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DNA Immunization of Mice against SIVmac239 Gag and Env Using Rev-Independent Expression Plasmids

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ABSTRACT

Simian immunodeficiency virus (SIV) structural gene expression, including *gag* and *env*, strictly depends on the interaction of the viral posttranscriptional regulator Rev with its target RNA, the Rev-responsive element (RRE). A small RNA element, termed the constitutive transport element (CTE), located in the 3′ portion of simian retrovirus 1 (SRV-1) mRNA, can efficiently substitute for the human immunodeficiency virus (HIV) Rev–RRE interaction, and thus render HIV expression and replication Rev independent. We tested the ability of the SRV-1 CTE to drive the expression of SIVmac239 *env* and *gag* from subgenomic constructs designed for possible use in vaccine trials. *In vitro* expression studies showed that when the SRV-1 sequence is coupled to the SIV *gag* and *env* mRNAs, it functions in an orientation-dependent fashion, and leads to strong expression of SIV Gag and Env in human and monkey cell lines; levels of CTE-mediated protein expression were similar to those obtained with a functional Rev–RRE system. On the other hand, in murine fibroblast-like cells, SIV Gag and Env were expressed from constructs at relatively high levels even in the absence of Rev–RRE; nevertheless, their expression was increased by the presence of the SRV-1 CTE. As reported previously for HIV, the murine cell lines appeared to be defective for Rev–RRE activity, and required overexpression of Rev to induce a Rev response. Intramuscular injection of the *gag*-CTE and *env*-CTE constructs in BALB/c mice resulted in the expression of the corresponding mRNAs, and the production of anti-Gag and anti-Env antibodies, thus suggesting that these vectors might be used for genetic immunization approaches.

INTRODUCTION

The expression of the HIV and SIV *gag* and *env* genes, which are encoded in unspliced and singly spliced mRNAs, respectively, is a tightly regulated process involving the interaction between the viral posttranscriptional regulator Rev and the Rev-responsive element (RRE), its target RNA element, present on all incompletely spliced viral mRNAs. In the absence of the Rev protein, multiply spliced transcripts dominate and are preferentially transported into the cytoplasm, and Gag and Env protein synthesis is nearly undetectable. Rev binding to the RRE on unspliced and singly spliced viral mRNAs increased their stability, and transport into the cytoplasm, leading to an efficient translation of Gag and Env proteins.[1,2]

Two independent groups have described a novel artificial Rev-independent pathway of HIV *gag* and *env* gene expression that relies on a short RNA sequence, termed the CTE, encoded in the genomes of two closely related type D retroviruses, namely simian retrovirus 1 (SRV-1),[3] and Mason–Pfizer monkey virus.[4] When expressed on HIV mRNAs, this sequence can efficiently replace the Rev–RRE system, and lead to structural gene expression as well as viral replication in the absence of Rev.

We are presently generating subgenomic expression plasmids encoding SIVmac239 genes for possible use as vaccines in an SIV–macaque model for HIV infection. Considering the high degree of homology between HIV and SIV, we reasoned that the SRV-1 CTE might be capable of rescuing the *gag* and *env* mRNAs of SIV. To this end, we generated eukaryotic expression plasmids carrying either the *gag* or *env* gene of SIV-

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mac239 coupled to the CTE of SRV-1. Functional characterization of the plasmids in cell lines and in mice indicated the utility of this approach for achieving high-level *in vitro* expression of SIV structural genes in an Rev–RRE-independent manner, as well as the generation of an albeit limited *in vivo* response against the relevant antigens.

MATERIALS AND METHODS

Cloning procedures

The entire *gag* coding region, spanning nucleotides 2043 to 3792, was amplified by polymerase chain reaction (PCR) from plasmid p239SpSp5′, which contains the 5′ half of the SIVmac239 genome,[5–6] using primers Gag-for (5′ TGAGTACGGCTGAGTGAAGGCAGTAA 3′) and Gag-rev (5′ CAGGCTGTCCTTCAATATGAGCAGTG 3′). The resulting PCR product was blunt-end cloned into pBluescript KS +/− (Stratagene, La Jolla, CA) at the *Sma*I site. The *gag* gene was then subcloned into the *Kpn*I site of pBKCMV (Stratagene), which contains the cytomegalovirus (CMV) immediate early promoter to drive expression of cloned genes, as well as the neomycin-resistance gene driven by the simian virus 40 (SV40) promoter, to allow stable selection of transfected cells. To increase expression from this plasmid in eukaryotic cells, sequences between the *Nhe*I and the *Not*I sites of the polylinker were removed, according to manufacturer instructions, resulting in plasmid pBKCMV*gag*. A second CMV promoter-driven *gag* expression plasmid was generated by cloning the *gag* gene into the *Hin*dIII site of vector pHCMVHspA (Fig. 1). This vector contains sequences from the human CMV early promoter/enhancer region, a short multiple cloning site, and the poly(A) signal/site from the rat preproinsulin gene.[7]

