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Research Article

Molecular Characterization of Heterologous HIV-1gp120 Gene Expression Disruption in *Mycobacterium bovis* BCG Host Strain: A Critical Issue for Engineering Mycobacterial Based-Vaccine Vectors

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Mycobacterium bovis Bacillus Calmette-Guérin (BCG) as a live vector of recombinant bacterial vaccine is a promising system to be used. In this study, we evaluate the disrupted expression of heterologous HIV-1gp120 gene in BCG Pasteur host strain using replicative vectors pMV261 and pJH222. pJH222 carries a lysine complementing gene in BCG lysine auxotrophs. The HIV-1 gp120 gene expression was regulated by BCG hsp60 promoter (in plasmid pMV261) and *Mycobacteria* spp. α-antigen promoter (in plasmid pJH222). Among 14 rBCG:HIV-1gp120 (pMV261) colonies screened, 12 showed a partial deletion and two showed a complete deletion. However, deletion was not observed in all 10 rBCG:HIV-1gp120 (pJH222) colonies screened. In this study, we demonstrated that *E. coli*/Mycobacterial expression vectors bearing a weak promoter and lysine complementing gene in a recombinant lysine auxotroph of BCG could prevent genetic rearrangements and disruption of HIV 1gp120 gene expression, a key issue for engineering Mycobacterial based vaccine vectors.

1. Introduction

The need for a safe and effective human immunodeficiency virus (HIV) vaccine has never been greater. The acquired immune deficiency syndrome (AIDS) epidemic update released by UNAIDS on December 2009, documented that the overall number of people with HIV has stabilized, albeit at an unacceptably high level of about 33.4 million people living with HIV and 2.7 million individuals being newly infected with the virus in 2008. The global AIDS epidemics killed 2 million people in 2008, and the number of children orphaned by AIDS may reach 25 millions by 2010. About 85% of these new infections occur in developing countries where access to antiretrovirals is still an enormous challenge [1, 2].

There is strong evidence to support a role of cytotoxic T lymphocytes (CTLs) in the containment of HIV replication [3–6] and several vaccine approaches have been pursued to elicit anti-HIV CTL responses [7]. One promising approach is to use *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) as a live recombinant bacterial vaccine vector. BCG vaccine has been used to immunize more than two billion individuals against tuberculosis with a long record of effectiveness and safety for use in humans [8]. CTL induction against HIV-1 and simian immunodeficiency virus (SIV) *gag* or *env* antigens has been described following the immunization of mice or nonhuman primates with recombinant BCG (rBCG) expressing these antigens [8, 9]. More recently, recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) expressing HIVA immunogen has

been generated and shown to be stable and to induce durable and high-quality HIV-1-specific CD4+ and CD8+ T-cell responses in BALB/c mice. Furthermore, when the recombinant BCG vaccine was used in a priming-boosting regimen with heterologous components, the HIV-1-specific responses provided protection against surrogate virus challenge, and the recombinant BCG vaccine alone protected against aerosol challenge with *M. tuberculosis* [10].

Three issues are critical for engineering a stable and immunogenic mycobacterial-based vaccine vector: (i) antigen localization, (ii) codon optimization, and (iii) in vivo plasmid DNA stability and genetic rearrangements. The antigen secretion and fusion of foreign antigens to mycobacterial surface lipoproteins will provide these antigens with access to the major histocompatibility complex (MHC) class I pathway and subsequently enhance the immunogenicity and to prevent foreign proteins from becoming toxic to BCG [11]. The use of mycobacterial optimal codons will enhance the transcriptional/translational activity of the foreign gene. The plasmid stability in vivo is essential for heterologous gene expression [8].

Genetic rearrangements have been described occurring in eukaryotes and prokaryotes [12]. Homologous recombination, which takes place between repeated DNA sequences, is one of the most important mechanisms for bacterial genome rearrangements. Any repeated DNA sequences on a chromosome can induce homologous recombination, but insertion sequences (IS) are among the most abundant of these [13–15]. Mycobacteria have been reported to contain several IS. In *Mycobacterium tuberculosis*, it has been suggested that IS6110 is an important source of genome variation. However, the role of such IS elements has not been thoroughly explored in heterologous gene expression in mycobacteria [16–18].

Expression of heterologous antigen in recombinant BCG necessarily imposes a metabolic burden. The extent of this burden will determine the degree to which fitness of the recombinant BCG is compromised. Thus, the relative level of fitness of the recombinant and any derived mutants will determine the rate at which the inserted element (structural instability) or its expression (functional instability) is lost from the bacterial population [19].

Husson et al. constructed a plasmid shuttle vector that allows insertion of foreign DNA and stable integration in the mycobacterial genome by homologous recombination. This shuttle vector was developed to express foreign antigens in *M. smegmatis* [20]. The structural instability of several recombinant plasmids expressing the cirscumsporozoite protein (CSP aa 18–391) from *Plasmodium falciparum* was described by Haeseleer [21]. The analysis in *M. smegmatis* and BCG of numerous rearranged plasmids showed that the presence of a functional specific expression cassette was responsible for the plasmid instability and that the observed deletions seemed to come from recombination between homologous and nonhomologous sequences [21].

