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Prokaryotic Expression and Purification of HIV-1 Vif and hAPOBEC3G, Preparation of Polyclonal Antibodies*

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Abstract: To prepare HIV-1 Vif and hAPOBEC3G and to produce their antibodies, the full length gene fragment of HIV-1 vif was amplified by PCR from a plasmid of HIV-1 NL4.3 cDNA, and the APOBEC3G gene was obtained by RT-PCR from the total RNA of H9 cells. The resulting DNA construct was cloned into a prokaryotic expression vector (pET-32a). Recombinant pET-vif and pET-APOBEC3G were expressed respectively in Eserichia coli BL21 (DE3) as an insoluble protein. The vector also contained a six-histidine tag at the C-terminus for convenient purification and detection. To express and purify the HIV-1 Vif and hAPOBEC3G in *E.coli* cells, the accuracy of inserted gene and specificity of proteins were detected by the two enzyme digestion method, SDS-PAGE, and Western blotting. Rabbits were then immunized by Vif or APOBEC3G protein and serum samples were tested by indirect ELISA to determine the level of antibodies. Immunoenzyme and immunofluorescence assays were performed to identify the specificity of polyclonal antibodies. The titer of the anti-Vif antibodies was 1:204800, and that of the anti- APOBEC3G antibodies was 1:102400. Thus the antibodies could detect the antigen expression in the cells, demonstrating that fusion proteins with high purity and their corresponding polyclonal antibodies with high titer and specificity were achieved.

Key words: Human immunodeficiency virus type 1 (HIV-1); Viral infectivity factor; hAPOBEC3G; Protein purification; Polyclonal antibody

The viral infectivity factor (*vif*) gene which is located in the genome of human immunodeficiency virus (HIV) was originally discovered in the mid 1980s and was initially called *sor* (10, 3). The gene product is a 23 kDa basic protein encoded by all lentiviruses, except for the equine infectious anemia virus, and was later renamed as Vif. Compared to the *rev* and *gag* sequences, the *vif* sequence is highly conserved across clades (6). *APOBEC3G*, the apoli-protein B mRNA-editing enzyme-catalytic polypeptide-like-3G, which is arranged in tandem on chromosome 22, includes eight exons and seven introns. The length of the *APOBEC3G* cDNA is 1 155 bp, and encodes 384 amino acid residues (9). APOBEC3G has been identified as a mediator of anti-HIV-1 activity (1, 2, 7), and its activity has been shown to be suppressed by

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Vif (5, 11). The interaction between HIV-1 Vif and APOBEC3G provides a potential novel target for *vif* and its subsequent products in the HIV life cycle. In this study, HIV *vif* and *hAPOBEC3G* were each cloned into a prokaryotic expression vector (pET-32a) and the resulting recombinant pET-vif and pET-APOBEC3G were highly expressed respectively in *E. coli* BL21 (DE3) as an insoluble protein. In addition, rabbits were immunized with the purified protein to produce polycloned antibodies. These products have potential for use in studying the construction of HIV-1 Vif and as identifying potential new targets for HIV drugs.

MATERIALS AND METHODS

Reagents

Restriction endonucleases, T4 DNA ligase, Taq DNA polymerase and Agarose Gel DNA Fragment Recovery Kit were obtained form the Takara Company (Dalian, China). Anti-His-Tag monoclonal antibody and goat anti-mouse IgG/HRP were purchased from New England Biolabs. All other chemicals were of analytical reagent grade.

Cloning of *vif* by PCR

For PCR amplification of *vif*, a forward primer (5'-tgcaccatggaaaacagatggcaggtgat-3') and reverse primer (5'-ccgctcgagggtgtccattcattgtat-3') were used, which overhang the *Nco* I and *Xho* I sites (underlined) respectively. In a 50μL reaction system, the template used for *vif* open reading frame (ORF) amplification by polymerase chain reaction (PCR) was 1 μL plasmid of HIV-1 NL4.3 cDNA. Am- plification was accomplished using the following reaction conditions: initial heating at 94℃ for 5 min; then 30 cycles consisting of denaturation at 94℃ for 45 s, annealing at 55℃ for 45 s and elongation at 72℃ for 1 min; final extension at 72℃ for 10 min. The gene products were ligated to the cloning vector pGEM-T. The DNA sequence was determined using an automated sequencer.

