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SHORT COMMUNICATION

Rev/RRE-Independent Mason–Pfizer Monkey Virus Constitutive Transport Element-Dependent Propagation of SIVmac239 Vectors Using a Single Round of Replication Assay

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In a step toward creating live-attenuated or DNA subunit vaccines for AIDS, the replication of simian immunodeficiency virus (SIV) was studied independently of the Rev and RRE (Rev-responsive element) regulatory system, over a single round. To accomplish this, the *env* gene of an SIV vector was made defective by the insertion of a SV40 promoter/enhancer *hygromycin B phosphotransferase* gene cassette. Using this vector as the backbone, molecular clones of SIV were generated that contained a mutated Rev, Rev(−), a deleted RRE, RRE(−), or both, Rev(−)RRE(−). It has been shown recently that human immunodeficiency virus type 1 (HIV-1) Rev and RRE functions can be replaced *in vitro* by a *cis*-acting sequence, constitutive transport element (CTE), from simian type D retroviruses. To determine whether such a *cis*-acting element from Mason–Pfizer monkey virus (MPMV) would substitute for SIV Rev and RRE functions, the MPMV CTE was inserted either into the Nef ORF or at the junction of *vpx* and *vpr* of our Rev(−), RRE(−), and Rev(−)RRE(−) SIV molecular clones. Cell-free viral stocks harvested from Cos cells following transfections of these molecular clones revealed that these stocks were infectious over a single round of replication; however, their replication was attenuated 16-fold compared to that of wild-type virus. In addition, our experiments revealed that CTE functions in a position-dependent manner such that its insertion at the junction of *vpx* and *vpr* attenuated SIV replication 8- to 12-fold compared to the attenuation observed when it was inserted in the *nef* region. Our results demonstrate that MPMV CTE is capable of substituting for SIV Rev and RRE functions, resulting in an attenuated ability to produce infectious virus.

Transport of messages from the nucleus to the cytoplasm is an essential step in the retroviral life cycle. If not accomplished properly, replication-defective viruses can result since nuclear-sequestered messages cannot be translated (for review, see *5*). Human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) have developed a highly efficient mechanism, the Rev/RRE (Rev responsive element) system, to regulate the expression of incompletely spliced and unspliced mRNAs. In the absence of the Rev/RRE regulatory system, these messages remain trapped in the nucleus until they are spliced, exported out of the nucleus, or degraded (*9, 10, 20, 21*). Binding of Rev to its *cis*-acting responsive element, RRE, found in these messages allows the expression of such messages (*1, 6, 10, 16, 29;* for more references, see *5*). Rev may accomplish this by binding to cellular cofactors involved in the export of messages from the nucleus to the cytoplasm (*2, 11, 13, 30*), resulting in enough structural gene products to allow a complete replication cycle to take place.

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Recently, two independent studies have shown that *cis*-acting elements present in the 3′ region of type D retroviruses, Mason–Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1), can substitute for the HIV-1 Rev/RRE regulatory system (*3, 32*). Once these constitutive transport elements (CTEs) are inserted before the polyadenylation sequences of either HIV-1 *gag*/*pol* or *env* expression vectors (*3*) or in the *nef* gene of a Rev(−) full-length HIV-1 proviral DNA (*3, 32*), the expression of the unspliced and singly spliced genes and even the replication of the virus become independent of the Rev/RRE regulatory system, though in an attenuated manner. Since no other viral protein is required for MPMV CTE function, Bray *et al.* (*3*) suggested that MPMV CTE may interact with cellular factors to allow nucleocytoplasmic transport of viral mRNAs. Similar *cis*-acting elements have been discovered in hepatitis B (*17, 18*) and rous sarcoma viruses (*23*).

