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Attenuated and Wild-Type HIV-1 Infections and Long Terminal Repeat-Mediated Gene Expression from Plasmids Delivered by Gene Gun to Human Skin *ex Vivo* and Macaques *in Vivo*

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Gene expression from HIV-based gene therapy vectors or live-attenuated HIV-1 vaccines requires RNA transcription supported by the HIV-1 promoter, the long terminal repeat (LTR). Delivery of live-attenuated HIV-1 vaccines as plasmid DNA would overcome problems associated with production of attenuated HIV-1 strains. We investigated the expression of reporter plasmids and proviral HIV-1 constructs driven by either the HIV-1 LTR or LTRs with deletions in the U3 enhancer regions. LTR-driven plasmids were inoculated by gene gun into both human epidermis *ex vivo* and macaques *in vivo*. The HIV-1 LTR drove reporter gene expression in human and macaque skin, although with 15- to 20-fold less efficiency compared to the immediate-early cytomegalovirus promoter. A deleted LTR derived from a naturally attenuated HIV-1 strain infecting a member of the well-characterized Sydney Blood Bank Cohort of long-term nonprogressors was 5-fold less efficient in expression of the reporter gene compared to wild-type LTR. Delivery of proviral wild-type HIV-1 DNA constructs to human skin resulted in recovery of HIV-1 from cells emigrating from the epidermis, providing an *ex vivo* model of the infectivity of proviral HIV-1 DNA. However, delivery of proviral HIV-1 DNA containing deletions in either the LTR, Nef, or the secondary viral transcription activator,Vpr, significantly reduced HIV-1 replication in this model. The early coexpression of Tat from a second plasmid did not restore replication. Thus, although attenuated lentiviral vaccines might be deliverable as proviral DNA constructs in primate subjects, significant improvements are needed to enhance the efficiency of this method.

Key Words: HIV-1; vaccines; tat; live-attenuated.

INTRODUCTION

The HIV-1 pandemic continues to escalate, particularly in less developed countries, and HIV vaccine research remains a global priority. Among candidate HIV-1 vaccines that currently show promise are naked DNA vaccines expressing HIV-1 antigens and live-attenuated HIV-1 vaccines (Barouch *et al.*, 2000; Boyer *et al.*, 1997; Daniel *et al.*, 1992).

HIV-1 DNA vaccines, by themselves, provide only partial protection from AIDS-inducing viruses in animal models in comparison to live-attenuated lentiviral vaccines (Barouch *et al.*, 2000; Daniel *et al.*, 1992; Lu *et al.*, 1996). Most DNA vaccines utilize the immediate-early cytomegalovirus (CMV) promoter to drive antigen expression. The CMV promoter is a strong, constitutive promoter in many fibroblastoid tissue culture cells, but is comparatively weak in T-lymphocytes that have not been activated with mitogen and may have little advantage over the HIV-1 promoter, the long-terminal-repeat (LTR) (Hunninghake *et al.*, 1989; Sambucetti *et al.*, 1989). Indeed, the HIV-1 LTR might initiate more effective antigen expression in important antigen-presenting cells such as macrophages or dendritic cells (Pope *et al.*, 1994). Additionally, since viral transcriptional transactivators such as Tat can enhance the LTR promoter, codelivery of DNA vaccines encoding Tat could increase LTR-driven gene expression in primate tissues. In this study, we evaluated the efficiency of gene-gun-delivered DNA vaccines and HIV-1 proviral DNA utilizing the LTR promoter in both macaques and human skin.

Human subjects infected with HIV-1 strains attenuated *in vivo* by the deletion of portions of the *nef* and LTR genes or of *vpr* have been described (Alexander *et al.*, 2000; Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995). The 12 individuals that constitute the Sydney Blood Bank Cohort were infected by a common blood donor and have HIV-1 strains with a common deletion in the U3 region of the LTR, in addition to various flanking deletions in *nef* and U3, that account for the attenuated phenotype observed (Deacon *et al.*, 1995). Infection of macaques with *nef*/LTR-deleted SIV provides protective immunity from virulent SIV challenge (Daniel *et al.*, 1992; Wyand *et al.*, 1996). However, *nef*/LTR-deleted SIV and HIV-1 strains are only

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partially attenuated *in vivo,* resulting in disease in a proportion of SIV-infected macaques and HIV-1-infected humans (Baba *et al.,* 1995; Learmont *et al.,* 1999). Despite these limitations, should modified attenuated lentivirus vaccines eventually prove both safe and efficacious, delivering such viruses to large numbers of people in undeveloped countries poses logistical problems. Manufacturing difficulties involved in the production of live lentiviruses would be overcome if an infectious attenuated HIV-1 vaccine could be manufactured as proviral DNA. A DNA-based vaccine would be inexpensive to manufacture, quality control would be greatly simplified, and such a vaccine would resist degradation during transport. To date, examination of limited numbers of inoculated macaques suggests that wild-type SIV can be delivered as proviral DNA intramuscularly (Letvin *et al.,* 1991; Liska *et al.,* 1999). However, delivery of *nef*/LTR-deleted attenuated SIV or HIV-1 could be difficult since the transcriptional efficiency of the LTR could be compromised as a result of deletions in the promoter and enhancer regions. Although delivery of attenuated HIV-1 would be practical when delivered as DNA, it may be important to optimize this method of delivery. We compared the efficiency of reporter gene expression by an HIV-1 LTR plasmid containing U3-region deletions to wild-type HIV-1 LTR and CMV expression plasmids in human and macaque skin. In addition, we addressed directly the effect of deletions in LTR *nef* and *vpr* genes on the levels of expression of HIV-1 in human skin explants after gene-gun transfection of HIV-1 provirus.

