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Mason–Pfizer Monkey Virus (MPMV) Constitutive Transport Element (CTE) Functions in a Position-Dependent Manner

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The Mason–Pfizer monkey virus (MPMV) constitutive transport element (CTE) is a *cis*-acting RNA element located in the 3′ untranslated region (UTR) of the viral genome. The HIV-1 and SIV Rev/RRE regulatory system can be replaced with MPMV CTE (Bray *et al.,* 1994; Zolotukhin *et al.,* 1994; Rizvi *et al.,* 1996a); similarly, CTE function can also be replaced by the HIV or SIV Rev/RRE regulatory system (Rizvi *et al.,* 1996b; Ernst *et al.,* 1997). In addition, we have shown that in the context of the SIV genome, position is important for CTE function (Rizvi *et al.,* 1996a). To determine the importance of position for CTE function in the context of the MPMV genome, MPMV molecular clones were generated by deleting CTE or removing it from the 3′ UTR and placing it in the approximately 40 bp of intervening sequences between the *pol* termination codon and *env* initiation codon. A test of these molecular clones in a single round of replication assay revealed that deletion or displacement of CTE in the intervening sequences between *pol* and *env* completely abrogated virus replication. Western blot analysis of cell lysates and pelleted culture supernatants revealed negligible amounts of Pr78 Gag/Pol precursor and the processed $p27^{gag}$ when CTE was deleted or displaced. Slot blot analysis of fractionated RNAs revealed entrapment of the viral Gag/Pol mRNA in the nucleus with CTE deletion or displacement. Upon reinsertion of CTE in the original genomic position of clones with the deleted or displaced CTE, virus replication, Gag/Pol protein production, and nucleocytoplasmic transport of viral mRNA were restored to normal levels. Displacement of CTE to the 5′ UTR immediately upstream of the Gag initiation codon also resulted in aberrant Gag/Pol protein production and nucleocytoplasmic transport of viral RNA. Reinsertion of CTE at the original genomic position of the clone with CTE displacement at the 5′ UTR restored normal Gag/Pol protein production and RNA transport, demonstrating that the 3′ terminal position of CTE is important for its function. To explore why the 3′ terminal location of CTE is important, heterologous DNA sequences of increasing lengths were inserted between CTE and the polyadenylation (poly(A)) signal of the virus to augment the distance between the two *cis*-acting elements. Test of these constructs revealed that CTE function was progressively lost with incremental increase in distance between CTE and poly(A). To explore this relationship further, CTE was displaced to the *env* region ~2000 bp upstream of the poly(A) signal which abrogated CTE function. However, cloning of poly(A) signal to ~200 bp downstream of CTE in the *env* region (the natural distance between CTE and poly(A)) restored CTE function. Together, these results demonstrate that the close proximity of CTE to the poly(A) signal is important for CTE function, suggesting a functional interaction between CTE and the polyadenylation machinery.

INTRODUCTION

MPMV is a simple type D retrovirus that contains the canonical three open reading frames (ORFs) found in all retroviruses — 5′-*gag-pol-env*-3′ (Sonigo *et al.,* 1986). Although it lacks the plethora of accessory genes found in the more complex human and simian immunodeficiency viruses (HIV and SIV) thought to be responsible for pathogenicity of these viruses (Temin, 1993), MPMV can cause a complicated disease like simian acquired immunodeficiency syndrome (SAIDS) in infant rhesus macaques (Daniel *et al.,* 1984; Marx *et al.,* 1984; Stromberg *et al.,* 1984). In this regard, MPMV is similar to other simple type D retroviruses that also cause SAIDS, simian retrovirus type 1 and type 2 (SRV-1 and SRV-2) (Power *et al.,* 1986; Thayer *et al.,* 1987).

The 3′ terminal untranslated region (UTR) of MPMV and SRV-1 contains a *cis*-acting RNA element, the constitutive transport element (CTE), which has been shown to be critical for the replication of MPMV by affecting the nucleocytoplasmic transport and/or stability of genomic, unspliced viral message (Bray *et al.,* 1994; Zolotukhin *et al.,* 1994; Rizvi *et al.,* 1996a,b; Ernst *et al.,* 1997). Thus, CTE functions in a manner analogous to the Rev/Rev responsive element (RRE) regulatory system of complex retroviruses such as HIV and SIV to control viral gene expression (Bray *et al.,* 1994; Zolotukhin *et al.,* 1994; Rizvi *et al.,* 1996a). However, CTE-like positive regulatory elements are not unique to type D retroviruses. It is becoming increasingly apparent that viruses that do not contain accessory regulatory systems like Rev/RRE seem to contain CTE-like positive regulatory elements in their ge-

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nomes to regulate gene expression. These include other simple retroviruses such as Rous sarcoma virus (RSV; Ogert *et al.,* 1996), as well as RNA and DNA viruses such as influenza virus (Alonso-Caplen and Krug, 1991), Hepatitis B virus (HBV; Huang and Liang, 1993; Huang and Yen, 1994), herpes simplex virus (HSV; Liu and Mertz, 1995), and human foamy virus (HFV; Dr. Bryan Cullen, personal communication). In addition, Rev/RRE and CTE have been shown to overcome the negative inhibitory effects of *cis*-acting repressor sequences found in human papillomavirus types 1 and 16 (HPV-1 and 16; Tan and Schwartz, 1995; Tan *et al.,* 1995), allowing viral gene expression to take place. Most recently, a CTE-like element has been identified in a murine intracisternal-A particle retroelement inserted within the mouse *osteocalcin* gene that can substitute for the Rev/RRE regulatory system of HIV-1 (Tabernero *et al.,* 1997), revealing the broad ability of CTE-like elements to regulate gene expression.

The 3′ terminal location of CTE may be important for CTE function (Rizvi *et al.,* 1996a). In our earlier studies, we have shown that MPMV CTE can replace the SIV Rev/RRE regulatory system, though in a position-dependent manner (Rizvi *et al.,* 1996a). When CTE was inserted at the junction of *vpx* and *vpr* (in a more central position of the viral genome), virus replication was lowered 8- to 12-fold compared to the insertion of CTE in the *nef* gene of the virus (a more terminal location of the viral genome) in the absence of Rev/RRE (Rizvi *et al.,* 1996a). Insertion of CTE at the junction of *vpx* and *vpr* in an SIV molecular clone that maintained Rev/RRE did not affect viral replication in a negative manner. These studies suggested that, at least in the context of the SIV genome, position is important for CTE function. In the current study, we have analyzed the requirement of the 3′ terminal location of MPMV CTE for its function in more detail. Data presented here reveal that CTE functions only at the 3′ UTR of the viral genome and attempts to displace it either in the middle or at the 5′ UTR of the virus result in disruption of viral gene expression and aberrant nucleocytoplasmic transport of viral RNA.