Kumar et al. monitored the role of such genetic rearrangement events in the expression of cloned *Escherichia coli lacZ* gene in *Mycobacterium smegmatis* host strain using integrative (pMV361::*lacZ*) and episomal (pMV261::*lacZ*) vectors. The *lacZ* gene present in both vectors was the mutable target within the vector and simple color detection assay in mycobacteria was used to screen the genetic rearrangements during replication in *Mycobacterium smegmatis*. They reported that a loss of *lacZ* phenotype was due to the insertion of an IS element in the *lacZ* gene of the integrative vector (frequency 1.7×10^{-5}), whereas in the case of the replicative vector, the loss of *lacZ* phenotype was due to deletions of different sizes in the *lacZ* gene and the hsp60 promoter region (frequency 2×10^{-3}) [22].

In this study we evaluated the expression of disrupted heterologous HIV-1gp120 gene, from SHIV-HXBc2P 3.2 clone (GenBank accession number AF041850), by genetic rearrangements in *Mycobacterium bovis* BCG host strain using a replicative vector (pMV261) regulated by BCG hsp60 promoter (strong promoter). To compare the HIV-1gp120 gene expression and plasmid DNA stability in vivo, the replicative (pJH222) and integrative (pJH223) vectors carrying a wild-type lysine-complementing gene in the lysine auxotroph of BCG host strain were used. In these vectors, the HIV-1gp120 gene expression was regulated by *Mycobacteria spp.* α-antigen promoter (weak promoter). We have demonstrated that the use of weak promoters (*Mycobacteria spp.* α-antigen promoter) to regulate HIV-1gp120 gene expression and BCG lysine auxotrophs complemented with a lysine gene do, in fact, prevent the disruption of gene expression caused by genetic rearrangements.

2. Materials and Methods

2.1. Bacterial Strains and Culture Methods. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth (Sigma-Aldrich, USA) or on LB agar plates (Sigma-Aldrich, USA) at 37°C. LB was supplemented with kanamycin (40 μg/mL) (Sigma-Aldrich, USA). Mycobacterial cultures were grown in Middlebrook 7H9 broth (Becton-Dickinson, USA) or on Middlebrook 7H10 agar medium (Becton-Dickinson, USA) supplemented with 10% albumin-dextrose-catalase (ADC) (Becton-Dickinson, USA) and containing 0.05% Tween 80 (Sigma-Aldrich, USA) and kanamycin (25 μg/mL). The L-lysine monohydrochloride was purchased from Sigma-Aldrich (USA), dissolved in distilled water and used at a concentration of 40 μg/mL.

2.2. Construction of Expression Vectors. Tables 2 and 3 listed the parental *E. coli*/mycobacterial shuttle vectors used for cloning the HIV-1gp120 gene from SHIV-HXBc2P 3.2 clone (GenBank accession number AF041850) and the constructs. Plasmid DNA vectors pMV261 (4,480 bp), pJH222 (6,423 bp), and pJH223 (6,313 bp) were used as parental plasmids for all plasmid DNA constructs obtained as described below. The entire DNA coding sequence of HIV1gp120 (1,578 bp) antigen was synthesized by polymerase chain reaction (PCR), using oligonucleotide primers specific for HIV-1 gp120 gene and cloned into the different *E.coli*/mycobacterial shuttle vectors.

Table 1: List of bacterial strains used in this study.

Bacterial strains	Relevant characteristics	Reference or source
Mycobacterium bovis BCG	1173 P2 Pasteur strain	[23]
Mycobacterium bovis BCG mc^2 1604	Pasteur ΔlysA5::res	[24]
Escherichia coli JM109	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 ($rK^- mk^+$) *supE*44 *relA*1 Δ(*lac-proAB*)/F′ [*traD*36*proAB*$^+$ *lac I* q *lacZ*ΔM15]	GIBCO BRL

Table 2: List of parental *E. coli*/mycobacterial shuttle vectors used in this study.

Plasmid DNAs	Relevant characteristics	Reference or source
pMV261	*E. coli*/mycobacterial shuttle vector Multicopy extrachromosomal vector Kanamycin resistant	[25]
pJH222	pMV261 derivative Multicopy extrachromosomal vector Kanamycin resistant Lysine A complementing gene	Kindly provided by Bloom and Jacobs laboratory
PJH223	pMV361 derivative Monocopy integrative vector Kanamycin resistant Lysine A complementing gene	Kindly provided by Bloom and Jacobs laboratory

Plasmid DNA pMV261 is a replicative vector (multicopy, extrachromosomal) that contains a DNA cassette encoding kanamycin resistance (Tn903-derived *aph* gene), an *E. coli* origin of replication (*oriE*), a mycobacterial plasmid DNA origin of replication (*oriM*), an expression cassette containing a mycobacterial promoter, a multiple cloning site, and a transcriptional terminator. For pMV261::HIVgp120, the HIVgp120 gene was fused to the first six codons of the cytoplasmically expressed BCG hsp60 protein. For immunodetection purposes, we fused downstream of the HIV-1 gp120 gene with the influenza virus hemagglutinin epitope (HA) coding sequence and six residues of histidine. The HIV-1 gp120 gene expression was regulated by BCG hsp60 promoter. The primers were designed to incorporate BamHI (forward 5′-CAAGGATCCGAAATTGTGGGTCACAGTC-3′) and HindIII (reverse 5′-CAGAAGCTTCTAGTGGTG-GTGGTGGT-3′) sites at the 5′ and 3′ termini of the amplified DNA fragment to be cloned into the pMV261 plasmid DNA at 4,326 bp-BamHI/4,346 bp-HindIII.