The ability of CTE to obviate the need for the Rev/RRE regulatory system in the case of HIV-1 prompted us to test this phenomenon in the case of SIV. We were interested in generating infectious molecular clones of SIV that could be used as pathogenically attenuated yet replication-competent vaccines. The studies conducted earlier for HIV-1 had weaknesses that resulted in many

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unanswered questions (*3, 32*). Foremost among these, both groups had used viral replication assays based on multiple rounds of replication; the use of such assays, especially for long-term virus propagation, can lead to reversion of introduced mutations, generation of second-site revertants, and potential loss of unstable genetic elements that may lead to ambiguity in the interpretation of results. In addition, the cumulative effects of several rounds of replication have the potential of masking subtle differences that may exist, while magnifying others that may be present at low levels. For example, reversion of even one molecule of functional Rev could account for the results obtained by Bray *et al.* (*3*) since sequences encoding RRE were still present in their molecular clones. Zolotukhin *et al.* (*32*) had introduced double mutations in Rev and multiple mutations in RRE to circumvent this problem. However, even partial reversion of mutations in Rev or compensatory second-site mutations in RRE could have led to virus replication. The fact that Zolotukhin *et al.* (*32*) had found 11 point mutations in RRE, even by Day 14, further substantiates such concerns. In addition, there were discrepancies between the two studies; for example, Bray *et al.* (*3*) reported an attenuation of viral replication of two orders of magnitude, whereas Zolotukhin *et al.* (*32*) observed an attenuation of 5- to 10-fold. Thus, we decided to systematically and quantitatively measure the effect of CTE on SIV replication and reevaluate its role in HIV-1 replication in the absence of the Rev/RRE regulatory system using a single round of replication assay.

To study the role of CTE in the Rev/RRE-independent replication of SIV, we established a *trans* complementation assay based on assays developed earlier to study propagation and pseudotyping of replication-defective SIV (*25–27*) and HIV-1 vectors (*7, 14, 24*) without the generation of replication-competent viruses in a single round of replication. This assay used a previously described SIV vector, pTR140, containing the SV40 promoter/enhancer *hygromycin B phosphotransferase* (*hyg*^{r}) gene "cassette" (SV-Hyg^r cassette) inserted in the *env* region (*25, 26*). Propagation of this vector was dependent upon complementation in *trans* by an *env*-expression plasmid. A series of molecular clones was created by modifying pTR140 defective in Rev, in RRE, or in both. MPMV CTE was inserted at the *Bgl*II site in the *nef* gene of these vectors. In order to mutate the SIV Rev ORF, a premature stop codon was introduced at amino acid 12 in the second exon in Rev. This mutation in Rev changed a lysine to isoleucine in exon 2 in Tat, a modification that does not affect Tat function (Scott Wong, personal communication). To disrupt the RRE, a deletion mutation was introduced that completely eliminated sequences encompassing RRE. We were able to delete RRE without consideration for the overlapping env ORF since *env* was provided in *trans* by a separate expression plasmid. Study of CTE in the complete absence of RRE is important since RRE is known to contain sequences inhibitory to the transport of unspliced messages (*4, 22*). Analyses such as these could not be accomplished in earlier published reports due to the overlap of RRE and the Env ORF (*3, 32*). To avoid any possible homologous recombination between the RRE-containing sequences carried by the homologous *env* expression plasmid and the sequences in the vector, a heterologous murine amphotropic *env* (pSV-A-MLV-env) was used to pseudotype SIV particles. Pseudotyping by the murine amphotropic *env* has been demonstrated to yield higher titers of virus than *trans* complementation with the homologous *env* in HIV-1 (*7, 14, 24*). Finally, by deleting RRE completely, this assay also allowed us to use Rev in *trans* to study any possible Rev/CTE interactions that may exist.

SIVmac239 has been cloned in two halves with *env, rev,* RRE, and *nef* residing in the 3′ half (p239SpE3′) of the virus (*19*). To generate the full-length viral genome, the 5′ half (p239SpSp5′) was ligated *in vitro* with the 3′ half, following cleavage by *Bst*BI (Fig. 1) as described previously (*25, 26*). Transfection of Cos cells with the ligated vector DNA along with a plasmid expressing murine amphotropic *env* (pSV-A-MLV-env) resulted in the production of infectious SIV particles from the Cos cells via genetic complementation. Stocks of these viruses were used to infect HeLaT4 cells and the infected cells were selected in media containing hygromycin B. Only cells with successful integration of vector DNA and expressing the *hyg*^{r} gene gave rise to hygromycin resistant (Hyg^r) colonies. The number of Hyg^r colonies that survived directly reflected the ability of a particular pseudotyped particle to infect and integrate in the cellular genome. Thus, by using a single round of replication assay, we were able to evaluate the ability of MPMV CTE to substitute for the Rev/RRE regulatory system in the replication of SIV in a controlled and quantitative manner.