Other than CTE, the 3′ U3 sequences of MPMV contain *cis*-acting sequences important for efficient polyadenylation of viral transcripts. Therefore, we explored the possibility of whether the close proximity of CTE to the poly(A) signal (~200 bp) in the viral genome is important for efficient CTE function. Incremental increase in the spatial distance between CTE and the poly(A) signal revealed a progressive loss of CTE function. To test if it was important to maintain the original distance between CTE and the poly(A) signal for efficient CTE function, CTE was displaced to the *env* region ~2000 bp upstream of the poly(A) signal, abrogating CTE function. However, cloning of the poly(A) signal to ~200 bp downstream of the displaced CTE in *env* restored CTE function, demonstrating that the close proximity of CTE to the poly(A) signal is important for efficient CTE function and suggesting a functional interaction between CTE and the polyadenylation machinery.

MATERIALS AND METHODS

Numbering system

Nucleotide designations for MPMV are based on GenBank Accession No. M12349 (Sonigo *et al.,* 1986).

Vector construction

Using splice overlap extension polymerase chain reaction (SOE PCR) (Ho *et al.,* 1989), vector pK1(RV)−CTE was created by deleting CTE between nt 8006 and 8192 while keeping the polypurine tract intact in pKAL001 (described in Rizvi *et al.,* 1996b). This was followed by the insertion of a fragment containing the SV40 early promoter/enhancer driving the *hygromycin B phosphotransferase* gene (*hyg*^{r}) (SV-Hyg^r cassette) at the *Nhe*I site (nt 7181) in *env,* truncating *env* and resulting in pK1(RV)−CTE HYG^R. CTE was reinserted in pK1(RV)−CTE HYG^R at the original position to create pK1(RV)+CTE HYG^R (Fig. 1).

To displace CTE in the *pol-env* spacer region [after the *env* splice acceptor (SA) and before the *env* initiation codon], pdisC2(aSA)HYG^R was created by inserting CTE between nt 6241 and 6242. To accomplish this, two rounds of PCR were conducted as shown in Fig. 2. Briefly, in the first round, three separate PCR amplifications were conducted using the primers shown (initial denaturation at 94° for 5 min, followed by a cycling program of 94° for 45 sec, 50° for 45 sec, and 72° for 2 min for 30 cycles followed by a final extension step at 72° for 7 min). The resulting three PCR products (products A, B, and C) were gel purified and combined to conduct the second round of PCR using primers at either ends of products A and C (initial denaturation at 94° for 5 min, followed by a cycling program of 94° for 45 sec, 42° for 45 sec, and 72° for 2 min for 30 cycles followed by a final extension step at 72° for 7 min) (Fig. 2). All PCR amplifications were conducted in 100-μl volumes using the GeneAmp PCR reagent kit with native *Taq* polymerase in a Perkin–Elmer Cetus 9600 block thermocycler (Perkin–Elmer, Foster City, CA). The final PCR product resulted in the insertion of CTE after the *env* SA and immediately upstream of the *env* initiation codon. This product was cleaved with *Nco*I and *Nhe*I and cloned back into pK1(RV)−CTE HYG^R, resulting in pdisC2(aSA)HYG^R. As a control, CTE was reinserted in this vector at the original position while maintaining the CTE at the displaced position, creating pdisC2(aSA)+CTE HYG^R.

To displace CTE at the 5′ UTR just upstream of the Gag initiation codon, an *Eco*RV site was created between nt 889 and 890 by SOE PCR in K1(RV)−CTE HYG^R. Next, CTE was inserted at the *Eco*RV site, creating pKAL83 (−CTE)disC HYG^R. As a control, CTE was reinserted at

the original position of this clone while maintaining the displaced CTE at the 5′ end, creating pKAL83disC HYG^R. All mutations were verified by "*fmol* DNA Cycle Sequencing System" (Promega, Madison, WI) using the end-labeled primer method.

To increase the distance between CTE and the polyadenylation sequence, SOE PCR was used to create an *Eco*RV site in pKAL036 (Rizvi *et al.,* 1996b) at the end of CTE at nt 8240, generating pKAL036(*Eco*RV) (Fig. 5A). Heterologous sequences of various lengths (250, 500, 750, and 1000 bp) were PCR-amplified with flanking *Eco*RV sites and inserted at the *Eco*RV site in pKAL 036(*Eco*RV) creating pKAL036(*Eco*RV)250, pKAL036 (*Eco*RV)500, pKAL036(*Eco*RV)750, and pKAL036(*Eco*RV) 1000, respectively (Fig. 5A).

CTE was also displaced to the *env* region of the CTE(−) clone, pK1(RV)−CTE HYG^R, at the *Hin*cII site (nt 6478), creating pKAL135 (Fig. 6A). To clone the poly(A) signal to ~200 bp of the displaced CTE in the *env,* MPMV sequences containing the poly(A) signal were inserted at the *Spe*I site (nt 6680), 202 bp downstream from the displaced CTE in *env* creating pKAL136. As a control, CTE was reinserted at the *Eco*RV site (nt 8240) in pKAL135, creating pKAL137.

All plasmids were constructed employing standard molecular cloning techniques (Maniatis *et al.,* 1982) through several stages of cloning (details can be obtained from the authors upon request).

Transfection and infections of cells

Cos cells were used to produce viral stocks by a modification of the DEAE–dextran method (Cullen, 1988) as described earlier (Rizvi *et al.,* 1996b). Viral stocks were used in the single round of replication assays as described earlier (Rizvi *et al.,* 1996b). Since all the MPMV vectors contained disrupted *env* genes due to the insertion of the SV-Hyg^r cassette, the propagation of the vectors was dependent on *trans* complementation by *env.* Therefore, MPMV vectors were cotransfected individually with a murine amphotropic Env-gp expressing plasmid, pSV-A-MLV-env (Page *et al.,* 1990).

