

Genetic Alteration of *Mycobacterium smegmatis* To Improve *Mycobacterium*-Mediated Transfer of Plasmid DNA into Mammalian Cells and DNA Immunization[∇]

Yongkai Mo,^{1†} Natalie M. Quanquin,^{1†} William H. Vecino,^{1‡} Uma Devi Ranganathan,¹ Lydia Tesfa,¹ William Bourn,² Keith M. Derbyshire,^{3,4} Norman L. Letvin,⁵ William R. Jacobs, Jr.,^{1,6} and Glenn J. Fennelly^{7,8*}

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461¹; Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory, 7925, Cape Town, South Africa²; Division of Infectious Disease, Wadsworth Center, New York State Department of Health,³ and Department of Biomedical Sciences, University at Albany,⁴ Albany, New York 12201; Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115⁵; Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York 10461⁶; Department of Pediatrics, Albert Einstein College of Medicine, Bronx, New York 10461⁷; and The Lewis M. Fraad Department of Pediatrics, Jacobi Medical Center, Bronx, New York 10461⁸

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Mycobacteria target and persist within phagocytic monocytes and are strong adjuvants, making them attractive candidate vectors for DNA vaccines. We characterized the ability of mycobacteria to deliver transgenes to mammalian cells and the effects of various bacterial chromosomal mutations on the efficiency of transfer in vivo and in vitro. First, we observed green fluorescent protein expression via microscopy and fluorescence-activated cell sorting analysis after infection of phagocytic and nonphagocytic cell lines by *Mycobacterium smegmatis* or *M. bovis* BCG harboring a plasmid encoding the fluorescence gene under the control of a eukaryotic promoter. Next, we compared the efficiencies of gene transfer using *M. smegmatis* or BCG containing chromosomal insertions or deletions that cause early lysis, hyperconjugation, or an increased plasmid copy number. We observed a significant—albeit only 1.7-fold—increase in the level of plasmid transfer to eukaryotic cells infected with *M. smegmatis* hyperconjugation mutants. *M. smegmatis* strains that overexpressed replication proteins (Rep) of pAL5000, a plasmid whose replicon is incorporated in many mycobacterial constructs, generated a 10-fold increase in plasmid copy number and 3.5-fold and 3-fold increases in gene transfer efficiency to HeLa cells and J774 cells, respectively. Although BCG strains overexpressing Rep could not be recovered, BCG harboring a plasmid with a copy-up mutation in *oriM* resulted in a threefold increase in gene transfer to J774 cells. Moreover, *M. smegmatis* strains overexpressing Rep enhanced gene transfer in vivo compared with a wild-type control. Immunization of mice with mycobacteria harboring a plasmid (pgp120_h^E) encoding human immunodeficiency virus gp120 elicited gp120-specific CD8 T-cell responses among splenocytes and peripheral blood mononuclear cells that were up to twofold ($P < 0.05$) and threefold ($P < 0.001$) higher, respectively, in strains supporting higher copy numbers. The magnitude of these responses was approximately one-half of that observed after intramuscular immunization with pgp120_h^E. *M. smegmatis* and other nonpathogenic mycobacteria are promising candidate vectors for DNA vaccine delivery.

Injection of plasmid DNA vaccines encoding protective antigens under the control of a eukaryotic promoter induces protective T- and B-cell responses in mice (43, 45) and subhuman primates (32) and is being studied in phase I trials in humans (42). Despite the promise of DNA vaccination, the widespread use of DNA as an inexpensive and effective immunogen in humans may be limited by requirements for large inocula of highly purified DNA and/or the coadministration of expensive adjuvants. In an attempt to improve the efficiency of DNA vaccination, bacteria have recently been studied as gene

delivery vectors through a process called “bactofection” (29), in which bacteria harboring antigen-encoding plasmids enter a mammalian cell and release the plasmids for uptake into the nucleus. Consequently, plasmid-encoded genes are expressed endogenously and, therefore, ensure that the protein is correctly folded and modified. In addition, protein-encoded secretion signals allow appropriate delivery for antigen presentation, thus overcoming many of the limitations of recombinant antigen expression in prokaryotes. Importantly, the bacterial vector both maintains and amplifies the DNA vaccine plasmids and acts as a natural adjuvant to enhance immune responses. In contrast to direct DNA vaccination techniques, this approach would obviate the need for large-scale production (and purification) of plasmids and adjuvants and therefore would be less costly (18). For example, intranasal or oral vaccination with bacteria that target the digestive tract (5, 9) would eliminate the need for DNA processing and needle injection, making the process simpler, less expensive, and more acceptable. Previously, we and others have demonstrated that attenuated

* Corresponding author. Mailing address: The Lewis M. Fraad Department of Pediatrics, Jacobi Medical Center, Bronx, NY 10461. Phone: (718) 918-4026. Fax: (718) 518-0366. E-mail: fennelly@aecom.yu.edu.

† Y.M. and N.M.Q. contributed equally to this work.

‡ Present address: PeerView Institute for Medical Education, 315 Bleecker Street, Suite 182, New York, NY 10014.

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intracellular pathogens such as *Shigella flexneri* (10, 33), *Listeria monocytogenes* (15), and invasive *Escherichia coli* (16) are effective vectors for DNA vaccination and that *Salmonella enterica* serovar Typhimurium bacterofection is more immunogenic than live recombinant *S. enterica* serovar Typhimurium expressing heterologous antigens for protective T-cell responses against a heterologous challenge (6). Our aim in the present study was to test the feasibility of using mycobacteria as a novel vector for DNA vaccine delivery. An attenuated mycobacterium vaccine vector would have several advantages over other bacterial species currently being tested for gene delivery. Such vectors are nonpathogenic, yet powerful adjuvants (12, 13, 31) with strong antitumor activity (17, 24). *Mycobacterium bovis* BCG, currently the most widely administered vaccine in the world, is safe for infants and can be given orally. We have observed previously that recombinant *Mycobacterium smegmatis*, a promising vaccine vector, generates T cells against human immunodeficiency virus (HIV) (5, 47). Nevertheless, the exclusive residence of these cells in the vacuoles of infected antigen-presenting cells may restrict their ability to release plasmids directly to the host cell cytoplasm, and the low plasmid copy number of pAL5000 (the replicon used most often in genetic manipulation of mycobacteria, which permits replication of only five copies per bacterium) (40) may limit the use of mycobacteria as vectors for DNA plasmid transfer. Despite these limitations, other workers have demonstrated recently that mucosal delivery of *M. smegmatis* harboring a eukaryotic expression plasmid encoding interleukin-12 (IL-12) and granulysin enhances Th1-specific immune responses in mice that are comparable to responses after BCG Pasteur immunization (46).

In the present study we observed, for the first time, the eukaryotic expression of reporter genes within eukaryotic nuclei that had been delivered by BCG. This expression was detected following infection of eukaryotic cell cultures with BCG harboring a plasmid encoding enhanced green fluorescent protein (GFP) under the control of a eukaryotic promoter.

We tested several approaches to improve the ability of mycobacteria to transfer plasmids to mammalian cells, including the use of lysis-susceptible, hyperconjugating, and increased-plasmid-copy-number mutants. Although early lysis of mycobacteria had no effect on the efficiency of gene transfer to mammalian cells, we observed a statistically significant, albeit only moderate (1.7-fold), increase in the level of plasmid transfer to eukaryotic cells infected with hyperconjugating *M. smegmatis* mutants compared to the level of plasmid transfer to eukaryotic cells infected with wild-type *M. smegmatis*.

The pAL5000 copy number is limited by the availability of two plasmid-encoded proteins, RepA and RepB, that recognize the plasmid origin of replication (*oriM*). To overcome negative autoregulation of pAL5000, we overexpressed these proteins in *trans* from the chromosome in *M. smegmatis*. We observed that *M. smegmatis* strains that overexpressed Rep proteins (referred to as Rep^{High} *M. smegmatis*) increased the plasmid copy number up to 10-fold and transferred genes to HeLa or J774 cells upon infection up to 3.5-fold more frequently than a control *M. smegmatis* strain (Rep^{Wt} *M. smegmatis*). Vaccination with Rep^{High} *M. smegmatis* strains harboring an *oriM*-based plasmid encoding HIV type 1 (HIV-1)

gp120 under the control of a eukaryotic promoter generated gp120-specific CD8 T-cell responses among peripheral blood mononuclear cells (PBMCs) in mice at an up-to-threefold-higher frequency than vaccination with Rep^{Wt} *M. smegmatis* harboring the same plasmid. These observations encourage the further development of mycobacteria as efficient DNA vaccine delivery vectors.

MATERIALS AND METHODS

Plasmid purification and construction. Table 1A lists the plasmids used in this work. To create a mycobacterial GFP expression plasmid, the *egfp* gene was subcloned from pEGFP-N1 (Clontech, Mountain View, CA) on an EcoRI fragment and ligated into the EcoRI site of pMV261, downstream of the *M. bovis* heat shock protein 60 promoter (*P*_{hsp60}) (40), to create pGFP_k^P. To create a eukaryotic GFP expression plasmid that replicates in mycobacteria, the cytomegalovirus (CMV) immediate-early promoter/enhancer (*P*_{CMV}) (41) and the region encoding enhanced GFP linked to the simian virus 40 late polyadenylation signal [*egfp*-SV40-poly(A)] were subcloned from pEGFP-N1 on an AseI-XbaI-digested fragment, blunt ended, and ligated into pMV206 digested with XbaI and HpaI to create pGFP_h^E. pGFP_h^E/RFP encoded the red fluorescent protein DsRed2 downstream of the *Mycobacterium marinum* *msp12* promoter (*P*_{msp12::dsRed2}) replicated by PCR from pYUB1086 (a generous gift from L. Ramakrishnan) with primers 5'-AAAAAAGCGTGCATCCGTGGC-3' and 5'-GCTGTACGC GTGAAGCAGACAG-3', digested with MluI, and ligated into the unique MluI site of pGFP_h^E.

