PROTOCOL

Expression of Human Immunodeficiency Virus Genes Using Baculovirus Expression System

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Abstract

The structural protein genes of HIV-1 and HIV-2 have been expressed in *Spodoptera frugiperda* (SF) cells using baculovirus expression system. The noncoding flanking sequences of HIV structural genes were removed and a putative ribosome binding site was placed in front of the open reading frame of each gene by using crossover linker mutagenesis. The coding sequences of the *gag*, *pol*, *env*, and *vif* proteins were inserted into *Autographa californica* nuclear polyhedrosis virus (AcNPV) so that HIV genes were under the control of the AcNPV polyhedrin promoter. All recombinant AcNPV-infected SF cells express high levels of HIV structural proteins. Detailed strategies of recombinant AcNPV construction for high level protein expression are presented.

Index Entries: Human immunodeficiency virus; viral proteins; AIDs; gene expression; baculovirus expression system; vaccines.

1. Introduction

The acquired immune deficiency syndrome (AIDS) is caused by human immunodeficiency virus type 1 or 2 (HIV-1 or HIV-2). The disease is characterized by a high susceptibility to opportunistic infections or to malignant diseases, such as Kaposi's sarcoma. The major immunologic abnormality is the selective depletion of $CD4^+$ T-cells together with decreased capacity to secrete or respond to lymphokines, such as interleukin 2. During infection, HIV induces both a humoral and cell-mediated immune response to its viral components. However, the response cannot cope with progressive infection. An antibody response to the virion surface proteins, *env* (gp120, gp41), and the core protein, *gag* (p24, p17), is observed in most instances 4–8 wk after infection with the virus. The antibody response clears the virus from the circulation, but does not eliminate cells that become latently infected. As infected individuals progress from the asymptomatic state to produce AIDS-related complex (ARC) and subsequently to full-blown AIDS, anti-p24 titers are reduced and the viral antigen appears in the circulation of patients. The significance of this reduction in p24 antibody for disease progression is presently not known. High levels of antibodies to *env* gene products are also found in HIV-1 positive patients, but no real relationship has been noted between their titers or their neutralizing capacities and progression to AIDS *(1,2)*. Therefore, vaccination of individuals with *env* protein alone may be successful in eliciting an antibody response, but may not be good enough to control the disease process. We have also discovered that the anti-*vif* response is only observed in sera of asymptomatic patients *(3)*. The antibodies in patients with ARC and AIDS have no detectable anti-*vif* activity, as measured by Western blot. A number of laboratories speculate that the loss of anti-*vif* antibody correlates with disease progression.

A cell-mediated immune response to the various viral antigens also appears during HIV-1 infection. Two cell-mediated immune mechanisms are thought to play important roles in the clearance of virus infected cells, antibody-dependent cellular cytotoxicity (ADCC), and cytotoxic

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T-lymphocyte (CTL) responses, which are part of the cell-mediated immune response. In viral infections, ADCC seems to be responsible for the lysis of infected cells that have bound antiviral antibody directed against gp120/gp160 expressed on the cell surface *(4)*. In another study, it was found that antibody reactivity with p24 nucleoprotein of the virus correlated better with ADCC activity than reactivity to gp120/160 *(5)*, suggesting that antibody to this internal protein might also play a role in ADCC. Patients with AIDS have lower ADCC activity than sera from HIV positive healthy individuals *(5–8)* whereas others have found no significant reduction in ADCC activity of effector cells isolated from AIDS patients *(9)*.

The cytotoxic T-cell response to both envelope and internal proteins of viruses, in general, have been shown to be important in recovery from disease and possibly in elimination of virus from the host *(10,11)* Walker et al. *(12,13)* demonstrated that cytotoxic T-cells to *env*, *gag*, and *pol* can be detected in AIDS patients, but how those responses relate to disease has not been elucidated. This information is very important in understanding the pathogenesis of the disease and can serve to help in vaccine development.

The goal of my group is to develop an AIDS vaccine through the introduction of HIV-specific structural proteins into the body in order to induce both humoral and cell-mediated immune responses. Our approach is to produce a vaccine containing viral surface proteins, internal structural proteins, and proteins involved in transcription and replication of HIV. Majority of candidate vaccines, already licensed for human clinical trial by the US Food and Drug Administration, contain an HIV surface glycoprotein, gp120, or its precursor gp160, which may produce neutralizing antibodies against HIV. However, it is not clear if such a vaccine will work because some apparently healthy, yet HIV-infected individuals who already have detectable levels of antibodies against surface proteins still develop AIDS. We will assess the properties of these proteins in terms of being superantigens, since this class of antigen can causes depletion of specific T-cell subsets *(14)*. In the subsequent work, we have expressed high levels of the *gag* precursor, envelope (*env*), polymerase (*pol*), and *vif* proteins from both HIV-1 and HIV-2 in *Spodoptera frugiperda* (Sf9) cells using recombinant *Autographa claifornica* nuclear polyhedrosis virus.

2. Materials

1. Sf9 insect cells can be obtained form the American Type Culture Collection (Rockville, MD).
2. TC100 medium or Grace's supplemented medium can be obtained from Gibco Life Technologies (Grand Island, NY).
3. The pAcYM1 vector came from David Bishop (NERC Inst. of Virology, Oxford, UK) *(15)*.
4. SeaPlaque agarose was purchased from FMC Bioproducts (Rockland, ME).
5. Pooled HIV-positive human antisera were obtained from the National Institutes of Allergy and Infectious Diseases, AIDS Research and Reference Reagent Program (Bethesda, MD).
6. [^{35}S]-Methionine can be purchased from Amersham (Arlington Heights, IL).
7. The plasmid p1BM containing *gag-pol* came from R. C. Gallo (NIH, Bethesda, MD).
8. Plasmid HXB-2D containing the entire HIV-1 genome *(16)* was obtained from R. C. Gallo (NIH, Bethesda, MD).
9. Acrylamide, *bis*-acrylamide, sodium dodecyl sulfate (SDS), TEMED, and TRIS base, were purchased from Bio-Rad (Hercules, CA).

3. Methods

The structural protein genes of HIV-1 and HIV-2 have been expressed in large quantity in Sf9 cells using a baculovirus expression system. In the baculovirus expression system, the polyhedrin gene of the baculovirus is replaced with a gene from HIV to make a recombinant baculovirus that in infected cells directs production of desired proteins. As a result, infected Sf9 cells produce large quantities of group-specific antigens, *gag*, reverse transcriptase, *pol*, envelope glycoprotein, gp120, and *vif* protein.

3.1. Expression of gag Protein

The construct designed to express the HIV-2 *gag* precursor proteins is shown in **Fig. 1**. The transfer vector pAcYM1-gag was constructed by using crossover linker mutagenesis in order to achieve high-level expression of the HIV-2 *gag* protein precursor. The crossover linker mutagenesis is a convenient method *(17,18)* to delete noncoding