Protein analysis

Viral proteins were analyzed by harvesting viral particles from transfected supernatants as described earlier (Rizvi *et al.,* 1996b). Cellular proteins were prepared from transfected cells by lysing washed cells in the radioimmunoprecipitation assay buffer (50 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) in the presence of 1 m*M* phenylmethylsulfonyl fluoride and 50 μl/ml β-mercaptoethanol. Viral and cellular proteins were analyzed by the Enhanced Chemiluminescence kit (Amersham, Arlington Heights, IL) using a polyclonal antiserum against MPMV Gag/Pol polyprotein, Pr78.

RNA fractionation and slot blot analysis

To analyze the effect of CTE displacement on the nucleocytoplasmic trafficking of viral messages, cellular RNAs were fractionated into nuclear and cytoplasmic fractions from transfected Cos cells as described earlier (Rizvi *et al.,* 1996b). Fractionated RNAs were transferred to nitrocellulose filters using slot blot apparatus and probed using both a *pol*-specific probe and a probe specific for a cellular housekeeping gene for β-*actin* (Rizvi *et al.,* 1996b).

RESULTS

Effect of CTE displacement to the *pol-env* spacer region of the viral genome on MPMV replication

To determine the effect of CTE displacement on virus replication, we took advantage of our previously established single round of replication assay for SIV (Rizvi and Panganiban, 1992) and MPMV (Rizvi *et al.,* 1996b). Toward this end, an *env*-defective MPMV vector was created containing an insertion of the *hyg^r* gene "cassette" expressed from an SV40 promoter/enhancer in the *env* gene. Using this vector, a series of CTE-deletion and -displacement molecular clones were created by either deleting CTE from the original position in the 3′ UTR alone or simultaneously moving it to the intervening ~40 bp between the *pol* termination codon and the initiation codon for *env* (the *pol-env* spacer region) in the MPMV genome (Figs. 1 and 2). The SA for MPMV *env* also lies in this spacer region (Dr. Eric Hunter, personal communication), and such a location of the SA was exploited to insert CTE either before or after the *env* SA.

Genetic complementation of the *env*-defective vectors with a plasmid expressing the amphotropic Env-gp (Page *et al.,* 1990) resulted in virus particles containing a replication-defective vector RNA. The virus particles produced were infectious for one round of replication since they lacked the *env* gene for further propagation. The single round of replication assay allowed us to study the significance of the 3′ terminal location of CTE in the genomic context in a clear and quantitative manner. In addition, since the viral particles produced could undergo only a single replication event, our assay also prevented any selection pressures from being exerted on the CTE-displacement mutants to delete the displaced sequences.

Test of the mutants containing the deleted CTE [pK1(RV)−CTE HYG^R] or displaced CTE [pdisC2(aSA) HYG^R] after the *env* SA revealed that either deletion of CTE or its move to the middle of the viral genome completely abrogated virus replication as seen by the lack of Hyg^r colonies (Fig. 1 and Table 1). However, when CTE was reinserted at its original position in pK1(RV)−CTE HYG^R, creating pK1(RV)+CTE HYG^R, or in pdisC2(aSA) HYG^R while maintaining the CTE at the dis-

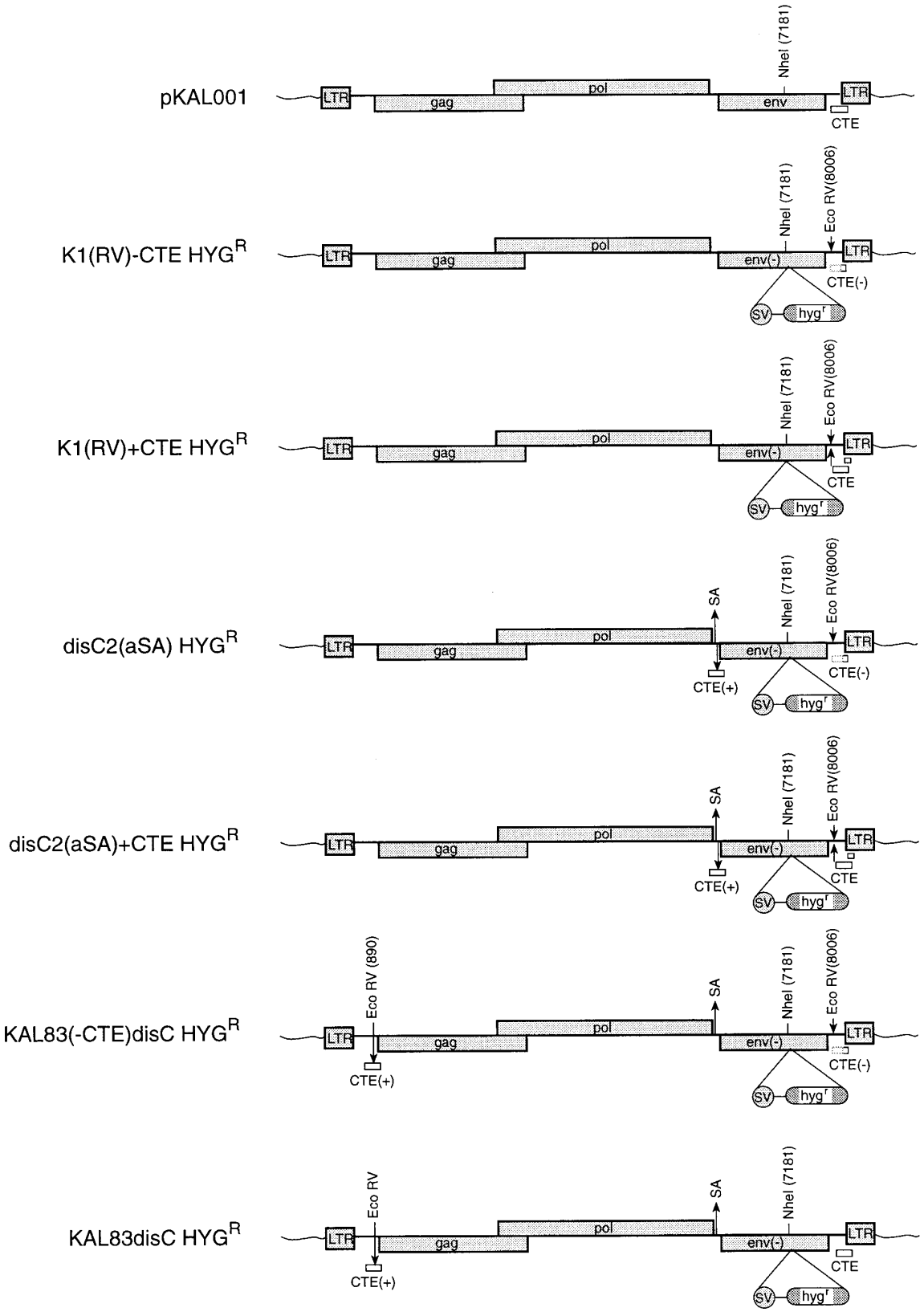

FIG. 1. Schematic representation of the MPMV genome (pKAL001) with open reading frames (ORFs) and MPMV CTE-displacement vectors with the insertion of the "SV-Hyg^r cassette" in the *env* region. Details of the origin and construction of these plasmids are presented under Materials and Methods. The shaded parts of CTE indicate CTE removal. SA, splice acceptor; SV, simian virus 40 early promoter; hyg^r, *hygromycin B phosphotransferase* gene.