RESULTS

HIV-1 LTR-mediated transcription in macaques *in vivo*

We compared the efficiency of LTR-mediated gene expression from DNA vaccines delivered to macaques by gene gun, with or without the codelivery of Tat, to gene expression mediated by the CMV immediate-early (IE) promoter commonly used by DNA vaccines. The β-galactosidase (β-gal) reporter plasmids pCMVβgal, pLTRNL4-3βgal, and pLTRNL4-3βgal plus pCMVtat, or pCMVtat control plasmid expressing only Tat, were applied to four limbs of two macaques by gene gun (Table 1). Animals subsequently underwent skin biopsies at 2 days and a regional lymph node biopsy at 14 days from all four limbs. Two days following immunization, β-gal expression was readily observed by microscopy of stained tissue sections of pCMVβgal-immunized skin biopsies of both monkeys (Table 1, Figs. 2C and 2D). β-gal-expressing cells were detected following gene-gun delivery of the pLTRNL4-3βgal construct, but this occurred with 15-fold less efficiency than the pCMVβgal expression. Codelivery of pCMVtat with the pLTRNL4-3βgal plasmid resulted in marginally (2- to 3-fold) more β-gal expression than administration of pLTRNL4-3βgal alone.

TABLE 1

In Vivo Expression in Macaque Skin of HIV-1 NL4-3 LTR or CMV IE Promoter-Driven Reporter Constructs

Plasmid administered (1 μg to skin by gene gun)	Limb administered to	β-gal-expressing cells/500 gold beads in macaque skin biopsy 2 days after plasmid inoculation	
		Monkey 1[a]	Monkey 6[a]
pCMVtat	Left arm	0, 0	0, 0*
pCMVβgal	Right arm	82, 104	69, 43
pLTR NL4-3βgal	Left leg	4, 6	3, 7
pLTR NL4-3βgal + pCMVtat	Right leg	9, 15	12, 12

[a] Two separate histologic sections from each biopsy were counted.
* There was a statistically significant difference in β-gal expression dependent on the plasmid administered ($P < 0.001$, ANOVA). Individual differences between pairs of the four groups were also all significant ($P < 0.01$, Student's t test).

Since significant marker gene expression was observed in the skin, draining lymph nodes from each immunized limb were sampled at autopsy and examined for protein expression 2 weeks after DNA inoculation. β-gal-positive cells were observed in the lymph nodes draining limbs receiving β-gal immunization from both immunized animals, but not in lymph nodes from the control limbs draining the pCMVtat inoculated site. Lymph node cells positive for β-gal were detectable but rare (3–10 cells) in the 10 histologic sections screened from lymph node cells draining the three sites of β-gal immunization (pCMVβgal, pLTRNL4-3βgal, and p LTRNL4-3βgal plus pCMVtat), without significant differences between groups.

Wild-type and attenuated LTR-mediated transcription in human skin *ex vivo*

The efficiency of the HIV-1 LTR promoter in human skin is not known. Additionally, it is not known whether plasmids bearing deletions in the U3 enhancer region of the LTR, such as those derived from the Sydney Blood Bank Cohort of long-term slow progressors, can mediate transcription in primary human skin tissues. We delivered the LTR reporter constructs as DNA to human skin explant cultures and subsequently assessed β-gal expression. LTR NL4-3-driven plasmids expressed β-gal in a smaller number of cells than the CMV IE promoter reporter plasmid by a factor of approximately 12 (Table 2). A deleted LTR-driven reporter construct derived from a member of the Sydney Blood Bank Cohort (pLTRC18mbcβgal) that lacks one of the GAGA elements of the negative regulatory element as well as the COUP, AP-1, NRT-1, Ets-1, and Lef-1 transcription factor binding elements resulted in detectable gene expression (Table 2, Figs. 1, 2A, and 2B). However, pLTRC18mbcβgal detectable β-gal gene ex-

TABLE 2

Administration of Wild-Type and Deleted LTR Reporter Constructs into Human Skin Explants

Plasmid administered	β-gal-positive cells following 1 μg DNA injected into 1 cm^2 of human skin by gene gun[a]
pCMVtat	0, 0, 0*
pCMVβgal	547, 782, 881
pLTRNL4-3βgal	39, 48, 92
pLTRC18mbcβgal	6, 11, 15

[a] Three separate skin pieces were inoculated and counted for each plasmid.

* There was a statistically significant difference in β-gal expression dependent on the plasmid administered ($P < 0.001$, ANOVA). Individual differences between pairs of the four groups were also all significant ($P < 0.04$, Student's t test).

pression was found in approximately fivefold fewer cells in the human skin explants compared to the wild-type HIV-1NL4-3 LTR reporter plasmid. Morphologically, the vast majority of transfected cells in the epidermis were keratinocytes utilizing either CMV or LTR promoters (Fig. 2B), and this was confirmed by flow cytometry of enhanced green fluorescent protein (EGFP)-transfected epidermal cells (data not shown).