The SIV *env* gene was PCR amplified from plasmid p239SpE3′, which contains the 3′ half of the SIVmac239 genome,[5,6] using primers Env-for (5′ TGAGCAGTCACGAAAGAGAAGAAGAACTC 3′) and Env-rev (5′ ACTGCCCCTGATTGTATTTCTGTCCC 3′). The resulting 2740-bp fragment was digested with *Xba*I and *Hin*dIII, and inserted between the *Xba*I–*Hin*dIII sites of pHCMVHspA, yielding plasmid pHCMV*env* (Fig. 1).

The 173-bp-long SRV-1 CTE, spanning nucleotides 7620 to 7792 of the SRV-1 genome,[8] was obtained from plasmid pS12 as a restriction fragment, and cloned between the SIV *gag* or *env* gene and the poly(A) signal/site in both the pBKCMV- and pHCMV-derived vectors, resulting in plasmids pBKCMV*gag*-CTE, pBKCMV*gag*-CTE(AS), pHCMV*gag*-CTE, pHCMV-*gag*-CTE(AS), pHCMV*env*-CTE, and pHCMV*env*-CTE(AS) (Fig. 1). Restriction analysis and sequencing confirmed both the identity and orientation of the various cloned fragments described above.

Cell lines and transfections

gag and *env* expression plasmids were transiently transfected into cell lines HLtat, cos-7, and T6 by the calcium phosphate coprecipitation method, as previously described.[9] HLtat is a HeLa-derived cell line that constitutively produces Tat protein[10]; cos-7 is a green African monkey-derived fibroblast-like cell line, and T6 is a fibroblast-like cell line derived from a human T cell lymphotropic virus type I (HTLV-I) tax-transgenic C57BL/6 mouse.