The *repA* and/or *repB* gene of pAL5000 was amplified from pMV261 by PCR, using the following oligonucleotide primers: for *repAB*, 5'-TAAGGATCCGTT GTGGGGTGGCCCTCAG-3' and 5'-CCATCGATTAGAACACGCGGTGG ATTGTC-3'; for *repA*, 5'-CCATCGATTATAGCAATGCCTCCATGGCTG AC-3'; and for *repB*, 5'-TAAGGATCCATGAGCGACGGCTACAGCGAC-3'. BamHI and ClaI sites present in the primers were used to clone each fragment into the integrative plasmid pMV361 downstream of *P*_{hsp60} to generate pAB, pA, and pB, containing *repA* and *repB*, *repA*, and *repB*, respectively.

A CMV promoter from pcDNA3.1(-) (Invitrogen, Carlsbad, CA) was cloned into pYUB1058 to allow HIV-1 gp120 expression from a mycobacterial plasmid. *P*_{CMV} was cloned on an NruI-SmaI fragment into pYUB1058 digested with PvuII and EcoRV to create pCMV_{oriM}. The HIV-1 IIIb (HXBc2)-derived gp120 envelope gene, optimized for human codon usage, was subcloned from plasmid pVR1012x/s(VRC2000)-gp120 (generously provided by Gary Nabel, Vaccine Research Center, National Institute of Allergy and Infectious Diseases). gp120 was inserted downstream of *P*_{CMV} in pCMV_{oriM} cleaved with EcoRV and BamHI to generate pgp120_h^E. The gp120 coding sequence was confirmed by sequence analysis.

The recently characterized, increased-copy-number mycobacterial plasmid pHIGH100 (accession number EF21638) was derived from p16R1 (14) by mutation of *oriM* such that a higher level of replication in mycobacteria was achieved (3a). *oriM* from pHIGH100 was cut by SfoI and EcoRV and cloned into pYUB1143 and pYUB1146, which were linearized by MluI and filled in by the Klenow fragment, to generate pHIGFP_h^E and pHIGp120_h^E (which contain the *P*_{CMV::gfp} and *P*_{CMV::gp120} expression cassettes, respectively).

Bacterial strains and culture conditions. *E. coli* DH5α was used for routine manipulations of plasmid DNA, which was purified using QIAGEN midiprep columns (QIAGEN, Inc., Valencia, CA). *E. coli* transformants were grown at 37°C in LB media supplemented with kanamycin (40 µg/ml) and/or hygromycin (150 µg/ml) as appropriate to select for plasmid transformants. Plasmid pgp120_h^E DNA for intramuscular injection was produced in *E. coli* and purified with a QIAGEN Maxiprep kit by following the manufacturer's instructions. Table 1 lists the mycobacterial strains used in this work. Mycobacteria were grown in Middlebrook 7H9 broth (Becton Dickinson, Franklin Lakes, NJ) with 0.05% Tween 80 at 37°C. Cultures of auxotrophic mycobacteria were supplemented with 40 µg/ml of lysine, 0.1 µg/ml of diaminopimelic acid, or 48 µg/ml of pantothenate (Sigma Chemical Co., St. Louis, MO). Plasmids were electroporated into competent mycobacterial cells as previously described (35, 44). Cultures were inoculated from individual colonies grown on Middlebrook 7H10 medium plates or subcultured from frozen stocks of previously screened clones, with appropriate antibiotic selection (20 µg/ml of kanamycin, 50 µg/ml of hygromycin, and/or 20 µg/ml of apramycin) and supplements. Samples were grown to late-log phase (optical density at 600 nm, 1) and diluted in phosphate-buffered saline (PBS)-Tween for administration to eukaryotic cell cultures. Cell counts were verified by plating serial dilutions of the inocula.

TABLE 1. Plasmids and strains^a

Plasmid or strain	Abbreviation	Relevant characteristics	Reference or source
Plasmids			
pMV206		Shuttle vector, Km ^r	40
pMV261		Shuttle vector, Km ^r	40
pMV361		Shuttle vector, Km ^r	40
pYUB1058		pMV206, Hy ^r	40
pYUB1060	pMV261 _h	pMV261, Hy ^r	40
pYUB1063		pMV206, Ap ^r	This study
pYUB1082	pGFP _k ^P	pMV261 with P _{hsp60} :: <i>egfp</i> , Km ^r	Hsu ^b
pYUB1083	pGFP _h ^E	pYUB1058 with P _{CMV} :: <i>egfp</i> , Hy ^r	This study
pYUB1084	pGFP _a ^E	pYUB1063 with P _{CMV} :: <i>egfp</i> , Ap ^r	This study
pYUB1085	pGFP _h ^E /RFP	pYUB1058 with P _{msp12} :: <i>dsRed2</i> P _{CMV} :: <i>egfp</i> , Hy ^r	This study
pYUB1086		pMV261 with P _{msp12} :: <i>dsRed2</i> , Km ^r	Ramakrishnan ^c
pYUB1143	pGFP _h ^P	pYUB1060 with P _{hsp60} :: <i>egfp</i> , Hy ^r	Koo ^d
pYUB1145	pCMV _{oriM}	pYUB1058 with P _{CMV} , Hy ^r	This study
pYUB1146	pgp120 _h ^E	pYUB1058 with P _{CMV} :: <i>gp120</i> , Hy ^r	This study
pYUB1147	pAB	pMV361 with P _{hsp60} :: <i>repA</i> and <i>repB</i> , Km ^r	This study
pYUB1148	pA	pMV361 with P _{hsp60} :: <i>repA</i> , Km ^r	This study
pYUB1149	pB	pMV361 with P _{hsp60} :: <i>repB</i> , Km ^r	This study
pHIGH100	pHI	p16R1 with copy-number-up mutations in <i>oriM</i> , Hy ^r	Bourn ^e
pHIGFP _h ^E		pYUB1143 with pHIGH100 <i>oriM</i> , Hy ^r	This study
pHlgp120 _h ^E		pYUB1146 with pHIGH100 <i>oriM</i> , Hy ^r	This study
<i>M. smegmatis</i> strains			
mc ² 155	155	Efficient plasmid transformation (<i>ept-1</i>)	35
mc ² 1278		<i>ask1</i> :: <i>aph</i> , diaminoalmitate auxotroph, Km ^r	27
mc ² 4519		<i>Desat6-cfp10</i>	Lawrence ^f
mc ² 4554	155(pMV261 _h)	mc ² 155 with pYUB1060, Hy ^r	40
mc ² 4556	155(pGFP _k ^P)	mc ² 155 with pYUB1082, Km ^r	Hsu ^b
mc ² 4557	155(pGFP _h ^E)	mc ² 155 with pYUB1083, Hy ^r	This study
mc ² 4559	155(pGFP _h ^E /RFP)	mc ² 155 with pYUB1085, Hy ^r	This study
mKD211	211	Transposon mutation in <i>Ms_orf</i> , Km ^r	11
mc ² 4565	211 _{Esx1}	mKD211 complemented with <i>M. tuberculosis</i> <i>esx-1</i> , Km ^r Hy ^r	11
mc ² 4566		Transposon mutation in <i>Ms4898</i> , Km ^r	Derbyshire ^g
mc ² 4571	211(pGFP _h ^E)	mKD211 with pYUB1083, Km ^r Hy ^r	This study
mc ² 4572	211 _{Esx1} (pGFP _a ^E)	mc ² 4565 with pYUB1084, Km ^r Hy ^r Ap ^r	This study
mc ² 4574		mc ² 4566 with pYUB1083, Km ^r Hy ^r	Derbyshire ^g
mc ² 4575		mc ² 1278 with pYUB1083, diaminoalmitate auxotroph, Km ^r Hy ^r	This study
mc ² 4576		mc ² 4519 with pYUB1083, Hy ^r	This study
mc ² 5100	155 _{AB}	<i>attB</i> ::pYUB1147, Km ^r	This study
mc ² 5101	155 _A	<i>attB</i> ::pYUB1148, Km ^r	This study
mc ² 5102	155 _B	<i>attB</i> ::pYUB1149, Km ^r	This study
mc ² 5103	155 _N	<i>attB</i> ::pMV361, Km ^r	This study
mc ² 5104	155 _{AB} (pGFP _h ^P)	mc ² 5100 with pYUB1143, Km ^r Hy ^r	This study
mc ² 5105	155 _A (pGFP _h ^P)	mc ² 5101 with pYUB1143, Km ^r Hy ^r	This study
mc ² 5106	155 _B (pGFP _h ^P)	mc ² 5102 with pYUB1143, Km ^r Hy ^r	This study
mc ² 5107	155 _N (pGFP _h ^P)	mc ² 5103 with pYUB1143, Km ^r Hy ^r	This study
mc ² 5109	155 _{AB} (pGFP _h ^E)	mc ² 5100 with pYUB1083, Km ^r Hy ^r	This study
mc ² 5110	155 _A (pGFP _h ^E)	mc ² 5101 with pYUB1083, Km ^r Hy ^r	This study
mc ² 5111	155 _B (pGFP _h ^E)	mc ² 5102 with pYUB1083, Km ^r Hy ^r	This study
mc ² 5112	155 _N (pGFP _h ^E)	mc ² 5103 with pYUB1083, Km ^r Hy ^r	This study
mc ² 5114	155 _{AB} (gp120 _h ^E)	mc ² 5100 with pYUB1146, Km ^r Hy ^r	This study
mc ² 5115	155 _A (gp120 _h ^E)	mc ² 5101 with pYUB1146, Km ^r Hy ^r	This study
mc ² 5117	155 _N (pCMV _{oriM})	mc ² 155 with pYUB1145, Hy ^r	This study
mc ² 5118	155 _N (gp120 _h ^E)	mc ² 5103 with pYUB1146, Km ^r Hy ^r	This study
mc ² 5119	155(pHI)	mc ² 155 with pHI, Hy ^r	Bourn ^e
mc ² 5120	155(pHIGFP _h ^E)	mc ² 5119 with pYUB1083, Hy ^r	This study
mc ² 5121	155(pHlgp120 _h ^E)	mc ² 5119 with pYUB1146, Hy ^r	This study
<i>M. bovis</i> BCG strains			
Pasteur	BCG	Vaccine strain	28
mc ² 1604		<i>ΔlysA</i> , lysine auxotroph	This study
mc ² 4580	BCG(pMV261)	Pasteur BCG with pMV261, Km ^r	This study
mc ² 4582	BCG(pGFP _h ^E)	Pasteur BCG with pYUB1083, Hy ^r	This study
mc ² 4585		mc ² 1604 with pYUB1083, lysine auxotroph, Hy ^r	This study
mc ² 6000		<i>ΔpanCD</i> , pantothenate auxotroph, Hy ^r	Sambandamurthy ^h
mc ² 5122	BCG(pHI)	BCG with pHI, Hy ^r	This study
mc ² 5123	BCG(pHIGFP _h ^E)	BCG with pHIGFP _h ^E , Hy ^r	This study
mc ² 5124	BCG(pHlgp120 _h ^E)	BCG with pHlgp120 _h ^E , Hy ^r	This study