Plasmid DNA pJH222, a derivative of pMV261, contains also the *lys*A complementing gene, under the regulatory control of BCG *hsp60* promoter. For the pJH222::HIV-1 gp120 construct, the HIV-1 gp120 gene was fused to the 5′ region encoding the 19 kDa lipoprotein signal sequence from *Mycobacterium tuberculosis*, and the HA epitope and six residues of histidine were fused downstream. The HIV-1gp120 gene expression was under the control of *Mycobacteria spp*. α-antigen promoter. The primers were designed to incorporate HindIII (forward 5′-CAGAAGCTTGGGCCC-GAAAAA-3′) and PstI (reverse 5′-CAACTGCAGCTAGTG-GTGGT-3′) sites at the 5′ and 3′ termini of the amplified DNA fragment to be cloned into the pJH222 plasmid DNA (335 bp-HindIII/341 bp PstI).

Plasmid DNA pJH223 is an integrative vector (single-copy, integrated). It contains a DNA cassette encoding kanamycin resistance (Tn903-derived *aph* gene), an *E. coli* origin of replication (*oriE*), a DNA segment carrying the attachment site (*attP*) and the integrase (*int*) gene from the mycobacteriophage L5 [26], the *lys*A complementing gene under the regulatory control of BCG hsp60 promoter, and an expression cassette containing a mycobacterial promoter, a multiple cloning site and a transcriptional terminator. The pJH223::HIV-1 gp120 plasmid DNA was constructed using the same method as for pJH222::HIV-1 gp120. The primers were designed to incorporate HindIII sites (forward 5′-CAGAAGCTTGGGCCCGAAAAA-3′ and reverse 5′-CAA-AAGCTTCTGCAGCTAGTGGTGG-3′) at the 5′ and 3′ termini of the amplified DNA fragment to be cloned into the pJH223 plasmid DNA (2,134 bp-HindIII).

The correct cloning of the HIV-1gp120 gene into the plasmid DNA and the deletion of the ∼900 bp fragment were confirmed by DNA sequencing, PCR and enzyme restriction analysis performed following standard protocols [27].

2.3. Electroporation of Mycobacteria. The BCG wild type host strain was transformed with pMV261::HIV-1 gp120 plasmid DNA and lysine auxotroph of BCG was transformed with pJH222::HIV-1gp120 and pJH223::HIV1gp120 plasmid DNA by electroporation. BCG cultures were grown to an OD of 0.9 (600 nm), pelleted at 3,000 rpm, washed twice by resuspension and centrifugation (3,000 rpm) in 10% glycerol at 4°C and finally resuspended in 1/20th of the original culture volume with cold 10% glycerol. Then 100 μL of the cold BCG suspension was mixed with plasmid DNA (50–500 ng) in a

Table 3: Constructs obtained using *E. coli*/mycobacterial shuttle expression vectors to express HIV-1gp120 antigen (HXBc2).

Constructs	Promoter	Secretion signal	Resistance Gene	Complementing gene	Tag
pMV261:HIV-1 gp120	BCG hsp60	None	Kanamycin	None	HA His
pJH222:HIV-1 gp120	α-antigen of *Mycobacterium spp*	19 kDa lipoprotein (*M. tuberculosis*)	Kanamycin	Lysine	HA His
pJH223:HIV-1 gp120	α-antigen of *Mycobacterium spp*	19 kDa lipoprotein (*M. tuberculosis*)	Kanamycin	Lysine	HA His

HA: hemagglutinin epitope tag from influenza virus; His: Histidine residues.

prechilled 0.2 cm electroporation cuvette and transformed using the Biorad Gene Pulser electroporator at 2.5 kV, 25 mF, and 1,000 Ω. After electroporation 1 ml of 7H9 medium, supplemented with ADC and containing 0.05% Tween 80, was added and incubated at 37°C for 12 hours before plating on Middlebrook agar 7H10 medium supplemented with 10% ADC and containing 0.05% Tween 80 and kanamycin (25 μg/mL).

2.4. Detection of the Fragment DNA Deletion. After transformation by electroporation, the rBCG colonies were used as templates for the PCR analysis. Different rBCG colonies were inoculated into 50 μL of distilled water, vortexed and 1 μL was used for the PCR analysis. Specific primers for cloning the HIV-1 gp120 gene into pMV261, pJH222 and pJH223 vectors were used. The plasmid DNA before BCG transformation (pre-BCG) was used as positive control for the PCR analysis.

2.5. Mycobacterial Plasmid DNA Isolation for DNA Sequencing and Enzyme Restriction Mapping. The QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) was used for *E. coli* and mycobacteria plasmid DNA purification. Different rBCG colonies that showed the DNA fragment deletion by PCR analysis were inoculated in mycobacterial broth culture up to an OD of 0.9 (600 nm). This BCG culture was used for mycobacterial plasmid DNA isolation. For mycobacteria, the standard QIAprep Spin Miniprep protocol was used with a slight modification: after resuspension in buffer P1, lysozyme was added at a concentration of 10 mg/mL and samples were incubated at 37°C overnight. For improving the purified plasmid yield, the mycobacterial plasmid DNAs from selected rBCG colonies were transformed in *E. coli* JM109 using 5 μL of the isolated plasmid DNAs, and purified according to the manufacturer's instructions for DNA sequencing and enzyme restriction mapping. The plasmid DNA before BCG transformation (pre-BCG) was used as positive control for the enzyme restriction analysis. The nucleotide sequences were obtained using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI PRISM 3100 Genetic Analyzer. Multiple DNA sequence alignments were performed using the Clustal software.