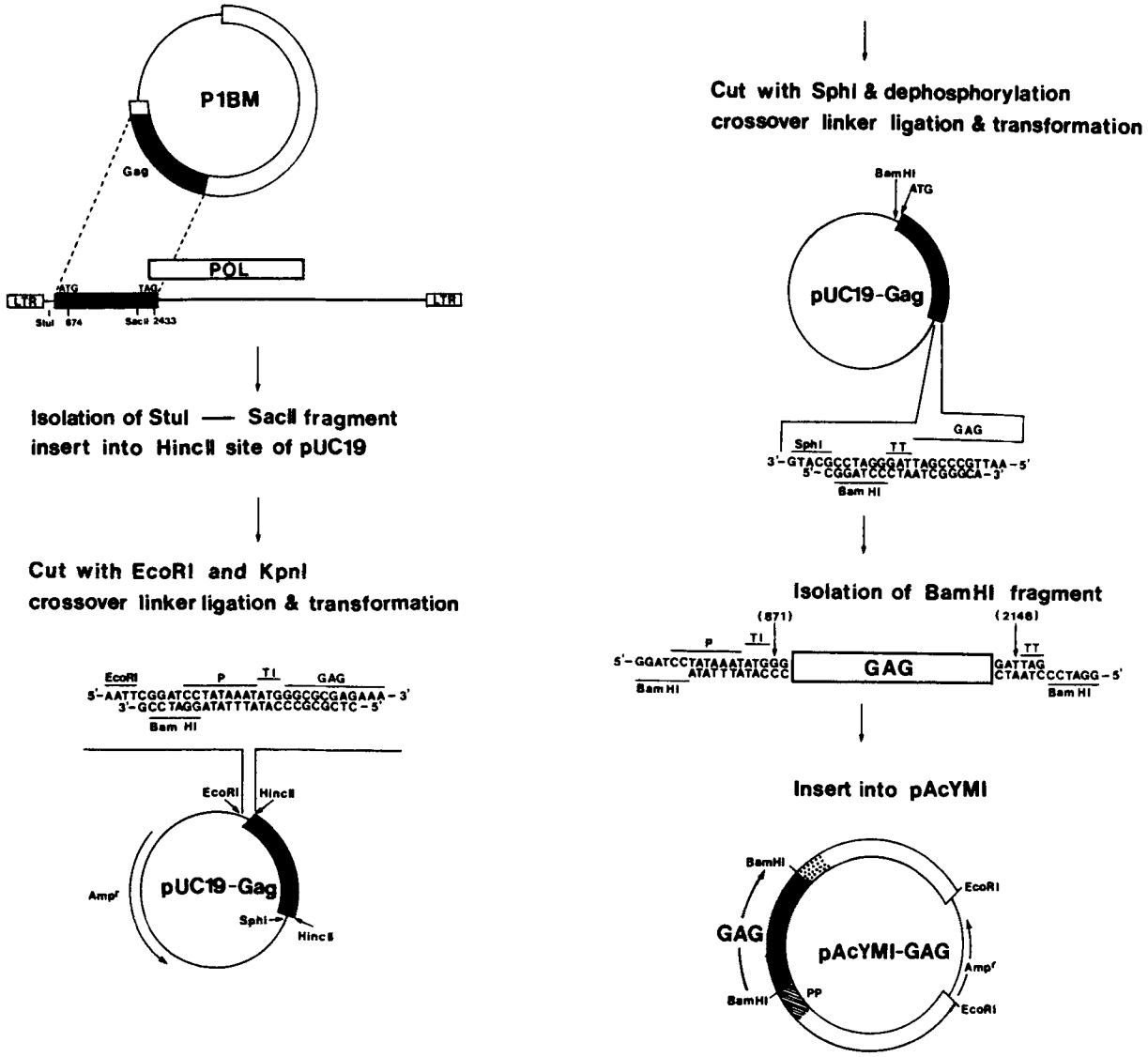


Fig. 1. Construction of recombinant baculovirus containing the HIV-2 *gag* gene. The *gag* gene was isolated from p1BM ***(19)***, which contains the entire HIV-2_{NIH-Z} sequence, by *Stu*I and *Sac*II digestion. A 1.6-kb *Stu*I–*Sac*II fragment of the p1BM genome was purified and subcloned into the *Hin*cII site of pUC19. pUC19-*gag* was digested with *Eco*RI and *Kpn*I, and ligated with a synthetic oligonucleotide crossover linker containing an *Eco*RI sticky end, a *Bam*HI site, a putative Sf9 ribosome binding site (P), and translation initiation codon ATG (TI), followed by 11 additional nucleotides from the coding sequence representing the N-terminus of the *gag* protein. pUC19-*gag* was further modified to delete the *gag-pol* overlapping sequences and to add translation termination codon (TT) and a *Bam*HI site. The pUC19-*gag* was digested with *Sph*I and ligated with another crossover linker containing the translation termination codon (TAG) (TT), which deletes 93 amino acids at the C terminus of the *gag* protein, and introduces a *Bam*HI site and an *Sph*I sticky end. The *Bam*HI fragment was inserted into the *Bam*HI site of the transfer vector pAcYM1 ***(15)***. The transfer vector pAcYM1-GAG was used to cotransfect Sf9 cells with wild-type AcNPV DNA and recombinant baculovirus, AcNPV-HIV-2*gag*, was isolated. Modified from Luo et al. ***(20)***.

flanking sequences and to add essential sequences, such as the putative ribosome binding site. Recombinant baculovirus-expressing *gag* has been isolated, and the protein has been shown to produce 100-nm particles at the surface of infected insect cells that are secreted into the culture media.

1. The plasmid p1BM containing the *gag-pol* genes of HIV-2 was cut with *Stu*I and *Sac*II, and a DNA fragment containing *gag* and a small portion of *pol* at the 3' terminus were ligated into the *Hin*cII site of pUC 19 (**Fig. 1**). pUC19-*gag* was cut with *Eco*RI and *Kpn*I and ligated with the 5' oligonucleotide containing *Eco*RI/*Bam*HI/ Sf9 cell ribosome binding site/initiation codon/ and a short piece of the adjacent *gag* sequence. The duplicated *gag* sequence forms a loop structure that is removed by crossover repair following transformation into *E. coli*. The modified pUC19-*gag* plasmid containing the new ATG was subsequently modified at the 3' end of the gene in a similar manner using an oligonucleotide containing *Sph*I/*Bam*HI/termination codon TAG/a short piece of adjacent 3' *gag* sequence. In this way, the 5' terminus of the *pol* gene was deleted. The modified *gag* gene was cut from pUC19 as a *Bam*HI fragment and ligated into the *Bam*HI cloning site of the baculovirus expression vector, pAcYM1. This construct contains a 1278-bp open reading frame of the *gag* gene which can code for 425 amino acids.
2. Cotransfection of pAcYM1-*gag* and wild-type AcNPV viral DNA is performed with methods described in *Methods in Molecular Biology*, Volume 39, Chapter 6. Occlusion body-negative plaques were isolated by visual screening.
3. Sf9 cells are inoculated with the recombinant baculovirus (AcNPV-HIV-2 *gag*) and the infection was allowed to proceed for 1, 2, 3, and 4 d.
4. Sf9 cells (10^5) are harvested at the various times, lysed with SDS sample buffer, subjected to electrophoresis in 12.5% acrylamide gels containing SDS, and stained with Coomassie blue.
5. Lysate proteins can also be resolved by SDS-polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose, and detected with Western blots using pooled antisera from AIDS patients.
6. A band migrating at 41 kDa is seen in the extracts of Sf9 cells infected with the recombinant virus; this band is not seen in extracts from wild-type AcNPV or mock-infected cells.
7. The time-course experiment reveals that *gag* pr41 does not accumulate in infected cells. Electron microscopy also demonstrates that *gag* particles (100 nm diameter) budded from the surface of infected insect cells *(20)*. Sf9 cells are infected with recombinant virus at a multiplicity of infection of 5 PFU/cell and incubated at 27°C for 72 h. The cell culture supernatant is collected after centrifugation at 1000*g* for 20 min.
8. Particles in the clarified culture supernatant are collected by ultracentrifugation at 100,000*g* for 1.5 h, and the pellets are resuspended in PBS containing 0.1% Tween-20 and 10 µg/mL aprotinin at 4°C overnight. The suspension is subjected to SDS-polyacrylamide electrophoresis, and gels are either stained with Coomassie blue or analyzed with Western blots using antisera from AIDS patients (**Fig. 2**). The *gag* precursor

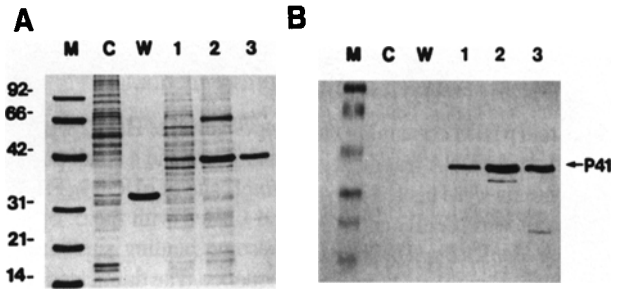


Fig. 2. *gag* Protein expression in recombinant AcNPV-HIV-2 *gag* virus-infected Sf9 cells and the detection of extracellular *gag* particles in cultured supernatant. The lysate of AcNPV-HIV-2 *gag* virus-infected Sf9 cells from 3 d postinfection was subjected to SDS-PAGE in 12.5% polyacrylamide gels, and proteins were stained with Coomassie blue. Sf9 cells were infected with recombinant baculoviruses at an multiplicity of infection (MOI) of 5 PFU/cell and incubated at 27°C for 72 h. The cell culture supernatant was collected after centrifugation at 1000*g* for 20 min. Secreted particles in the culture supernatant were collected by ultracentrifugation at 100,000*g* for 1.5 h, resuspended in PBS containing 0.1% Tween-20, 10 µg/mL aprotinin, and left at 4°C overnight. Coomassie blue-stained SDS-PAGE of intracellular and extracellular *gag* protein is shown in panel A. Panel B shows Western blot analysis of the *gag* protein with pooled serum from AIDS patients. *gag* Particles were isolated from a 20–60% sucrose gradient after treatment with 0.1% Tween-20 and 10 µg/mL aprotinin at 4°C overnight. M, marker protein; C, cell control; W, wild-type AcNPV-infected cells; Lane 1, cell lysate infected with AcNPV-HIV-2 gag virus; Lane 2, extracellular *gag* particles present in the pellet obtained by ultracentrifugation of cultured supernatant; Lane 3, purified *gag* particles. The arrow indicates the major *gag* pr41 protein.