Expression of infectious virions in human skin *ex vivo* after gene-gun transfection

The experiments utilizing the LTR-driven expression constructs showed that wild-type LTR was 10- to 15-fold more efficient at initiating gene expression in skin than an HIV promoter bearing U3 deletions found in attenuated HIV strains infecting the Sydney Blood Bank Cohort. We wished to test whether these LTR deletions and other attenuating deletions in the *nef* and *vpr* genes would diminish ongoing viral replication in the human skin explant model. To assess this, we expressed both T-cell tropic (T-tropic, pNL4-3) and macrophage tropic [M-tropic, pNL(AD8)] proviral HIV-1 strains with deletions in the *vpr, nef,* and/or U3 genes from proviral constructs that had a wild-type 5′ LTR. The progeny virus from the plasmid with a U3 deletion in the 3′ LTR would transduce the U3 deletion into the 5′ LTR after the first infection cycle. The two different *env* types were assessed since previous studies utilizing live HIV-1 virus suggested that M-tropic strains more efficiently infected Langerhans cells emigrating from human skin (Reece *et al.,* 1998).

The previously described method for detecting HIV production and transfer from the skin emigrants was used to determine HIV-1 replication after gene-gun transfection (Reece *et al.,* 1998). The epidermal cells emigrating from the epidermal sheets were cocultured with activated PBMCs and reverse transcriptase (Fig. 3) and PCR (Fig. 4) assays for virus were employed. After transfection of either the M-tropic pNL(AD8) or the T-tropic pNL4-3 molecular clones, HIV-1 replication was detected in the cocultures by reverse transcription assays, with no significant difference between the different strains with different tropisms (Fig. 3). The level of reverse transcriptase activity in cocultures of cells migrating from epidermis transfected with deleted provirus contructs was less than in cocultures from skin transfected with wild-type provirus. Deletions in *nef* reduced virus production but virus production was undetectable by reverse transcriptase in cocultures where provirus defective in both *nef* and U3 or provirus with a *vpr* premature termination

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                |-> U3        |>Sp1-4<|                                                                    |> 100
pLTR_NL4-3      TGGAAGGGCTAATTCACTCCCAAAGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGG
pLTR_ΔU3        TGGAAGGGCTAATTCACTCCCAAAGAAGACAAGATATCC.....................................................
pLTR_C18mbc     TGGAAGGGCTAATTCACTCACAGAGAAG.....................................................

                COUP/ AP-1<|       |>AP-1<|     |>       NRT-1      <|>myb<|> AP-1 <|                    |> GAGA  <|      199
pLTR_NL4-3      GCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAG.ATAAGGTAGAAGAGGCCAATAAAGGAGAGAACACCAG
pLTR_ΔU3        .....................................................................................
pLTR_C18mbc     ..............................................ATCAGTTGAACCAGAAGAAGATAGAAGAGGCC.ATGAAGAAGAAAACAACAA

                                                  |> GAGA  <|                                  |>USF<|        299
pLTR_NL4-3      CTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
pLTR_ΔU3        .....................................................................................
pLTR_C18mbc     ATTGTTCCGT.....................................TTGTTCCGTTGGGGACTTTCCAGGAGACGTGGCCTGAGTGACTAAGCCC..

                     |>Ets-1<|   |>Lef-1<|                        |> NFkB <|          |> NFkB <| |>Sp1-3<|     |>  390
pLTR_NL4-3      GAGCTGCATCCGGAGTACTTCAAGAACTGCTGACATCGAGCTTGCTACAAGGGACTTTCCG.........CTGGGGACTTTCCAGGGAGGCGTGGCCTGG
pLTR_ΔU3        ......CATCCGGAGTACTTCAAGAACTGCTGACATCGAGCTTGCTACAAGGGACTTTCCG.........CTGGGGACTTTCCAGGGAGGCGTGGCCTGG
pLTR_C18mbc     ..............................................CGCTGGGGACTTTCCGAAGAGGCGTGACGGGACTTTCCAAGGCGGCGTGGCCTGG

                Sp1-2<||>Sp1-1<|                  TATA                          |->  R                TAR          490
pLTR_NL4-3      GCGGGACTGGGGAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGC
pLTR_ΔU3        GCGGGACTGGGGAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGC
pLTR_C18mbc     GCGGGACTGGGGAGTGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTCTGCCTGTAC.GGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGC

                                                  532
pLTR_NL4-3      TCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAA
pLTR_ΔU3        TCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAA
pLTR_C18mbc     TCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAG
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FIG. 1. Genetic sequence of the LTR (U3/R) component employed as β-gal reporter constructs of HIV-1_{C18mbc} in comparison to HIV-1_{NL4-3}.