Cloning of *APOBEC3G* by RT-PCR

RNA was extracted from H9 cells using TRIzol reagent according to the manufacturer's specifications. *APOBEC3G* was amplified using these primers: (F): 5'-ccatggctatgaagcctcact-3', (R): 5'-ctcgagcgttttcctgatt ctg-3'. The first step of RT-PCR to synthese cDNA used the reaction condition: 42℃, 30 min. The second step used the reaction condition: initial heating at 94℃ for 5 min; followed by 35 cycles consisting of dena-turation at 94℃ for 50 s, annealing at 55℃ for 1 min and elongation at 72℃ for 3 min; and final extension at 72℃ for 10 min. The gene products were ligated to the cloning vector pGEM-T. The DNA sequence was determined using an automated sequencer.

Construction of expression vector for *vif* and *APOBEC3G*

The amplified PCR product was digested with *Nco* I and *Xho* I followed by subcloning into the prokaryotic expression vector pET-32a. This generated the recombinant plasmid pET-vif and pET-APOBEC3G respectively, in which the C-terminal domains of *vif* and *APOBEC3G* were fused with a His-tag for improved purification and immune identification. *E.coli* DH5α. competent cells were transformed with the ligation reaction, and the DNA was subsequently purified and checked by 1% agarose-gel electrophoresis after digesting with *Nco* I and *Xho* I. The samples were then sequenced to confirm the correct insert by the Shanghai Sangon Biology Technology Limited Company (sequencing primer is T7 termi-

nator primer). The results were compared with the expected sequence using the vector NTI 8.0 software.

Recombinant pET-vif and pET-APOBEC3G expression *E.coli*

E.coli BL21 (DE3) competent cells were transformed with pET-vif and pET-APOBEC3G respectively, and the transformant cells were cultivated at 37℃ with shaking at 190 r/min in 5mL of LB medium supplemented with 100 μg of ampicillin and 34 μg of chloramphenicol per mL. Cultures were induced for expression at an OD_{600} of 0.6～0.8 with 1 mmol/L IPTG, and continued growth with shaking for 4 h. Cells were then harvested by centrifugation at 12 000 r/min for 1 min. Total cellular pellets were analysed by 12% SDS-PAGE and Western blotting.

Purification of recombinant Vif and APOBEC3G

Identified pET-vif / BL21 (DE3) and p ETAPOBEC3G/ BL21 (DE3) cells were cultivated overnight. 8 mL Cultures were added to 800 mL 2×YT medium supplemented with 100 μg of ampicillin and 34μg of chloramphenicol per mL, and were cultivated at 37℃ with shaking at 190 r/min. Cultures were induced for expression at an OD_{600} of 0.6～0.8 with 1 mmol/L IPTG, and continued growth with shaking for 4 h. Cells were harvested by centrifugation at 5000 r/min for 20 min at 4℃. Cell pellets were thoroughly suspended in 10 mL of lysis buffer (10 mmol/L Tris-HCl, 100 mmol/L NaH_2PO_4, pH 8.0) per gram of cell paste. The cells were then sonicated at 50% amplitude on ice for 300 s (30 s on/30 s out) followed by centrifugation at 8 000 r/min for 30 min at 4℃. The pellet washing was repeated for a total of three washes and the final pellet was resuspended in 10 mL extraction buffer (10 mmol/L Tris-HCl, 100mmol/L NaH_2PO_4, and 7.5 mol/L guanidine-HCl, pH 8.0) per gram of cells, followed by incubation overnight at 15℃ with gentle mixing. Following the step, the sample was centrifuged at 12 000 g for 30 min at 4℃. 2 mL supernatant was then filtered through a 0.45 μm syringe filter and applied to an equilibrated gel filtration chromatography column (superdex75、200) using a 2 mL Superloop at l mL/min on the FPLC, and eluted by buffer B(100 mmol/L NaH_2PO_4, 10 mmol/L Tris, 8 mol/L Urea, pH 6.2). The purified productions were analyzed by 12% SDS-PAGE and Western blotting.