^a Abbreviations: Km and subscript k, kanamycin; Hy and subscript h, hygromycin; Ap and subscript a, apramycin; superscript P, prokaryotic promoter; superscript E, eukaryotic promoter; subscript N, contains integrative pMV361 plasmid without *rep* insert.

^b Hsu, T. Hsu, laboratory of W. R. Jacobs, Jr. (unpublished).

^c Ramakrishnan, laboratory of L. Ramakrishnan (unpublished).

^d Koo, M. Koo, laboratory of W. R. Jacobs, Jr. (unpublished).

^e Bourn, W. Bourn, laboratory of P. van Helden (submitted) (GenBank accession no. EF216316).

^f Lawrence, K. Lawrence, laboratory of W. R. Jacobs, Jr. (unpublished).

^g Derbyshire, laboratory of K. Derbyshire (unpublished).

^h Sambandamurthy, V. Sambandamurthy, laboratory of W. R. Jacobs, Jr. (unpublished).

Measurement of plasmid copy number. The relative plasmid copy numbers of Rep^{High}, pHIGH100, and Rep^{Wt} derivatives were determined by comparing the amounts of plasmid DNA extracted from the derivatives. The results were corroborated by analyzing the distribution and intensity of GFP expression in populations of various Rep derivatives of *M. smegmatis* expressing GFP. *M. smegmatis* was grown in 6 ml of Middlebrook 7H9 medium to log phase (optical density at 600 nm, 0.8 ± 0.02) before plasmid extraction using a modified Qiaprep kit (QIAGEN) protocol. Briefly, pelleted *M. smegmatis* was resuspended with 250 μ l of P1 buffer containing 10 mg/ml lysozyme and incubated at 37°C for 4 h in the presence of RNase for 10 min, and then it was lysed at room temperature for 5 min with 300 μ l of P2 lysis buffer, which was then neutralized with 350 μ l of prechilled N3 buffer. Aliquots of serial twofold dilutions of extracted plasmid DNA were run on a 0.8% agarose gel and stained with ethidium bromide. Plasmid quantities were estimated using ImageJ software, version 1.34n (Wayne Rasband; <http://rsb.info.nih.gov/ij/>) after calibration with the DNA High Mass Ladder (Invitrogen). For estimation by fluorescence-activated cell sorting (FACS) analysis, a 500- μ l suspension of each clone was washed twice with an equal volume of PBS and then resuspended in 1 to 2 ml PBS. The distribution and intensity of GFP expression among 50,000 bacilli were determined by FACS analysis using a BD Biosciences FACScan flow cytometer and were analyzed by using CellQuest software (Becton Dickinson, Mountain View, CA).

Infection of mammalian cell cultures with mycobacteria. RAW 264.7 murine macrophage and HeLa (human cervical adenocarcinoma) cell lines were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% HEPES buffer, and 5% NCTC-109 medium (Gibco). J774 murine macrophage cells were grown in DMEM supplemented with 10% FBS. Cells were transferred at a concentration of 1×10^5 to 2×10^5 cells/well into a 48-well plate and incubated at 37°C in the presence of 10% CO₂ for 2 to 24 h before infection to generate semiconfluent lawns of cells. To determine the optimal inoculum for each cell line, freshly grown mycobacteria were added to the cells at a multiplicity of infection (MOI) of 1, 3, 10, 50, or 100 to obtain a total volume of no more than 350 μ l/well in a 48-well plate. To determine whether viable bacteria are required for gene transfer to mammalian cells, mycobacteria were killed by heating them to 80°C for 5 min prior to infection of RAW 264.7 cells. After certain infections, isoniazid (25 μ g/ml) was added immediately to induce premature lysis. Extracellular bacteria were killed by addition of 50 μ g/ml of gentamicin (Gibco). To compare the efficiency of lipid-mediated DNA plasmid transfection to the efficiency of bactofection, lipofectamine (Invitrogen) was diluted 1:50 in Opti-Mem medium (Gibco) and then incubated at room temperature for 5 min, added to 10 μ g of DNA in an equal volume of medium, and incubated for 20 min at room temperature before it was added to RAW 264.7 or HeLa cells. After incubation for 3 to 5 h, cells were washed three times with DMEM and resuspended in 0.5 to 2 ml of medium. Cells were examined by fluorescence microscopy and FACS analysis (after trypsinization and resuspension in 4% FBS-PBS) at various intervals for up to 5 days after infection.

Microscopic imaging and FACS analysis. Live RAW 264.7 and HeLa cell samples were examined after infection using an Olympus IX 81 microscope (Melville, NY) equipped with a Cooke Sensicam QE air-cooled charge-coupled device camera and a mercury lamp for fluorescence illumination. Images were collected using IPLab Spectrum software (Scanalytics, Rockville, MD) at a magnification of $\times 10$ or $\times 40$. Adobe Photoshop (Adobe Systems, San Diego, CA) was used to restore color and merge images captured using different fluorescence filters or normal light (phase-contrast) illumination. The fluorescence in a minimum of 10^5 cells per sample was measured by FACS using a FACScan or FACSCalibur cytometer and CellQuest software (Becton Dickinson). Data were further analyzed with the FloJo software (Tree Star, Inc., Ashland, OR).

Animals and immunization. Six-to-eight-week-old female BALB/c mice (Charles River Laboratories) were inoculated with 10^8 CFU of Rep^{High} *M. smegmatis* strains (which overexpress Rep proteins and elevate the plasmid copy number) harboring pgp120_h^E or of BCG or *M. smegmatis* strains harboring pHgp120^E via the intraperitoneal route (Table 1). The relative plasmid copy number per bacterium of pHgp120^E or Rep^{High} and Rep^{Wt} strains was confirmed prior to and after immunization using the agarose gel density method. To compare the immunogenicity of *M. smegmatis* having the wild-type plasmid copy number, groups of control mice were inoculated with the corresponding Rep^{Wt} *M. smegmatis* strain. To compare the effects of the usual route of DNA vaccination on tetrameric responses, intramuscular purified pgp120_h^E was administered at a dose of 50 μ g per mouse (25 μ g/gastrocnemius muscle) via intramuscular injection. To control for the nonspecific effects of mycobacteria or DNA vaccination on tetrameric responses, groups of mice were inoculated with 10^8 CFU of 155_N(pCMV_{oriM}) or BCG(pHI) or with purified pCMV_{oriM} DNA by the same method.

Tetramer staining and flow cytometric analysis. To determine the frequency of gp120-specific tetrameric responses in peripheral blood mononuclear cells

(PBMCs) and splenocytes, blood was obtained from the retroorbital plexus and spleens were harvested 7 days after inoculation. H-2D^d tetrameric complexes folded with the P18 peptide (RGPGRFAVTI) (5), a sequence found in the V3 loop of HIV-1 HXBc2 envelope protein, were prepared as described previously (5). Fresh blood samples (200 μ l from each mouse) or splenocyte suspensions (recovered after passage through a 70- μ m nylon cell strainer) were diluted in 3 ml RPMI medium with 40 U/ml heparin and layered over Ficol-Hypaque (lympholyte-M) before centrifugation at $400 \times g$ for 20 min at 20°C. The lymphocyte layer was carefully transferred to a fresh tube, diluted with 10 ml of PBS, and then pelleted and washed in 1 ml PBS with 2% FCS before resuspension in 100 μ l (final volume) of the solution. The cells were stained with P18-tetramer-phycoerythrin, vortexed briefly, and incubated at 20°C for 20 min, and this was followed by staining with APC-CD8 for 20 min at 20°C. To control for nonspecific fluorescence, samples were incubated with no monoclonal antibody, with only APC-CD8, and with only phycoerythrin-CD4. The cells were washed with 5 ml PBS at room temperature, resuspended in 2% formaldehyde in PBS, vortexed, and analyzed with a FACSCalibur cytometer. A minimum of 10^4 cells were analyzed for each sample.

Statistical analysis. Statistical tests were performed using the Student *t* test or one-way analysis of variance with Dunnett's posttest by Prism 4.01 for Windows (GraphPad Software, Inc., San Diego, CA). *P* values of <0.05 were considered significant.