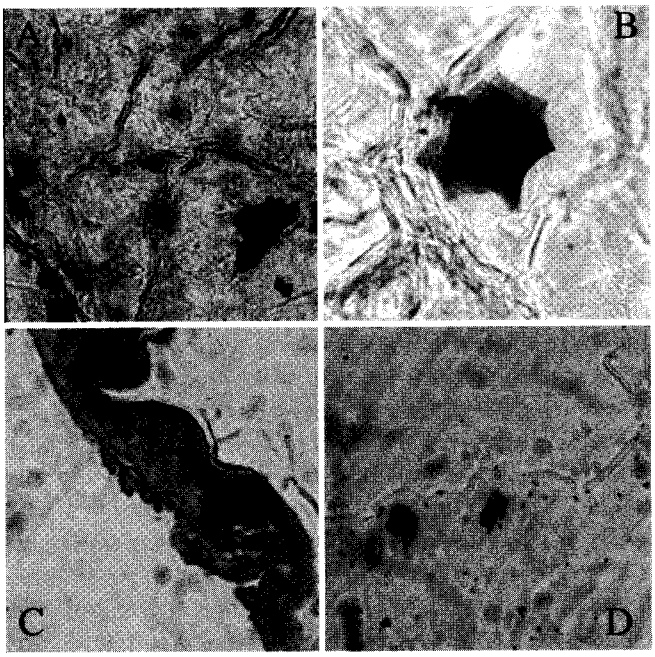

FIG. 2. Expression of a marker gene in human and primate skin following immunization with DNA using HIV-1 LTR promoter. A gene gun delivered 1 μg of DNA attached to 1- to 3-μm gold beads into human epidermal skin explants *ex vivo* (A and B) and monkey skin *in vivo* (C and D). Expression of β-galactosidase protein was assessed by X-gal staining of whole tissues. C was also stained with hematoxylin and eosin to show epidermal tissues. The gold beads can been seen as small dense particles within the epidermis and most β-galactosidase-expressing cells contain one or more gold beads. The DNA plasmids were as follows: (A) pLTRNL4-3βgal, (B) pLTRC18mbc-βgal (a cell with morphology typical of a keratinocyte is shown), (C) pLTRNL4-3βgal, (D) pLTR NL4-3βgal + pCMVtat.

mutation was used. The more sensitive PCR assay showed low levels of infection in cocultures following transfection with proviral DNA containing the *vpr* mutation, but not in cocultures following transfection with the nef and U3 deletions (Fig. 4). The addition of pCMVtat by cotransfection together with the wild-type or deleted proviral constructs did not increase or rescue HIV-1 replication (Fig. 3, right-hand panels, Fig. 4).

DISCUSSION

This study of HIV-1 LTR-mediated protein expression in human and macaque skin found that 5- to 10-fold fewer cells expressed β-gal under direction of the LTR promoter compared to the CMV promoter in both human and macaque skin. In macaques, codelivery of HIV-1 tat DNA utilizing the CMV promoter resulted in a modest (2- to 4-fold) increase in the number of cells expressing β-gal from the LTR promoter *in vivo*. Both the CMV and the LTR promoters resulted in small but detectable numbers of β-gal-positive cells in draining lymph nodes sampled 2 weeks after inoculation. The insignificant differences in lymph node expression seen between LTR and CMV-promoter expression may reflect the small numbers of transfected cells observed or differences in expression of these promoters in lymph node tissues (Leonard *et al.*, 1989). The finding of reporter gene expression in lymph nodes has important implications for live-attenuated HIV vaccines delivered as DNA to the skin, since these viruses are likely to be required to reach the lymph node to efficiently expand.

In human skin explants transfected with DNA *ex vivo*, an LTR derived from a Sydney Blood Bank Cohort member lacking a number of transcription factor binding elements resulted in approximately fivefold less efficient gene expression compared to the wild-type LTR construct. Use of the human skin explant model was consistent with observations in macaque skin, suggesting that this explant model could be used to assess the relative efficiency of gene transfer and infectivity of HIV-1 in human skin *in vivo*. When human skin was transfected with a series of proviral HIV-1 DNA constructs, infectivity of the skin emigrants was readily detectable from both T-tropic and M-tropic wild-type constructs. The deletion of sequence within *nef* did not completely diminish viral

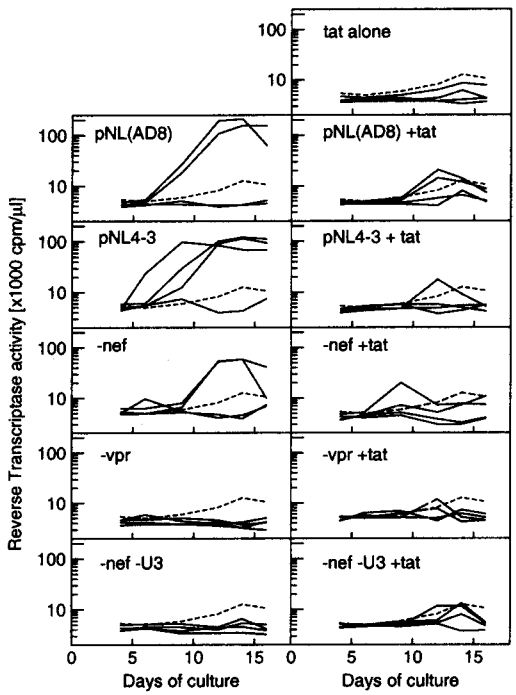

FIG. 3. Virus production in cocultures of activated T-cells and cells emigrating from transfected skin explants. Skin was transfected with M-tropic pNL(AD8) or T-tropic pNL4-3 proviral HIV-1 constructs or pNL(AD8) constructs containing deletions in *nef* (pNL(AD8)Δnef2.1, labeled -nef), deletions in *nef* plus U3 (pNL(AD8)ΔnefΔU3, labeled -nef -U3), and a premature termination mutation in *vpr* (pNL(AD8)ΔR, labeled -vpr). Cells migrating from the epidermis were cocultured with activated PBMCs and reverse transcriptase activity was monitored. The kinetics of HIV-1 replication for each of four replicate transfections with each construct is shown. The dashed line indicates the cutoff for a negative value and is the mean + 3 SD for cocultures of emigrants from skin transfected with pCMVtat alone. The right-hand panels correspond to the cultures from skin cotransfected with pCMVtat (+tat) and the infectious clone indicated on the left.