Western blotting analysis for recombinant protein

For Western blotting, samples were separated using 12% SDS-PAGE and then transferred to pre-cut nitrocellulose membrane. The membranes were blocked for l h at ambient temperature in phosphate- buffered saline (PBS) with 5% nonfat dried milk, and then incubated for l h with rat anti-His monoclonal antibody (1:200). The membranes were rinsed thrice with PBST (1×PBS, 0.05% Tween 20, pH 7.5), followed by incubation with goat anti-rat IgG antibodies conjugated to horseradish peroxidase (HRP) (1:400) for l h at ambient temperature. The Vif and APOBEC3G samples were visualized by incubation with DAB (0.06 mg/mL) and 0.1% H_2O_2.

Protein concentration detection

Concentration of protein was detected by the Bradford method.

Preparation polyclonal antibodies

Polyclonal anti-recombinant protein antibodies were raised in two rabbits. Rabbit sera were taken prior to immunization as negative control. Rabbits were immunized on days l and 28 with 500μg recombinant protein in their legs. Each immunization was done with l mL 500 μg/mL recombinant protein, which

was diluted to 2 mL with Freund's complete adjuvants. Serum samples were collected two weeks after the last injection and stored at-70℃. Polyclonal antibodies in sera were tested by indirect ELISA.

Indirect ELISA

Microtiter plates were coated overnight at 4℃ with 60 ng Vif and 15ng APOBEC3G per well in coating buffer (carbonate-buffer, pH 9.6). Unbound antigen was removed by washing with PBST solution (1×PBS, 0.2%Tween 20). 2% bovine serum albumin (BSA) wan added to block the coated wells for 2 h at 37℃. Serum samples diluted 200-204 800 fold were added to the coated wells and incubated for 90 min at 37℃ in a humidified atmosphere. Wells were washed with PBST solution and incubated with 100 μL of HRP-conjugated goat anti-rabbit immunoglobulins. After 60 min of incubation at 37℃, wells were washed and the antigen-antibody complexes were detected by the addition of 100 μL of A and B solution. After 15 min of incubation at room temperature, the enzymatic reaction was stopped by adding 50 μL of 2 mol/L sulfuric acid to each well. Optical densities (*OD*) were measurd at 450/630 nm. The cutoff value was set two standard deviations above the average of negative control sample which was taken before the immunization.

Construction of pEGFP-vif and transient cell transfection

Expression vectors pEGFP-vif expressing HIV-l Vif. The *vif* gene sequence was amplified by PCR from the HIV plasmid NL4.3 cDNA. For DNA transfection, 6-well plates were seeded with Hela-CD4-LTR-β-gal-CXCR4 cells at a density of 1×10^5 cells/well in 2 mL of DMEM cell culture medium and maintained at 37℃ and 5% CO_2.The following day, the optimal amount of pEGFP-vif for transfection was determined to be 6 μg. Lipofectamine 2000 (In-vitrogen, Carlsbad, California) was used essentially according to the manufacturer's instructions, at 3 μL Lipofectamine in FBS-and antibiotic-free DMEM. At 6 h post-transfection, the medium of the cells was replaced with fresh DMEM containing 10% FBS.

Cell preparation

Hela-CD4-LTR-β-gal-CXCR4 cells transfected by pEGFP-vif, H9 cells and MT4 cells were fixed in 4% formalin for 10 min at room temperature.

Immunoenzyme and immunofluorescence assay

Cells were incubated with polyclonal antibodies for l hour at 37℃. After washing three times with PBS, cells were incubated with HRP-conjugated or TRITC-conjugated goat anti-rabbit antibodies for 45 minutes at 37℃. Immunostaining was revealed by exposure to fresh DAB solution for 5 min, and rinsed with distilled water. The result of immunofluoresence was observed directly by fluorescent microscope.