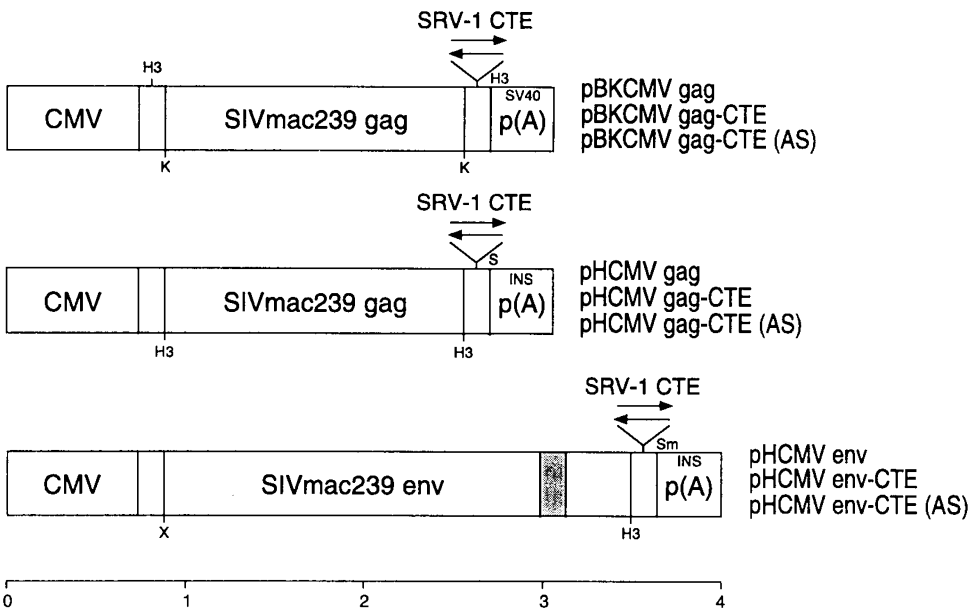

FIG. 1. Schematic representation of the BKCMV- and HCMV-based SIV *gag/env* expression plasmids. The structure of the relevant part of the constructs is shown. Both plasmids contain the human cytomegalovirus (HCMV) early promoter/enhancer sequences and poly(A) sequences from either the rat preproinsulin gene (in the case of HCMV constructs) or SV40 (BKCMV constructs). The SRV-1 CTE was cloned between the 3′ end of the *gag* or *env* gene and the poly(A) signal, in both directions. Position of restriction sites relevant for cloning are shown: H3, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I. Approximate size is indicated at the bottom of the scheme. The dashed box indicates the RRE sequence present in the *env* gene. AS, Antisense.

(D. Saggioro *et al.*, in preparation). These cell lines were selected because of their high permissiveness to transfection by this method, as evaluated in preliminary experiments by transient transfection with pCMVgal, a CMV promoter-driven plasmid expressing β-galactosidase (β-Gal; data not shown).

Radioimmunoprecipitation analysis

Eighteen to 24 hr posttransfection, cells were metabolically labeled for 7 hr with a mixture of [^{35}S]methionine and [^{35}S]cysteine (Promix [Amersham, Arlington Heights, IL]; 14,000 μCi/ml, prepared in methionine and cysteine-deficient Dulbecco's modified Eagle's medium [DMEM]) and lysed in radioimmunoprecipitation assay (RIPA) buffer (140 mM NaCl–8 mM Na_2HPO_4–2 mM NaH_2PO_4–1% Nonidet P-40–0.5% sodium deoxycholate–0.05% sodium dodecyl sulfate [SDS]) as described.[11] As a control for transfection efficiency among the different samples, pCMVgal was invariably cotransfected, and β-Gal activity of cell lysates was measured in a β counter (Packard, Downers Grove, IL). SIV proteins were then immunoprecipitated overnight at 4°C from the cell lysates using a pooled serum from SIV-infected macaques and protein A–Sepharose; bound proteins were released by boiling in 2-mercaptoethanol-containing buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) through 10% polyacrylamide gels. To reduce nonspecific binding to cellular proteins, before incubation with the test lysates the sera were combined with the protein A–Sepharose, and preadsorbed for 2 hr with an unlabeled cell lysate from the nontransfected cell line. Immunoprecipitation of the transfected cell lysates using sera from SIV-uninfected animals did not yield any specific signal (data not shown).

Reverse transcriptase-polymerase chain reaction assay

Total RNA was isolated from frozen muscle using Trizol (GIBCO-BRL, Gaithersburg, MD), according to manufacturer instructions. One microgram of total RNA was digested with RNase-free DNase I (Boehringer GmbH, Mannheim, Germany) and used for the synthesis of first-strand cDNA using reverse transcriptase (Perkin-Elmer, Emeryville, CA) and oligo(dT) primer by methods described elsewhere.[12] Aliquots of the cDNA samples were then amplified with either *gag*- or *env*-specific primers, whose sequences are listed here:

Gag-for: 5′ AGCCGTCAGGATCAGATATTGC 3′
Gag-rev: 5′ CCCTCTTTCCCACAATTCCAAC 3′
Env-for: 5′ ACGCGTCGAC-TAATATCACCATGAGT-GCAGAGG 3′
Env-rev: 5′ ACGCGTCGAC-ACTCTTGCCAAGTCT-CATTGTTC 3′

The *gag* primers amplify a 510-bp product from pHCMVgag-CTE, whereas the *env* primers amplify a 505-bp product from pHCMVenv-CTE. The PCR was performed in a 50-μl volume containing a 0.2 μM concentration of each primer and 0.7 U of *Taq* polymerase (Perkin-Elmer), under the following conditions: denaturation at 94°C for 1 min, annealing at 65°C for 30 sec, and extension at 72°C for 30 sec, for 40 cycles. The amplified products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Serological analysis