Table 1 reveals the results of several *trans* complementation experiments performed using the Rev(−), RRE(−), and Rev(−)RRE(−) SIV molecular clones with or without the insertion of MPMV CTE. The control vector, pTR140, containing all intact genes except *env,* was able to replicate well, giving rise to over 3000 colony-forming units (CFU)/ml of viral stock. However, when RRE was deleted, as for pTR216, or when Rev was mutated, as for pTR238, or when both Rev and RRE were disrupted, as for pTR244, no colonies were observed. This was expected since mutations in either Rev or RRE disrupt the Rev/RRE regulatory system essential for the replication of the virus (*9, 10, 20, 21*). However, when the Rev(−) vector pTR238 was complemented with Rev in *trans,* virus replication was restored, though not to the wild-type levels (1050 vs 3168 CFU/ml). To make sure that the Rev mutation did not affect Tat function, Tat was provided in *trans;* however, no increase in viral titers was observed (data not shown). These results could have been due to several factors: first, several independent DNAs were used in the transfection procedure, causing

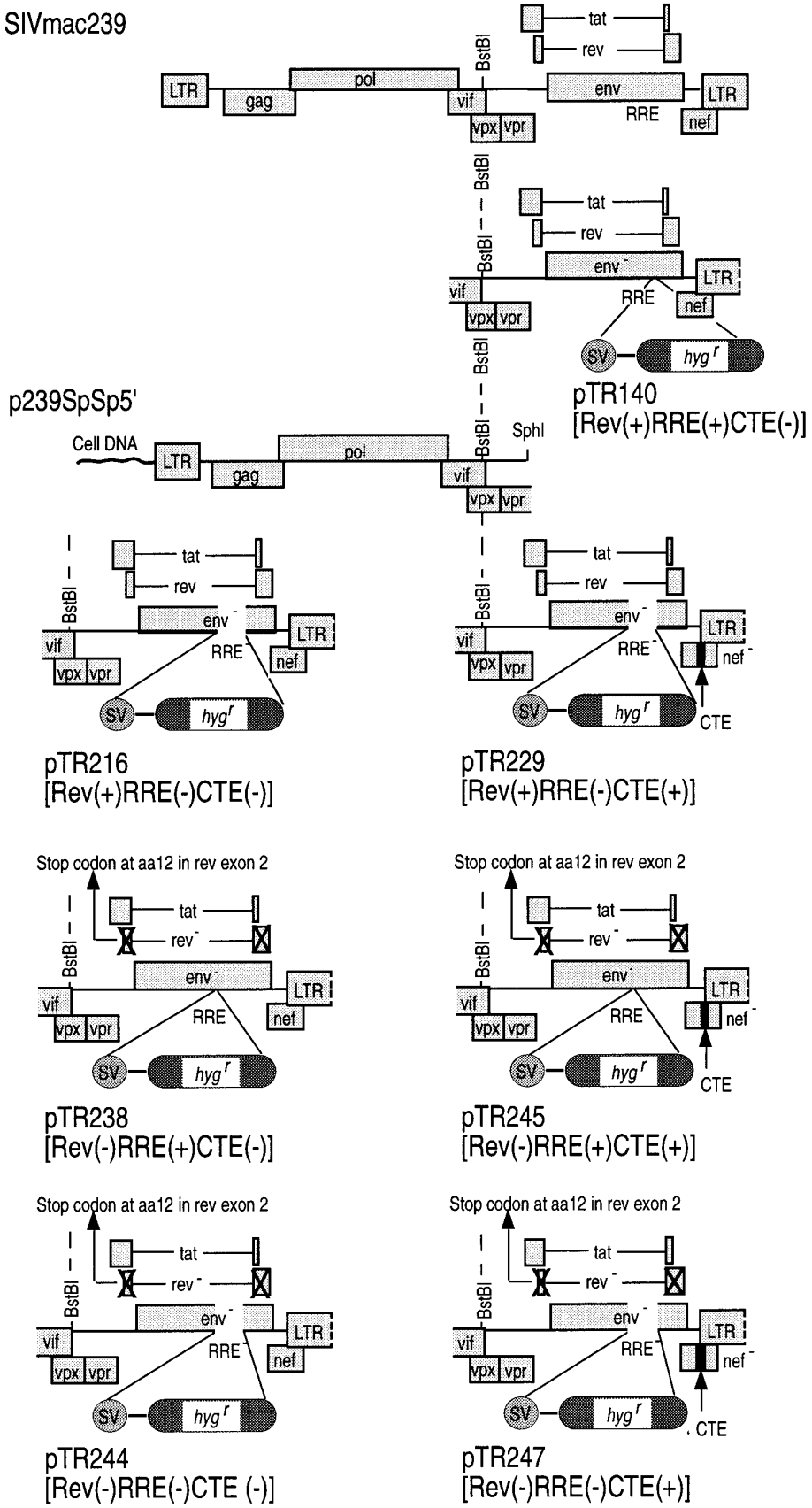

FIG. 1. Schematic representation of the SIVmac239 genome with open reading frames (ORFs) and SIV vectors with the insertion of MPMV CTE in the *nef* region. Details of the origin and construction of these plasmids can be obtained from the authors upon request. The broken lines represent the site of *in vitro* ligation of the SIV vectors with p239SpSp5′ at the *Bst*BI site. RRE, Rev-responsive element; SV, SV 40 early promoter; *hyg*^{r}, *hygromycin B phosphotransferase* gene.

TABLE 1