2.6. Western Blot Analysis. BCG transformants were grown to mid-logarithmic phase in liquid 7H9 medium supplemented with 10% ADC and containing 0.05% Tween 80 and kanamycin (25 μg/ml). rBCG cultures were centrifuged at 3,000 rpm for 10 minutes at 4°C. Pellets were washed twice in Phosphate Buffered Saline (PBS) plus 0.02% Tween 80 and resuspended in 1 ml of extraction buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.6% sodium dodecyl sulfate), and added with 5 μl of 100x protease inhibitor cocktail (1 mg/ml aprotinin, 1 mg/ml E-64, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 50 mg/ml pefabloc SC, and 10 ml dimethyl sulfoxide). Cells were sonicated for 4 minutes on ice with a Branson sonicator at output control seven and duty cycle of 50%. Extracts were centrifuged at 13,000 rpm for 10 minutes at 4°C and supernatants were collected. Proteins were separated on 15% SDS-polyacrylamide gel. After electroblotting, nitrocellulose membranes were first probed with a 1 : 1000 dilution of mouse monoclonal antibody HA.11 (Covance, USA) directed to influenza virus hemagglutinin epitope (YPYDVPDYA) followed by a 1 : 2000 dilution of horseradish peroxidase (HRP)-conjugated antibodies. The membranes were developed using chemoluminiscent ECL (GE Healthcare, USA) as HRP substrate.

3. Results

3.1. PCR Detected a ~900 bp-DNA Fragment Deletion in the HIV-1gp120 Gene. As shown in Figure 1, PCR analysis of rBCG:HIV-1gp120(pMV261) colonies detected a partial deletion of the HIV-1gp120 DNA coding sequence using the specific primers that were used for cloning the DNA sequence into the pMV261 vector. This deletion was reproduced in 12 out of 14 rBCG:HIV-1 gp120 (pMV261) colonies analyzed (data not shown). In addition, this partial deletion was observed only by PCR of plasmid DNA from BCG colonies in which the HIV-1gp120 DNA fragment was inserted into the pMV261 vector and the gene expression was regulated by the hsp60 promoter from BCG (Figure 1, lane 2). The deletion was not detected when the DNA fragment encoding the HIV-1gp120 antigen was cloned into pJH222 and pJH223 vectors under *Mycobacteria spp.* α-antigen promoter regulation (Figure 1, lanes 4 and 6). The deletion was not detected in all 10 rBCG:HIV-1gp120 (pJH222) colonies screened by PCR (data not shown).

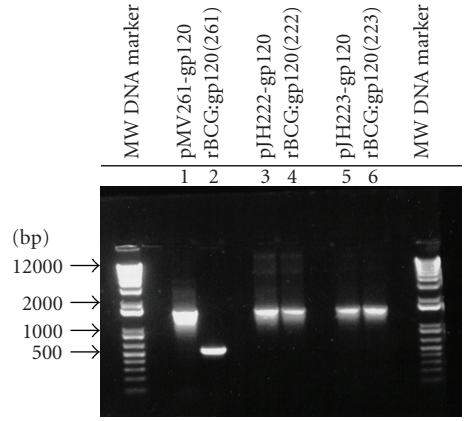

Figure 1: PCR analysis of the HIV-1gp120 gene cloned into pMV261, pJH222 and pJH223 vectors. *Lane 1, 3 and 5* are PCR products of the HIV-1gp120 gene (1578 bp) cloned into pMV261 (*lane 1*), pJH222 (*lane 3*) and pJH223 (*lane 5*) vectors, respectively. Plasmid DNA before BCG transformation (pre-BCG) were used as positive controls (lane 1, 3 and 5). *Lane 2, 4 and 6* are PCR products from the rBCG:HIV-1gp120 (pMV261), rBCG:HIV-1gp120 (pJH222) and rBCG:HIV-1gp120 (pJH223) colonies.