RESULTS

***M. smegmatis* and *M. bovis* BCG transfer reporter plasmids to eukaryotic cell lines.** Mycobacteria are promising vectors for DNA vaccine delivery. Our goal is to develop an attenuated *Mycobacterium tuberculosis*, mutant BCG, or *M. smegmatis* vector for DNA immunization. To develop a system to measure the efficiency with which various mycobacterial strains can deliver DNA plasmids to eukaryotic cells, an extrachromosomal mycobacterial reporter plasmid, pGFP_h^E (the superscript E indicates that the plasmid contains the eukaryotic promoter P_{CMV} expressing *egfp*; the subscript h indicates that the plasmid encodes resistance to hygromycin) was constructed and transformed into *M. smegmatis* (Fig. 1A). No fluorescence of *M. smegmatis* strain 155(pGFP_h^E) was detected by FACS, confirming the absence of cryptic mycobacterial promoters upstream of *egfp* (Fig. 1C). By contrast, GFP expression was readily detected in strain 155(pGFP_k^P), which contained the mycobacterial P_{hsp60}::*egfp* expression cassette (the superscript P indicates that the plasmid contains the prokaryotic promoter P_{hsp60} expressing *egfp*; the subscript k indicates that the plasmid encodes resistance to kanamycin) (Fig. 1B and D).

Macrophages and dendritic cells are the primary targets during mycobacterial infection. To determine whether mycobacterial infection, DNA transfer into the nucleus, and subsequent eukaryotic GFP expression (and fluorescence) could be observed in a macrophage cell line, RAW 264.7 cells were infected with strains 155(pGFP_k^P) and 155(pGFP_h^E), which harbor the P_{hsp60}::*egfp* and P_{CMV}::*egfp* expression cassettes, respectively. Twenty-four hours after infection with 155(pGFP_k^P) at an MOI of 10, one or more internalized bacteria were observed by fluorescence microscopy in $96.5\% \pm 1.6\%$ of the RAW 264.7 cells (data not shown). However, a striking difference in the patterns of GFP expression in RAW 264.7 and HeLa cells was observed after infection by the two strains. In RAW 264.7 and HeLa cells infected with 155(pGFP_k^P), individual mycobacteria expressing GFP were visible (Fig. 2A and 2D, respectively). By contrast, a small percentage of RAW 264.7 and HeLa cells infected with 155(pGFP_h^E) fluoresced throughout the cytoplasm, indicating that host cells expressed GFP (Fig. 2B and 2E, respectively). No eukaryotic GFP ex-

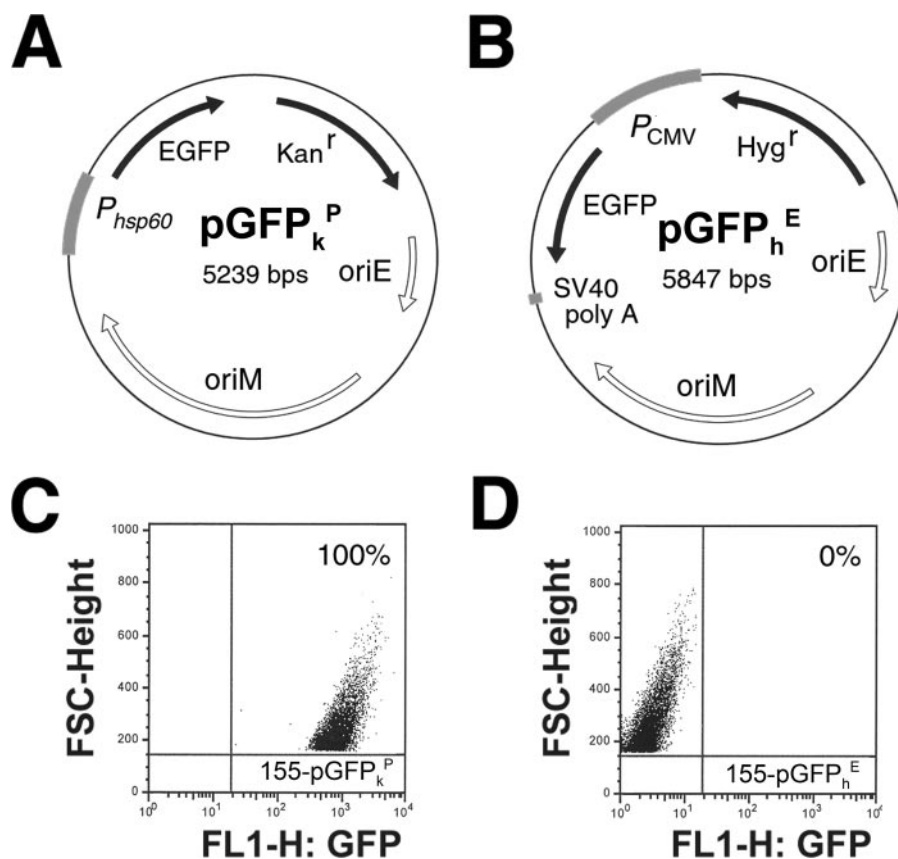


FIG. 1. *M. smegmatis* expresses GFP from a mycobacterial promoter but not from a eukaryotic promoter. Plasmids that replicate in mycobacteria encoding GFP under the control of a eukaryotic immediate-early CMV promoter/enhancer (A) or the mycobacterial *hsp60* promoter (B) were constructed. Abbreviations: oriE, origin of replication in *E. coli*; oriM, origin of replication in mycobacteria; Kan^r, kanamycin resistance gene; Hyg^r, hygromycin resistance gene; EGFP, enhanced GFP; SV40 poly A, simian virus 40 late polyadenylation signal. *M. smegmatis* strains transformed with pGFP_h^E [strain 155(pGFP_h^E)] or pGFP_k^P [strain 155(pGFP_k^P)] were cultured and analyzed by flow cytometry for GFP expression (C and D). FSC, forward scatter.

pression was detected within RAW 264.7 cells infected with heat-killed 155(pGFP_h^E), suggesting that viable bacteria are required for gene transfer to mammalian cells (data not shown).

BCG or other mycobacteria harboring DNA plasmid vaccines could be given orally. DNA has been observed to be transiently expressed by tissue-specific epithelial cells, and DNA vaccines can induce immune responses after intranasal or oral administration; dendritic cells may capture antigens from mucosal epithelial or epidermal tissues and migrate to draining lymph nodes for antigen presentation to T cells in vivo (8). To determine whether HeLa cells permit plasmid transfer from mycobacteria, HeLa cells were infected with 155(pGFP_k^P) or 155(pGFP_h^E). To compensate for the lower bacterial uptake by this nonphagocytic cell line, a 5- to 10-fold-higher MOI was used in HeLa cell infections than in the RAW 264.7 cell infection experiments. After infection with 155(pGFP_h^E), a significantly higher proportion of HeLa cells (Fig. 2F, right panel) than of RAW 264.7 cells (Fig. 2C, right panel) expressed GFP, despite the fact that the efficiency of uptake of mycobacteria by HeLa cells (Fig. 2F, middle panel) was significantly less than the efficiency of uptake by RAW 264.7 cells (Fig. 2C, middle panel). Transfection with purified pGFP_h^E DNA and lipofectamine yielded only slightly higher rates of

GFP expression than mycobacterial bactofection in RAW 264.7 cells yielded (0.0385% ± 0.01%) after 24 h.

To more precisely identify cells with internalized bacteria that expressed the GFP transgene, RAW 264.7 and HeLa cells were infected with *M. smegmatis* harboring plasmid pGFP_h^E/RFP (which contains the P_{CMV}::*egfp* and P_{msp12}::*dsRed2* expression cassettes). By using microscopy, it was observed that a single internalized red mycobacterium was sufficient to permit GFP expression by RAW 264.7 or HeLa cells (Fig. 3).

M. bovis BCG was demonstrated to mediate bactofection by infecting HeLa or J774 cells with BCG harboring pGFP_h^E (Fig. 4). To control for nonspecific fluorescence, HeLa or J774 cells were infected with BCG Pasteur harboring the vector pMV261. After 24 h of infection, FACS analysis revealed that statistically significant (*P* < 0.05) percentages of HeLa cells (0.093% ± 0.032%) and J774 cells (0.023% ± 0.0058%) expressed GFP compared to the background fluorescence cells infected with BCG harboring pMV261 (Fig. 4).

Lysis-susceptible mycobacteria do not improve DNA transfer. Mutants of *Shigella* that are auxotrophic for diaminopalmitate undergo rapid lysis after they invade mammalian cells and are very efficient vectors for bactofection (33). To test whether lysis-susceptible mycobacteria mediate bactofection more efficiently than wild-type parent strains, RAW 264.7 and HeLa