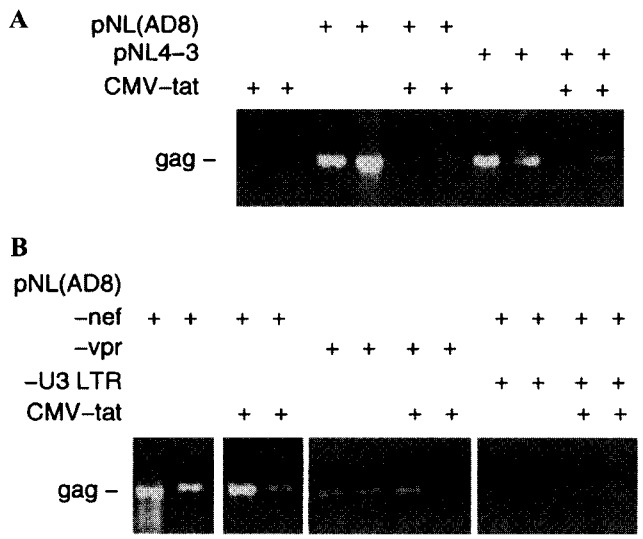

FIG. 4. PCR amplification of HIV-1 provirus in cocultures of T-cells and human skin emigrants. PCR for HIV-1 gag was performed on cells harvested from day 16 of culture. Results for two skin samples are shown for each plasmid construct. (A) Cocultures of skin transfected with M-tropic provirus pNL(AD8) and for T-tropic provirus pNL4-3. (B) Cocultures of skin transfected with pNL(AD8) with deletions of *nef*, combined deletions of both *nef* and U3, and a premature termination mutation in *vpr*. Each set of transfections was also assessed following the cotransfection of pCMVtat.

replication in the cocultures; however, a termination mutation in the *vpr* gene reduced replication such that it was detected only by PCR, and a combined *nef* and U3 deletion completed abrogated infectivity of the proviral contruct in the skin explant model. The poor transfer of attenuated HIV-1 viruses from emigrating Langerhans cells to stimulated PBMCs seen in this system is likely the result of reduced production in the keratinocytes and Langerhans cells (LCs), reduced transfer from the transfected keratinocytes to LCs, or reduced transfer from the emigrating LCs to the activated PBMCs.

Our results suggest that the protein expression levels and immunogenicity of DNA vaccines in either humans or other primates are unlikely to be improved by utilizing the LTR promoter, consistent with recently published data in macaque models (Galvin *et al.*, 2000). The observation that the LTR-mediated gene expression in human and macaque tissues does occur is, however, consistent with reports of infections initiated in macaques by administration of full-length wild-type proviral SIV DNA via intramuscular inoculation (Ilyinskii *et al.*, 1997; Letvin *et al.*, 1991; Liska *et al.*, 1999). Recent data suggest that wild-type and LTR-deleted SIV infections can also be initiated by administering proviral SIV DNA to the skin utilizing only 15 μg of DNA delivered epidermally via the gene gun (S. Kent *et al.*, unpublished data). Our results here suggest that more efficient generation of lentiviral infections could be mediated by generating constructs using the heterologous CMV promoter in place of the 5′ LTR. Theoretically, the coadministration of Tat under the control of a CMV promoter could also enhance infectivity of LTR-driven constructs, and a modestly higher proportion of cells supported gene expression with coadministration of Tat (Table 1). However, no increase in infectivity was observed when Tat was codelivered with deleted proviral HIV-1 constructs to human skin (Figs. 3 and 4). Coexpression of both *tat* and green fluorescent protein (GFP) in the skin had no effect on the total number of LCs emigrating from the epidermis or GFP-transfected LCs, suggesting that tat was not having an effect on LC viability or mobilization from the epidermis (data not shown). The reduced infectivity following cotransfection with tat could reflect competition between the CMV and the LTR promoters or comparative inefficiency of Tat to enhance replication of virus in the second round of viral replication.

The observation that a deleted LTR derived from the Sydney Blood Bank Cohort was less efficient than the wild-type LTR suggests that the loss of critical transcription factor binding elements may play a role in the attenuation of the infection observed in the Sydney Blood Bank Cohort in addition to defects in Nef protein function and stability. These observations on deleted LTRs in human skin *ex vivo* are consistent with preliminary observations following transfection of immortalized cell lines *in vitro* (P. Thompson *et al.*, unpublished data) and SIV-LTR studies (Ilyinskii *et al.*, 1994, 1997; Ilyinskii and Desrosiers, 1996; Pohlmann *et al.*, 1998). Our results suggest that utilizing proviral DNA may be an inefficient way to deliver attenuated HIV-1 (or SIV) vaccines that contain deletions in the LTR, especially in conjunction with other deletions. Additional manipulations, such as using the CMV promoter instead of the 5′ LTR to drive the initial round of virus production, may enhance the initial replication from these deleted lentiviral constructs in primary primate tissues and facilitate a more efficient vaccination. Delivery of *nef*/LTR-deleted full-length SIV plasmids as proviral DNA is now being assessed in macaques *in vivo*.