RESULTS

Identification of the pET-vif and pET-APOBEC3G

Two fragments were obtained from the recombinant plasmid of pET-vif by digesting with *Nco* I and *Xho* I, and the bulks of two fuagments were about 576 bp and 5.9 kb respectively(Fig. 1). By the same method, digested pET-APOBEC3G had two fragments with 1152 bp and 5.9 kb (Fig. 2).Plasmid pET-vif and pET-APBOEC3G were sequenced by the Shanghai Sangon Biology Technology Limited Company. Using Vector NTI AlignX software, the pET-vif sequencing result was compared with the HIV-1

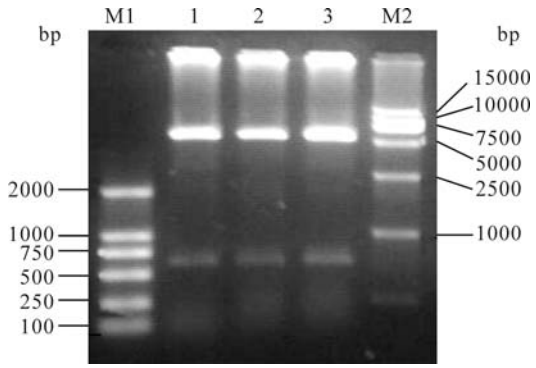

Fig. 1. Analysis of recombinant plasmid pET-vif by *Nco* I & *Xho* I digestion. 1-3, pET-vif; M1/M2, DNA ladders.

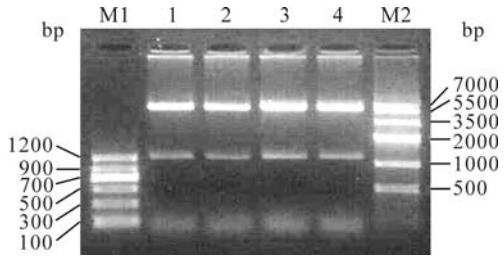

Fig. 2. Analysis of recombinant plasmid pET-APOBEC3G by *Nco* I & *Xho* I digestion. 1-4, pET-APOBEC3G; M1/M2, DNA ladders.

NL4.3 *vif* sequence(GenBank No.: AF070521). This indicated that the homologous fragment of 576 bp had high similarity to the expected sequence and the recombinant plasmid pET-vif was correctly constructed. The pET-APOBEC3G sequencing result was compared with the *APOBEC3G* sequence (GenBank No.: NM-021822) and it was similarly demonstrated that the homologous fragment of 1 152 bp had high similarity and the recombinant pET-APOBEC3G gene was correctly constructed.

Expression of Vif and APOBEC3G fusion protein

Extracts of *E.coli* BL21 (DE3) cells transformed with the recombinant plasmid and induced by IPTG treatment were analysed by polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue and Western blotting. Fig. 3 shows strong expression of the Vif-His fusion protein of about 45 kDa (lane 4, arrowed), which was consistent with the expected molecular mass of the desired protein. In the lane containing the blank control, the band of the Vif fusion protein was not detected (lane 3). There were faint expression before induction (lane 1), and after induction a band of about 22 kDa was visible which was consistent with the enhanced solubility of the protein (lane 2, arrowed). Its sequence was then introduced immediately upstream of the vif sequence in the vector. The clearly visible band of APOBEC3G fusion protein was present in the total cell extracts of *E. coli* BL21 (DE3) cells transformed with pET-APOBEC3G after induction (lane 6, arrowed) but not the cell extracts before induction (lane 5). The relative amount of Vif was estimated by densitometry of the stained gel, and was found to be about 17.7% of the total protein; similarly the relative amount of APOBEC3G was 22.3%

Purification and identification of recombinant Vif and APOBEC3G

Total cell content after sonication, including both the supernatant fluid (soluble-protein fraction) and the pellet (inclusion-body fraction), were analysed by SDS-PAGE and Western blotting. Most of the expressed Vif protein accumulated in inclusion bodies (data not shown). After the inclusion bodies were washed and dissolved, the supernant was passed through gel filtration chromatography and eluted by buffer B. The purified proteins were analysed by 12% SDS-PAGE and Western blotting. Figure 4A shows the Vif fusion protein was ~ 45 kDa (lane 2) and APOBEC3G was ~68 kDa (lane 7). These results were consistent with the expected molecular mass of