Twenty 8-week-old BALB/c mice were immunized three times over a 4-month period by intramuscular (i.m.) injection of either SIVmac239 *gag* or *env* expression vectors carrying the SRV-1-CTE (100 μg of DNA/animal); 10 additional mice served as controls, and were injected i.m. with equal doses of pCMVgal. Blood samples were obtained from the retroorbital plexus of the animals, 2 weeks after the first immunization, and at monthly intervals thereafter. SIVmac239 antibody titers were determined in the sera of immunized animals by end-point dilution, using a solid-phase radioimmunoassay (RIA), as previously described[13] with minor modifications. Briefly, SIVmac antigen (ABI, Columbia, MD; 10 μg/ml in carbonate buffer, pH 9.6) was coupled to the wells of flat-bottom plates (Optiplate; Packard), which were then washed and saturated with 3% bovine serum albumin (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS, pH 7.4); the test samples were added in duplicate (50 μl/well), and incubated overnight at 4°C. The plates were washed, ^{125}I-labeled sheep anti-mouse Ig $F(ab')_2$ (Amersham; specific activity, 19–74 TBq/mmol) was added, and the plates were left to stand for 4 hr at room temperature. The wells were finally washed thoroughly with PBS, 25 μl of Microscint-20 (Packard) per well was added, and the plates counted in an B9912V TopCount β counter (Packard). The antibody contents of the sera were expressed as the reciprocal of the last positive dilution, where values were scored as positive when bound radioactivity exceeded three times background values.

RESULTS

In vitro *characterization of SIV* gag *expression constructs*

We initially tested the different pBKCMV- and pHCMVHspA-derived SIV *gag*-expressing plasmids, either lacking or containing the SRV-1-CTE downstream of the *gag* gene, by transfection into the cos-7 cell line. We observed that the 56-kDa Gag precursor protein was undetectable by RIPA following transfection of pBKCMV*gag*, which lacks the CTE (Fig. 2A, lane 1); this was not surprising, because the *gag* mRNA of several primate lentiviruses is known to contain several instability elements, which promote rapid degradation of the mRNA in the absence of the Rev–RRE rescue system.[14,15] On the other hand, pHCMV*gag* produced higher basal levels of p56 compared with pBKCMV*gag* (Fig. 2A, lane 3). This difference might reflect an influence of the plasmid backbone on the expression of certain foreign genes. Substantially higher levels of p56 expression were obtained using pBKCMV*gag*-CTE or pHCMV*gag*-CTE, both of which contained the CTE downstream of the *gag* gene. Again, pHCMV*gag*-CTE was more efficient in expressing p56 compared with the pBKCMV-derived plasmid (Fig. 2A, lanes 2 and 4).

Transfection of an HCMV*gag* construct carrying the CTE in the antisense orientation with respect to *gag* yielded only background levels of *gag* expression (not shown), thus suggesting that the effects of the CTE are orientation dependent, as reported by others.[4,8] The higher levels of *gag* expression ob-

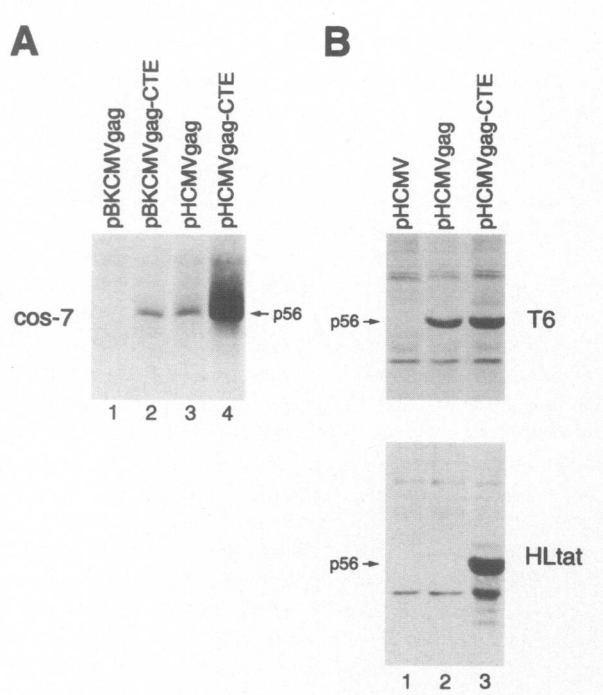

FIG. 2. Comparison of Gag expression from various plasmids. The indicated cell lines were transiently transfected with the indicated constructs, metabolically labeled with [^{35}S]methionine plus [^{35}S]cysteine, and subjected to RIPA as described in Materials and Methods. The position of the precursor Gag protein (p56) is shown. Cells transfected with pHCMV lacking the insert served as control. (A) compares pBKCMV and pHCMV *gag* expression vectors in cos-7 cells. (B) shows Gag expression in human HLtat and murine T6 cell lines transfected with the HCMV constructs.

tained using the HCMVHspA-derived plasmids prompted us to use this vector in all subsequent experiments.