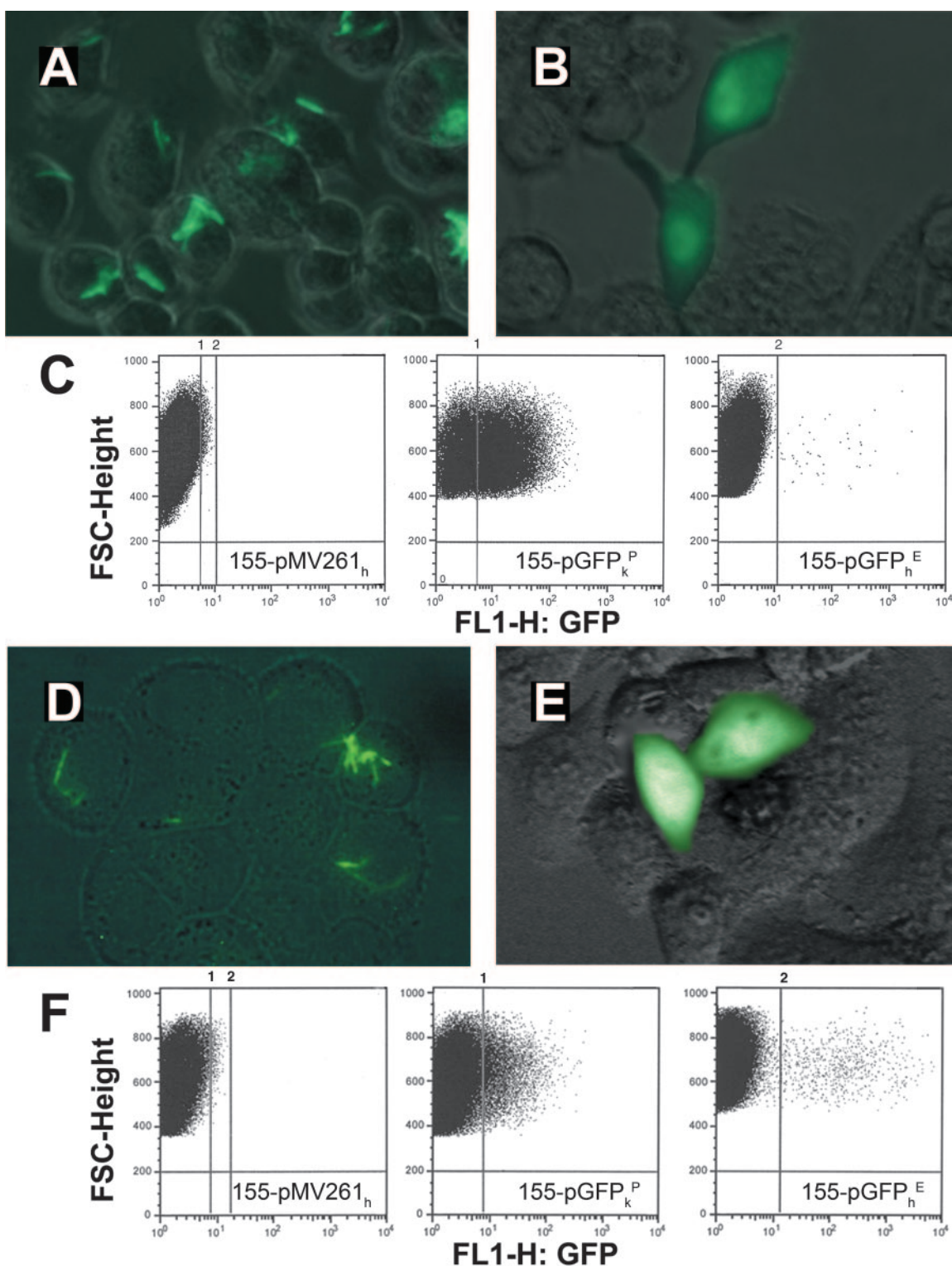


FIG. 2. Eukaryotic GFP expression is observed in RAW 264.7 and HeLa cells infected with 155(pGFP_h^E). RAW 264.7 cells were infected with 155(pGFP_k^P) (A) or 155(pGFP_h^E) (B) at an MOI of 10. HeLa cells were infected with 155(pGFP_k^P) (D) or 155(pGFP_h^E) (E) at an MOI of 100. Both groups were observed by fluorescence microscopy 24 h postinfection, and the original images were taken at a magnification of $\times 40$. Cells infected with 155(pMV261_h), 155(pGFP_k^P), or 155(pGFP_h^E) at an MOI of 10 (RAW 264.7 cells) (C) or 50 (HeLa cells) (F) were also collected 5 days postinfection for flow cytometric analysis. Data representative of a minimum of three experiments are shown, and the quadrant axes are aligned to reduce the background in high-percentage (axis 1) and low-percentage (axis 2) GFP-expressing samples. FSC, forward scatter.

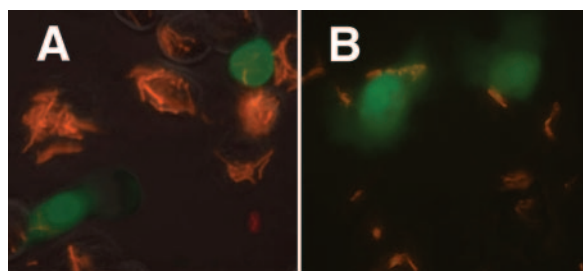


FIG. 3. Eukaryotic GFP expression in RAW 264.7 and HeLa cells requires the presence of intracellular *M. smegmatis* 155(pGFP_h^E/RFP). RAW 264.7 cells were infected with *M. smegmatis* 155(pGFP_h^E/RFP) at an MOI of 10 (A), and HeLa cells were infected at an MOI of 100 (B). Fluorescing mycobacteria (red) in RAW 264.7 cells expressing GFP (green) were observed 24 h after infection. Red and green images were combined, and enhanced emission was recorded using phase-contrast and red and green filter fluorescence microscopy. The original images were taken at a magnification of $\times 40$.

cells were infected with pGFP_h^E-containing *M. bovis* BCG strain mc²1604 or mc²6000 or *M. smegmatis* strain mc²1278 (which are auxotrophic for lysine, pantothenate, and diaminopalmitate, respectively, and undergo lysis soon after infection of mammalian cells). Isoniazid also causes bacterial lysis after only a few generations due to inhibition of cell wall synthesis; therefore, RAW 264.7 and HeLa cells were also treated with isoniazid after infection with pGFP_h^E-containing *M. smegmatis* and *M. bovis* BCG. Neither the premature lysis mutants nor isoniazid treatment increased gene transfer frequency compared to that observed with the wild type (data not shown). Therefore, early lysis of mycobacteria does not appear to improve the delivery of DNA into the host cell.

Hyperconjugation mutants of *M. smegmatis* transfer DNA plasmids to mammalian cells more efficiently than the wild type. We predicted that an *M. smegmatis* conjugation system that mediates DNA transfer to other mycobacteria (26) may contribute to plasmid transfer to mammalian cells. It was recently dis-

covered that transposon insertion mutations within and near the *Esx-1* locus of the *M. smegmatis* chromosome lead to a hyperconjugation phenotype (11). The *Esx-1* locus is thought to encode a specialized secretory apparatus responsible for secreting at least two proteins (*EsxA* and *EsxB*), which are encoded within the *Esx* locus (4, 7). The insertions are predicted to disrupt *Esx-1* functions that normally suppress conjugation, perhaps by interfering with *EsxA* and *EsxB* secretion. Complementation of the mutants with the wild-type *Esx-1* region of *M. tuberculosis* reduces or eliminates the hyperconjugative phenotype (11).

HeLa cells were infected with a hyperconjugating *M. smegmatis* mutant (MKD211) harboring pGFP_h^E. We observed a significantly higher frequency of GFP expression in HeLa cells after infection with MKD211 than after infection with the wild-type parent strain ($P = 0.04$) (Fig. 5). Complementation of the mutation with the *M. tuberculosis* *Esx-1* locus suppressed the increased transfer of pGFP_h^E to infected cells relative to the mutant and wild-type strains (Fig. 5). In addition, an *M. smegmatis* mutant with disruption of the *lpqM* gene that conjugates 1,000-fold less than the wild type was still able to mediate bactofection, albeit inefficiently (data not shown).

A primary role of *Esx-1* is the secretion of a heterodimer of *EsxA* and *EsxB* (4, 7). To determine whether *EsxA* and *EsxB* suppress plasmid transfer into nuclei, an *M. smegmatis* mutant with a precise deletion of the *esxA* and *esxB* genes (strain mc²4519) was transformed with pGFP_h^E to generate mc²4576. Surprisingly, the same frequency of GFP expression was observed among HeLa cells infected with strain mc²4576 and among HeLa cells infected with mc²4557 [155(pGFP_h^E)] (data not shown). Together, these results suggest that the secreted forms of *EsxA* and *EsxB* do not inhibit bactofection but that it is the disruption of the *Esx-1* translocation apparatus that alleviates bactofection inhibition.

Overexpression of Rep proteins increases the copy number of *oriM*-based plasmids in *M. smegmatis* and enhances the gene transfer frequency to mammalian cells. A potential limitation of the use of mycobacteria as vectors for DNA vaccines is that

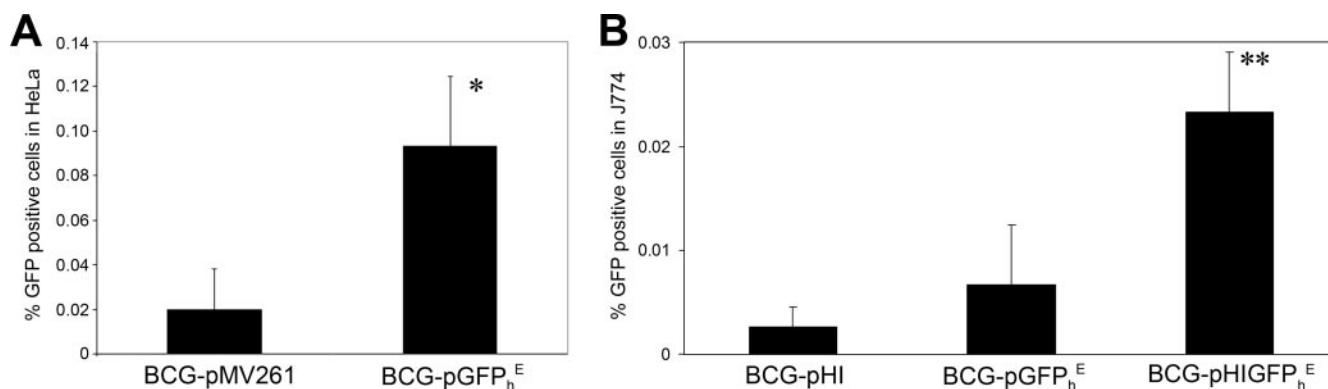


FIG. 4. *M. bovis* BCG mediates plasmid transfer to infected HeLa (A) and J774 (B) cells. (A) HeLa cells were infected with BCG(pGFP_h^E) [BCG Pasteur harboring pGFP_h^E that contains the eukaryotic P_{CMV}::*egfp* expression cassette] at an MOI of 50 and analyzed by flow cytometry 24 h after infection. The mean (and standard error of the mean) proportion of HeLa cells expressing GFP is shown based on the results of three experiments. The proportion of HeLa cells expressing GFP by 24 h after infection with BCG(pGFP_h^E) was significantly higher than the background frequencies after infection with control strain BCG(pGFP_h^E) (one asterisk, $P < 0.05$). (B) J774 cells were infected with BCG harboring plasmid pHI [BCG(pHI)], pGFP_h^E [BCG(pGFP_h^E)], or pHIGFP_h^E [BCG(pHIGFP_h^E)] at an MOI of 50 and analyzed by flow cytometry 24 h after infection. The mean (and standard error of the mean) proportion of J774 cells expressing GFP is shown based on the results of three experiments. The proportion of J774 cells expressing GFP by 24 h after infection with BCG(pHIGFP_h^E) was significantly higher than the proportion after infection with control strain BCG(pGFP_h^E) (two asterisks, $P < 0.01$).