MATERIALS AND METHODS

Plasmid constructs

The coding sequence for β-gal or the red-shifted EGFP reporters was expressed from plasmids driven by the CMV immediate-early promoter, the HIV-1_{NL4-3} LTR, or the HIV-1_{C18mbc} LTR (Adachi *et al.*, 1986; Deacon *et al.*, 1995). The pEGFP-N1 plasmid expressing GFP from the CMV promoter was purchased from Clontech (Palo Alto, CA). The plasmid pCMVβgal was prepared by inserting the β-gal coding fragment excised from pSVβgal with *Hin*dIII to *Bam*HI into the same sites of pEGFP-N1. The pLTRNL4-3-EGFP plasmid was prepared by replacing the *Ase*I to *Hin*dIII fragment containing the CMV promoter from pEGFP-N1 with the *Xho*I to *Hin*dIII 3′ LTR fragment from the pNL4-3 proviral plasmid (Adachi *et al.*, 1986).

The plasmid pLTRNL4-3βgal was prepared by replacing the *Hin*dIII to *Xba*I EGFP coding fragment of $pLTR_{NL4-3}$-EGFP with the *Hin*dIII to *Bam*HI β-gal fragment from pSVβgal. The pLTRC18mbc-EGFP and pLTRC18mbc-βgal plasmids were prepared from the attenuated Sydney Blood Bank Cohort virus, $HIV\text{-}1_{C18mbc}$ (GenBank Accession No. U37270), which contains deletions in the negative regulatory element of the U3 region (Fig. 1) (Deacon *et al.*, 1995). The $HIV\text{-}1_{C18mbc}$ LTR was amplified by PCR using primers that included suitable restriction sites and products cloned first into pCRII (Invitrogen, San Diego, CA) and then subcloned to replace the $HIV\text{-}1_{NL4\text{-}3}$ LTR in the respective reporter plasmids.

HIV proviral plasmids included both pNL4-3 and pNL(AD8). pNL(AD8) replaces the *Kpn*I to *Bsm*I *env* fragment of the CXCR4-tropic pNL4-3 with *env* from the M-tropic ADA strain that uses the CCR5 coreceptor from the pAD8.1 proviral clone (Englund *et al.*, 1995; Freed and Martin, 1994; Theodore *et al.*, 1996).

Proviral plasmids deleted by 267 bp in U3 (Fig. 1) and/or 222 bp in *nef* were first prepared in the NL4-3 background by removing the 5′ *Bst*EII site in the $HIV\text{-}1_{NL4\text{-}3}$ 3′ half plasmids, p210-8 and p210-5 [(Gibbs *et al.*, 1994), obtained from the AIDS Reference Reagent Program (ARRP), Rockville, MD, 2486, 2485], by cutting with *Nco*I and *Nru*I, end-filling with Klenow, and religating the blunt ends. The 5′ half of $HIV\text{-}1_{NL4\text{-}3}$ was added to the 3′ half clones by purifying the *Bst*EII and *Eco*RI fragment from p83-2 (ARRP 2497) and cloning this between the same sites in the 3′ half plasmids. The M-tropic *env* was substituted into these T-tropic clones by exchanging the *Eco*RI to *Bam*HI fragment from pNL(AD8), creating the pNL(AD8)Δnef2.1 and pNL(AD8)ΔnefΔU3 plasmids. The pNL(AD8)ΔR plasmid has a premature termination in *vpr* that results from the frameshift resulting from the end-filling and religation of the *Eco*RI site in the pNL(AD8) plasmid.

Plasmid DNA was precipitated onto 0.95-μm gold beads and delivered to shaved epidermis using a He-driven Accell gene gun using 1 μg of DNA per shot as previously described (Kent *et al.*, 1998). For the delivery of both pCMVtat and pLTRNL4-3βgal, DNA was coprecipitated at 1 μg/shot for each plasmid onto the same gold beads. Inoculations of both macaques and human skin employed a He pressure of 350 psi.

Macaques

Two pigtailed macaques (*Macaca nemestrina*), M1 and M6, were used previously for an HIV-1 infection experiment 12 months prior to the reporter DNA inoculations described in this report (Kent *et al.*, 1997). All procedures were performed under ketamine anesthesia and were approved by the Institutional Animal Ethics Committee. The animals were immunized with 1 μg of four different preparations of DNA (pCMVtat. pCMVβgal, pLTRNL4-3βgal, or pLTRNL4-3βgal + pCMVtat) attached to gold beads to shaved skin of the four limbs (Table 1). Two days later, the inoculated site was biopsied using a 5-mm punch skin biopsy. Fourteen days after immunization, the animals were euthanized and the lymph node most proximal to the immunization site of the draining the axillary and inguinal lymph node groups was biopsied.