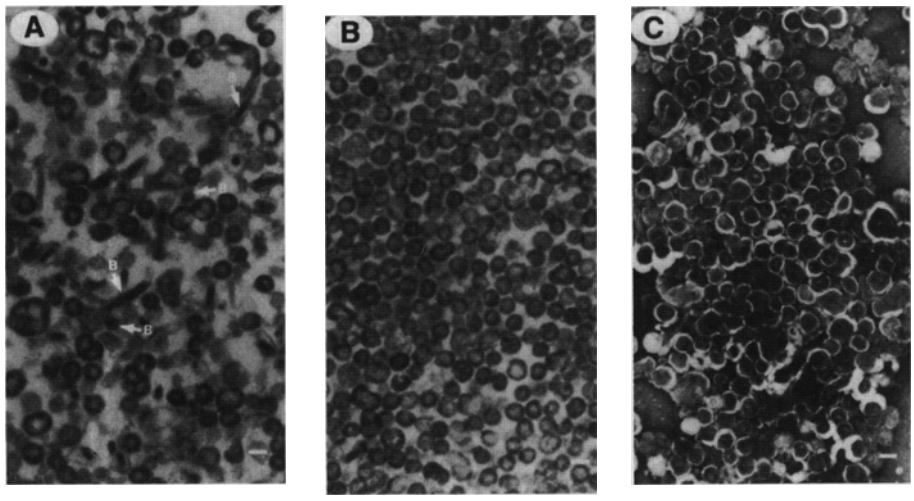


Fig. 3. Transmission electron microscopy of HIV-2 *gag* particles. **(A)** Pelleted material from infected Sf9 cell supernatants showing both *gag* and baculovirus particles (arrow B shows baculovirus in longitudinal and transverse section). **(B)** Thin section of purified *gag* particles from 20–60% sucrose gradient. **(C)** Negative staining of purified *gag* particles fixed with glutaraldehyde and osmium tetroxide. The white bars in the figure represent 100 nm.

protein (41 kDa) and a 26-kDa degradation product are evident.

9. Pelleted *gag* particles are purified by ultracentrifugation on a 20–60% (w/v) sucrose gradient at 100,000*g* overnight. Two well-separated bands are evident; the higher band sediments at about 40% sucrose, and the lower band sediments at about 52% sucrose. Electron microscopy reveals the upper band to be the *gag* p41 particles whereas the lower band contains particles that had the typical rod-like structure of baculoviruses (**Fig. 3**). The identity of the upper band can be confirmed biochemically through PAGE and Western blot analyses (**Fig. 2**).
10. The level of production of total *gag* p41 is estimated to be 30–50 mg/5 × 10^8 cells/L of culture fluid (*see* **Note 1**).

3.2. Expression of pol Protein

We have used the same strategy to express three different cassettes of the *pol* gene:

1. A deleted polymerase (Dpol) cassette containing a deleted protease, the reverse transcriptase, and integrase coding regions was constructed first as shown in **Fig. 4** starting from the plasmid pHXB-20. This cassette started from the first AUG codon in the *pol* open reading frame, deleting 103 amino acids at the amino terminus of the protease, but retaining 64 amino acids at the carboxyl terminus of the protease. In addition, we also included the putative ribosome binding site CCTATAAAT in front of the translation initiation codon. To make this construct, the *Bgl*II-*Sal*I fragment containing the HIV-1 pol coding region was isolated and inserted into the *Bam*HI and *Sal*I sites of pUC18. The resulting plasmid was cut with *Sst*I, the ends were dephosphorylated, and the linearized construct was annealed to an oligonucleotide containing *Sst*I/*Bam*HI/Sf9 cell ribosome binding site (CCTATAAAT)/initiation codon/15 nucleotides of the 5' terminus of the Dpol gene. Deletion of the 5' noncoding sequences (i.e., 103 amino acids of the protease) was accomplished by crossover linker ligation. Similarly, the noncoding sequences of the *pol* gene at the 3' end were removed by crossover linker ligation with an oligonucleotide containing *Sph*I/*Bam*HI/termination codon/15 nucleotides from the 3' terminus of the Dpol gene. A *Bam*HI fragment containing the coding sequence of Dpol was isolated from pUC18-Dpol3 and inserted into the *Bam*HI site of pAcYM1. Through this process, we deleted all noncoding sequences at both 5' and 3' termini of the *pol*