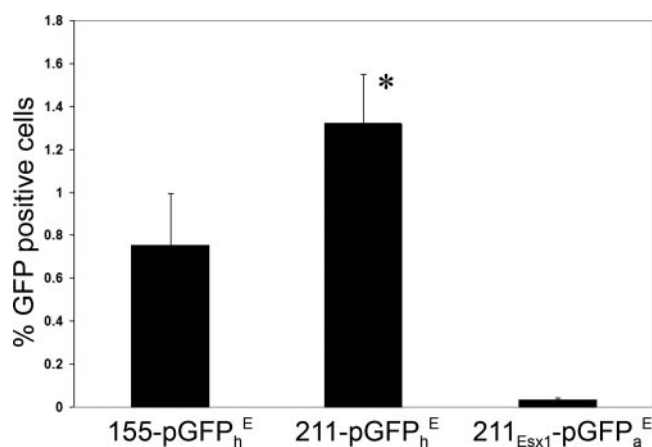


FIG. 5. Hyperconjugative *M. smegmatis* strain 211(pGFP_h^E) mediates transfer of pGFP_h^E to HeLa cells more efficiently than wild-type *M. smegmatis*. HeLa cells were infected with 211(pGFP_h^E), 155(pGFP_h^E) (wild-type *M. smegmatis* harboring pGFP_h^E), or 211_{Esx1}(pGFP_a^E) [211(pGFP_h^E) complemented with the *Esx-1* region of *M. tuberculosis*] at an MOI of 100 and collected 4 to 5 days postinfection for flow cytometric analysis. The *P* value for a comparison of the percentage of HeLa cells expressing GFP after infection with 155(pGFP_h^E) and the percentage of HeLa cells expressing GFP after infection with 211(pGFP_h^E) was <0.05 (indicated by an asterisk). The data are representative of a minimum of three experiments. The mean (and standard error of the mean) peak intensity of GFP expression is shown for each strain.

mycobacterial plasmids exist at a low copy number, restricting the amount of transferable plasmid. pAL5000, the best-characterized mycobacterial plasmid, is present at a level of only five copies per bacterium (40). pAL5000 encodes two proteins, RepA and RepB, which are thought to form an essential replication initiation complex that recognizes and initiates replication from the origin of replication, *oriM* (2, 3, 37). A negative regulatory circuit appears to control mRNA synthesis of RepA and RepB (38) and thus reduce their expression, which directly impacts the initiation of replication and copy number.

We investigated whether overexpression of Rep proteins resulted in increased replication of pAL5000. Three *M. smegmatis* Rep^{High} strain derivatives were constructed expressing either RepA, RepB, or RepAB from the chromosome (designated 155_A, 155_B, and 155_{AB}). pGFP_h^P was introduced into each strain, and the relative quantities of pGFP_h^P extracted from the strains were compared (Fig. 6). In each strain, elevated expression of Rep proteins resulted in increased yields of plasmid DNA that were up to 10-fold greater than the wild-type yields (Fig. 6A and 6B).

FACS analyses with the Rep^{High} derivatives demonstrated that an increase in copy number correlated with an increase in the level of mycobacterial GFP expression from pGFP_h^P. The maximal intensity of GFP expression among populations of Rep^{High} *M. smegmatis* was four- to sixfold higher than that among the wild-type control population (Fig. 6C and 6D).

The ability of Rep^{High} strains to transfer pGFP_h^E into HeLa or J774 cells was examined. The frequency of transfer of pGFP_h^E into HeLa cell nuclei infected with a RepA^{High} strain was up to 3.5-fold higher by day 3 postinfection and up to 3-fold higher by 12 h postinfection in J774 cells than the frequency of transfer with the controls (Fig. 7). These results suggest that an

increased plasmid copy number in *M. smegmatis* correlates with enhanced plasmid transfer to mammalian cells.

We were unable to recover BCG clones that express the RepA and/or RepB protein *in trans* from the chromosome in BCG. As an alternative to Rep overexpression, BCG was transformed with high-copy-number plasmid pHIGFP_h^E, derived from the copy-number-up mutant plasmid pHIGH100. Plasmid pHIGH100 is maintained at a level of 32 to 64 copies per bacterium and is stably maintained in BCG (Bourn et al., submitted). The ability of the resulting strain, designated BCG(pHIGFP_h^E), to transfer pHIGFP_h^E into phagocytic J774 cells compared to that of BCG harboring the regular-copy-number plasmid pGFP_h^E [strain BCG (pGFP_h^E)] was examined. The frequency of GFP-expressing J774 cells infected with BCG(pHIGFP_h^E) was up to 3.5-fold higher by 24 h postinfection than the frequency of GFP-expressing J774 cells infected with BCG(pGFP_h^E) (Fig. 4B). The BCG(pHIGFP_h^E) strain was not tested in HeLa cells. The frequency of GFP-expressing J774 cells infected with strain 155 containing pHIGFP_h^E was up to 1.56-fold higher by 12 h postinfection than the frequency of GFP-expressing J774 cells infected with Rep^{High} strain 155_{AB}(pGFP_h^E) (0.20% versus 0.13%), although the difference was not statistically significant (*P* > 0.05) (data not shown).

Immunization with recombinant *M. smegmatis* strains harboring an HIV gp120 eukaryotic expression plasmid generates gp120-specific CD8 T cells in mice. Several candidate immunogens, including recombinant live attenuated viruses (23) and bacteria (22), are being studied for the generation of T-cell responses against HIV. To determine the effect of a higher plasmid copy number on the frequency of gp120-specific T-cell responses, we compared the frequencies of gp120-specific tetrameric responses after immunization with recombinant *M. smegmatis* and BCG strains harboring pHIGp120_h^E. The frequency of P18 tetramer staining among CD8⁺ T cells from mice immunized with strain 155_{AB}(pgp120_h^E) was approximately twofold higher (*P* < 0.05) for splenocytes and threefold higher (*P* < 0.001) for PBMCs (0.12% ± 0.065%) than the frequency in mice immunized with Rep^{Wt} strain 155_N(pgp120_h^E) (Fig. 8). The magnitude of the P18 tetrameric responses among PBMCs after 155_{AB}(pgp120_h^E) immunization was approximately one-half (48.2%) the magnitude detected after intramuscular pgp120_h^E immunization (Fig. 8). Agarose gel analysis of plasmids recovered from *M. smegmatis* prior to inoculation confirmed that strain 155_{AB}(pgp120_h^E) had a fivefold-higher copy number of pgp120_h^E per bacterium on average than strain 155_N(pgp120_h^E) (data not shown). Surprisingly, no gp120-specific responses above the background level were detected among splenocytes 1 week after immunization with BCG(pHIGp120_h^E) in control BCG-immunized mice (data not shown). Also, the frequency of tetrameric responses among splenocytes 1 week after immunization were 2.19-fold higher after intraperitoneal inoculation of Rep^{Wt} strain 155_N(pgp120_h^E) than after inoculation of 155(pHIGp120_h^E) (data not shown).

DISCUSSION

Intracellular bacterial vectors harboring eukaryotic expression plasmids may be more effective immunogens than bacterial vectors harboring prokaryotic expression plasmids. Although attenuated strains of *Shigella flexneri* (34), *L. monocytogenes* (21), *Salmonella* (36), *M. smegmatis* (5, 20), and *M.*