Human skin

Human skin was obtained with patient consent from the Victoria Plastic Surgery Unit as normal skin otherwise discarded at the time of breast reduction surgery. Skin was stored at 4°C and processed within 2 h of collection as previously described (Reece *et al.*, 1998). After incubation for 30 min on ice in RPMI medium containing 250 μg/ml gentamicin (Durlock, Melbourne, Victoria, Australia) the full-thickness skin was spread on a sterile surface and transfected by a gene gun (Helios, Bio-Rad) at a pressure of 350 psi. Split-thickness skin was prepared from the transfected skin using a Froud skin graft knife. Pieces of split-thickness skin approximately 1 cm^2 in surface area were placed in culture in individual wells of a 6-well plate (Nunc, Naperville, IL) and cultured in RF10 consisting of RPMI 1640 (PA Biologicals, Sydney, New South Wales, Australia) supplemented with 10% heat-inactivated fetal bovine serum (PA Biologicals), 20 μg/ml gentamicin, 10 mM HEPES (Trace Biologicals, Sydney, New South Wales, Australia), and 2 mM glutamine (Trace Biologicals).

Epidermal sheets were prepared from human skin explants by culture at 4°C for 6 h in RPMI containing 5 mg/ml dispase (Worthington, Lakewood, NJ). After being washed in PBS the epidermis was separated from the dermis and processed for detection of β-gal.

β-Galactosidase expression in tissues

To assess comparative β-gal expression in tissues, the skin or lymph nodes were fixed with 2% glutaraldehyde for 2 h, washed, and stained with X-gal (1 mg/ml, Sigma), 5 mM potassium ferrocyanide, and ferricyanide for 48 h at 37°C with shaking. Blue cells were counted either by microscopy on separate histologic sections of macaque skin or using an inverted microscope on the entire 1-cm^2 sheet of human epidermis. β-gal staining was expressed as either the number of blue (transfected) cells per 1 cm^2 of skin that received 1 μg of DNA (human skin) or, since the entire region of the gene-gun delivery to macaque skin was not biopsied (a 5-mm punch biopsy was taken), the number of blue cells/500 gold beads counted (macaque skin).

Detection of virus production from transfected skin explants

Skin explants were transfected with plasmids coding for HIV proviral constructs as described above. Split skin from the site of transfection was prepared using the Froud skin graft knife and then placed in culture in 6-well plates overnight. Split skin sheets were dispase treated as above and the epidermal sheets were placed in culture in 24-well plates. After a further day, 10^6 human PBMCs previously activated with staphylococcal enterotoxin B (40 ng/ml, Sigma) were added to each well to expand any production of HIV-1. Epidermal skin sheets were removed on day 3 and supernatants were harvested from day 4 through 16. Virus production was determined by a reverse transcriptase assay described previously (Reece *et al.,* 1998) and modified to use a PhospholmagerTM (FLA 2000, Fuji Photo Film Co., Tokyo, Japan) to count incorporated radioisotope. On day 16, cells were harvested and lysed, and PCR for HIV-1 was carried out using the HIV-1gag-specific primers A2, gggggacatcaagcagccatgcaaatg, and B2, actccctgacatgctgtcatcatttcttc (Reece *et al.,* 1998).

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REFERENCES

Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M. A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59,** 284–291.

Alexander, L., Weiskopf, E., Greenough, T. C., Gaddis, N., Auerbach, M., Malim, M., O'brien, S., Walker, B. D., Sullivan, J., and Desrosiers, R. C. (2000). Unusual polymorphisms in human immunodeficiency virus type 1 associated with nonprogressive infection. *J. Virol.* **74,** 4361–4376.

Baba, T. W., Jeong, Y. S., Pennick, D., Bronson, R., Greene, M. F., and Ruprecht, R. M. (1995). Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* **267,** 1820–1825.

Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., and Lewis, M. G. (2000). Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290,** 486–492.

Boyer, J. D., Ugen, K., Wang, B., Agadjanyan, M., Gilbert, L., Bagarazzi, M., Chattergoon, M., Frost, P., Javadian, A., Williams, W. V., Refaeli, Y., Ciccarelli, R., Mccallus, D., Coney, L., and Weiner, D. B. (1997). Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. *Nat. Med.* **3,** 526–532.

Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K., and Desrosiers, R. C. (1992). Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258,** 1938–1941.

Deacon, N. J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D. J., Mcphee, D. A., Greenway, A. L., Ellett, A., Chatfield, C., Lawson, V. A., Crowe, S., Maerz, A., Sonza, S., Learmont, J., Sullivan, J. S., Cunningham, A., Dwyer, D., Dowton, D., and Mills, J. (1995). Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* **270,** 988–991.

Englund, G., Theodore, T. S., Freed, E. O., Engleman, A., and Martin, M. A. (1995). Integration is required for productive infection of monocyte-derived macrophages by human immunodeficiency virus type 1. *J. Virol.* **69,** 3216–3219.

Freed, E. O., and Martin, M. A. (1994). HIV-1 infection of non-dividing cells. *Nature* **369,** 107–108.

Galvin, T. A., Muller, J., and Khan, A. S. (2000). Effect of different promoters on immune responses elicited by HIV-1 gag/env multigenic DNA vaccine in *Macaca mulatta* and *Macaca nemestrina*. *Vaccine* **18,** 2566–2583.

Gibbs, J. S., Regier, D. A., and Desrosiers, R. C. (1994). Construction and in vitro properties of HIV-1 mutants with deletions in "nonessential" genes. *AIDS Res. Hum. Retroviruses* **10,** 343–350.