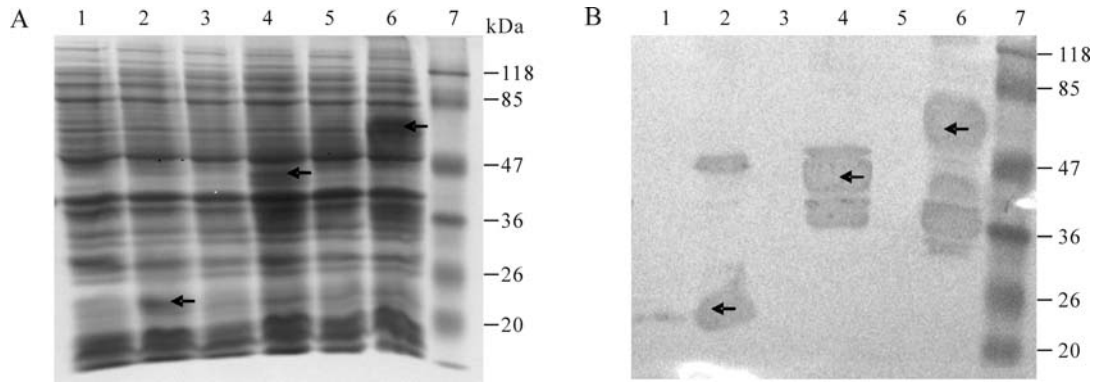

Fig. 3. SDS-PAGE and Western blotting analysis of expressed protein by pET-Vif and pET-APOBEC3G. A:1, pET-32a total cell extract (uninduced) ;2, pET-32a total cell extract (induced); 3, pET-Vif total cell extract (uninduced); 4, pET-Vif total cell extract (induced); 5, pET-APOBEC3G total cell extract (uninduced); 6, pET-APOBEC3G total cell extract (induced). B: 1-6, Western blotting of A1-6 respectively; 7:Protein marker.

the each protein. Furthermore, Western blotting analysis of these fractions using an anti-His monoclonal antibody confirms this result (arrowed if Fig. 4B). The concentration of purified Vif protein was 0.45 mg/mL, and that of APOBEC3G was 0.87 mg/mL.

Immunogenicity evaluation of recombinant Vif and APOBEC3G

After repeated inoculation, the sera of rabbits were collected and antibodies to anti-Vif and anti- APOBEC3G measured by indirect ELISA.To confirm appropriate quantities of the antigen, serial dilutions of recombinant protein were made down to 60 ng/well for the Vif protein and 15 ng/well for the APOBEC3G. The antibody titer was calculated from the mean of four wells .The sera before immunization were used as negative control. The highest antibodies level was elicited after the immunization. The titer of antibodies to anti-Vif reached 1:204 800, and to anti-APOBE-