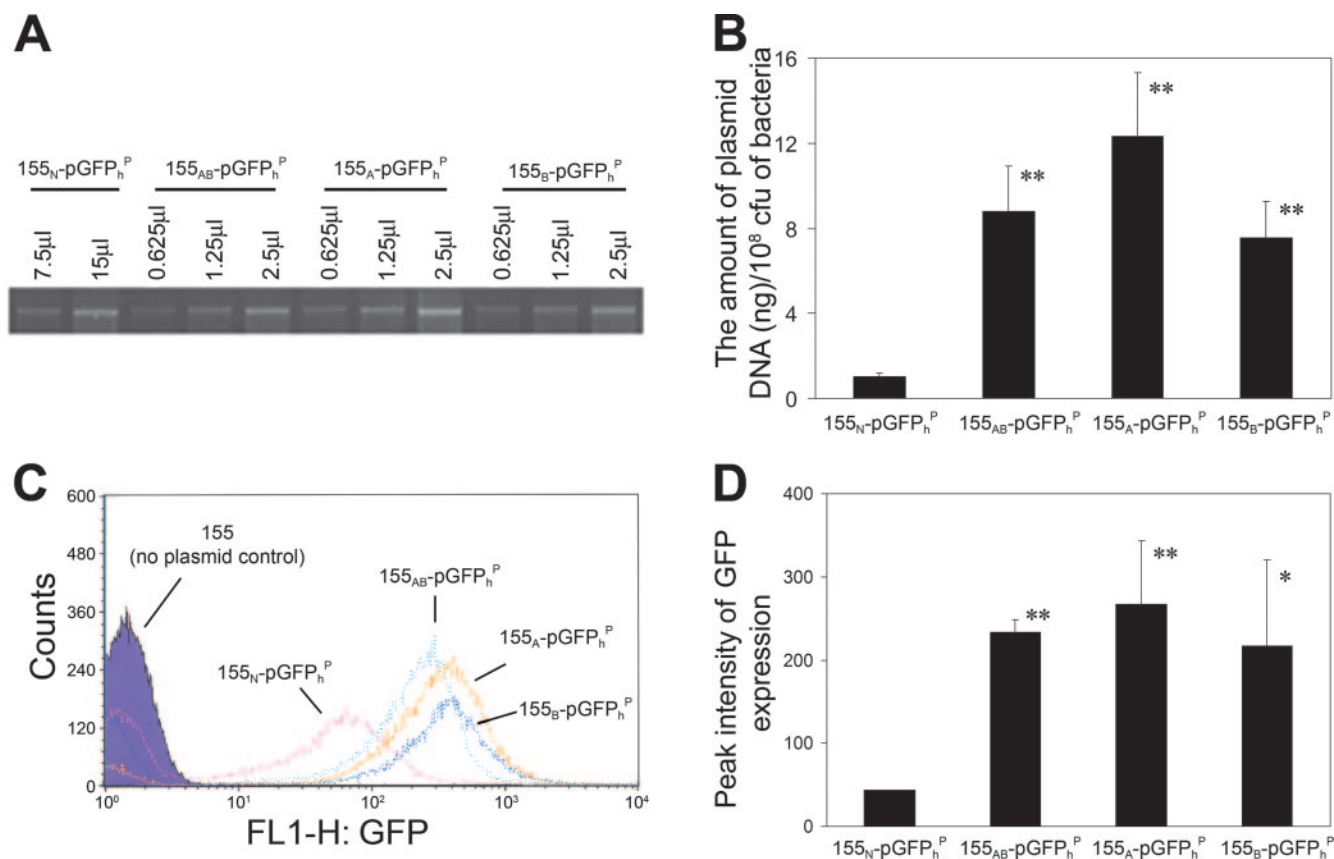


FIG. 6. Measurement of relative copy number of plasmid pGFP_h^P per bacterium in various recombinant Rep^{High} and Rep^{Wt} *M. smegmatis* strains. Following extraction of pGFP_h^P from 10⁸ CFU of each strain, DNA was eluted with 40 μl of distilled H₂O, and aliquots of each sample were loaded on a 0.7% agarose gel. (A) Agarose gel comparing relative amounts of pGFP isolated from each strain. The sample volumes loaded are indicated above the lanes. The Rep^{Wt} strain 155_N contains pMV361 without the *repAB* gene insert. (B) Estimates of the amount of plasmid DNA recovered from 10⁸ CFU of each strain based upon quantification of the intensity of plasmid bands on the agarose gel by the software ImageJ, version 1.34n. Two asterisks indicate that the *P* value is <0.01 for comparisons of Rep^{High} strains 155_{AB}(pGFP_h^P), 155_A(pGFP_h^P), and 155_B(pGFP_h^P) with strain 155_N(pGFP_h^P). The mean (and standard error of the mean) amounts of plasmid DNA shown are based on the results of three experiments. (C) Representative flow cytometry analysis of various Rep^{High} *M. smegmatis* strains harboring pGFP_h^P (which contains the P_{hsp60::egfp} cassette) indicated by different colors. A population consisting of 50,000 bacteria of each strain was sorted by flow cytometry. Strain mc²155 without a plasmid (indicated by purple shading) served as a negative control. (D) Peak intensity of GFP expression. The peak intensity of GFP expression by populations of each strain was measured by FACS. The mean (and standard error of the mean) peak intensities of GFP expression shown are based on the results of three experiments. The *P* values for comparisons of the peak GFP intensities with that of Rep^{Wt} strain 155_N(pGFP_h^P) were <0.01 for Rep^{High} strains 155_{AB}(pGFP_h^P) and 155_A(pGFP_h^P) (two asterisks) and <0.05 for 155_B(pGFP_h^P) (one asterisk).

bovis bacillus Calmette-Guérin (48) which express heterologous antigens have shown promise as vaccine vectors, their efficacy is limited by reduced expression and incomplete processing of full-length recombinant polypeptides within the bacteria (9) and the failure to engender strong immune responses to nonsecreted recombinant antigens (19, 30). By contrast, an attenuated bacterial vector that can deliver a DNA vaccine into mammalian cells has the distinct advantage of ensuring correct endogenous expression and processing of recombinant polypeptides for major histocompatibility complex class I and II presentation to T cells, as well as proper glycosylation and folding of conformational B-cell epitopes, facilitating a robust antigen-specific immune response. *M. smegmatis* is a promising recombinant candidate vaccine vector. We observed previously that recombinant *M. smegmatis* generates heterologous antigen-specific T-cell responses despite its rapid clearance in mice (5, 47). Other workers have demonstrated that *M. smegmatis*

can mediate plasmid delivery to and subsequent transgene expression by infected mice (46). We demonstrate here for the first time that BCG can also transfer genes to mammalian cells and define several critical parameters affecting bactofection determined through genetic manipulation of *M. smegmatis*.

Macrophages are the natural target and host for mycobacteria in vivo. Despite the high rate of infection (>95%) of RAW 264.7 murine macrophage cells by recombinant *M. smegmatis* observed in the present study, only a very low proportion (0.036% ± 0.1%) of the infected cells expressed GFP. Nevertheless, the efficiency of mycobacterial bactofection in RAW 264.7 cells was comparable to that observed after lipofectamine transfection (0.0385% ± 0.01%). A higher proportion of HeLa cells than of RAW 264.7 cells infected with *M. smegmatis* expressed GFP, despite a much lower frequency of bacterial uptake in HeLa cells than in RAW 264.7 cells. Although the overall efficiency of mycobacterial pGFP_h^E bacto-

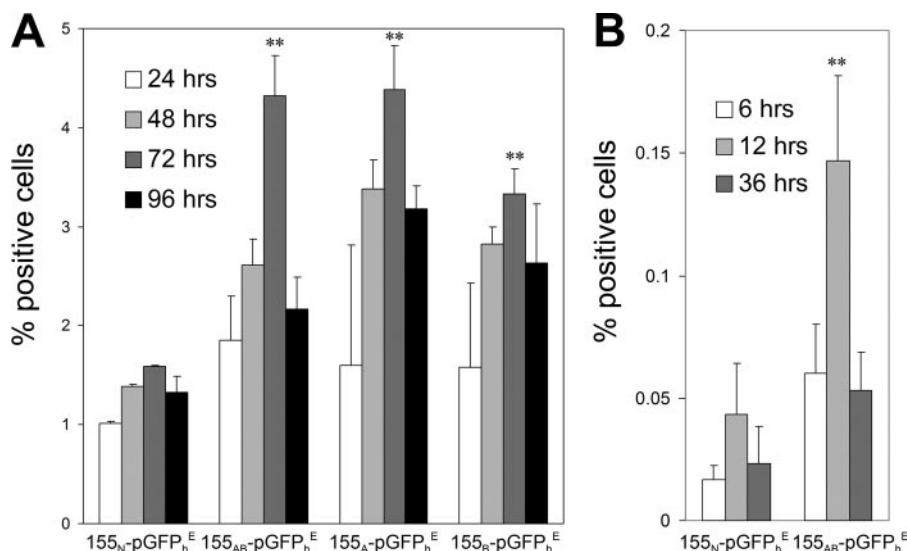


FIG. 7. Rep^{High} *M. smegmatis* strains mediate bactofection in HeLa (A) or J774 (B) cells more effectively than Rep^{Wt} *M. smegmatis*. HeLa cells (A) or J774 cells (B) were infected with Rep^{High} *M. smegmatis* strain 155_{AB}(pGFP_h^E), 155_A(pGFP_h^E), or 155_B(pGFP_h^E) or with Rep^{Wt} strain 155_N(pGFP_h^E). The proportion of HeLa cells expressing GFP was measured at different time points by FACS after infection. The mean (and standard error of the mean) proportions of HeLa or J774 cells expressing GFP shown are based on the results of three experiments. The proportion of HeLa cells expressing GFP by 72 h after infection with Rep^{High} strains 155_{AB}(pGFP_h^E), 155_A(pGFP_h^E), and 155_B(pGFP_h^E) was significantly higher than the proportion after infection with Rep^{Wt} strain 155_N(pGFP_h^E) (two asterisks, $P < 0.01$); the proportion of J774 cells expressing GFP by 12 h after infection with Rep^{High} strain 155_{AB}(pGFP_h^E) was significantly higher than the proportion after infection with Rep^{Wt} strain 155_N(pGFP_h^E) (two asterisks, $P < 0.01$).

fection per cell was fivefold lower than the efficiency of lipofectamine transfection, we estimated that the efficiency of the former was 100,000-fold higher per microgram of plasmid DNA. Notably, microscopic analysis of RAW 264.7 and HeLa cells infected with *M. smegmatis* harboring the dual-color fluorescence plasmid pGFP_h^E/RFP revealed that infection with a

single mycobacterium was sufficient for GFP transgene expression in the mammalian cells.