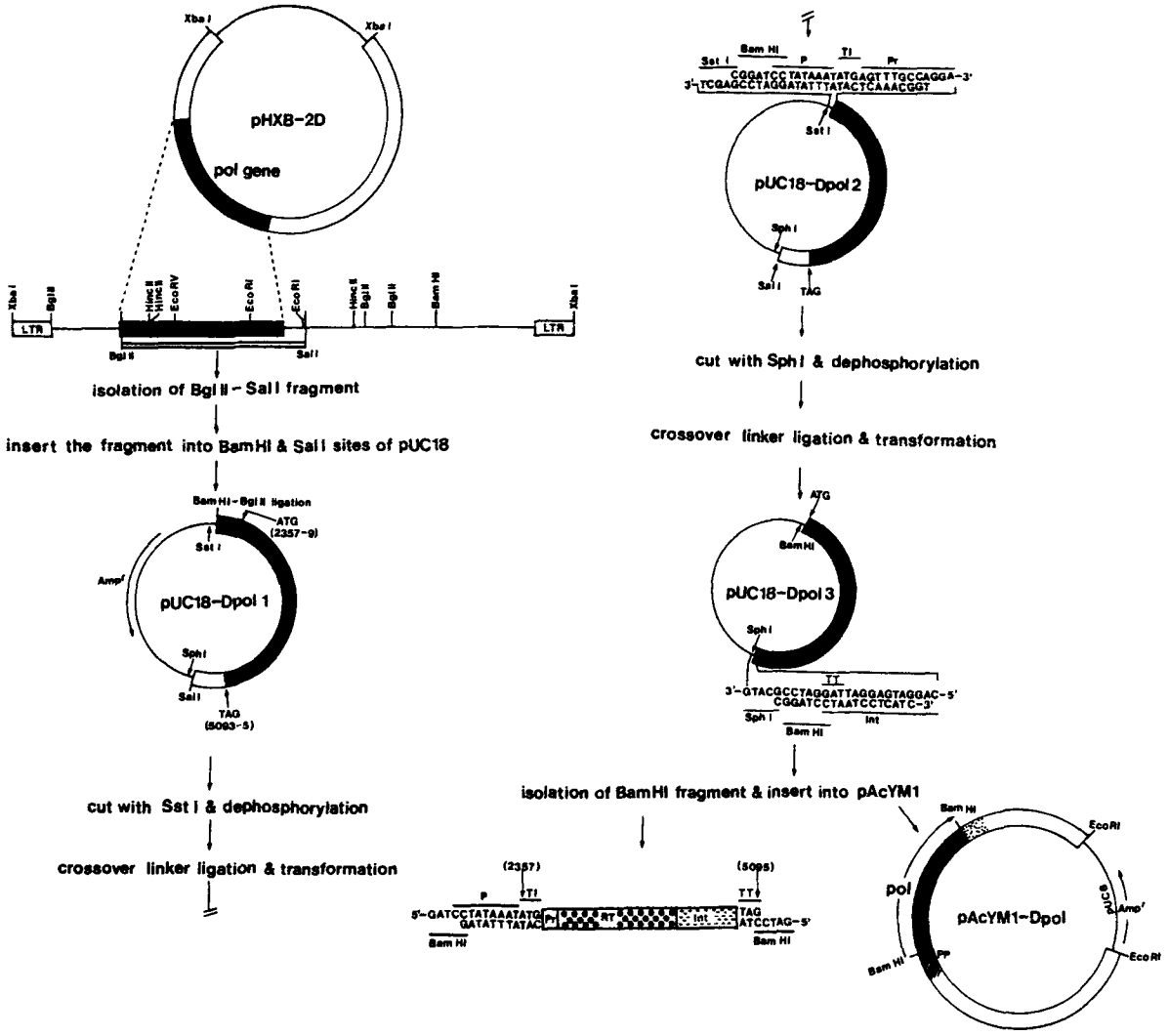


Fig. 4. Construction of the HIV-1 *pol* open reading frame. The Dpol cassette shows deletion of 273 bp from the 5' terminus of the *pol* open reading frame. The *Bgl*II -*Sal*I fragment of plasmid pHXB-2D ***(16)*** containing the HIV-1 *pol* coding region was isolated and inserted into *Bam*HI and *Sal*I sites of pUC18. The resulting recombinant plasmid pUC18Dpol 1 was cut with *Sst*I and dephosphorylated. A synthetic double-strand crossover linker containing an *Sst*I cohesive end, a *Bam*HI site, the putative ribosome binding site, CCTATAAAT, which was derived from nucleotides -9 to -1 of the polyhedrin gene ***(20)***, and 15 nucleotides of homology searching sequence that overlap with the 5' terminus of the Dpol gene was ligated to the *Sst*I site, and the resulting construct was used to transform *E. coli*. Recombinant plasmid (pUC18Dpol 2) was isolated, digested with *Sph*I, dephosphorylated, and ligated with another crossover linker DNA containing *Sph*I cohesive end, a *Bam*HI site, and 15 nucleotides of a homology searching sequence that recognizes the 3' terminus of the *pol* gene. The resulting recombinant plasmid (pUC18-Dpol 3) contains the putative ribosome binding site (P) followed by the *pol* open reading frame starting with the first ATG (TI) in the *pol* gene and ending with the translation termination codon TAG (TT). This Dpol cassette is flanked with *Bam*HI sites. The *Bam*HI fragment was isolated, inserted into the *Bam*HI site of the pAcYM1 baculovirus transfer vector ***(15)***, and pAcYM1-Dpol DNA was used to cotransfect Sf9 cells along with wild-type AcNPV DNA to produce recombinant baculovirus.

gene, and added *Bam*HI restriction sites at either end. This cassette contains genetic information for 912 amino acids.

2. When we express the Dpol cassette in Sf9 cells, large quantities (approx 80–100 mg/5 × 10^8 cells/L culture) of unprocessed 100-kDa protein

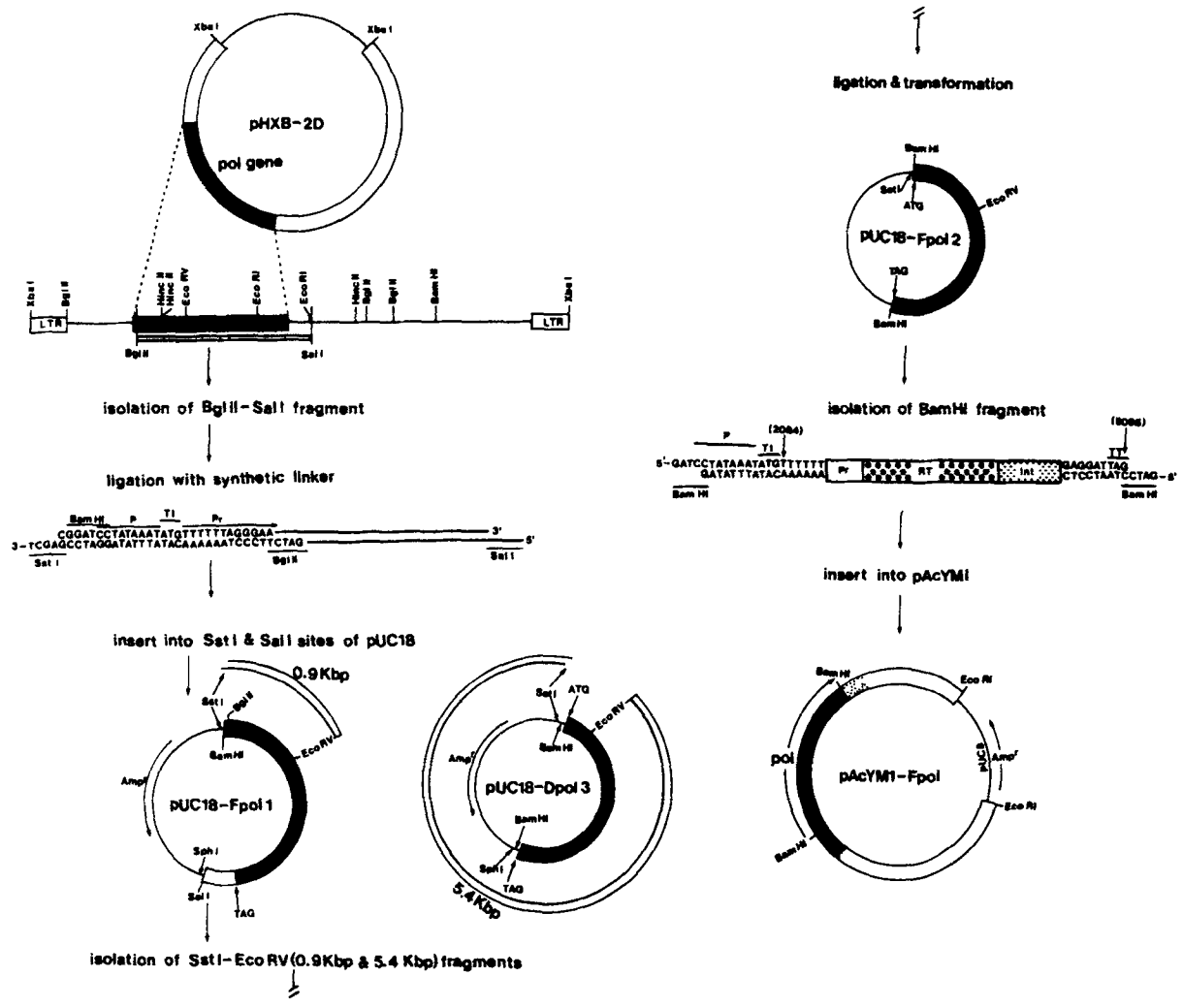


Fig. 5. Construction of recombinant AcNPV HIV-1 *pol* containing the entire open reading frame. The full-length *pol* gene (Fpol) was constructed as follows: the *Bgl*II-*Sal*I fragment of plasmid pHXB-2D was isolated and ligated with a synthetic double-stranded DNA linker that provides 12 nucleotides missing immediately upstream from the *Bgl*II site plus a translation initiation codon (TI), a putative ribosome binding site (P), a *Bam*HI site, and an *Sst*I cohesive end. The DNA was inserted into the *Sst*I and *Sal*I sites of pUC18. The resulting recombinant plasmid (pUC18-Fpol 1) was cut with *Sst*I and *Eco*RV, and a 900-bp fragment was isolated. The 900-bp *Sst*I-*Eco*RV fragment and a 5.4-kbp *Sst*I-*Eco*RV fragment of pUC18-Dpol 3 (**Fig. 4**) were ligated and used to transform *E. coli*. The resultant plasmid (pUC18-Fpol 2) contains the putative ribosome binding site (P) followed by the newly introduced translation initiation codon (TI), the full-length *pol* open reading frame, and the translation termination codon (TT), and flanked by a *Bam*HI site at either end. This cassette (Fpol) was isolated and inserted into the *Bam*HI site of the pAcYM1 baculovirus transfer vector and was used to cotransfect Sf9 cells along with wild-type AcNPV DNA to produce recombinant baculovirus.

accumulate in recombinant AcNPV-infected Sf9 cells (*see* **Fig. 7** later in this article). The 100-kDa protein is immunoreactive with antibodies present in HIV-1 and HIV-2 positive human sera (**Fig. 7B**) *(22)*.