Effect of MPMV CTE on the Propagation of Rev(−), RRE(−), and Rev(−)RRE(−) SIV Vectors[a]

Vector	Rev/RRE phenotype	Presence or absence of MPMV CTE in		Titer (CFU/ml ± SD)[b] HIV-1 Rev	
		nef	*vpx/vpr*	(+)	(−)
pTR140	Rev(+)RRE(+)	CTE(−)	—	ND[c]	3168 ± 32
pTR216	Rev(+)RRE(−)	CTE(−)	—	ND	0
pTR229	Rev(+)RRE(−)	CTE(+)	—	ND	197 ± 3
pTR230	Rev(+)RRE(−)	CTE(+)[d]	—	ND	0
pTR238	Rev(−)RRE(+)	CTE(−)	—	1050 ± 50	0
pTR245	Rev(−)RRE(+)	CTE(+)	—	869 ± 10	193 ± 3
pTR244	Rev(−)RRE(−)	CTE(−)	—	ND	0
pTR247	Rev(−)RRE(−)	CTE(+)	—	ND	199 ± 1
pTR304	Rev(+)RRE(+)	—	CTE(+)	ND	3093 ± 11
pTR255	Rev(+)RRE(−)	—	CTE(+)	ND	22 ± 3
pTR258	Rev(−)RRE(−)	—	CTE(+)	ND	16 ± 2

[a] All vectors were *in vitro* ligated individually with p239SpSp5′ following *Bst*BI digestion as described earlier (*25, 26*). *In vitro*-ligated samples were cotransfected with pSV-A-MLV-env.

[b] All data represent experiments performed in parallel and are based on a minimum of three independent experiments with two duplicate samples per experiment. No Hyg^r colonies were observed for mock, pSV-A-MLV-env alone, or any of the vectors when transfected without pSV-A-MLV-env.

[c] ND, Not done.

[d] MPMV CTE was inserted in the antisense orientation.

possible competition among the DNAs for their expression. Second, Rev was provided in *trans* rather than *cis,* and as a result it may not have functioned optimally. Finally, HIV-1 Rev was provided instead of SIV Rev, leading to suboptimal rescue of virus replication. Regardless of the lower efficiency of rescue by Rev, these results indicate that the *trans* complementation system could be used to study the effects of MPMV CTE in replacing Rev/RRE functions in a single round of replication.