Hunninghake, G. W., Monick, M. M., Liu, B., and Stinski, M. F. (1989). The promoter-regulatory region of the major immediate-early gene of human cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements. *J. Virol.* **63,** 3026–3033.

Ilyinskii, P. O., Daniel, M. D., Simon, M. A., Lackner, A. A., and Desrosiers, R. C. (1994). The role of upstream U3 sequences in the pathogenesis of simian immunodeficiency virus-induced AIDS in rhesus monkeys. *J. Virol.* **68,** 5933–5944.

Ilyinskii, P. O., and Desrosiers, R. C. (1996). Efficient transcription and replication of simian immunodeficiency virus in the absence of NF-κB and Sp1 binding elements. *J. Virol.* **70,** 3118–3126.

Ilyinskii, P. O., Simon, M. A., Czajak, S. C., Lackner, A. A., and Desrosiers, R. C. (1997). Induction of AIDS by simian immunodeficiency virus lacking NF-κB and SP1 binding elements. *J. Virol.* **71,** 1880–1887.

Kent, S. J., Woodward, A., and Zhao, A. (1997). Human immunodeficiency virus type 1 (HIV-1)-specific T cell responses correlate with control of acute HIV-1 infection in macaques. *J. Infect. Dis.* **176,** 1188–1197.

Kent, S. J., Zhao, A., Best, S., Chandler, J. D., Boyle, D. B., and Ramshaw, I. A. (1998). Enhanced T cell immunogenicity and protective efficacy from a HIV-1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpoxvirus. *J. Virol.* **72,** 10180–10188.

Kirchhoff, F., Greenough, T. C., Brettler, D. B., Sullivan, J. L., and Desrosiers, R. C. (1995). Brief report: Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N. Engl. J. Med.* **332,** 228–232.

Learmont, J. C., Geczy, A. F., Mills, J., Ashton, L. J., Raynes-Greenow, C. H., Garsia, R. J., Dyer, W. B., Mcintyre, L., Oelrichs, R. B., Rhodes, D. I., Deacon, N. J., Sullivan, J. S., Mcphee, D. A., Crowe, S., Solomon, A. E., Chatfield, C., Cooke, I. R., Blasdall, S., and Kuipers, H. (1999). Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1—A report from the Sydney Blood Bank Cohort. *N. Engl. J. Med.* **340,** 1715–1722.

Leonard, J., Khillan, J. S., Gendelman, H. E., Adachi, A., Lorenzo, S., Westphal, H., Martin, M. A., and Meltzer, M. S. (1989). The human immunodeficiency virus long terminal repeat is preferentially expressed in Langerhans cells in transgenic mice. *AIDS Res. Hum. Retroviruses* **5,** 421–430.

Letvin, N. L., Lord, C. I., King, N. W., Wyand, M. S., Myrick, K. V., and Haseltine, W. A. (1991). Risks of handling HIV. *Nature* **349,** 573.

Liska, V., Khimani, A. H., Hofmann-Lehmann, R., Fink, A. N., Vlasak, J., and Ruprecht, R. M. (1999). Viremia and AIDS in rhesus macaques

after intramuscular inoculation of plasmid DNA encoding full-length SIVmac239. *AIDS Res. Hum. Retroviruses* **15,** 445–450.

Lu, S., Arthos, J., Montefiori, D. C., Yasutomi, Y., Manson, K., Mustafa, F., Johnson, E., Santoro, J. C., Wissink, J., Mullins, J. I., Haynes, J. R., Letvin, N. L., Wyand, M., and Robinson, H. L. (1996). Simian immunodeficiency virus DNA vaccine trial in macaques. *J. Virol.* **70,** 3978–3991.

Pohlmann, S., Floss, S., Ilyinskii, P. O., Stamminger, T., and Kirchhoff, F. (1998). Sequences just upstream of the simian immunodeficiency virus core enhancer allow efficient replication in the absence of NF-κB and Sp1 binding elements. *J. Virol.* **72,** 5589–5598.

Pope, M., Betjes, M. G., Romani, N., Hirmand, H., Cameron, P. U., Hoffman, L., Gezelter, S., Schuler, G., and Steinman, R. M. (1994). Conjugates of dendritic cells and memory T lymphocytes from skin facilitate productive infection with HIV-1. *Cell* **78,** 389–398.

Reece, J. C., Handley, A. J., Anstee, E. J., Morrison, W. A., Crowe, S. M., and Cameron, P. U. (1998). HIV-1 selection by epidermal dendritic cells during transmission across human skin. *J. Exp. Med.* **187,** 1623–1631.

Sambucetti, L. C., Cherrington, J. M., Wilkinson, G. W., and Mocarski, E. S. (1989). NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. *EMBO J.* **8,** 4251–4258.

Theodore, T. S., Englund, G., Buckler-White, A., Buckler, C. E., Martin, M. A., and Peden, K. W. (1996). Construction and characterization of a stable full-length macrophage-tropic HIV type 1 molecular clone that directs the production of high titers of progeny virions. *AIDS Res. Hum. Retroviruses* **12,** 191–194.

Wyand, M. S., Manson, K. H., Garcia, M. M., Montefiori, D., and Desrosiers, R. C. (1996). Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J. Virol.* **70,** 3724–3733.