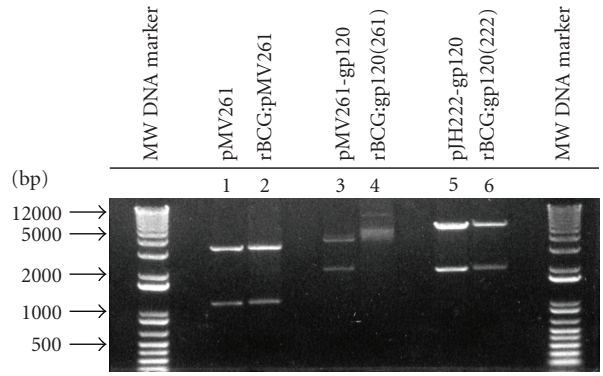

Figure 2: Enzymatic restriction analysis of pMV261::HIV-1gp120 and pJH222::HIV-1gp120 plasmid DNA after BCG transformation. *Lane 1,* pMV261 plasmid DNA without HIV-1 gp120 DNA insert digested with BglII; *L*ane 2 pMV261 plasmid DNA without HIV-1 gp120 DNA insert from BCG colonies, digested with BglII, *Lane 3,* pMV261-HIV-1gp120 plasmid DNA digested with SacII; *lane 4*, pMV261-HIV-1gp120 plasmid DNA from rBCG:HIV-1gp120 colonies, digested with SacII; *lane 5,* pJH222-HIV-1gp120 plasmid DNA digested with HpaI and *lane 6*, pJH222-HIV-1gp120 plasmid DNA from rBCG:HIV-1 gp120 colonies, digested with HpaI. Plasmid DNAs before BCG transformation (pre-BCG) were used as positive controls (lane 1, 3 and 5).

3.2. Enzymatic Restriction Analysis Revealed a ~900 bp-DNA Fragment Deletion. We also compared the restriction enzyme digestion profile of the pMV261 and pJH222 plasmid DNA vectors with and without heterologous HIV-1gp120 DNA insert, pre- and post-BCG transformation (Figure 2). The pMV261 plasmid DNA vector without the DNA insert corresponding to HIV-1gp120 gene did not show any difference in the enzymatic restriction profiles from pre- and post-BCG transformations (Figure 2**,** lanes 1 and 2). The same result was obtained for the pJH222 plasmid DNA vector containing the DNA insert (Figure 2, lanes 5 and 6). In contrast, the pMV261 containing the HIV-1gp120 insert showed different enzymatic restriction profiles from pre- and post-BCG transformations (Figure 2, lanes 3 and 4).

3.3. The ~900 bp-Partial DNA Fragment Deletion Corresponds to the HIV-1gp120 Core. Following sequence analysis and molecular characterization of the deleted DNA fragment in the 10 rBCG:HIV-1gp120 (pMV261) mutant colonies, we demonstrated that the partial deletion (~900 bp) corresponds to gp120 core rich in potential glycosylation sites and the variable regions V3, V4, and V5 of HIV-1 *env*, a key structure involved in the HIV union to its receptor (CD4) and co-receptor on the host cell surface. Strikingly, the deleted DNA fragment in the rBCG mutants contained the DNA sequence coding for HIV-1 envelope immunodominant CTL, murine H-2D^{d}-restricted epitope P18-I10 (Figure 3). Deletion in HIV-1gp120 gene was not observed in any rBCG:HIV-1gp120 (pJH222) and rBCG:HIV1gp120 (pJH223) colonies that carry the pJH222:HIV-1gp120 and pJH223:HIV1gp120 vectors containing the *Mycobacteria spp*. α-antigen promoter to regulate the HIV1gp120 gene expression, indicating that the deletions were not due solely to the toxic effects of HIV-1gp120 expression during growth of the recombinant BCG strains.

3.4. A Truncated Protein Was Expressed in rBCG Mutants That Show the ~900 bp-DNA Fragment Deletion. Expression of HIV-1gp120 protein by rBCG:HIV-1gp120 (pMV261 and pJH222) strains was confirmed by SDS-PAGE and western blot analysis of whole-cell BCG lysates. The chimeric 19 kDa lipoprotein signal sequence-HIV-1gp120-Flu-His recombinant protein, with a relative molecular mass (Mr) of 67 kiloDalton (kDa) was present in lysates of rBCG:HIV-1gp120 (pJH222) bacterial cells containing the 1.82 kilobase-pair (kbp) HIV-1gp120 DNA coding sequence in pJH222 vector. The apparent Mr of HIV-1gp120 protein was consistent with that predicted by the gene sequence without post-translation modification. By contrast, when all rBCG:HIV-1gp120 (pMV261) mutant colonies were analyzed by western blot, we detected a band of 28 kDa (truncated protein) instead of the expected 67 kDa protein (Figure 4).

4. Discussion

Mycobacterium tuberculosis infection is a major cause of human morbidity and mortality, and vaccine is the most cost-effective intervention to prevent disease. M*ycobacterium bovis* BCG is a widely used vaccine against tuberculosis, and a single dose given at birth confers long-lasting immunity. *Mycobacterium bovis* BCG has been suggested as an ideal delivery system for expression of foreign antigens due to the long persistence of BCG in the immunized host. Thus, *Mycobacterium bovis* BCG not only is used as vaccine against

