COMPARISON OF THE BIOLOGICAL ACTIVITIES OF HUMAN IMMUNODEFICIENCY VIRUS 1 P24 AND GP41 EXPRESSED IN *SPODOPTERA FRUGIPERDA* CELLS BY USE OF BAC-TO-BAC SYSTEM

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Summary. – Recombinant transposing plasmids pFH24 and pFH41 were constructed by cloning the human immunodeficiency virus 1 (HIV-1) p24 and gp41 genes, respectively, into the transposing vector pFastBacHTa. Recombinant bacmids rBH24 and rBH41 were obtained by transposing pPolh/p24 and pPolh/gp41 expression cassettes from recombinant plasmids pFH24 and pFH41, respectively. Recombinant viruses rAcH24 and rAcH41 were generated by transfection of the *Spodoptera frugiperda* (Sf9) cells with the DNAs of plasmids rBH24 and rBH41, respectively. Analysis of the expressed p24 or gp41 proteins with an antiserum to HIV-1 (HIV-1 antiserum) by an enzyme-linked immunosorbent assay (ELISA) and dot blot assay showed high biological activity of these proteins; p24 was more active than gp41. Also a Western blot analysis showed stronger bands for p24 than for gp41. The high reactivities of p24 and gp41 with the HIV-1 antiserum suggest that these proteins could also be used as specific standard antigens in HIV-1 diagnostics.

Key words: HIV-1; p24; gp41 genes; proteins; bacmid, baculovirus; Sf9 cells

Introduction

HIV, the causative agent of acquired immunodeficiency syndrome (AIDS), contains p24, gp41, and gp120 proteins. p24 is the major core antigen produced by proteolytic cleavage of the precussor protein p55. The transmembrane envelope glycoprotein gp41 and the extracellular envelope glycoprotein gp120 are produced by the proteolytic cleavage of a precussor protein, gp160. p24 protects the two identical genomic RNA molecules and all the enzymes (reverse transcriptase, protease, and integrase) responsible for virus replication. gp41 helps in the fusion of the virus with cell membrane after binding to the cluster determinant 4 (CD4) molecule (Dalgleish *et al.*, 1984). gp41 also plays a role in the antibody neutralization (Klatzman *et al.*, 1984). gp41 secreted during the course of HIV infection is toxic to HIV-infected cells, thereby causing cell death (Gallaher, 1987). gp120 plays an important role in the first step of virus infection, because it serves as a high-affinity ligand for the T-cell receptor CD4 (Dalgleish *et al.*, 1988). The p24, gp41, and gp120 antigens are important for HIV diagnosis because most people who are HIV-seropositive have antibodies to them.

The baculovirus vector expression system has been used to express a wide variety of genes of viruses, fungi, plants, and animals in large quantities in insect cells under the control of a strong polyhedrin gene promoter (pPolh) of the Autographa californica nucleopolyhedrosis virus (AcNPV) (Luckow and Summers, 1988). Most of the recombinant proteins produced in this system have been shown to be

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Abbreviations: AcNPV = Autographa californica nucleopolyhedrosis virus; AIDS = acquired immunodeficiency syndrome; CPE = cytopathic effect; ELISA = enzyme-linked immunosorbent assay; FBS = fetal bovine serum; HIV-1 = human immunodeficiency virus 1; HIV-1 antiserum = antiserum to HIV-1; MCS = multiple cloning site; Ni-NTA = nitrilo-tri-acetic acid; PBS = phosphate-buffered saline; p.i. = post infection; pPolh = plyhedrin gene promoter; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TEM = transmission electron microscope

immunologically, antigenically, and functionally similar to their authentic counterparts, because the insect cells are able to carry out many types of posttranslational modification (Luckow and Summers,1988).

The Bac-to-Bac system has been developed for a rapid and efficient generation of recombinant baculoviruses (AcNPV, Luckow *et al.*, 1993). It is based on a site-specific transposition (transposon Tn7) of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *Escherichia coli* cells. Recombinant bacmids are constructed by transposing a mini-att Tn7 element from the donor plasmid pFastBacHTa to a mini-att Tn7 attachment site on the bacmid when the Tn7 transposition functions are provided in trans by a helper plasmid. Recombinant bacmid DNA can be rapidly isolated from small scale cultures and then used to transfect insect cells. Virus stocks (>10^7 PFU/ml) harvested from the transfected cells can then be used to infect fresh insect cells for protein expression, purification, and functional analysis.