Transfection of the human HLtat cell line with pHCMV*gag* did not yield detectable levels of Gag protein (Fig. 2B, lane 2). In contrast, the T6 murine cell line showed high basal levels of Gag protein production following transfection with this plasmid (Fig. 2B, lane 2). A dramatic increase in p56 production was obtained by transfecting HLtat cells with pHCMV*gag1*-CTE (about 100-fold); T6 cells showed a comparatively smaller increase in Gag production (about 3-fold) (Fig. 2B, lanes 2 and 3, respectively). These observations suggest that some murine cell lines could be able to support Rev–RRE-independent expression of SIV Gag, and offer a slightly less optimal environment for CTE function, compared with primate cells.

In vitro *characterization of SIV* env *expression constructs*

We next tested the ability of the CTE to augment expression of the SIV *env* gene. Transfection of the HCMV*env* plasmid into HLtat cells resulted in the expression of the gp130/160 Env proteins at moderate levels, in the absence of both the Rev protein and the SRV-1 element (Fig. 3, lane 2). Cotransfection of this construct and pCMV*rev*, a plasmid encoding the Rev protein of HIV, strongly increased Env expression in the HLtat cell line (Fig. 3, lane 3). This was indeed an expected result, given that the *env* construct carries the RRE sequence within the *env* gene,[16] and that HIV Rev is known to substitute functionally for SIV Rev.[17] Transfection of the HCMV*env* construct carrying the SRV-1 CTE yielded levels of gp130/160 expression that were similar to those obtained in the presence of Rev (Fig. 3, lane 4). Quantitative analysis by scanning densitometry disclosed that Env expression was increased sevenfold in the presence of Rev protein, and fourfold when the *cis*-acting SRV-1-CTE was present on the 3′ end of the *env* mRNA (average of three experiments). To analyze the effect of multiple CTE copies on Env expression, we transfected HLtat cells with an HCMV*env* construct carrying two copies of the CTE in tandem in the sense orientation; no further increase in Env expression was detected compared with a construct containing a single CTE (data not shown). Cotransfection of pCMV*rev* with pHCMV*env*-CTE resulted in a further increase in Env production (Fig. 3, lane 5); this observation confirmed that Env expression remained Rev sensitive in the context of the SRV-1 CTE, as reported by others.[3,4]

Expression of the Env proteins was also tested in cos-7 and T6 cell lines (Fig. 3). Whereas Env expression in cos-7 cells was similar to that observed in HLtat cells, some differences

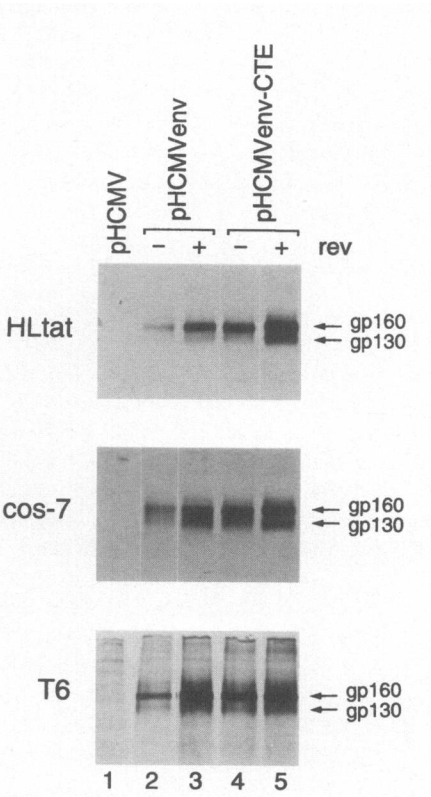

FIG. 3. Comparison of Env expression by various plasmids in the presence or absence of Rev. The cell lines were transiently transfected with the indicated constructs, either alone (–) or with (+) pCMV*rev*; the cells were then metabolically labeled with [^{35}S]methionine plus [^{35}S]cysteine, and subjected to RIPA, as described in Materials and Methods. The positions of the precursor (gp160) and the cleaved Env protein (gp130) are indicated. Cells transfected with pHCMV lacking the insert served as a mock control.