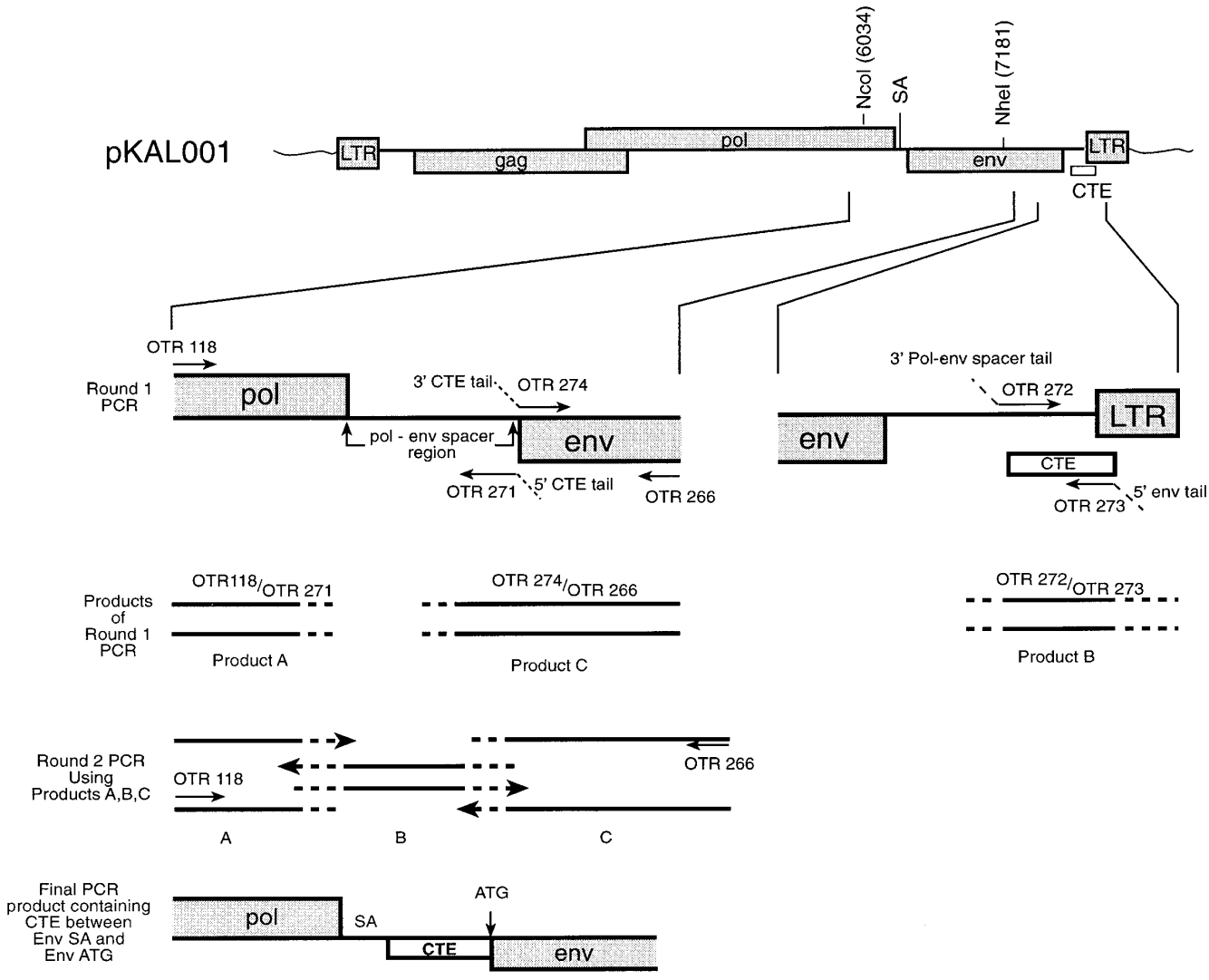

FIG. 2. Schematic representation of the PCR strategy used to displace CTE in the MPMV genome in the *pol-env* spacer region after the Env splice acceptor (SA). The top schematic represents the wild-type MPMV genome, pKAL001, followed by the location of oligonucleotides used to amplify different regions of MPMV genome. Portions of hybrid primers that do not anneal are shown with offset portions. Two rounds of PCR were performed to displace CTE, with the first round of PCR representing three separate reactions (see Materials and Methods for details).

placed position (pdisC2(aSA)+CTE HYG^R), viral titers were restored to wild-type levels (Table 1). Similar results were obtained when CTE was displaced before the *env* SA (data not shown). These results confirm our previous findings that CTE is critical for MPMV replication and further suggest that the genomic position of CTE is equally crucial for its function.

Effect of CTE displacement on Gag/Pol protein production

To further test if the terminal location of CTE was important for its function, CTE was also displaced to the 5′ UTR of the viral genome after the major splice donor, creating pKAL83(−CTE)disC HYG^R (Fig. 1). Western blot analysis of cell lysates and pelleted culture supernatants for the production of Gag/Pol proteins revealed highly reduced levels of Pr78 Gag/Pol precursor and the processed $p27^{gag}$ when CTE was either deleted or displaced both in the *pol-env* spacer region and at the 5′ UTR (Fig. 3). Reinsertion of CTE at the original position while maintaining the CTE at the displaced position (either at the *pol-env* spacer region or at the 5′ UTR) restored Gag/Pol production to normal levels in both the cell lysates and the pelleted particles (Fig. 3). These results clearly suggest that the position of CTE plays an important role in virus replication.