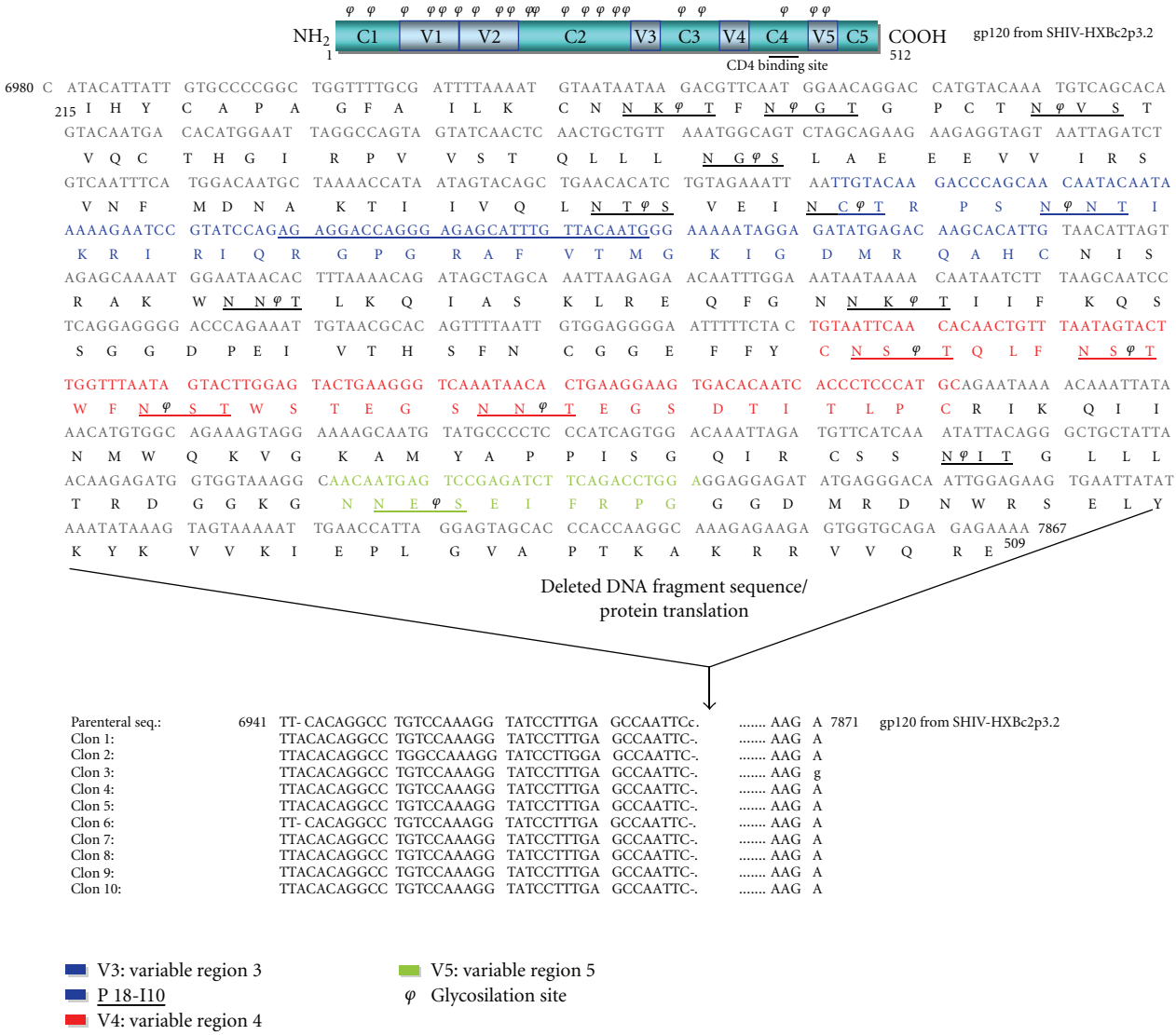

Figure 3: Multiple HIV-1gp120 DNA sequence alignment carrying the ~900-bp deletion from the mutant rBCG:HIV-1gp120 (pMV261) colonies. HIV-1gp120 DNA sequences of 10 mutant rBCG:HIV-1gp120 (pMV261) colonies were aligned and compared with the parental nucleotide sequence of the HIV-1gp120 gene. The ~900 bp deleted fragment (sequence and position shown in the scheme) structurally corresponds to the gp120 core (see protein translation).

tuberculosis, but also offers great potential for innovative approaches for development of polyvalent vaccines [25, 28–30]. In vivo genetic stability and persistence are of special importance for the use of live bacterial vaccines. A mutant BCG which is rapidly eliminated by the host immune system is unlikely to be an effective vaccine [31]. The rearrangement of DNA is one of the fundamental properties of life, including both prokaryotes and eukaryotes, with great biological significance. Some of these DNA rearrangements are important in controlling gene expression in specific cell types; others may play an evolutionary role by contributing to genetic diversity.

Some studies using episomal vectors in rBCG reported high levels of stability in vitro. For example, plasmids responsible for expressing *M. Tuberculosis* α-antigen in BCG were stable over six consecutive 4-week cultures without selection and changes in the level of expression [32]. An α-antigen-HIV-1 V3J1 chimeric protein was secreted by rBCG for at least 450 passages in vitro [33]. However, in vitro instability of episomal vectors has also been reported regularly. Lim et al. [34] described that expression of SIV mac 251 *env* (aminoacids, aa, 1-245) by rBCG was stable in culture (pBlaF promoter), but rBCG harboring the same vector containing aa 1-521 or aa 215-521 were unstable. Chawla and Das Gupta [35] reported that the disruption of pAL5000-derived vectors in *M.smegmatis* through transposition of an insertion sequence upstream of the kanamycin resistance gene led to structural instability and large deletions. Kumar et al. studied the stability of the expression of *Escherichia coli* β-galactosidase under the