The aim of this work was (1) to express the HIV-1 p24 and gp41 genes in Sf9 cells by use of the Bac-to-Bac expression system and to compare the reactivity of these proteins with a HIV-1 antiserum in ELISA and dot blot assay, and (2) a large scale production of the expressed proteins for use as specific standard antigens for HIV-1 diagnostics by ELISA, dot blot assay and Western blot analysis.

Materials and Methods

Plasmids, bacterial strains, cells, and reagents. The plasmids pHIV24 and pHIV41 containing p24 and gp41 genes of HIV-1, respectively were provided by Dr. S. Zhonghe, Academy of Military Medical Sciences, Beijing, P.R. China. The Grace's insect cell culture medium, fetal bovine serum (FBS), the Cellfectin Reagent, the plasmid pFastBacHTa containing a stretch of 6 histidines, the *E. coli* DH10Bac cells containing bacmid, and the helper plasmid were purchased from Gibco-BRL. The nitrilo-tri-acetic acid (Ni-NTA) resin was obtained from Qiagen. Restriction endonucleases and the T4 DNA ligase were obtained from Promega. X-gal, IPTG, and all antibiotics used in this work were obtained from Hua-Mei Co., P.R. China. The Sf9 cell line was obtained from the China Center for Type Culture Collection (CCTCC), Beijing, P.R. China. Monolayer cultures of Sf9 cells infected with the recombinant viruses were maintained in the Grace's insect cell culture medium supplemented with 10% of FBS. The *E. coli* TG1 and DH5a bacterial strains originated from our laboratory.

Cloning into the transposing vector pFastBacHTa. DNA fragments containing HIV-1 p24 (0.649 kb) and gp41 (1.145 kb) genes were cleaved out from the plasmids pHIV24 and pHIV41 with *Bam*H1+*Eco*R1 and *Eco*R1+*Sal*1, respectively, and recovered from the gels using the DE-81 filter. The transposing vector pFastBacHTa containing the pPolh of AcNPV, the multiple cloning site (MCS) extending from *Ehe*I (nt 4119) to *Hind*III (nt 4218) sites, the 6 His motif, and a SV40 poly(A) signal was digested with *Bam*HI+*Eco*RI, *Eco*RI+*Sal*I, and purified. The purified fragments containing p24 and gp41 genes, respectively were ligated to the purified transposing vector pFastBacHTa by T4 DNA ligase. The ligation mixtures were transfected into *E. coli* TG1 cells by the calcium chloride method. The transfected cells were spread evenly over the surface of a Luria-Bertanii (LB) agar plate containing 100 mg/ml ampicillin and 7 μg/ml gentamicin and incubated at 37°C overnight for selection of pFastBacHTa recombinants.

Transposition between recombinant pFastBacHTa and bacmid. DNAs of pFastBacHTa recombinants carrying p24 or gp41 genes of HIV-1 were prepared from the overnight cultures and transfected into the *E. coli* DH10Bac competent cells containing bacmid with a mini-att Tn7 attachment site and the helper plasmid. Transformation mixtures were shaken at 37°C for 4 hrs and diluted 10-fold (to 10^{-1}–10^{-3}) with LB medium. An aliquot (100 ml) of each dilution was spread evenly over the surface of LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 80 μg/ml X-gal, and 10 μg/ml IPTG. The plates were incubated at 37°C for 1–2 days for selection of recombinant bacmids.

Transfection of Sf9 cells with recombinant bacmid DNAs and purification of recombinant viruses. The recombinant bacmid DNAs containing p24 and gp41 genes, respectively, were prepared from overnight cultures of selected *E. coli* colonies and were used for transfection of Sf9 cells. For each transfection, 9 x 10^5 cells in 2 ml of the Grace's medium with 2% FBS were seeded per 35-mm well (of a 6-well plate) in 2 ml of Grace's medium supplemented with 10% of FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and were allowed to attach for 1 hr at 27°C. For each sample, 5 μl of DNA diluted in 100 ml of a serum-free medium was gently mixed with 6 ml of the Cellfectin reagent diluted in 100 ml of the serum-free medium. This mixture was incubated at room temperature for 45 mins to form lipid-DNA complexes. The mixture was then carefully poured onto cells and incubated at 27°C. After 5 hrs, the medium was removed and replaced with 2 ml of fresh medium supplemented with 10% of FBS. After 5–7 days at 27°C, the culture medium was harvested, clarified at 5,000 rpm for 5 mins, and then used to infect fresh cells at 27°C for expression of target proteins.