Effect of CTE displacement on nucleocytoplasmic transport and/or stability of viral Gag/Pol message

Since the most pronounced effect of CTE observed has been on the nucleocytoplasmic transport of viral messages (Rizvi *et al.,* 1996b; Ernst *et al.,* 1997), slot blot

TABLE 1

Effect of CTE Displacement on the Propagation of MPMV Vectors[a]

Vectors	CTE displacement	Titer (CFU/ml)[b]
Mock	—	0
pK1(RV) + CTE HYG^R	Original 3′ UTR	1296
pK1(RV) − CTE HYG^R	CTE(−)	0
pdiscC2(aSA) HYG^R	Between *pol* termination codon and *env* initiation codon	0
pdiscC2(aSA)+CTE HYG^R	Between *pol* termination codon and *env* initiation codon and reinsertion of CTE at the original 3′ UTR	1260

[a] No Hyg^r colonies were observed for any of the vectors when the *trans* complementation assay was performed without pSV-A-MLV-env or pSV-A-MLV-env by itself or mock transfected.

[b] Data are the means of two independent experiments with two duplicate samples per experiment.

analyses were conducted to assess how CTE displacement within the viral genome may affect this process. To accomplish this, cellular RNAs were harvested 48–72 hr posttransfection of the CTE-deletion and -displacement clones and fractionated into nuclear and cytoplasmic fractions. Slot blot analyses of fractionated RNAs using a *pol*-specific probe revealed that either deletion or displacement of CTE led to the entrapment of viral Gag/Pol mRNA in the nucleus with little or none of the RNA transported to the cytoplasm (Fig. 4A). To control for the amount of RNA loaded into each slot, a parallel blot was hybridized with a probe for the *β-actin* message, revealing that similar amounts of RNA were transferred onto nitrocellulose (Fig. 4B). When CTE was reinserted at the original position while maintaining the displaced CTEs, proper nucleocytoplasmic transport of the Gag/Pol mRNA was observed. However, we cannot rule out the possibility that displacement of CTE could have affected the stability of Gag/Pol mRNA as well. Thus, these data confirm that CTE regulates the cytoplasmic export and/or stability of viral mRNAs and reveal that the 3′ terminal position of CTE is significantly linked to the ability of the viral messages to be expressed.

Close proximity of CTE to the poly(A) signal is important for CTE function

Since the natural 3′ terminal location of CTE positions it closely to *cis*-acting sequences important for polyadenylation of viral transcripts, we explored the possibility that the distance between CTE and the poly(A) signal was important for CTE function. Toward this end, MPMV molecular clones were generated that increased the distance between CTE (nt 8006–8240) and poly(A) signal

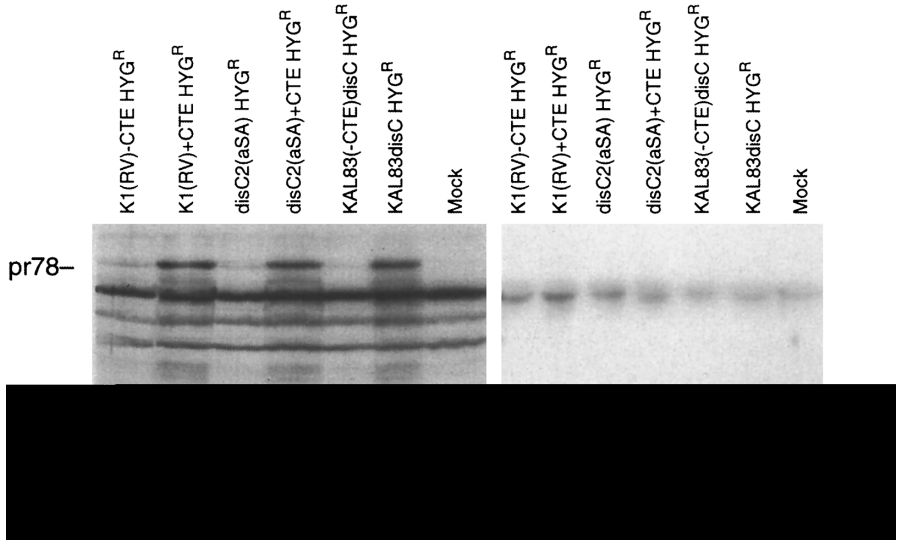

FIG. 3. Western blot analysis of cell lysates (left) and disrupted viral particles (right) prepared from Cos cultures transfected with MPMV CTE-deletion and -displacement molecular clones. The MPMV proteins were detected using a polyclonal antiserum against MPMV Gag/Pol polyprotein, Pr78, with an enhanced chemiluminescence kit. Mock, mock-transfected cells.