were noted in the T6 cell line. First, Env expression in the absence of Rev was higher than that found in other cell lines, in agreement with findings using *gag* constructs. Second, the two bands of the gp130/160 Env doublet had different intensities; gp130 was underrepresented compared with the precursor protein, suggesting that cleavage might be less efficient in the T6 cell line. Third, cotransfection of either pCMV*rev* or pHCMV*env*-CTE led only to a modest increase in the production of SIV Env proteins, compared with background expression (Fig. 3, lanes 3 and 4). Because Rev activity is thought to require protein accumulation beyond a given threshold,[18] we assayed the transfected T6 murine cell line for Rev expression by immunofluorescence. We observed that Rev protein was expressed at high levels in both the cytoplasm and the nucleus of transfected cells, thus ruling out that poor protein synthesis might have accounted for the defect in Rev function (data not shown). Taken together, our findings confirm that the Rev–RRE system is suboptimally active in rodent cells, as previously suggested by others,[19] and also show that SIV Env expression can be increased in these cells by the alternative SRV-1 CTE-based pathway.

In vivo *expression of SIV* gag *and* env *genes*

To verify whether the SRV-1-CTE based expression system would function *in vivo* in a small animal model, 2 groups of 10 adult BALB/c mice were immunized by i.m. injection with 100 μg of either pHCMV*gag*-CTE or pHCMV*env*-CTE; 10 mice injected with pCMVgal served as controls. The animals were subsequently boosted twice with 100 μg of the respective plasmid DNA; following the last boost, RNA was extracted from the muscles to detect *in vivo* expression of *gag* and *env*. As shown in Fig. 4, we were able to detect *gag* or *env* expression by RT-PCR in mice inoculated with the *gag* and *env* expression plasmids, respectively; the cDNA from mice that received the mock plasmid did not yield any PCR product (Fig. 4). Because contamination by small amounts of plasmid DNA would also generate identical PCR products, we also performed PCR on the same RNA samples treated with DNase I but not reverse transcribed into cDNA. These amplifications did not yield any product, thus confirming that DNA contamination of the samples did not occur (Fig. 4).

Generation of anti-Env and anti-Gag antibodies following plasmid DNA injection into mice

Sera were collected at regular intervals from the mice injected with the different DNA constructs; humoral responses obtained in four representative mice are depicted in Fig. 5. Anti-Gag antibodies were detected in 4 of 10 animals following the second boost at titers ranging between 1:50 and 1:400, while anti-Env antibodies were detected in 6 of 10 animals at titers ranging from 1:100 to 1:1600. No anti-Gag or anti-Env antibodies were detected in mice immunized with pCMVgal; nevertheless, these animals were normally responsive to foreign antigens, as anti-β-Gal antibodies were detected in 5 of 10 animals by ELISA following plasmid injection (not shown). The number of seroconverted animals and the time of seroconversion probably depend on the immunogenicity of the gene products in this mouse strain.

We also tested some mouse sera by RIPA. ^{35}S-labeled Env and Gag proteins were specifically precipitated from cos-7 cells transiently transfected with the relevant plasmids by antisera from mice that were vaccinated with the *gag* and *env* expression plasmids, respectively (Fig. 6). In contrast, serum from the mice immunized with the control plasmid pCMVgal showed no

