a given serum sample can be characterized. Judged by the sequence of HIV (19, 21), the *gag* gene products are not glycosylated in vivo. Therefore our recombinant proteins synthesized in bacteria should be similar to the native protein. Indeed, the reactivity of our proteins with patient sera indicates that epitopes similar to those on the native p24 protein are presented by our TrpE/Gag fusion proteins. However, it is not clear to what extent the recombinant peptides are folded back into the native conformation. Our analysis using Western blot and immunodot blot does not necessarily detect conformational epitopes. It is therefore likely that the domains we describe here are linear epitopes of the p24 protein backbone and that other antigenic domains formed by conformational folding of the native protein do exist but may not be detected by our analysis.

Our ability to distinguish different patterns of reactivity to p24 domains indicates that it is possible for individuals to react differently to the domains on the p24 protein. The C-terminal domains appear to be immunodominant; i.e., in all individuals with detectable anti-p24 antibodies, the antibodies recognized this region. In contrast, the amino-terminal domain may or may not be recognized. The nature of the antibody response to p24 may be influenced by the nature of the strain of HIV that an individual is infected with as well as the clinical state of the individual. Serological studies using clinically well characterized serum panels will be helpful in further clarifying the response to p24.

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