Recombinant virus particles containing HIV-1 p24 and gp41 genes, respectively, were purified from the culture medium after clarification by high speed pelleting (20,000 rpm for 1 hr), resuspended in phosphate-buffered saline pH 7.4 (PBS) and then observed under the transmission electron microscope (TEM).

p24 and gp41 expression and purification. To get good yields of the expressed target proteins, 0.1 ml of the culture supernatant (containing p24 or gp41 proteins) harvested from the transfected cells was diluted in 0.9 ml of fresh medium supplemented with 10% of FBS and then used to infect fresh cells at 27°C or at room temperature. After 1 hr when most cells were already infected with the recombinant virus, 2 ml of a fresh medium supplemented with 10% of FBS was added and the culture was incubated at 27°C. After 48, 72, and 96 hrs post infection (p.i.), the cells were harvested and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) for the expressed target proteins (Sambrook *et al.*,1989).

High yields and homogeneity (over 95%) of p24 and gp41 were obtained by affinity chromatography using the Ni-NTA resin

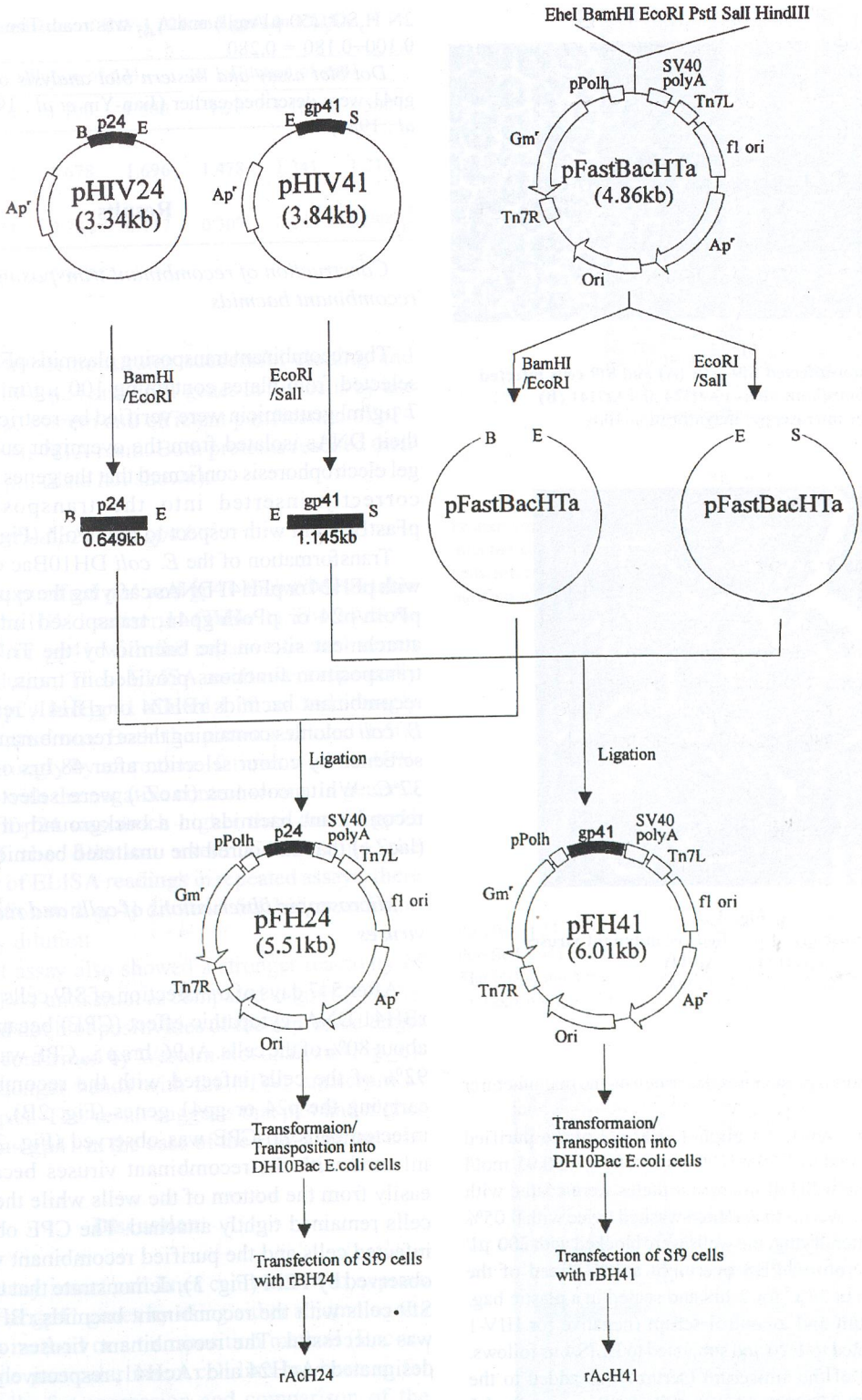

Fig. 1

Scheme of construction of recombinant transposing plasmids pFH24 and pFH41, recombinant bacmids rBH24 and rBH41, and recombinant viruses rAcH24 and rAcH41

B = *Bam*HI; E = *Eco*RI; S = *Sal*I; H = *Hind*III; Tn7R and Tn7L = transposons turned to right and left, respectively; Ap^r and Gm^r = ampicillin and gentamicin resistance gene markers, respectively; pFH24 and pFH41, rBH24 and rBH41, and rAcH24 and rAcH41 = recombinant transposing plasmids, recombinant bacmids, and recombinant viruses carrying p24 and gp41 genes, respectively.