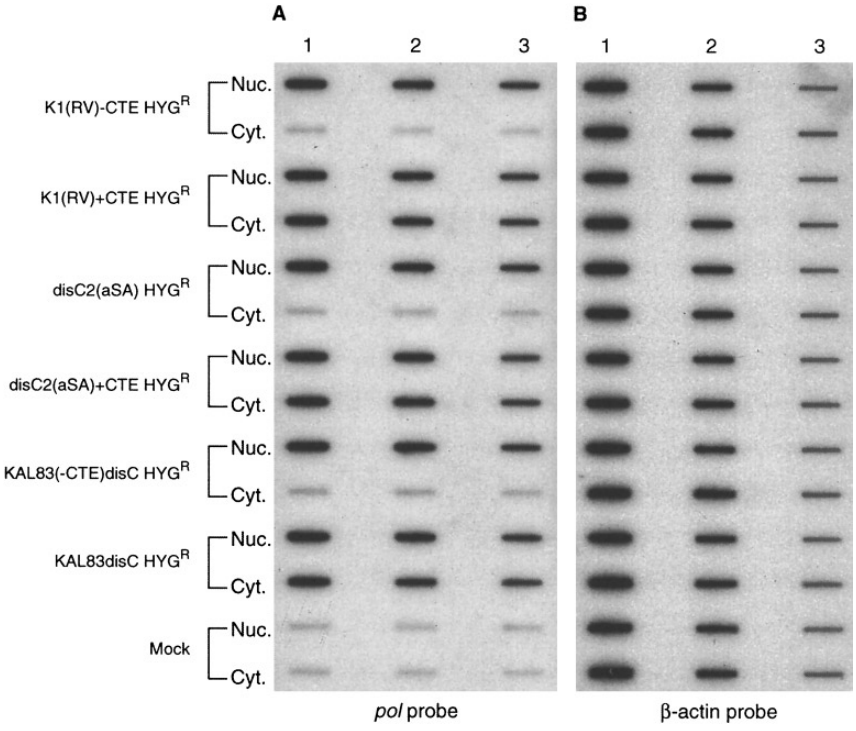

FIG. 4. Slot blot analysis of fractionated RNAs isolated from Cos cells transfected with MPMV vectors containing CTEs in the displaced or original position. Nuclear (Nuc.) and cytoplasmic (Cyt.) RNA fractions were transferred onto a nitrocellulose filter using the slot blot apparatus. (A) X-ray film of a filter probed with a ^{32}P-labeled 1.52-kb *pol*-specific DNA probe. Lanes 1, 2, and 3, twofold sequential dilutions of the nuclear and cytoplasmic RNAs. (B) X-ray film of a filter probed with a ^{32}P-labeled human *β-actin* cDNA control probe. Lanes 1, 2, and 3, fivefold sequential dilutions of the nuclear and cytoplasmic RNAs. Mock, mock-transfected cells.

(nt 8436–8444) from 196 to 1196 bp in the viral genome in increments of 250 bp (Fig. 5A). Upon transfection of these constructs in Cos cells, supernatants were harvested 72 hr posttransfection to prepare viral lysates for protein analysis and viral stocks for quantitation in the single round of replication assay as described earlier.

Western blot analysis of disrupted viral particles using anti-MPMV Gag/Pol sera revealed that increasing the natural distance between CTE and poly(A) signal progressively decreased the function of CTE as observed by lower amounts of p27^{gag} detected (Fig. 5B). Similar results were observed with cellular lysates prepared from transfected Cos cells (data not shown). To quantitate the effects of insertion of heterologous DNA sequences of various lengths between CTE and the poly(A) signal on CTE function, the harvested viral stocks were tested in the quantitative and sensitive single round of replication assay. Results of the single round of the replication assay correlated well with those obtained in the Western blot analyses; insertion of progressively longer extraneous DNA sequences between the CTE and the poly(A) signal led to a proportionally lower number of Hyg^r colonies (Table 2). Insertion of 250 bp reduced viral titers from 1296 colony forming units (CFU)/ml to 208 CFU/ml, a 6-fold reduction in titer, while an incremental increase of 250 bp decreased viral titers progressively from 29-fold (44 CFU/ml) for a 500-bp insertion to 108-fold (12 CFU/ml) for a 1000-bp insertion (Table 2). One potential caveat of this analysis could be that since CTE extends into the U3 region of the 3′ LTR, creation of the artificial *Eco*RV site in pKAL036(*Eco*RV) could have affected virus replication inadvertently. However, equal titers of virus (1296 CFU/ml each) were observed with a comparable clone, pK1(RV)+CTE HYG^R (Table 1), which does not contain the *Eco*RV site in the U3 region of the 3′ LTR. Thus, results of these analyses suggest that the close proximity of CTE to the poly(A) signal is important for efficient CTE function.

To examine the relationship between CTE and the poly(A) signal in more detail, we argued that if displacement of CTE away from the poly(A) signal disrupts CTE function, moving poly(A) signal close to displaced CTE should restore CTE function. To test this hypothesis, CTE was displaced ~2000 bp upstream of the poly(A) signal in the *env* region of MPMV, creating pKAL135 (Fig. 6A). As a control, CTE was reinserted in pKAL135 at the original genomic position while maintaining the displaced CTE in the *env* region, creating pKAL137. Western blot analysis of pelleted clarified supernantants revealed abrogation of particle production by the displacement of CTE in the *env* region, while reinsertion of CTE at the original position in this clone rescued particle production (Fig. 6B). The natural distance between CTE and the poly(A) sequence in the MPMV genome is approximately 200

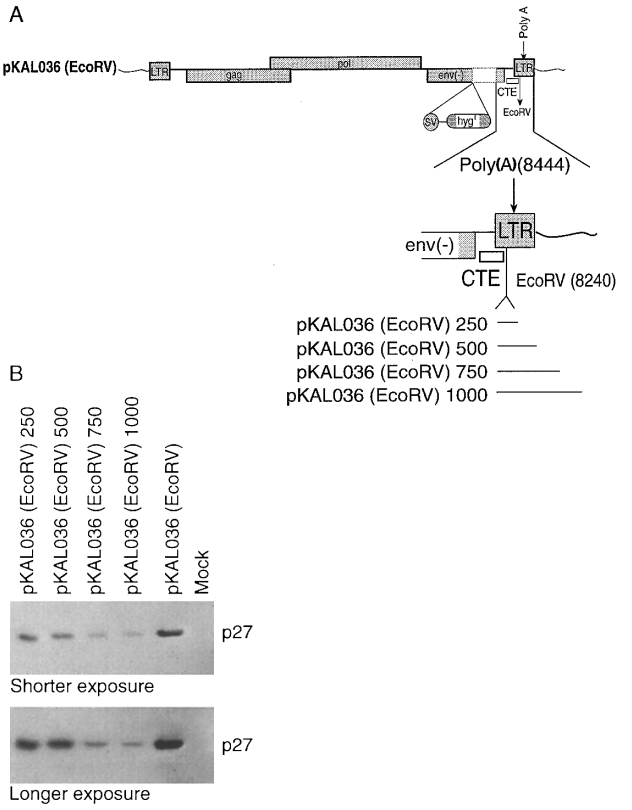

FIG. 5. Effect of insertion of heterologous DNA sequences of various lengths between CTE and polyadenylation sequences on CTE function. (A) Schematic representation of MPMV molecular clone, pKAL036 (*Eco*RV), containing insertions of 250, 500, 750, and 1000 bp at the *Eco*RV site introduced at the end of CTE. Details of the origin and construction of these plasmids are presented under Materials and Methods. Poly(A), polyadenylation signal. (B) Western blot analysis of pelleted viral particles from cultures transfected with MPMV vectors containing insertions of DNA fragments. The MPMV proteins were detected using a polyclonal antiserum against MPMV Gag/Pol polyprotein Pr78, with an enhanced chemiluminescence kit. (Top) A shorter exposure of the membrane; (bottom) a longer exposure. Mock, mock-transfected cells.

bp. Therefore, the poly(A) signal was cloned ~200 bp downstream of the displaced CTE in *env,* creating pKAL136 (Fig. 6A). Western blot analysis of particles produced by pKAL136 revealed that by moving poly(A) signal within the natural distance between CTE and poly(A) in *env* restored CTE function (Fig. 6B). Results of these analyses clearly demonstrate that the close proximity of CTE to the poly(A) signal is important for efficient CTE function and suggest functional interaction between CTE and the polyadenylation machinery.