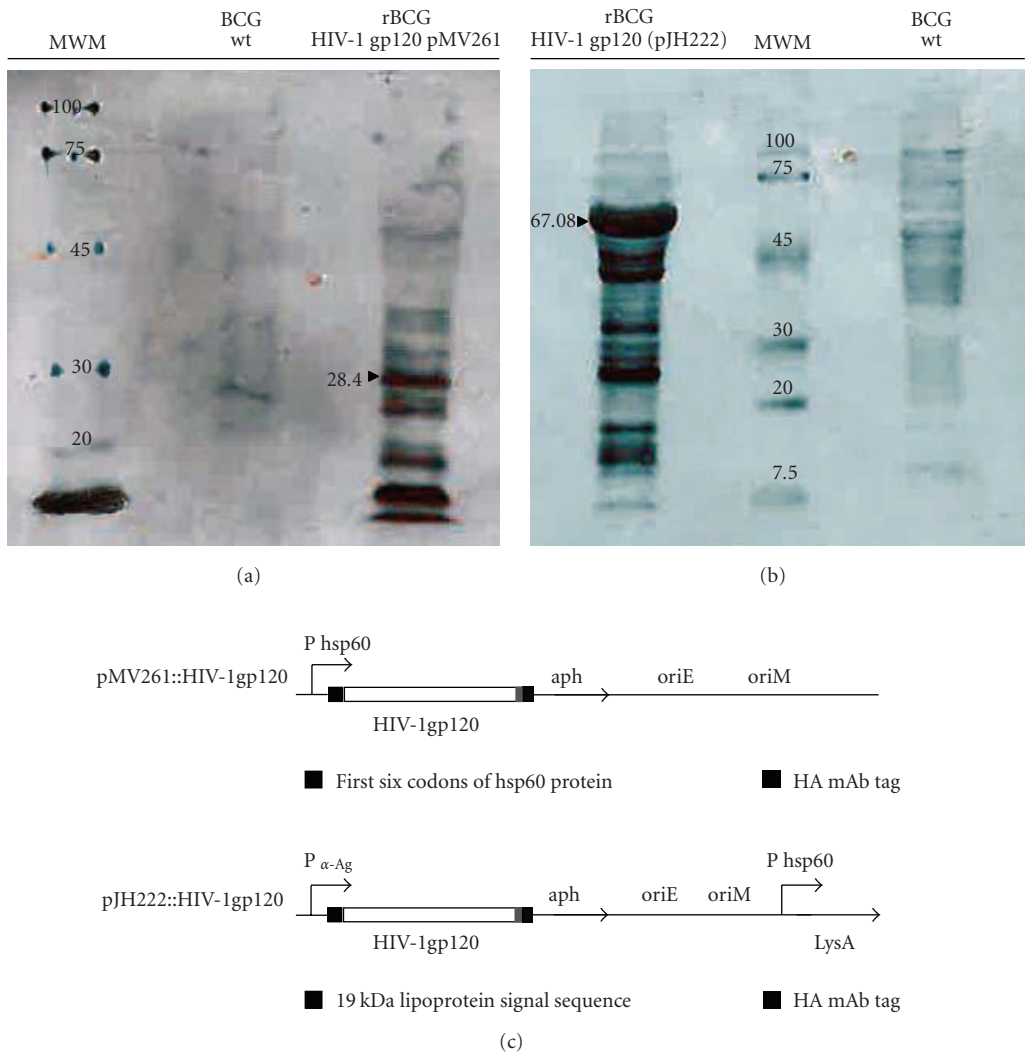

Figure 4: Expression of HIV-1gp120 protein in rBCG:HIV-1gp120 (pMV261) and rBCG:HIV-1gp120 (pJH222) strains by Western blot analysis of whole-cell BCG lysates. (a) A band of ~28 kDa was detected when analizing the rBCG:HIV-1gp120 (pMV261) colonies. (b) A 67 kDa protein was detected in lysates of rBCG:HIV-1gp120 (pJH222) bacterial cells containing the 1.82 kilobase (kb) HIV-1gp120 DNA coding sequence into pJH222 vector. The gp120 expression was determined using anti-HA MAb. Not transformed BCG (BCG wild type) were utilized as negative controls. (c) The HIV-1gp120 (HXBc2) gene was cloned into the E.coli/mycobacteria shuttle plasmids pMV261 and pJH222 (both multicopy and episomal). The gp120 *env* in the plasmids is under the control of BCG hsp60 promoter (P hsp60) and *M.Tuberculosis* α-antigen promoter (P α-Ag). Both plasmids contained kanamycin resistance gene (aph) and an *E.coli* origin of replication (ori E) and a mycobacterial origin of replication (oriM). In addition, pJH222 contained the complementing lysA gene. The HIV-1gp120 gene was fused to the first six codons of BCG hsp60 (pMV261) or fused to *M. tuberculosis* 19-kDa lipoprotein signal sequence (pJH222). MWM, molecular weight markers.