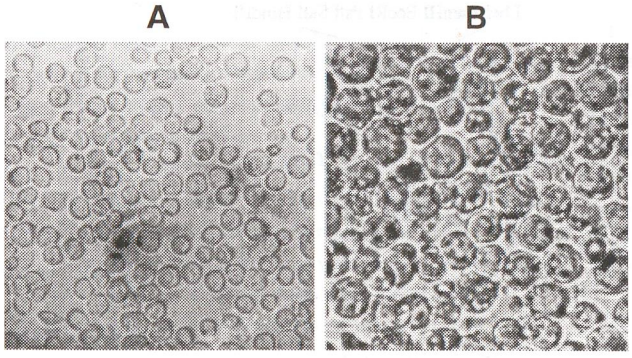

Fig. 2
Microscopy of non-infected Sf9 cells (A) and Sf9 cells infected with recombinant virus rAcH24 or rAcH41 (B)
Light microscopy; magnification 100x.

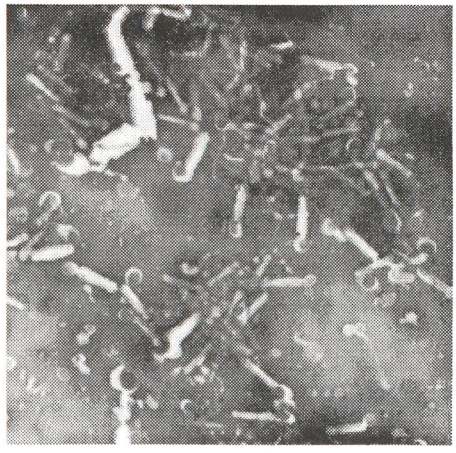

Fig. 3
Electron microscopy of purified recombinant viruses rAcH24 or rAcH41
Magnification 54,000x.

according to the standard procedures described by the manufacturer (Qiagen).

ELISA of p24 and gp41. An aliquot (100 µl) of the purified p24 or gp41 was diluted to 1:20, 1:100, and 1:500 with 0.05 mol/l $NaHCO_3$ pH 9.5. The wells of microtiter plates were coated with these dilutions at 4°C overnight and then washed twice with 0.05% Tween-20 in PBS. After drying, the wells were blocked with 200 µl/well of 20% calf serum in PBS overnight at 4°C, freed of the solution, dried again at 37°C for 2 hrs and sealed in a plastic bag.

A HIV-1 antiserum and a control serum (negative for HIV-1 antibodies) were diluted to 1:20 and subjected to ELISA as follows. An aliquot (100 µl) of the antiserum (serum) was added to the well and incubated at 37°C for 30 mins. The well was washed 5 times with the washing buffer and dried. An enzyme conjugate in a detection buffer (100 µl/well) was added and incubated at 37°C for 20 mins. The well was washed 5 times and dried. An enzyme substrate in a detection buffer (100 µl/well) was added and incubated at 37°C for 10 mins. The reaction was then stopped with 2N H_2SO_4 (50 µl/well) and A_{492} was read. The cut-off value was 0.100+0.180 = 0.280.

Dot blot assay and Western blot analysis of HIV-1 p24 and gp41 were described earlier (Jian-Yin *et al.*, 1989; Sambrook *et al.*, 1989).