When the MPMV CTE was inserted in the *nef* region of Rev(+)RRE(−) vector pTR229, the MPMV CTE was able to compensate for the defect in the Rev/RRE regulatory system and rescue vector propagation (Table 1). However, vector propagation was 16-fold less efficient compared with that of pTR140 (197 vs 3168 CFU/ml). As expected, the MPMV CTE was unable to compensate for the Rev/RRE system when inserted in the opposite orientation, as seen for pTR230 (Table 1); this result agreed with those of Bray *et al.* (*3*). Similar to pTR229, when MPMV CTE was inserted in the Rev(−)RRE(+) vector, pTR245, MPMV CTE was able to rescue infection, though again at a 16-fold lower efficiency compared to that of pTR140 (193 vs 3168 CFU/ml). When HIV-1 Rev was provided in *trans* with pTR245, virus could be rescued to approximately the same level as with pTR238 (869 vs 1050 CFU/ml; Table 1). When the MPMV CTE was inserted in the *nef* region of the Rev(−)RRE(−) double mutant, pTR247, virus replication was observed at a rate similar to that of pTR229 or pTR245 (199 vs 197 or 193 CFU/ml). These data strongly suggest that MPMV CTE can substitute for the function of the SIV Rev/RRE regulatory system though at a 16-fold lower efficiency.

The results presented above also revealed that no positive Rev-CTE interactions existed. We could study the existence of such an interaction since our system allowed us to completely delete RRE, something that could not be achieved in earlier reports showing Rev/RRE-independent replication of HIV-1 (*3, 32*). In the case of SIV, viral titers obtained with the Rev(+)RRE(−)CTE(+) vector, pTR229 (197 CFU/ml; Table 1), were the same as titers obtained with the Rev(−)RRE(−)CTE(+) vector, pTR247 (199 CFU/ml; Table 1). If a positive interaction between Rev and CTE existed, we should have observed higher titers with pTR229 [Rev(+)RRE(−)CTE(+)]. As a corollary, our results further show that Rev functions only in conjunction with RRE and does not seem to have any other roles in the viral life cycle except regulation of the expression of viral messages.

Next we examined whether MPMV CTE could substitute for Rev and RRE functions in a region different from *nef* where its insertion did not disrupt any known ORFs critical for SIV replication and pathogenesis *in vivo.* The MPMV CTE was inserted at the junction of *vpx* and *vpr* using splice overlap extension (SOE) PCR (Fig. 2) (*15*). *vpx* and *vpr* do not have any area of overlap; therefore, insertion of the CTE between the stop codon for *vpx* and the initiation codon for *vpr* did not disrupt *vpx, vpr,* or any other known ORFs. This was accomplished for the Rev(+)RRE(+) vector, pTR304, the Rev(+)RRE(−) vector, pTR255, and the double mutant Rev(−) RRE(−) vector, pTR258. Testing of these molecular clones in the *trans* complementation assay revealed that the control vector, pTR304, could replicate as well as the wild-type CTE(−) vector, pTR140, suggesting that insertion of CTE at the