control of hsp60 promoter in *Mycobacterium smegmatis* using integrative and replicative vectors. In the case of replicative vectors, they detected higher frequencies of *lacZ* expression inactivation as compared with the same *lacZ* cassette, when present in integrated state [22]. However, Springer et al. [36] reported a high-frequency loss of pMV361-based integrating vectors from *M. smegmatis* and BCG. Al-Zarouni and Dale investigated the effects of various combinations of posttranslational signals and promoters on heterologous gene expression and stability in different BCG strains. Plasmid DNAs were constructed using mycobacterial promoters (hsp60, 19-kDa antigen, 85A antigen, from *Mycobacterium tuberculosis* complex, and the 18-kDa antigen from *Mycobacterium leprae*) and post-translation signals (85A antigen secretion and 19-kDa antigen acylation signals), coupled with reporter genes. They provided evidence that the 85A secretion signal markedly enhanced the levels of cell-associated product, while the 19-kDa acylation signal had little effect on gene expression. This group also demonstrated that the hsp60 promoter caused plasmid DNA instability and showed that various deletions in the promoter region occurred during or soon after transformation, but not during subsequent growth of the transformants, nor with other promoters [37]. Gross

instability was also observed when the episomal vectors were used to express rotavirus VP6 protein under the control of hsp60 promoter [38]. The possibility of inserting foreign genes into the chromosome at precise positions to ensure the persistence of the heterologous genetic information in the recombinant vaccine strains would represent a crucial step in the development of *Mycobacterium bovis* BCG as a live vaccine vector for expression of heterologous antigens [28, 39]. Even though integrative plasmid DNAs have been shown to be more stable as they are incorporated into the chromosome via homologous recombination, Baulard and colleagues demonstrated that with replicating plasmid DNAs, relatively high levels of homologous recombination were obtained in fast- and slow-growing mycobacteria, and 100% of the selected clones underwent homologous recombination [40].

Thus, if BCG is to be used as a live bacterial carrier for generating novel vaccine candidates and as an immunotherapeutic agent, it is essential that a more genetically stable strain be developed. Sander and collaborators demonstrated that *Mycobacterium bovis* BCG *recA* mutants are a valuable tool for the further development of BCG as an antigen delivery system to express foreign antigens and as a source of a genetically stable vaccine against tuberculosis [41]. This was confirmed in a study performed by Keller and coworkers where it was observed that *recA* inactivation in BCG Russia strain was in part responsible for its high degree of genomic stability, resulting in a substrain that has fewer genetic alterations than other vaccine substrains with respect to *M. bovis* AF2122/07 wild-type [42].

Expression of HIV genes in BCG is not always possible. Lack of success in this respect may be due to overexpression of lethality or other forms of protein toxicity. Stover et al. were unable to express HIV-1 gp120 (hsp60 promoter) from an episomal vector, but could express it from an integrative vector [19, 25].

In our study, we have observed that, in the case of the replicative pMV261 vector, disruption of HIV-1gp120 gene expression was due to a consensus DNA fragment deletion in the HIV1gp120 gene detected in all ten different BCG clones. By contrast, we did not detect any HIV-1gp120 gene expression disruption in the replicative (pJH222) and integrative (pJH223) vectors. Sequence analysis indicated that the DNA fragment deletion contained several potential glycosylation sites, the variable regions V3, V4 and V5 and a CTL immunodominant epitope (P18-I10 peptide). Based on this observation, we hypothesized that the loss of expression of this immunodominant epitope could help mycobacteria to escape the host's immunological response and may represent part of an ongoing adaptation to survival in host environments that are screened by immunological defense mechanisms. Huber and collaborators have described a natural loss of expression of highly immunogenic proteins caused by a variety of genomic changes in *Mycobacterium ulcerans* that may confer a selective advantage to this emerging pathogen [43]. On the other hand, the deleted DNA sequence in the rBCG:HIV-1gp120(pMV261) mutant colonies analyzed in our study ($n = 10$) was highly conserved, which is in disagreement with Dennehy et al. that deletions in the VP6 heterologous gene (hsp60 promoter) in rBCG were random and different among clones examined [38].

We have demonstrated that the use of weak promoters (*Mycobacteria spp*. α-antigen promoter) to regulate HIV-1gp120 gene expression and BCG lysine auxotrophs complemented with a lysine gene do, in fact, prevent the disruption of gene expression caused by genetic rearrangements. The results showed that such genetic rearrangements can significantly affect the expression of foreign genes in mycobacteria. Thus, it could result in an rBCG that does not induce optimal immune responses to the HIV antigen.

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) as a live vector of recombinant bacterial vaccine is a promising system to be used. However, few promising approaches have emerged in the last five years to overcome the genetic instability in rBCG-based vaccines. To prevent the plasmid instability in vivo and in vitro and the genetic rearrangement by mycobacteria, different approaches should be considered: (i) the use of expression vectors containing small HIV DNA coding sequences, (ii) DNA fragments lacking glycosilation sites, (iii) the use of weak promoters, (iv) the use of BCG auxotrophic strains (containing the complementing gene in the expression vectors), (v) the use of inducible promoters, (vi) codon optimization of the recombinant gene, (vii) the choice of expression vector backbone and (viii) antigen secretion to enhance the immunogenicity and to prevent foreign proteins from becoming toxic to BCG.

In five years' time we will have a better picture of how the immune responses induced by rBCG could be enhanced, and what sort of route, doses, immunization schedule, timing for immunological assays are likely to induce optimal responses in mice and monkeys. Heterologous prime-boost regimen, different immunization schedule, routes and doses, should be performed to evaluate the potential for enhancing specific immune responses.

Mycobacterium Bovis BCG is a promising approach as a bacterial live recombinant vaccine vehicle. BCG has a long record of safe use in humans and is able to induce long-lasting immunity. In addition, it could be the best hope for protecting newborn infants, reducing the adult burden of HIV infection and protecting neonates against vertical transmission.

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