Results

Construction of recombinant transposing plasmids and recombinant bacmids

The recombinant transposing plasmids pFH24 and pFH41 selected from plates containing 100 µg/ml ampicillin and 7 µg/ml gentamicin were verified by restriction analysis of their DNAs isolated from the overnight cultures. Agarose gel electrophoresis confirmed that the genes of interest were correctly inserted into the transposing plasmids pFastBacHTa with respect to the pPolh (Fig. 1).

Transformation of the *E. coli* DH10Bac competent cells with pFH24 or pFH41 DNAs carrying the expression cassette pPolh/p24 or pPolh/gp41, transposed into the mini-att attachment site on the bacmid by the Tn7/R and Tn7/L transposition functions provided in trans, resulted in the recombinant bacmids rBH24 or rBH41, respectively. The *E. coli* colonies containing these recombinant bacmids were screened by colour selection after 48 hrs of incubation at 37°C. White colonies (lacZ-) were selected as positive recombinant bacmids on a background of blue colonies (lacZ+) that harboured the unaltered bacmid.

Microscopic observations of cells and recombinant viruses

After 5–7 days of transfection of Sf9 cells with rBH24 or rBH41 DNA, cytopathic effect (CPE) became apparent in about 80% of the cells. At 96 hrs p.i., CPE was seen in about 92% of the cells infected with the recombinant viruses carrying the p24 or gp41 genes (Fig. 2B), while in non-infected cells no CPE was observed (Fig. 2A). The cells infected with the recombinant viruses became detached easily from the bottom of the wells while the non-infected cells remained tightly attached. The CPE observed in the infected cells and the purified recombinant virus particles observed by TEM (Fig. 3), demonstrate that transfection of Sf9 cells with the recombinant bacmids rBH24 or rBH41 was successful. The recombinant viruses obtained were designated rAcH24 and rAcH41, respectively.

Expression of p24 and gp41

SDS-PAGE showed that p24 or gp41 were expressed in Sf9 cells with Mr of 24,000 and 41,000 (Fig. 4) due to posttranslational modifications (Luckow and Summers,

Table 1. Reactivities of HIV-1 p24 and gp41 in ELISA

Serum	Dilution of p24			Dilution of gp41		
	1:20	1:100	1:500	1:20	1:100	1:500
HIV-1 antiserum	1.832	1.678	1.696	1.478	1.341	1.217
Negative control serum	0.331	0.284	0.117	0.307	0.244	0.094

The values represent A_{492} readings.

1988). This result demonstrates successful cloning and expression of HIV-1 p24 and gp41 genes in Sf9 cells by use of the Bac-to-Bac system and efficient purification of p24 and gp41 by the Ni-NTA resin. Both proteins reached their peaks at 80 hrs p.i. (data not shown).

ELISA of purified p24 and gp41

ELISA of the purified p24 and gp41 showed detectable reactions with the HIV-1 antiserum (Table 1). There was no reaction of p24 or gp41 with the negative control serum even without dilution. These ELISA results were repeatedly identical. Both p24 and gp41 diluted 1:20 reacted strongly with the HIV-antiserum. Both the proteins diluted 1:500 reacted more strongly by more than 10 times with the HIV-antiserum than with the negative control serum. In general, the reactivity of p24 was much higher than that of gp41 regardless of the dilution used. Concerning the reproducibility of ELISA readings in repeated assays, there was slight variation with gp41 diluted 1:500 but no variation with p24 at any dilution.

The dot blot assay also showed a stronger reactivity of p24 with the HIV-1 antiserum as compared to gp41 (Fig. 5). The ELISA and dot blot positivities of the expressed target products were confirmed by Western blot analysis (Fig. 6). p24 showed stronger bands with the HIV-1 antiserum as compared to gp41. This result suggests that p24 had higher antigenicity than gp41 in the case of the HIV-1 antiserum.