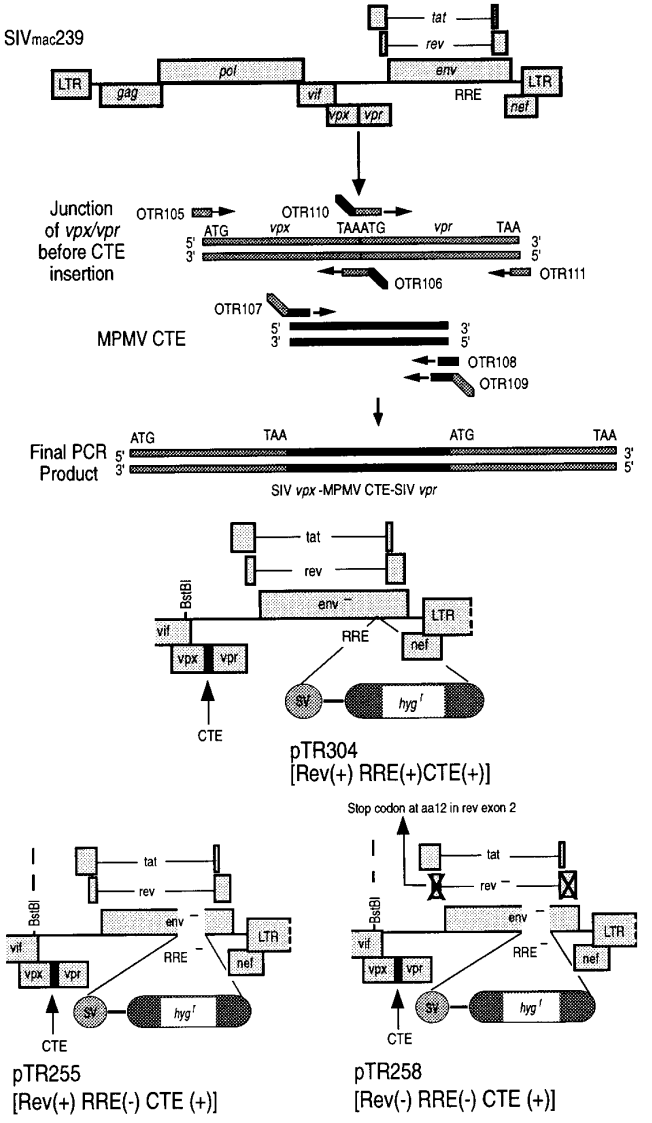

FIG. 2. Schematic representation of the SIVmac239 genome with open reading frames (ORFs) and SIV vectors with the insertion of MPMV CTE at the junction of *vpx* and *vpr* using splice overlap extension PCR. Four rounds of PCR were performed to insert MPMV CTE between the stop codon of SIV *vpx* (TAA) and the start codon of *vpr* (ATG) without disrupting either ORF. Details of the PCR amplification and construction of these plasmids can be obtained from the authors upon request. The MPMV CTE is shown as a black box. Annealing sites of PCR amplification primers used are shown with respect to the stop codon of *vpx* and start codon of *vpr.* Portions of hybrid primers that do not anneal are shown with offset portions. The broken lines represent the site of *in vitro* ligation of the SIV vectors with p239SpSp5′ at the *Bst*BI site. RRE, Rev-responsive element; SV, SV40 early promoter; *hygr, hygromycin B phosphotransferase* gene.

junction of *vpx* and *vpr* did not have any adverse effects on the replication of SIV (Table 1). However, insertion of MPMV CTE could rescue replication of Rev(+)RRE(−) vector pTR255 by only 1/140 (or 0.007) of the wild-type level and that of the Rev(−)RRE(−) double mutant, pTR258, by 1/200 (or 0.005) of the wild-type level (Table 1). These results suggest that the location of MPMV CTE is critical in its ability to substitute for the Rev/RRE regulatory system in the replication of SIV. Retention of the introduced CTE in the Nef ORF and at the junction of *vpx* and *vpr* and no reversion of Rev mutations were confirmed by PCR and subsequent sequence analyses (data not shown).

Since our *trans* complementation assay required the expression of *gag*/*pol* for particle formation, we next attempted to test whether the MPMV CTE could substitute for the function of Rev/RRE regulatory system in the context of a heterologous promoter. SIV *gag*/*pol* sequences were cloned into an expression plasmid containing the human cytomegalovirus (CMV) promoter and the bovine growth hormone polyadenylation sequences either with MPMV CTE (pRS102) or without (pRS106) (Fig. 3A). Cotransfection of these plasmids into Cos cells along with a control DNA expressing human growth hormone (hGH) revealed that SIV *gag*/*pol* expression was dependent upon the presence of CTE. In the absence of CTE, only 1.91 ng/ml of p27 antigen was released in the Cos supernatants compared to 30.5 ng/ml released in the presence