Limitations to the use of mycobacteria as vectors for DNA plasmid transfer include their exclusive residence in the vacuole of infected antigen-presenting cells, which restricts their ability to release plasmids directly to the host cell cytoplasm,

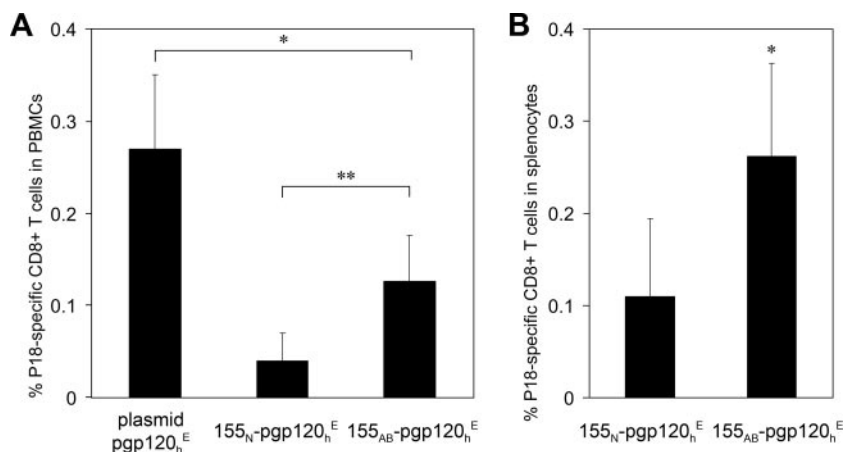


FIG. 8. Rep^{High} *M. smegmatis* strain 155_{AB}(pgp120_h^E) elicits a higher frequency than Rep^{Wt} *M. smegmatis* strain 155_N(pgp120_h^E); tetramer staining and flow cytometric analysis of PBMCs (A) and splenocytes (B) after immunization of BALB/c mice with purified gp120_h^E, Rep^{High}, or Rep^{Wt} strains harboring gp120_h^E. PBMCs or splenocytes were recovered from groups of three to five mice 7 days after immunization with 10⁸ bacilli. The gated population represented both CD8 and P18 tetramer-staining-positive T cells. The mean (and standard error of the mean) percent gp120 P18 tetramer-positive CD8 T cells among PBMCs or splenocytes is shown for each group of mice. After subtraction of background responses in control mice immunized with 155_N(pCMV_{oriM}) or 155_N(pCMV_{oriM}) (not shown), the frequencies of CD8 and P18 tetramer staining responses were significantly higher after Rep^{High} strain immunization than after Rep^{Wt} strain immunization (for PBMCs, $P < 0.01$ [two asterisks]; for splenocytes, $P < 0.05$ [one asterisk]). The frequencies of HIV-1 P18-specific CD8⁺ T-cell responses among PBMCs were significantly higher after intramuscular gp120_h^E immunization than after strain 155_{AB}(pgp120_h^E) immunization (one asterisk, $P < 0.05$).

and the low copy number of mycobacterial plasmids (about five copies per cell). To overcome these restrictions, we determined whether *M. smegmatis* with an enhanced-conjugation or premature-lysis phenotype or *M. smegmatis* or BCG with an increased-plasmid-replication phenotype would facilitate DNA plasmid transfer to the mammalian cell nucleus.

We did not observe an increase in the frequency of plasmid transfer in HeLa cells infected with mycobacterial auxotrophs that lysed prematurely compared to the cells infected with wild-type strains. This is in contrast to observations of other workers, who found that auxotrophic mutants of *Shigella* that lyse prematurely are highly effective vectors for plasmid gene transfer to mammalian cells (33). Unlike *Shigella* and *Listeria*, wild-type *M. smegmatis* and BCG do not escape the endosome. This suggests that DNA release, mediated by premature lysis, is not a limiting factor in gene transfer to mammalian cell nuclei. This observation, combined with the effect of copy number and the inhibitory influence of Esx-1, also indicates that the mechanism of bacterofection by *M. smegmatis* is fundamentally different than the mechanisms studied previously.

Consistent with our prediction that hyperconjugation in mycobacteria would correlate with an enhanced ability to transfer genes to mammalian cells, a significantly (albeit only 1.7-fold) higher frequency of GFP expression was observed in HeLa cells infected with a hyperconjugating mutant of *M. smegmatis* (11) than in cells infected with the wild-type parent. This effect was suppressed by genetic complementation of the mutant strain with the Esx-1 region from *M. tuberculosis*, suggesting that, similar to conjugation between mycobacteria, the Esx-1 apparatus secretes proteins that inhibit gene transfer to mammalian cells. We currently have no definitive explanation for why the presence of the *M. tuberculosis* Esx-1 apparatus would suppress bacterofection to almost background levels. One possibility is that the *M. tuberculosis* locus encodes proteins not present in *M. smegmatis* which have a greater inhibitory effect on bacterofection.

To distinguish between EsxA and EsxB inhibition and other effects of Esx-1 on bacterofection, the two genes encoding EsxA and EsxB were deleted; wild-type levels of bacterofection were observed (Fig. 5). This suggests that another protein(s) secreted by *M. smegmatis* Esx-1 normally suppresses bacterofection or that the apparatus itself interferes with plasmid transfer. Disruption of the entire locus would prevent either structural interference or secretion of other proteins and thus allow elevated levels of bacterofection. An *M. smegmatis* strain that contains a mutation that reduces its conjugation efficiency 1,000-fold was still able to transfect cells (although at reduced levels), suggesting that the conjugation system supports—but is not necessary for—the release of DNA into the host cell by mycobacteria.

The plasmid-encoded RepA and RepB proteins were overexpressed from the *hsp60* promoter to increase the pAL5000 copy number. Attempts by other workers to overexpress the pAL5000 Rep proteins in *trans* from a second episomal plasmid (39) in *M. smegmatis* resulted in a less-than-twofold increase in pAL5000 copy number. By contrast, we observed that overexpression of copies of *repA* and *repB* in *trans* from the chromosome consistently resulted in a 5- to 10-fold increase in the copy number of pAL5000-based plasmids in *M. smegmatis*. In addition, the episomal plasmid was maintained stably in

both the exponential and stationary phases of bacterial growth (data not shown). The most likely explanation for the difference in these and previous results is the location of the *repAB* genes supplied in *trans*. In the Rep^{High} strains 155_{AB}, 155_A, and 155_B, integration of the *repAB* genes into the chromosome ensured their constitutive and stable expression. By contrast, introducing a second plasmid also encoding RepAB may result in plasmid incompatibility, a lower copy number of both plasmid types, and inevitable plasmid loss.

pAL5000 encodes a negative regulatory circuit, which appears to control mRNA synthesis of RepA and RepB (38) from the native promoter. One explanation for our inability to recover BCG clones, which overexpress the Rep proteins from the constitutive *Hsp60* promoter, is that these proteins are toxic or interfere with replication of slowly growing mycobacteria, such as BCG. Indeed, we consistently observed that Rep^{High} *M. smegmatis* strains exhibited a significantly lower in vitro growth rate than the Rep^{Wt} strain (data not shown), suggesting that even in rapid growers RepAB overexpression exacts a toll on mycobacterial growth. We were surprised at the lack of an immune response induced by BCG(pHlgp120^E). This may have been due to the relative inefficiency with which BCG induces apoptosis in infected phagocytic cells compared to *M. smegmatis*. Of note, we observed that a markedly higher proportion of THP1 cell lines infected with *M. smegmatis* than of cells infected with BCG undergo apoptosis as detected by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling staining after 72 h (36.7 and 4.6%, respectively) (data not shown). After *M. smegmatis* induces apoptosis in infected phagocytic cells in vivo, vesicles carrying plasmid DNA may be taken up by bystander antigen-presenting cells. The basis for the inferior immunogenicity of *M. smegmatis* harboring pHlgp120_h^E compared to that of *M. smegmatis* Rep^{Wt} strain 155_N(pgp120_h^E) is not known. One possibility is that the episomal plasmids are maintained more stably in the former strain in vivo, resulting in a lower efficiency of plasmid transfer to macrophages; unlike pMV261, p16R1 and its derivative pHIGH100 has the full-length *rap* (14). This may lead to increased stability of the plasmid in mycobacteria.

No naturally occurring mycobacterial plasmid has been observed to be maintained at a level of more than five copies per bacterium on average. To our knowledge, this work demonstrates, for the first time, that plasmid replication to obtain ≥ 30 copies per bacterium can be achieved in mycobacteria by overexpression of copies of *repA* and *repB* in *trans* from the chromosome. Furthermore, the higher copy number is stably maintained in vitro and is associated with enhanced eukaryotic gene transfer into mammalian cells both in vitro and in vivo. The use of Rep^{High} strains should reduce, by up to 10-fold, the amount of *M. smegmatis* cells required for plasmid extraction compared to the amount of wild-type cells. This expands the utility of *M. smegmatis* as a surrogate system for the manipulation and study of genes from *M. tuberculosis* and other slowly growing mycobacteria. The effect of Rep overexpression in *M. smegmatis* hyperconjugation mutants or *M. smegmatis* strains that harbor pHIGH100 on plasmid copy number and transfection efficiency will be studied in future experiments; we anticipate that it should increase the efficiency of gene transfer further. Also, the effects of immunization with mutant strains

of BCG or attenuated *M. tuberculosis* strains that induce apoptosis in phagocytes will be studied.

M. smegmatis and *M. bovis* BCG have the advantage of being strong adjuvants compared to other attenuated bacterial vectors. Moreover, BCG, which is given to more than 85% of infants worldwide (1), is well tolerated and can be taken orally, making it an attractive vector for recombinant and/or DNA vaccines against other pathogens in neonates. In this context, it is noteworthy that BCG is one of the few vaccines used in humans that elicit strong Th1-type responses against heterologous and homologous antigens in infants (25). It has been demonstrated previously that *M. smegmatis* harboring plasmids encoding HIV gp120, expressed from a mycobacterial alpha-antigen promoter, generated T-cell responses in mice (5, 47) and primed for protein boosts of anti-Env neutralizing antibodies (47). This encourages the view that recombinant *M. smegmatis* should be further developed as a vaccine vector against HIV. The frequencies of gp120-specific CD8⁺ T-cell responses observed after immunization with strain 155_{AB} (pgp120_h^E) were significantly lower than those observed after intramuscular immunization with pgp120_h^E; however, the former method may generate more durable memory T cells, as has been observed after immunization with recombinant *M. smegmatis* expressing a recombinant antigen compared to intramuscular DNA vaccination (20). In future studies we will study the effects of dual priming with recombinant mycobacteria that express antigens as well as harbor a transgene that encodes a eukaryotic gp120 expression cassette.

We have identified bacterial genetic components that affect either conjugation or plasmid copy number and that enhance mycobacterial bactofection in vivo. In the future we will focus on a genetic approach to isolate mutants that increase the efficiency of mycobacterial bactofection and provide insight into the mechanism of gene transfer. Mycobacterial bactofection is a promising vaccination method with the potential for having a great impact on controlling infections that affect young infants and for which no vaccines exist (e.g., breast milk HIV transmission and respiratory syncytial virus) or for which existing vaccines are limited (e.g., live-attenuated measles vaccine, due to interference by passive maternal antibody).

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