3. The full-length (Fpol) cassette contains the full-length *pol* open reading frame, which consists of the virus-specific protease, reverse transcriptase, RNase H, and the endonuclease/integrase (**Fig. 5**). Since the *pol* open reading

frame does not have its own translation initiation site, we inserted an ATG in front of the *pol* open reading frame. The *Bgl*II-*Sal*I fragment of pHXB-2D was isolated and ligated with a synthetic double-stranded DNA oligonucleotide linker consisting of a translation codon and 12 nucleotides from the protease gene missing upstream of the *Bgl*II site, a ribosome binding site (CCTATAAAT), a *Bam*HI site, and an *Sst*I cohesive end, and inserted into pUC18 to give pUC18-Fpol1. A 900-bp *Sst*I-*Eco*RV fragment was inserted in place of the *Sst*I-*Eco*RV fragment in pUC18-Dpol3 to yield pUC18-Fpol2. The *Bam*HI fragment from this plasmid was ligated into pAcYM1, cotransfected into Sf9 cells with AcNPV DNA, and recombinant virus expressing Fpol was isolated.

4. The resulting recombinant AcNPV Fpol virus is capable of synthesizing a product of 1016 amino acids. When we express the Fpol gene in Sf9 cells using recombinant AcNPV, we cannot detect a precursor polyprotein band by staining with Coomassie blue (**Fig. 7A**). However, Western blot analysis with HIV-positive human sera shows 66-, 51-, and 34-kDa processed proteins (**Fig. 7B**). This result suggests that the HIV protease must be active and cleaves the precursor polyprotein to produce the final products p66, p51, and p34.
5. We observe that cells infected with recombinant baculovirus containing Fpol cassette lyse early in infection. We believe that HIV protease is extremely toxic to the cells, and therefore, cells cannot synthesize large quantities of the polyprotein.
6. A cassette containing only the reverse transcriptase (RT) has a deletion of both protease and endonuclease/integrase genes (**Fig. 6**). It is able to code for a protein of 563 amino acids that contains RT and RNase H activities *(21)* (**Fig. 6**). The plasmid PUC18-Dpol3 was cut with *Sst*I, dephosphorylated, and the 5' terminal protease portion of the gene was removed with crossover linker mutagenesis using an oligonucleotide containing an *Sst*I/*Bam*HI/Sf9 ribosome binding site (CCTATAAAT), an initiation codon, and 15 nucleotides from the 5' end of the RT. The integrase from the 3' end was removed by crossover linker mutagenesis using an oligonucleotide containing an *Sph*I cohesive end, a *Bam*HI site, a termination codon, and 15 nucleotides with homology to the 3' end of the RT. The *Bam*HI fragment containing the RT was inserted into pAcYM1 and used to generate AcNPV-RT recombinants.
7. AcNPV-RT expresses large amounts (approx 100 mg/5 × 10^6 cells/L) of a 66-kDa protein in Sf9 cells that is recognized by antibody present in HIV-1-positive human sera (**Fig. 7B**) (*see* **Note 2**).

3.3. *Expression of gp120* env *Protein*

1. Using the same strategy of construction (**Fig. 8**), we have expressed gp120 and gp130 of HIV-1 and HIV-2, respectively. The plasmid pHXB-2D, which contains the entire HIV-1 genome, was used as a source of envelope glycoprotein coding sequence *(16)*. A 2.1-kb *Sst*I-*Hin*dIII fragment from pHXB-2D was isolated, blunt-ended with Klenow fragment and T4 DNA polymerases, and inserted into the *Hin*cII site of pUC19. The resulting recombinant plasmid, pUC19-Env, was digested with *Eco*RI and ligated with a synthetic oligonucleotide crossover linker that contained an *Eco*RI sticky end, a *Bam*HI site, ribosome binding site (CCTATAAAT), initiation codon, and 12 nucleotides of homology with the 5' terminus of the pUC19-Env coding region. The gp41 coding sequence adjacent to the 3' end of gp120 was deleted by crossover linker mutagenesis using an oligonucleotide containing a *Hin*dIII stickly end, an internal *Bam*HI site, a translation termination codon, and 12 nucleotides with homology to the 3' terminal residue of gp120. The gp120 coding region was cut and removed with *Bam*HI and inserted into pAcYM1, cotransfected with AcNPV DNA into Sf9 insect cells, and used to generate recombinant AcNPV-gp120.
2. **Figure 9** shows the nonglycosylated form of gp120 and gp130 of HIV-1 and HIV-2, respectively (*see* **Note 3**). We have partially purified these cell-associated glycoprotein precursors and used them to immunize rabbits. Antirabbit sera against nonglycosylated gp120 of HIV-1 neutralizes HIV-1 infectivity (data not shown). We have subsequently included this protein as one of the components of an AIDS vaccine.

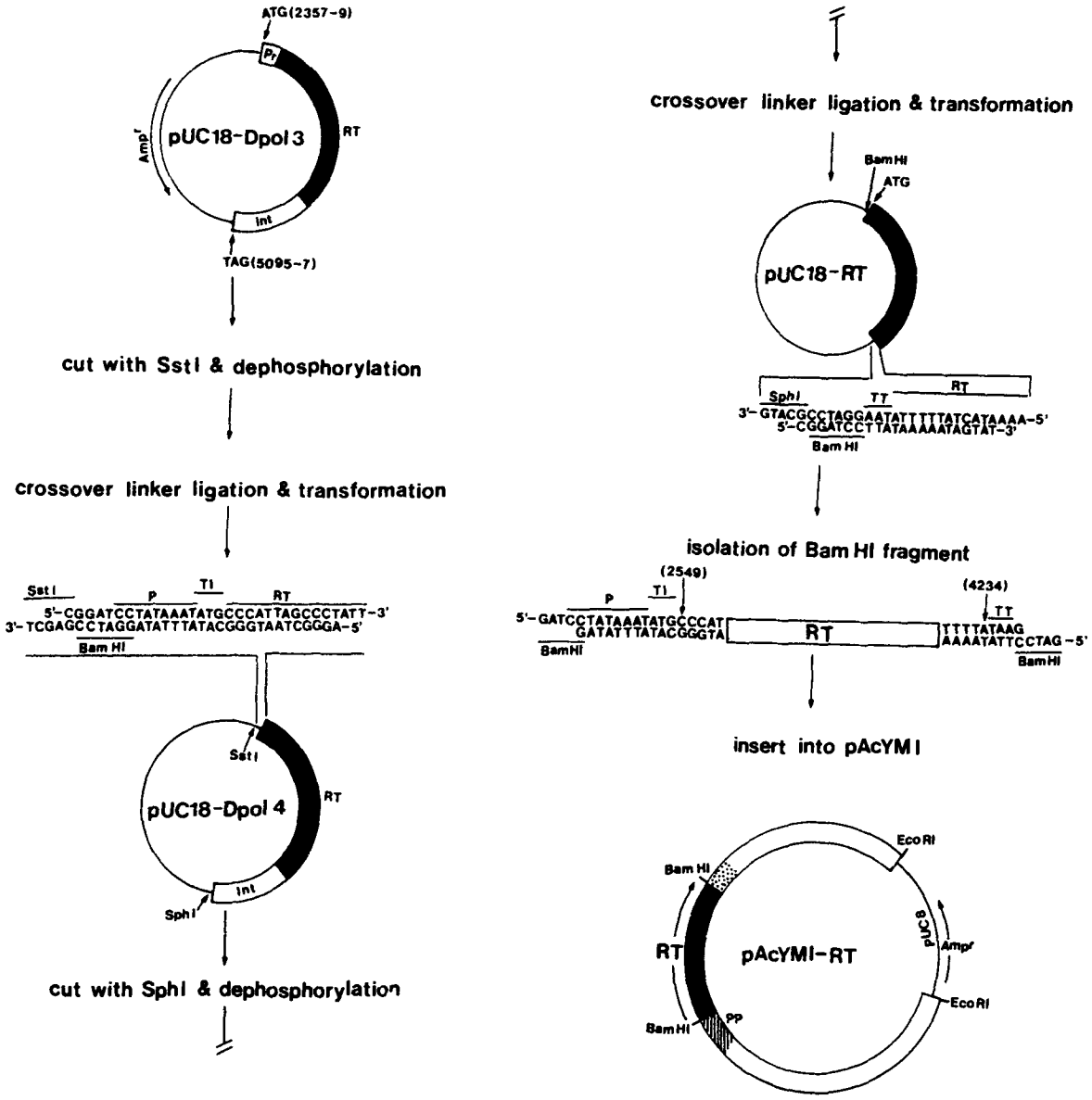
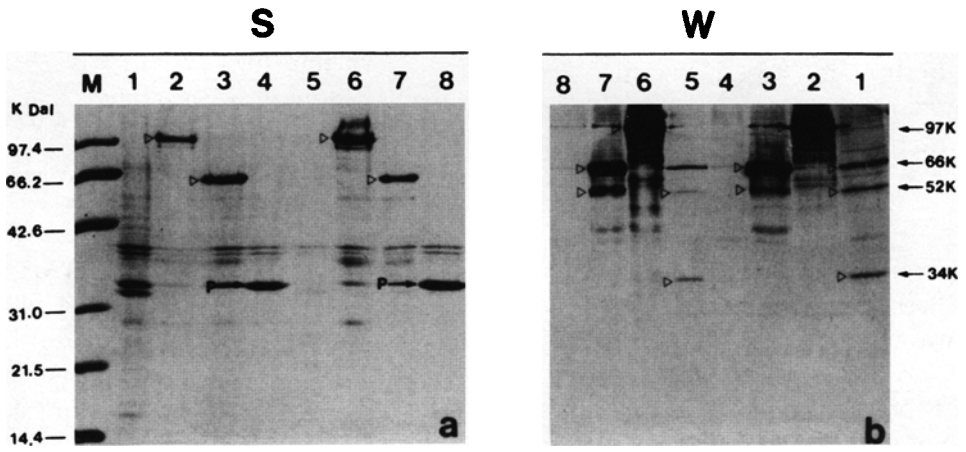