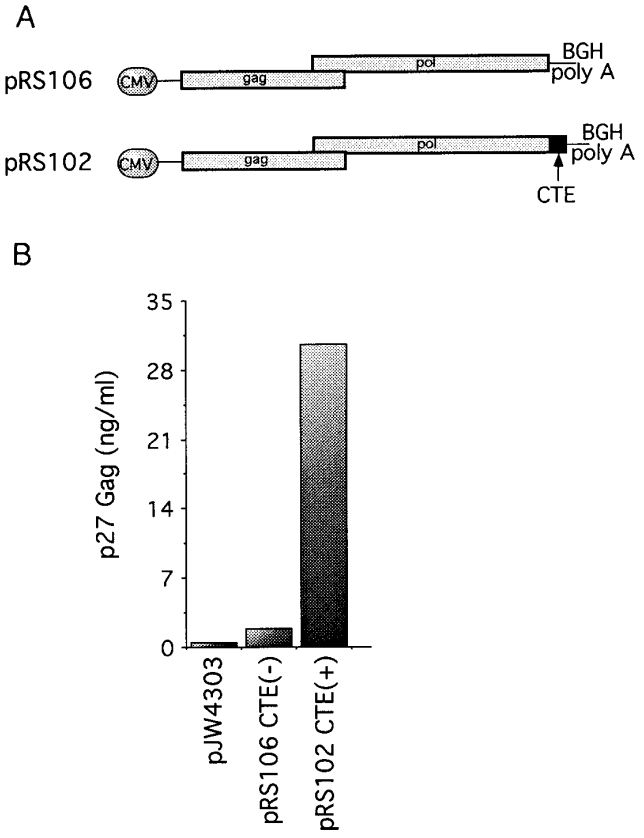

FIG. 3. Comparison of SIV *gag*/*pol* expression in the presence or absence of MPMV CTE. (A) SIVmac239 *gag*/*pol* expression constructs. Details of the origin and construction of these plasmids can be obtained from the authors upon request. CMV, human cytomegalovirus promoter; BGH, bovine growth hormone; poly A, polyadenylation sequences. (B) Quantitative measurement of the p27 Gag antigen released in the media 72 hr after transfection using the Coulter p27 Antigen Capture Assay. pJW4303, control plasmid.

TABLE 2

Effect of MPMV CTE on the Propagation of Rev(−), RRE(−), and Rev(−)RRE(−) HIV-1 Vectors[a,b]

Vector	Rev/RRE phenotype	Presence or absence of MPMV CTE in the *nef* region	Titer (CFU/ml ± SD)[c] HIV-1 Rev (+)	Titer (CFU/ml ± SD)[c] HIV-1 Rev (−)
pGB108	Rev(+)RRE(+)	CTE(−)	ND[d]	5864 ± 118
pTR211	Rev(+)RRE(−)	CTE(−)	ND	0
pTR226	Rev(+)RRE(−)	CTE(+)	ND	364 ± 5
pTR237	Rev(−)RRE(+)	CTE(−)	1580 ± 20	0
pTR233	Rev(−)RRE(+)	CTE(+)	1570 ± 26	355 ± 4
pTR236	Rev(−)RRE(−)	CTE(−)	ND	0
pTR241	Rev(−)RRE(−)	CTE(+)	ND	346 ± 5

[a] pGB108 was modified to create Rev(−), RRE(−), and Rev(−)RRE(−) vectors, the details of which can be obtained from the authors upon request.
[b] All vectors were cotransfected with pSV-A-MLV-env.
[c] All data represent experiments performed in parallel and are based on a minimum of three independent experiments with two duplicate samples per experiment. No Hyg^r colonies were observed for mock, pSV-A-MLV-env alone, or any of the vectors when transfected without pSV-A-MLV-env.
[d] ND, Not done.

of CTE (Fig. 3B). Similarly, we have successfully expressed SIV *gag* only from a heterologous promoter in the presence of MPMV CTE (data not shown). These results are in agreement with observations in the HIV-1 system in which the expression of HIV-1 Gag/Pol message was shown to be dependent upon the presence of CTE in the absence of Rev/RRE (*3*).