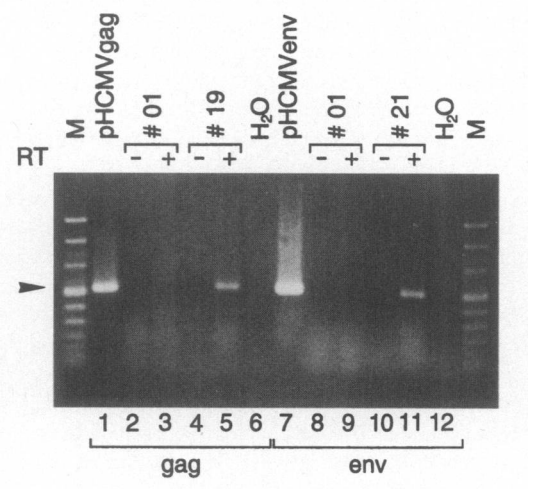

FIG. 4. Detection of *gag* and *env* transcripts in muscle by RT-PCR. RNA samples from two representative mice (19 and 21) and one control animal (01) were treated with DNAse I, and either reverse transcribed (RT^+ lanes) or not (RT^- lanes) before PCR amplification with either *gag*- or *env*-specific primers, as described in Materials and Methods. PCR products were separated on 2% agarose gels with molecular weight marker VIII (Boehringer Mannheim) as a size marker (lane M), and stained with ethidium bromide. Positive (pHCMV*gag* and pHCMV*env*) and negative (distilled water, H_2O) controls are also shown.

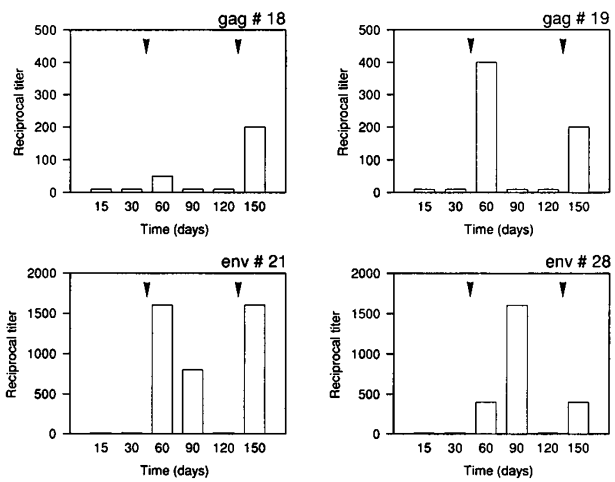

FIG. 5. Anti-Gag and anti-Env antibody titers of sera of mice immunized with DNA vaccines. Sera from 3 groups of 10 mice each immunized with either pHCMV*gag*-CTE, pHCMV*env*-CTE, or pCMVgal were tested for binding to SIV antigens by RIA as described in Materials and Methods. The mice were immunized at time 0 and then boosted twice as indicated (arrowheads); the titer was measured at the reported intervals. Results obtained in two representative *gag*-immunized mice (18 and 19) and two *env*-immunized mice (21 and 28) are shown.

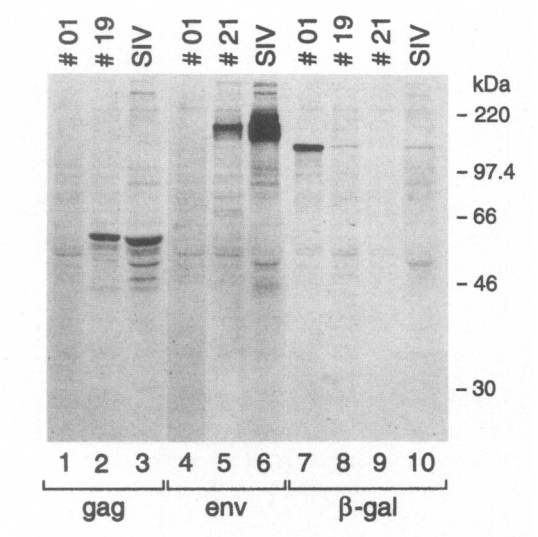

FIG. 6. Radioimmunoprecipitation of ^{35}S-labeled SIVmac239 Gag and Env proteins by DNA vaccine-inoculated mouse sera. Lanes 1, 4, and 7, serum from the control animal inoculated with pCMVgal; lanes 2 and 8, serum from mouse 19, inoculated with pHCMV*gag*-CTE; lanes 5 and 9, serum from mouse 21, inoculated with pHCMV*env*-CTE; lanes 3, 6, and 10, serum from an SIV-infected macaque. Molecular masses (kda) are reported on the right.

activity against SIV proteins, although it could immunoprecipitate β-Gal from cos-7 cells transfected with the β-Gal expressor (Fig. 6). These findings indicated that our constructs were able to express SIV *gag* and *env* gene products *in vivo* in a Rev-independent, SRV-1 CTE-dependent fashion at high enough levels to elicit an immune response.