Fig. 6. Construction of recombinant AcNPV containing 66-kDa RT gene. The pUC18-Dpol 3 from **Fig. 4** was cut with *Sst*I, dephosphorylated, and crossover linker mutagenesis was used to remove the 5' terminal protease portion of the gene and to add a *Bam*HI site, a putative ribosome binding site (P), and a translation initiation codon (TI) in front of the RT coding sequences. The resulting plasmid pUC18-Dpol 4 was then cut with *Sph*I, dephosphorylated, and ligated with another crossover linker containing an *Sph*I cohesive end, a *Bam*HI site, translation termination (TT) codon, and 15 nucleotides of homology searching sequence that recognizes the 3' terminus of the RT gene. The recombinant plasmid (pUC18-RT) contains the putative ribosome binding site (P) followed by a new translation initiation site (TI), the RT open reading frame, which also contains C-terminal RNase H coding sequences ending with a translation termination codon (TAA) (TT). This RT cassette is flanked with *Bam*HI sites. The *Bam*HI fragment was isolated, inserted into the *Bam*HI site of the pAcYM1 baculovirus transfer vector, and pAcYM1-RT DNA was used to cotransfect Sf9 cells along with wild-type AcNPV DNA to produce recombinant baculovirus.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8 7 6 5 4 3 2 1
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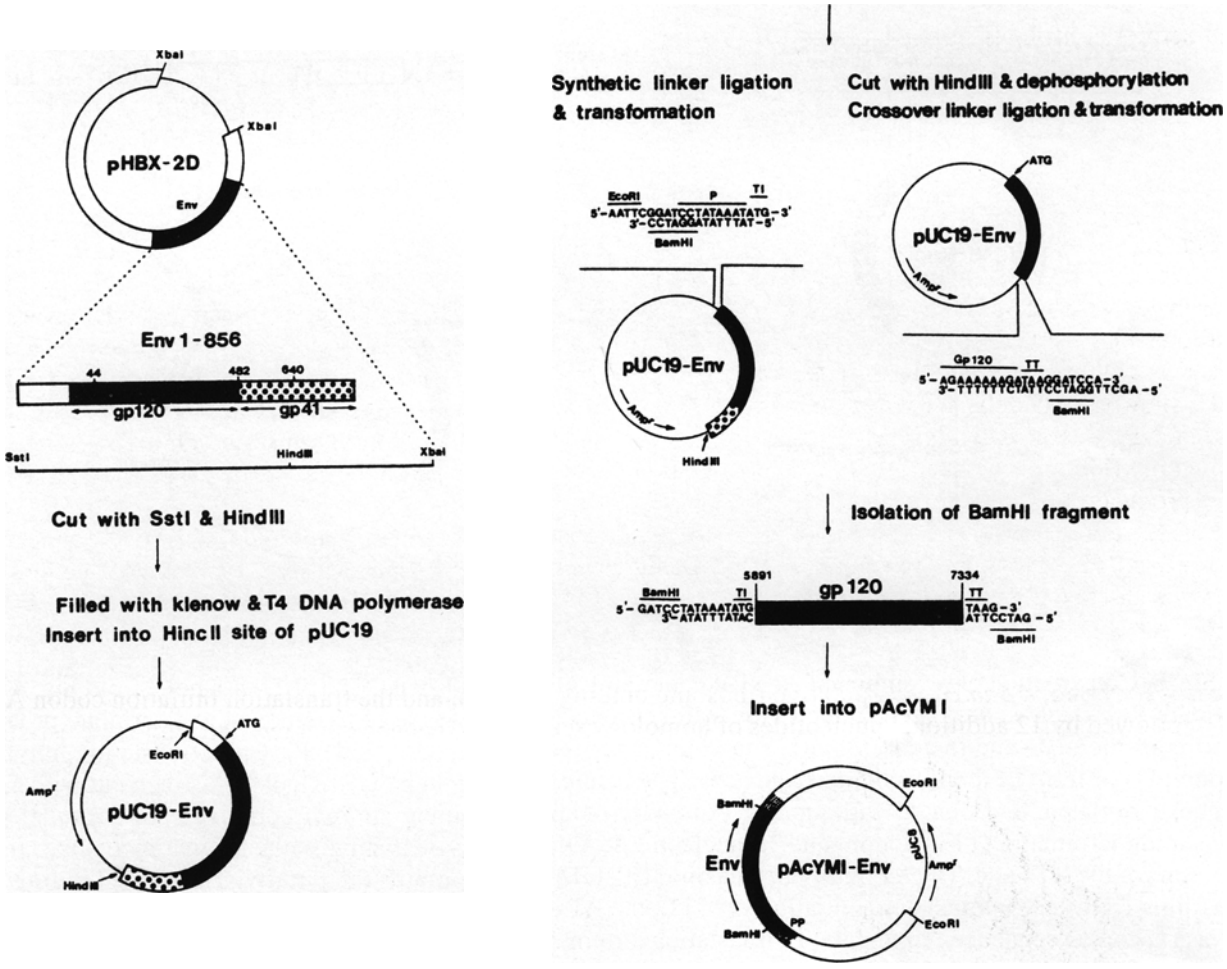
b

Fig. 7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Env
Env 1-856
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482
640
gp120
gp41
SstI
HindIII
Xbal
Cut with SstI & HindIII
Filled with klenow & T4 DNA polymerase
Insert into HincII site of pUC19
ATG
EcoRI
Amp
pUC19-Env
HindIII
Synthetic linker ligation
& transformation
Cut with HindIII & dephosphorylation
Crossover linker ligation & transformation
EcoRI
P
TI
5'-AATTCGGATCCTATAAATATG-3'
3'-CCTAGGATATTTAT-5'
BamHI
pUC19-Env
Amp
HindIII
ATG
pUC19-Env
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Gp120
TT
BamHI
Isolation of BamHI fragment
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BamHI
Insert into pAcYM I
EcoRI
BamHI
Env
pAcYMI-Env
pUC8
Amp
PP
BamHI
EcoRI

Fig. 8

3.4. Expression of vif Protein

In addition to structural proteins of HIV-1 and HIV-2, we have also constructed recombinant AcNPV containing the *vif* gene of HIV-1 using the same strategy of crossover linker mutagenesis (**Fig. 10**).