Discussion

Although many insect cell lines can be used to express the HIV-1 p24 or gp41 genes by using either a homologous recombination method or a transposition method (Bac-to-Bac system), no particular insect cell line has been so far used specifically for expression and comparison of the biological activities of these proteins in a HIV-1-positive serum by any diagnostic method for HIV-1. Here we report that by using the Bac-to-Bac system we have expressed the HIV-1 p24 and gp41 genes in Sf9 cells and compared the reactivities of the expressed products with the HIV-1-positive

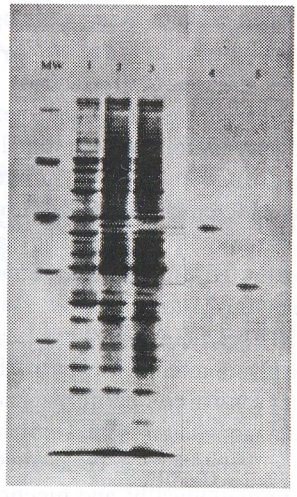

Fig. 4
SDS-PAGE
Protein size marker (97 K, 66 K, 43 K, 31 K, and 14 K, lane MW); non-infected cells (lane 1); cells infected with rAcH24, 80 hrs p.i. (lane 2); cells infected with rAcH41, 80 hrs p.i. (lane 3); purified gp41 (lane 4); purified p24 (lane 5).

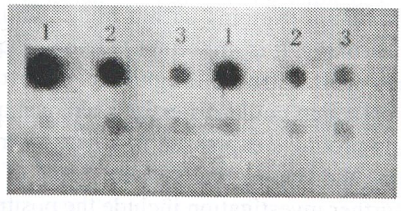

Fig. 5
Dot blot analysis
Purified p24 diluted 1:20, 1:100, and 1:500 (left vertical rows 1–3). Purified gp41 diluted 1:20, 1:100, and 1:500 (right vertical rows 1–3). The HIV-1 antiserum (top horizontal row) and the negative control serum (bottom horizontal row).

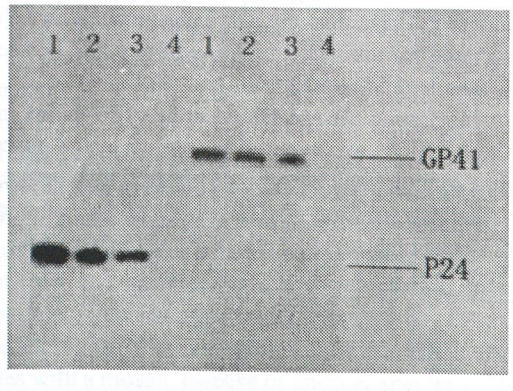

Fig. 6
Western blot analysis
Purified p24 diluted 1:20, 1:100, 1:500, and 1:20 (left lanes 1–4). Purified gp41 diluted 1:20, 1:100, 1:500, and 1:20 (right lanes 1–4). The HIV-1 antiserum (lanes 1–3) and the negative control serum (lanes 4).

serum by ELISA and dot blot assay. Although we expected that gp41 (a transmembrane envelope glycoprotein) is a stronger antigen than p24, it was not the case. The comparative analysis showed that the p24 expressed in this cell line reacted more strongly with the HIV-1 antiserum than gp41. We do not know exactly what caused this difference in the antigenicity of these proteins. The core protein may be genetically more stable than the envelope glycoproteins or the HIV-1 antiserum contained a higher titer of antibodies to p24 than to gp41. But, in general, in every or most HIV-positive sera there is always a higher level of envelope glycoproteins than that of the core proteins. Most of the recombinant proteins produced in the baculovirus vector expression system either by homologous recombination or transposition (Bac-to-Bac system) are antigenically, immunologically, and functionally similar to their authentic counterparts, because the insect cells are able to carry out many types of posttranslational modifications (Luckow and Summers, 1988). We found that p24 and gp41 of HIV-1 which are of diagnostic importance differred in their antigenicity when expressed in Sf9 cell line by the Bac-to-Bac system. It is possible that posttranslational modifications carried out in some insect cell lines may favour some recombinant proteins with a higher antigenicity as compared to others. It is important to know whether two or more genes which are of diagnostic importance for a particular pathogen could differ in biological activities of their protein products expressed in a particular insect cell line by using the Bac-to-Bac system. Some of the factors that need further investigation include the posttranslational modifications of the expressed products carried out by some insect cell lines and the level of the antibodies present in the serum.

The high expression of these proteins observed in this study suggests that Sf9 cells could be used for their mass production by use of the Bac-to-Bac system. Although the biological activities of the proteins expressed in this system were different, these proteins were antigenically similar to their authentic counterparts and therefore could be used as specific standard antigens for diagnosis of HIV-1 by ELISA or a dot blot assay. As we tested only one type of insect cells, the Sf9 line, we do not know whether the observed difference in biological activity of p24 and gp41 expressed by use of the Bac-to-Bac system would appear also in other types of insect cells.

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