Since the earlier published reports on Rev/RRE-independent replication of HIV-1 using CTE used multiple rounds of replication assays resulting in discrepancies in the extent of the ability of CTE to rescue replication of HIV-1, we wished to reevaluate those studies using a single round of replication assay. Toward this end, a set of *env*-defective HIV-1 clones containing the SV-Hyg^r cassette was constructed analogous to SIVmac239 defective for Rev, RRE, or both Rev and RRE with or without insertions of MPMV CTE. Table 2 reveals the results of several *trans* complementation experiments performed using these HIV-1 vectors with or without the MPMV CTE. The control vector pGB108 (described in *12*), which contains all intact HIV-1 genes except *env,* was able to replicate well, giving rise to 5864 CFU/ml of virus stock. As expected, when RRE was deleted in pGB108, creating pTR211, or when Rev was mutated, as in pTR237, or when both Rev and RRE were disrupted, as in pTR236, no colonies were observed (Table 2). Similar to the results obtained for the SIV vectors, when the RRE(+) vector, pTR237, was complemented with Rev in *trans,* viral replication was restored, though it was three- to fourfold lower than the wild-type levels (1580 vs 5864 CFU/ml). The higher titers observed for the HIV-1 vectors compared to SIV are probably due to the need for *in vitro* ligation of the two halves of SIV vectors to generate infectious virus, whereas no such need exists for HIV-1.

When the Rev- and RRE-defective viruses were complemented with MPMV CTE in *cis* by its insertion at the *Xho*I site in the *nef* gene, similar results were obtained as seen for the SIV vectors. Insertion of the CTE in the Rev(+)RRE(−) vector, pTR226, rescued viral replication from 0 to 364 CFU/ml (Table 2). This is 16-fold lower than the replication observed with pGB108, the wild-type vector (5864 CFU/ml). Similarly, when CTE was inserted in the Rev(−)RRE(+) vector, pTR233, viral titers of 355 CFU/ml were obtained, which again is 16-fold lower than the wild-type titer. Complementing pTR233 with Rev in *trans* increased viral titers to the same level as for pTR237 (1570 vs 1580 CFU/ml), consistent with results obtained with the SIV vectors. Finally, when the double mutant, pTR241, was complemented in *cis* by MPMV CTE insertion in the *nef* gene, the MPMV CTE was able to rescue vector propagation, but again the titers were 16-fold lower than the wild-type titers (346 vs 5864 CFU/ml). These results reveal very consistently that MPMV CTE can substitute for the Rev/RRE regulatory system for both SIV and HIV-1 with a 16-fold lower efficiency. These results also reveal that the extent of attenuation observed with substitution of the Rev/RRE system by CTE in HIV-1 is closer to results published by Zolotukhin *et al.* (*32*) than those of Bray *et al.* (*3*). Zolotukhin *et al.* (*32*), using SRV-1 CTE, observed a 5- to 10-fold lower efficiency of replication than did Bray *et al.* (*3*), who found a two orders of magnitude difference in the ability of MPMV CTE-containing molecular clones to replicate.

It is not clear exactly what the mechanism of CTE action is. It may serve to facilitate the export of unspliced or partially spliced mRNAs from the nucleus to the cytoplasm, as suggested by Bray *et al.* (*3*). However, MPMV does not contain any accessory genes analogous to Rev to carry out such a function. Thus, CTE may act as a nuclear export signal in the life cycle of MPMV by using cellular factors rather than viral *trans*-acting factors to promote expression of unspliced and partially spliced

mRNAs. This is consistent with our findings that by deleting CTE in the MPMV genome, unspliced mRNA was not transported properly to the cytoplasm, resulting in the abrogation of virus replication (*28*). However, when CTE function was replaced by SIV and HIV-1 Rev/RRE, proper export of messages from the nucleus to the cytoplasm was observed, leading to virus replication.

Recently, it has been suggested that a simple form of HIV-1 lacking accessory genes and expressing only *gag, pol,* and *env* may make a safe and effective vaccine against AIDS (*8, 31*). As a modification of vaccines based on simpler lentiviruses, we are focusing on DNA vaccines that use only components of viruses to boost immune responses *in vivo.* Toward this end, our SIV *gag/pol* expression plasmid containing MPMV CTE is currently being tested in DNA vaccine trials in macaques to mount protective immune responses against lethal viral challenges.

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