1. An *Eco*RI fragment was isolated from a plasmid containing the entire HIV-1 genome (pHXB); the fragment was rendered blunt-ended by filling it in with Klenow fragment DNA polymerase.
2. The blunt-ended fragment was inserted into the *Hin*cII site of pUC19 to produce pUC19-*vif*1.
3. The plasmid pUC19-*vif*1 was cut with *Bam*HI and *Xba*I, and ligated to an oligonucleotide consisting of *Sma*I/*Bam*HI/Sf9 ribosome binding site (CCTATAAAT)/initiation codon/12 nucleotides of the 5' end of *vif* coding sequence; transformation of *E. coli* was followed by deletion and crossover linker mutagenesis to yield pUC19-*vif*2.
4. The 3' of the vif coding region was modified by cutting pUC19-*vif*2 with *Pst*I and *Hin*dIII, ligating it to an oligonucleotide consisting of *Hin*dIII/*Bam*HI/termination codon/12 nucleotides of the 3' end of vif coding sequence to give pUC19-*vif*3.
5. A *Bam*HI fragment was removed from pUC19-*vif*3 and inserted into pAcYM1, the plasmid was cotransfected into Sf9 cells with AcNPV DNA, and recombinant virus was isolated.
6. **Figure 11** shows expression of the *vif* protein of HIV-1 in Sf9 cells. The *vif* proteins of HIV-1 and HIV-2 are extremely stable in recombinant baculovirus infected Sf9 cells.
7. The infected Sf9 cells synthesize *vif* protein until 3 d postinfection and *vif* protein remains in the cells without degradation for a week, whereas most of the cellular proteins degrade (**Fig. 12**). The level of *vif* protein expression is approx 150–200 mg/5 × 10^8 cells/L at 4 d after infection (*see* **Note 4**).

Fig. 7. *(opposite page)* Expression of *pol* genes in Sf9 cells by recombinant baculoviruses. Sf9 cells infected with recombinant baculoviruses were denatured and electrophoresed in 12.5% polyacrylamide gels. The proteins in gel were visualized by Coomassie blue staining **(A)** or Western Blot analysis **(B)** using standard National Institutes of Health HIV-positive human sera. Lanes 1, 2, and 3 represent the Sf9 cells harvested 72 h after infection with recombinant baculovirus carrying Fpol, Dpol, and RT gene cassettes, respectively. Lanes 5, 6, and 7 show the Sf9 cells harvested 96 h after infection using the same set of clones. Lanes 4 and 8 show wild-type AcNPV-infected cell lysate. P designates polyhedrin protein. Lane M contains molecular mass markers shown in kilodaltons. Lanes 1 and 5 in Panel B show processed proteins of 66-, 51-, and 34-kDa processed *pol* gene products.

Fig. 8. *(opposite page)* Construction of recombinant baculovirus containing the HIV-1 gp120 gene. The plasmid pHXB-2D containing an entire HIV-1 genome was used as a source of HIV-1 envelope glycoprotein coding sequence ***(16)***. A 2.1 kb *Sst*I–*Hin*dIII fragment from pHXB-2D was isolated, filled with Klenow and T4 DNA polymerase, and inserted into the *Hin*cII site of pUC19. The resulting recombinant plasmid, pUC19-Env was digested with *Eco*RI and ligated with a synthetic oligonucleotide crossover linker containing an *Eco*RI sticky end, a *Bam*HI site, putative ribosome binding site (P), and the translation initiation codon ATG (TI), followed by 12 additional nucleotides of homology searching sequence that overlap with the 5' terminus of the pUC19-Env gene. pUC19-Env was further modified to delete the remaining gp41 *env* coding sequence in the pUC19-Env gene, and an inframe stop codon was introduced. The pUC19-Env was digested with *Pst* I and *Hin*dIII, and ligated with another crossover linker DNA containing *a Hin*dIII sticky end, an internal *Bam*HI site, a translation termination codon (TT), and 12 nucleotide of homology searching sequence that recognizes the 3' terminal residues in the gp120 gene. The resulting recombinant plasmid contains the putative ribosome binding site (P) followed by the gp120 open reading frame, starting with the first ATG (TI) at the beginning of the signal sequence of the *env* gene and ending with the translation termination codon TAA (TT). This cassette was inserted into pAcYM1 (pAcYM1-Env) and used to cotransfect *S. frugiperda* cells to isolate recombinant AcNPV.

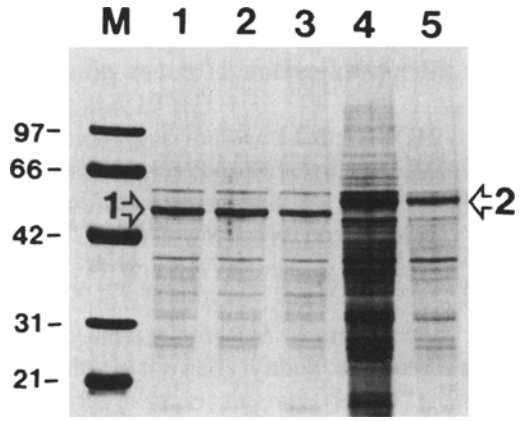
M 1 2 3 4 5
97-
66-
42-
31-
21-
1
2

Fig. 9

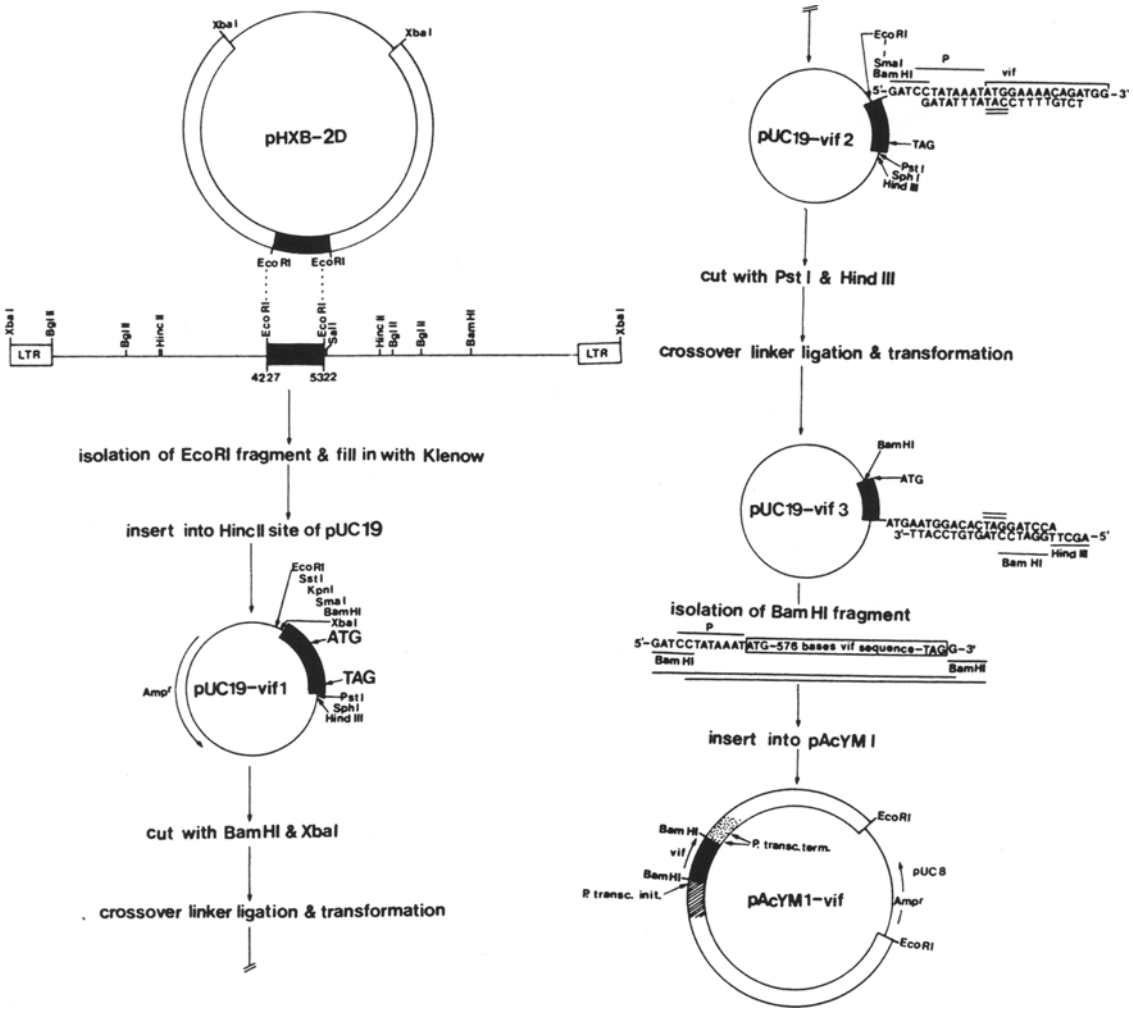
pHXB-2D
Xba I
EcoRI
LTR
4227
5322
isolation of EcoRI fragment & fill in with Klenow
insert into HincII site of pUC19
pUC19-vif1
ATG
TAG
cut with BamHI & XbaI
crossover linker ligation & transformation
pUC19-vif 2
cut with Pst I & Hind III
crossover linker ligation & transformation
pUC19-vif 3
isolation of BamHI fragment
insert into pAcYM I
pAcYM1-vif
P transc. term.
P transc. init.
pUC 8
Amp r