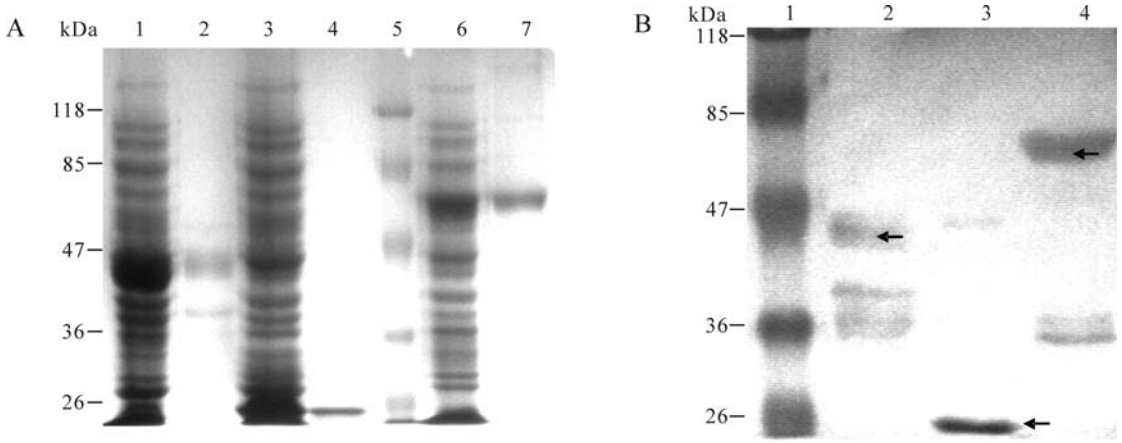

Fig. 4. SDS-PAGE and Western blotting analysis of purified fusion protein. A: 1, pET-Vif total cell extract (induced); 2, Purified fusion protein of Vif; 3, pET-32a total cell extract (induced); 4, Purified protein of enhancing solubility; 5, Protein marker; 6, pET-APOBEC3G total cell extract (induced); 7, Purified fusion protein of APOBEC3G. B: 1, Protein marker; 2, Purified fusion protein of Vif; 3, Purified protein of enhancing solubility; 4, Purified fusion protein of APOBEC3G.

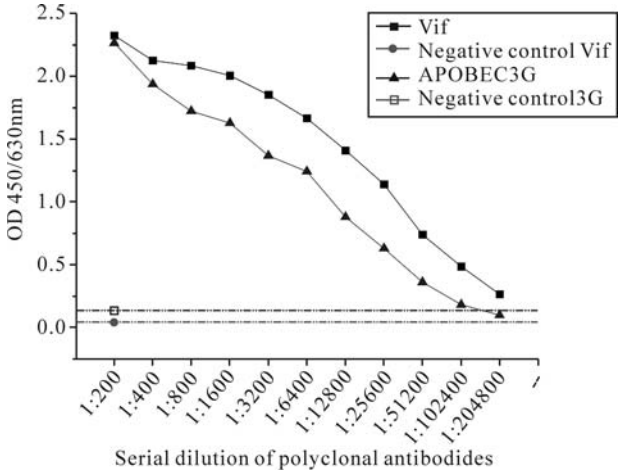

Fig. 5. Titer of polycloned antibodies detected by indirect ELISA.

C3G reached 1:102 400 (Fig. 5). In this study, the cutoff value of Vif was 0.038, and that of APOBEC3G was 0.134.

Identification of the pEGFP-vif

The recombinant plasmid of pEGFP-vif was generated by cloning the corresponding *vif* coding sequence obtained from the HIV-1 NL4.3 into the pEGFP-C3 vector. pEGFP-vif was digested with *Xho* I and *Bam*H I. Two fragments were obtained at about 576 bp and 4.7 kb respectively (Fig. 6). pEGFP-vif was then sequenced, and the result was compared with the submitted HIV-1 NL4.3 *vif* sequence (GenBank No.: AF070521). The result demonstrated that the homologous fragment of 576 bp maintained high similarity.

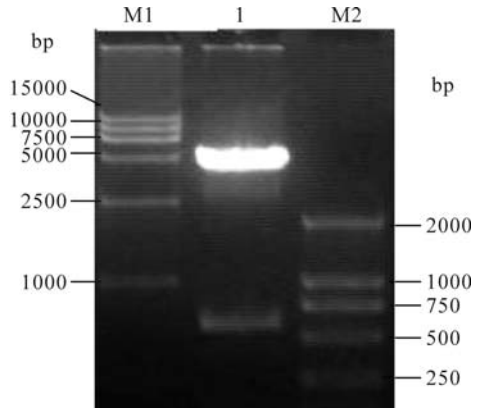

Fig. 6. Analysis of recombinant plasmid pEGFP-vif by *Xho* I & *Bam*H I digestion. 1, pEGFP-vif; M1/M2, DNA ladders.