DISCUSSION

The data presented in this report demonstrate that the 3′ terminal position of MPMV CTE, specifically the distance between CTE and the poly(A) signal, is important for efficient CTE function in the genomic context. These observations are in contrast to observations made in the case of HIV-1 where relocation of RRE from the original position in the Env ORF to the Gag or Nef ORF did not affect the expression of the Gag/Pol message or protein (Campbell *et al.,* 1996). However, relocation of RRE to the Gag ORF did affect Env-gp expression, leading to a reduction in Env mRNA translocated to the cytoplasm. Thus, apparently in the case of HIV-1, position is not as critical for RRE function as long as the RRE is present within the mRNA or pre-mRNA. CTE also needs to be present in the transcribed region (Tabernero *et al.,* 1996); however, our results show that it needs to be in close proximity to the 3′ end of the genome. The difference between RRE and CTE may reflect mechanistic differences in the two regulatory systems. For instance, HIV-1 encodes its own transactivator, Rev, to interact with RRE as opposed to CTE, which is thought to function most likely through interaction with cellular factor(s). Rev is a nuclear protein that probably binds the RNA substrates prior to splicing, making the position of RRE within the mRNA or pre-mRNA less critical. On the other hand, it is not known if a protein(s) interacting with CTE is nuclear or cytoplasmic and at what point during the transcript processing it binds the viral RNAs.

It is possible that being an RNA element with higher order structure, CTE does not fold appropriately in any other context except at the 3′ end of the viral genome, and its displacement in the viral genome alters its proper folding, disrupting CTE function. However, this possibility is discounted by the fact that data from our and other groups have shown successful displacement of CTE in HIV-1/SIV genomic and subgenomic contexts (Bray *et al.,* 1994; Zolotukhin *et al.,* 1994; Rizvi *et al.,* 1996a; Tabernero *et al.,* 1996; Ernst *et al.,* 1997). We have also successfully expressed the homologous MPMV *gag*/*pol* genes from a CMV-based expression vector using the same sequences of CTE cloned immediately downstream of the *pol* termination codon and upstream of the bovine growth hormone poly(A) signal (manuscript in preparation). It is precisely in this way that MPMV CTE was initially shown to substitute for the function of HIV-1 Rev/RRE for *env* expression from an expression vector (Bray *et al.,* 1994). In addition, CTE has been used to express truncated HIV-1 *gag* in the absence of Rev. In these constructs, the extensively folded HIV-1 RRE sequences were cloned 5′ to the CTE without affecting CTE RNA folding (Tabernero *et al.,* 1996; Ernst *et al.,* 1997). Similarly, in the study on the secondary RNA structure analysis of CTE by Ernst *et al.* (1997), chimeras between HIV-1 RRE and CTE were constructed that could maintain CTE function as long as the core CTE structure was used to generate the chimera. Together, these studies strongly suggest that CTE RNA forms a stable higher order structure in which the folding is not easily perturbed by neighboring sequences even if they have the potential of forming strong secondary RNA structures. These observations support our hypothesis that the loss of CTE function in

TABLE 2

Effect of Insertion of Heterologous DNA Sequences of Various Lengths on the Propagation of MPMV Vectors[a]

Vector	Sequences inserted between CTE and poly(A)	Titer (CFU/ml)[b]	Fold reduction in titer[c]
Mock	—	0	—
pKAL036 (*Eco*RV)	*Eco*RV site only	1296	—
pKAL036 (*Eco*RV) 250	*Eco*RV site and 250 bp	208	6
pKAL036 (*Eco*RV) 500	*Eco*RV site and 500 bp	44	29
pKAL036 (*Eco*RV) 750	*Eco*RV site and 750 bp	23	56
pKAL036 (*Eco*RV) 1000	*Eco*RV site and 1000 bp	12	108

[a] No Hyg^r colonies were observed for any of the vectors when the *trans* complementation assay was performed without pSV-A-MLV-env or pSV-A-MLV-env by itself or mock transfected.

[b] Data are the means of two duplicate samples.

[c] Values were calculated in comparison to titers observed for pKAL036(*Eco*RV).

the displaced contexts is due to disruption of close proximity of CTE to the poly(A) signal and not due to inappropriate folding of the CTE RNA. Further experiments are in progress to elucidate the requirement of close proximity of the poly(A) signal for efficient CTE function.

Aberrant nucleocytoplasmic transport of viral messages is a classic hallmark of CTE dysfunction. In the earlier studies, we and others have attributed the decrease in the amount of cytoplasmic RNA observed in the absence of CTE to defects in the nucleocytoplasmic transport of viral messages (Bray *et al.,* 1994; Rizvi *et al.,* 1996b; Ernst *et al.,* 1997). However, none of these studies conducted any analyses on RNA stability to exclude the possibility that the aberrant RNA transport observed could have resulted from increased instability of viral messages in the absence of CTE. In the present study, the fact that reinsertion of CTE restored CTE function in both CTE deletion and displacements indicates that if RNA stability was also being impacted, CTE was responsible for such effects and RNA stability was not independent of CTE function. Thus, if stability of viral messages was being affected independently of CTE function, reinsertion of CTE in the deletion or displacement mutants would not have restored CTE function. Similarly, in the case of mutants containing heterologous sequences of various lengths between CTE and the poly(A) signal, such sequences could have affected RNA stability; however, the fact that when both CTE and poly(A) signal were displaced to the *env* region, CTE function was restored suggests that the aberrant gene expression observed was due primarily to defects in CTE function, which could include effects on RNA stability, if any.