Fig. 10

4. Notes

1. The *gag* p41 particles are highly immunogenic and produce good levels of antibodies. This protein has previously been shown to induce CTL response ***(13,22)***.
2. The truncated HIV-2 *gag* gene was fused with the neutralizing domain (V3) of gp120 *env* gene sequences from HIV-1 and HIV-2. These fused genes express chimeric proteins that form virus-like particles. Antisera against these particles neutralize homologous HIV infectivity ***(23)***. Our results show that precursor *gag* protein has potential as a carrier for the presentation of foreign epitopes in good immunological context. The *gag* protein is highly immunogenic and has the ability to carry large foreign inserts; as such, it offers an attractive approach for HIV vaccine development ***(23)***.
3. Since HIV-1 and HIV-2 have a high degree of sequence homology in *pol* gene, HIV-1 *pol* precursor protein can interact with HIV-2 positive-patient sera ***(24)***. RT has been shown to induce a cytotoxic T-cell response ***(13)***.
4. We have observed that the gp120 polypeptide backbone of HIV-1 and the gp130 backbone of HIV-2 can be expressed at high levels if we delete the signal sequences of the protein; however, these proteins are cell-associated, and the majority of the proteins are not glycosylated.
5. We found that antibody against *vif* is produced early in HIV-1 infection, and disappears as disease progresses to ARC and AIDS. Our results suggest that continuous presence of anti-*vif* antibody may be crucial to stopping progression of the disease. We hypothesize that disappearance of anti-*vif* antibody is partially responsible for disease progression. Thus, I recommend to include *vif* protein as one of the components of an AIDS vaccine.
6. In summary, we have expressed high levels of *gag* particles, *pol* proteins, nonglycosylated gp120, and *vif* proteins of both HIV-1 and HIV-2 in Sf9 cells using recombinant baculoviruses. Different combinations of these structural proteins are being tested for their ability to protect nonhuman primates against HIV-2 or SIV infection. Our preliminary results show that the rhesus macaque immunized with the combination of proteins produces high levels of antibodies against these proteins. We are currently investigating the CTL responses to these proteins.

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Fig. 9. *(opposite page)* Coomassie blue-stained SDS-PAGE of the envelope glycoprotein backbone. Lanes 1, 2, and 3 show nonglycosylated gp120 of HIV-1 (arrow with 1), and lanes 5 and 6 show nonglycosylated gp120 of HIV-2 (arrow with 2). The HIV-2 gp120 was expressed by a recombinant baculovirus containing the HIV-2 glycoprotein gene that was constructed with the same strategy as shown in **Fig. 8**.

Fig. 10. *(opposite page)* Construction of recombinant AcNPV containing the HIV-1 *vif* gene. A *Eco*RI fragment was isolated from the infectious clone pHXB-2D and filled in with Klenow. The fragment was inserted into the *Hin*cII site of pUC19 to produce pUC19-*vif*1. pUC19-*vif*1 was cut with *Bam*HI and *Xba*I and a double-stranded crossover linker was used to construct pUC19-*vif*2. The downstream sequence was modified by cutting pUC19-*vif*2 with *Pst*I and *Hin*dIII and further crossover linker mutagenesis. The resulting plasmid (pUC19-*vif*3) contains the putative ribosome binding site (P) and open reading frame of *vif* gene, which ends with the translation termination codon TAG. This cassette was flanked with *Bam*HI sites. The *Bam*HI fragment was inserted into pAcYM1 (pAcYM1-*vif*). The plasmid pAcYMl-*vif* was cotransfected with wild-type AcNPV DNA to isolate the recombinant baculovirus containing the *vif* gene.

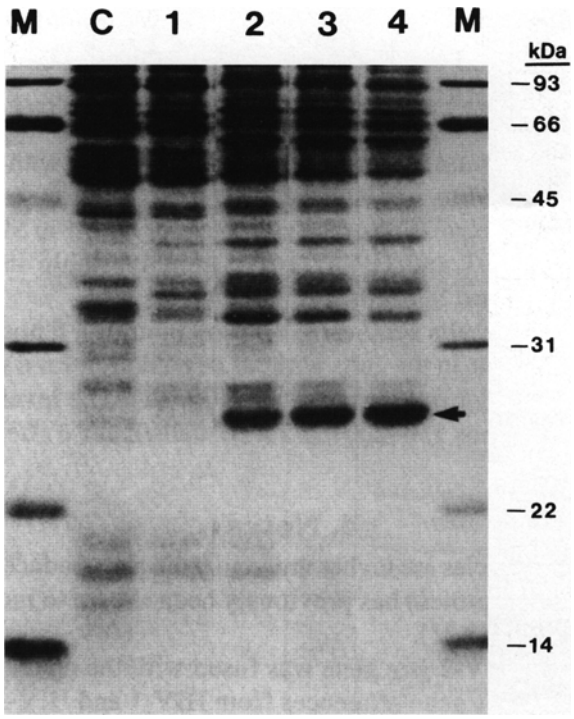

Fig. 11. Expression of HIV-1 *vif* protein in Sf9 cells by recombinant baculovirus. Sf9 cells infected with recombinant baculoviruses containing the *vif* gene were harvested 24 (Lane 1), 48 (Lane 2), 72 (Lane 3), and 96 (Lane 4) h after infection. Lysates of the infected cells were denatured and electrophoresed in 12.5% polyacrylamide gels. The proteins in gel were visualized by Coomassie blue staining. Lanes M contain markers.

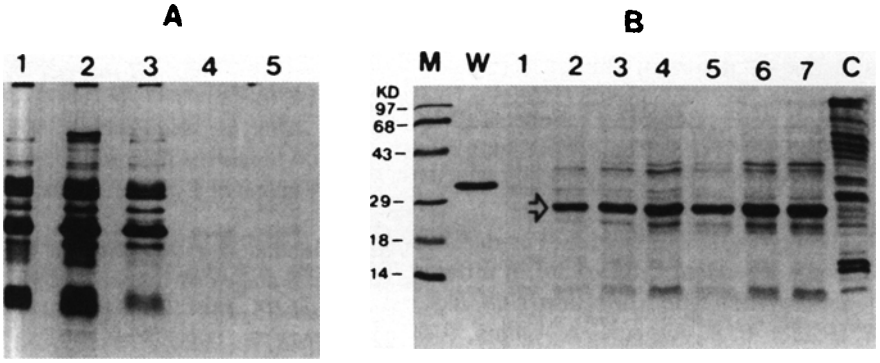

Fig. 12. Expression of HIV-2 *vif* in Sf9 cells by recombinant baculovirus. Sf9 cells infected with recombinant baculovirus containing the HIV-2 *vif* gene were denatured and electrophoresed in 12.5% polyacrylamide gels. **(A)** Methionine-labeled total cellular protein analyzed by SDS-PAGE. Lanes 1, 2, 3, 4, and 5 represent ^{35}S-methionine labeled protein on d 1, 2, 3, 4, and 5 postinfection. **(B)** Lysates of AcNPV HIV-2 *vif* recombinant virus infected Sf9 cells from d 1, 2, 3, 4, 5, 6, and 7 postinfection were subjected to SDS-PAGE in 12.5% polyacrylamide gels, and the proteins were stained with Coomassie blue. The arrow shows the accumulation of HIV-2 *vif* protein in recombinant baculovirus-infected cells. Lane W represents the 33-kDa polyhedrin protein from wild-type AcNPV-infected cell lysates. Lane C contains uninfected Sf9 cell lysate. Lane M contains molecular-mass markers.

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