DISCUSSION

This study demonstrates that the SRV-1 CTE efficiently replaces the Rev–RRE interaction in subgenomic constructs expressing the *gag* and *env* genes of SIVmac239. These data confirm and extend findings by Rizvi *et al.*, who constructed and propagated SIV molecular clones in which the Rev–RRE system was replaced by Mason–Pfizer monkey virus CTE, thus suggesting their applicability to live attenuated vaccines.[20] SRV-1 CTE-enhanced expression of SIV *gag* and *env* genes was observed in human, monkey, and mouse cell lines; we also showed for the first time that the SRV-1-CTE can be useful to engineer the *in vivo* expression of SIV gene products in the absence of Rev.

It was proposed that the simian lentivirus CTE might interact with largely unidentified cellular proteins that allow nucleocytoplasmic transport of viral mRNAs[8]; one of these cellular cofactors of CTE was identified as adenosine 5′-triphosphate-dependent RNA helicase A.[21] Our findings suggest that these factors might be relatively conserved among different species. Although the CTE might be expected to be most active in monkey cells, on the whole it was instead most active in HLtat cells, moderately active in cos-7 cells, and slightly active in murine T6 cells.

The experiments with murine cell lines provide some interesting observations regarding the expression of lentiviral genes in rodents. Little is known about SIV gene expression in murine cells,[22,23] a most studies have addressed the HIV system. Previous *in vitro* data generally suggested that both Tat and Rev of HIV might not function properly in rodent cells[19,24]; on the other hand, *in vivo* studies with subgenomic constructs described HIV structural gene expression in mice, as indicated by an immunologic response to the relevant retroviral gene products.[25,26]

Our findings confirm that the Rev–RRE system is suboptimally active in a murine cell line, in that Env expression was only moderately increased when Rev was grossly overexpressed. The most striking finding, however, was the demonstration of relatively high levels of Gag and Env expression in the absence of both Rev and the SRV-1 CTE in the murine cell line T6. Some background expression, like that found in the primate cell lines in this study, was not completely unexpected, and probably related either to the strong CMV promoter or to the vector backbone. Indeed, such background expression apparently was not observed by others working with HIV *env* expression constructs carrying weaker promoters, including the viral LTR[3] and the SV40 promoter.[4,27] Furthermore, in the case of the *gag* constructs, we observed that vector backbone clearly influenced protein expression. An alternative explanation for the particularly strong expression of both *gag* and *env* gene products in the absence of Rev in T6 cells is that the inhibitory sequences present on both SIV *gag* and *env* mRNAs, and partially responsible for their nuclear retention and degradation,[28] might be less efficiently recognized in murine cells, possibly owing to species-specific differences in the structural features of the RNA element, or to differences in putative cellular factors mediating the destabilizing effect. Nevertheless, SIV gene expression was also increased *in vitro* by the SRV-1 CTE element in T6 cells, as well as in the murine PA317 cell line (not shown). The similar results obtained with T6 and PA317 cells argue against a role for Tax protein, which is present in the former cells, in the observed high basal level of Gag and Env expression. The finding that PA317 cells support a high level of Env expression in the context of the SRV-1 CTE is of practical interest because PA317 is commonly used as a packaging cell line that releases amphotropic Moloney murine leukemia virus (MoMLV) particles. Further development of our Env-CTE expression system might lead to a strategy for producing SIV/MoMLV pseudotypes, which could be used to target foreign genes to CD4$^+$ cells by exploiting the Env–CD4 interaction.[28a]

A potentially important application of these constructs would be the development of a vaccine for SIV. Among the many possible vaccine strategies, to date the injection of naked DNA seems to be promising.[29] The feasibility of immunizing against HIV Env proteins by means of naked DNA was previously demonstrated in a murine model[25] and in monkeys.[30,31] Moreover, work in monkeys indicates that DNA vaccination with SIV constructs is possible, and induces both antibodies and virus-specific cytotoxic T lymphocytes (CTLs).[32] However, the DNA immunization protocol of Yatsutomi *et al.* did not prevent infection by SIVmac251, nor did it protect against CD4$^+$ cell loss,[32] thus suggesting that some improvement in this technology might be needed. Our *in vivo* findings constitute a lim-

ited but encouraging demonstration of the ability of CTE-containing constructs to evoke a humoral immune response against SIV antigens, and delineate the potential applicability of DNA inoculation as a vaccination approach to lentiviruses.

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