Specificity evaluation of anti-Vif polyclonal antibodies

Hela-CD4-LTR-β-gal-CXCR4 cells were transfected with pEGEP-vif，which expresses HIV-1 Vif with a GFP tag, and the cells transfected with pEGFP-C3 only express green fluorescent protein. Observed by fluorescent microscope, Hela-CD4-LTR-β-gal-CXCR4 cells with green signal (Fig. 7A) demonstrated pEGFP-vif had been transfected into cells, and HIV-1 Vif and been expressed successfully. Figure 7B showed green fluorescent protein had been expressed, as the negative control. After immunofuorescence staining, the cells which expressed Vif showed saffron yellow fluorescence (Fig. 7C), and there was no change in cells which expressed green fluorescent protein only (Fig. 7D).

Specificity evaluation of anti-APOBEC3G polyclonal antibodies

Human cells can be divided into two categories based on whether they are able to support the replication of Vif-deficient HIV-1 (permissive cells) or unable to do so (non-permissive cells) (8). H9 cells (non-per-mis-sive T cells) expressed endogenous hA3G, whereas MT4 cells (permissive cells) failed to express hA3G. Immunoenzyme assay studies of H9 and MT4 cells (Fig. 8) revealed that H9 cells were positive for anti-APOBEC3G polyclonal antibodies, and MT4 cells were negative.

DISCUSSION

The viral infectivity of HIV is modulated by Vif.

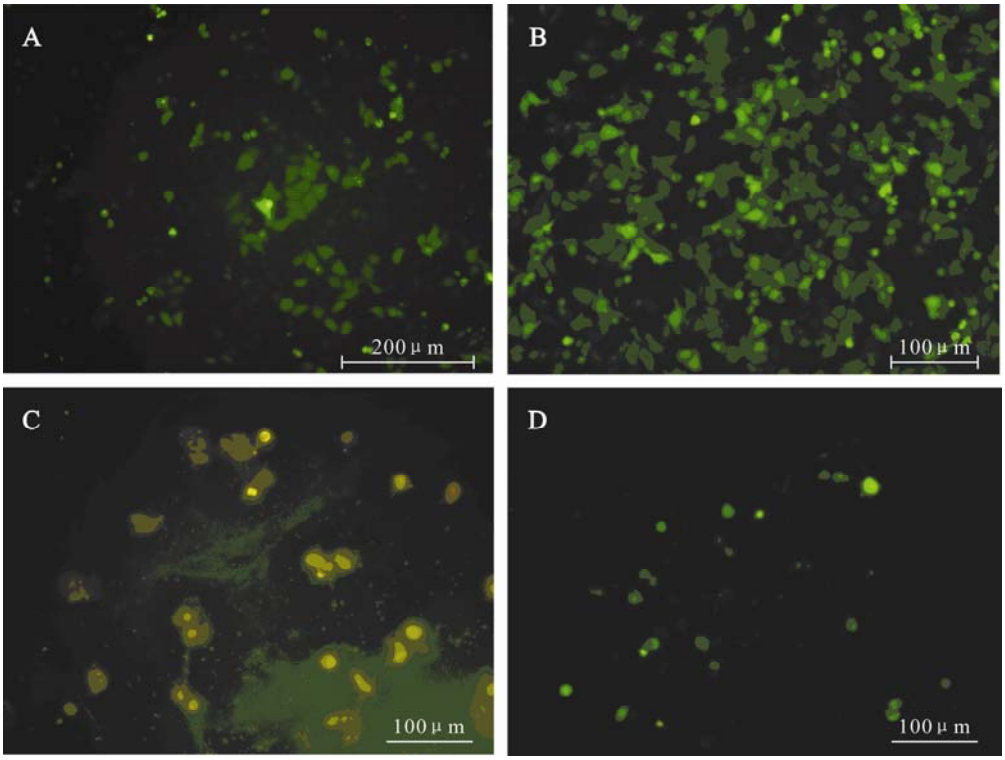

Fig. 7. Micrographs of immunofluorescence assay. A: Hela-CD4-LTR-β-gal-CXCR4 cells transfected with pEGFP-vif. B: Hela-CD4-LTR-β-gal-CXCR4 cells transfected with pEGFP-C3. C: Cells expressing Vif indicating fluorescent staining. D: Cells expressing GFP indicating fluorescent staining.

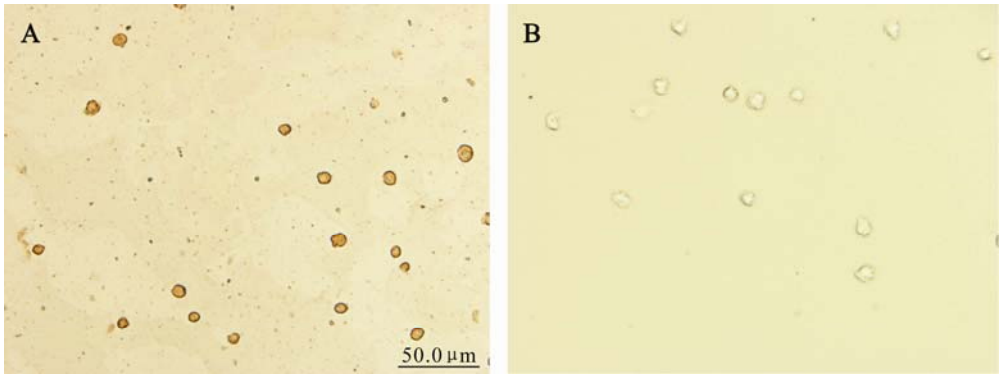

Fig. 8. Results of immunoenzyme assay. A: Stained H9 cells are clearly visible. B: No staining of MT4 cells is observed.

HIV-1 Vif is a 192-aminoacid protein conserved in all known lentiviruses with the exception of equine infectious anemia virus. Moreover, since Vif is essential for viral infection, its molecular characterization should provide information on potential antiretroviral strategies. The purpose of this study was to obtain the Vif protein and confirm its immunogenicity and antigenicity.

The T7-RNA-polymerase-promoter expression system is a tightly controlled bacterial expression system. The 22 kDa protein located before the Vif could enhance expression and solubility. In our experiments, *vif* or *APOBEC3G* DNA was inserted into the T7-promoter-controlled bacterial expression vector pET-32a and

the resulting Vif or APOBEC3G was successfully expressed at high levels within a few hours of IPTG inductino. Total cell content after sonication, including both the supernatant fluid (soluble-protein fraction) and the pellet (inclusionbody fraction), were analysed by SDS-PAGE and Western blotting. Most of the expressed Vif or APOBEC3G protein was accumulated in inclusion bodies. The recombinant protein could not be detected in the soluble fraction. The expressed recombinant protein in the inclusion-body contained histidine residues as a C-terminal marker sequence to facilitate purification and detection, which were beneficial in achieving high purity protein. SDS-PAGE and Western blotting indicated that no band wsa present priot to IPTG induction. After induction, the strong expression of a Vif-His fusion protein of about 45 kDa was detected corresponding to the expected molecular mass of the desired protein. However, there were faint bands below the expected band and this may be due to the instability of the expressed Vif and its susceptibility to proteolytic degradation (4), which is consistent with earlier observations in eukaryotic expression (12). An alternative explanation is that overexpressed Vif accumulated in *E. coli*, which promoted its degradtion. Nevertheless, it has been demonstrated a homogenous Vif protein with high purity was obtained using this technique.

The purified protein was used to immune the rabbits, and achived polyclonal antibodies which had high titer and excellent specificity.The titer of anti-Vif antibodies in srea of rabbits after immunization with this recombinant protein reached up to 1: 204800, and that of anti-APOBEC3G antibodies reached to 1:102400. These results ensure the immunogenicity and antigenicity of the purified recombinant proteins.

Immunoenzyme staining demonstrated that the anti-APOBEC3G polyclonal antibodies could detect APOBEC3G in H9 cells, but not in MT4 cells which did not express APOBEC3G. Anti-Vif polyclonal antibodies could detct HIV-1 Vif in Hela-CD4-LTR-β-gal-CXCR4 cells by immunofluorescence assay. The present study demonstrates the specificity of antibodies.

In summary, the results described above show that we have been able to obtain large amounts of purified protein using the pET-Vif or pET-APOBEC3G construct and can prepare high effectivity antibodies. The producion of Vif will be a subject of further structure study and a target of an anti-HIV durg.

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