One explanation why the 3′ terminal location of CTE may be important for function could be due to a possible linkage between proper 3′-end formation of viral transcripts and CTE function. Polyadenylation, or 3′-end formation, is a highly regulated biochemical process requiring sequence-specific recognition of the *cis*-acting poly(A) signal in substrate mRNAs by cellular factors. This is followed by endonucleolytic cleavage of the pre-mRNAs at a defined site and by the addition of a polyadenylated tail (260–300 bp) to the nascently formed 3′-end of the transcript (reviewed by Keller, 1995; Wahle, 1995). The poly(A) signal that directs the cleavage and polyadenylation of pre-mRNAs consists of the highly conserved AAUAAA hexanucleotide located within 10–30 bp upstream of the cleavage site and a "G/U or U" rich cluster located 5–50 bp downstream of the cleavage site. In addition to the AAUAAA hexamer and downstream elements that form the minimal sequences required for cleavage and polyadenylation, *cis*-acting "U rich" sequences located upstream of the conserved poly(A) hexamer have been shown to be important for efficient 3′-end formation in several viral (Carswell and Alwine, 1989; DeZazzo and Imperiale, 1989; Russnak and Ganem, 1990; Brown *et al.,* 1991; Prescott and Falck-Pedersen, 1994) and cellular (Moreira *et al.,* 1995) genes. Binding of appropriate cellular factors to the poly(A) signal in a concerted fashion results in the successful cleavage and polyadenylation of pre-mRNAs, leading to their export from the nucleus.

Several studies in the literature have suggested that nucleocytoplasmic transport, stability, and translatability of messages are affected by proper 3′-end formation (Bernstein *et al.,* 1989; Jackson and Standart, 1990; Eckner *et al.,* 1991). In fact, a recent study has suggested that not only is polyadenylation required for the nuclear export of mRNA, but that the Rev/RRE regulatory system of HIV-1 cannot carry out its function without proper polyadenylation (Huang and Carmichael, 1996). In this case, the authors speculate that binding of polyadenylation factors to poly(A) sequences and Rev to RRE may help stabilize the substrate mRNAs and protect them from premature degradation. In the case of CTE, we speculate that there may be a requirement for actual physical/functional interaction between CTE and/or CTE binding factors with the polyadenylation machinery so that as CTE is displaced away from the poly(A) signal, CTE function is also

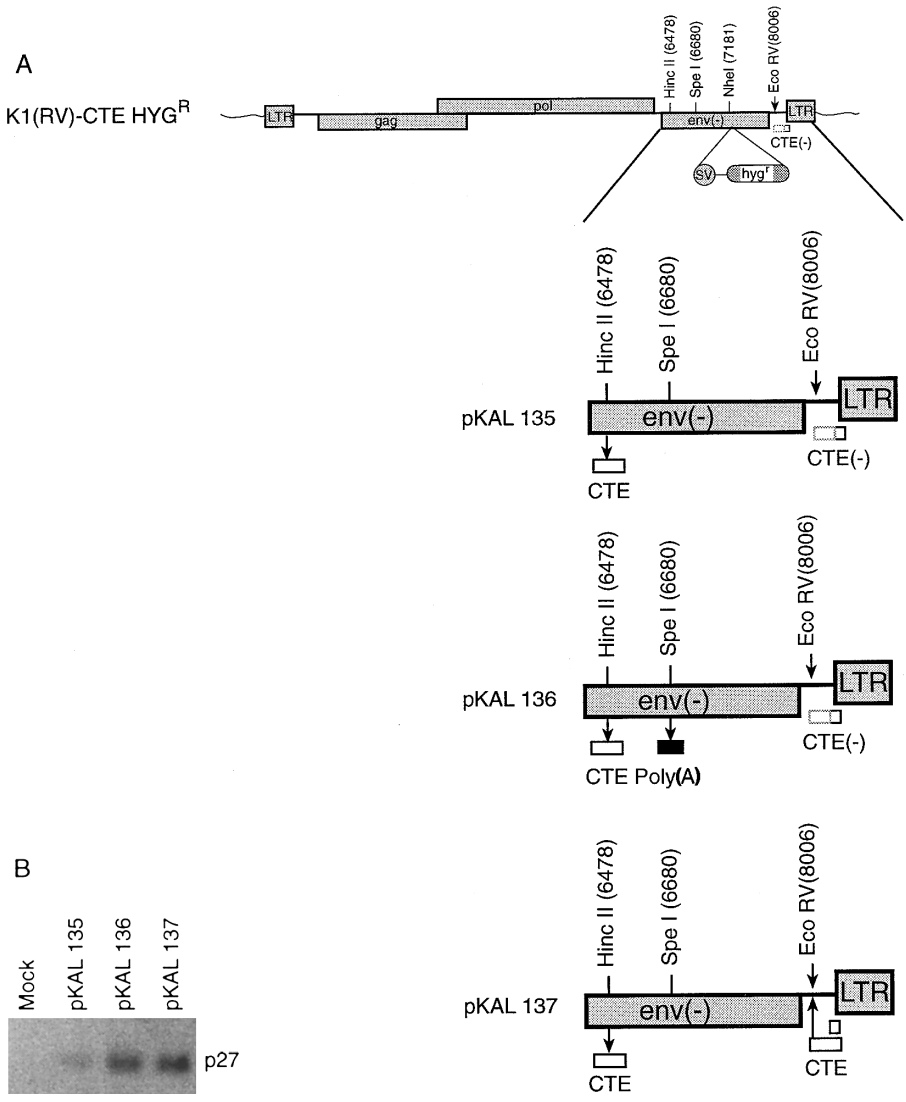

FIG. 6. Requirement of close proximity of CTE to poly(A) signal for CTE function. (A) Schematic representation of MPMV molecular clones containing displaced CTE and poly(A) signal. Only the 3′ half of the molecular clones are shown for clarity. Poly(A), polyadenylation signal. (B) Western blot analysis of pelleted viral particles from cultures transfected with MPMV vectors containing the displaced CTE and poly(A) signal. The MPMV proteins were detected using a polyclonal antiserum against MPMV Gag/Pol polyprotein Pr78, with an enhanced chemiluminescence kit. Mock, mock-transfected cells.

disrupted. Thus, unlike HIV-1 RRE, the spatial distance between CTE and poly(A) signal may also be more critical, especially in view of the fact that CTE is naturally found in close proximity to the poly(A) signal (within ~200 bp). In addition, as demonstrated in this report, CTE does not function when displaced to other regions of the viral genome, nor can it tolerate increases in the inherent distance between itself and the poly(A) signal. Furthermore, when the poly(A) signal itself is moved closer to a CTE displaced in the *env* region, CTE function is restored, suggesting a link between polyadenylation and CTE function. Experiments are currently underway to explore the relationship between proper 3′-end formation and CTE function.

The powerful ability of CTE and CTE-like elements to regulate posttranscriptional processes (Alonso-Caplen and Krug, 1991; Huang *et al.,* 1993; Bray *et al.,* 1994; Zolotukhin *et al.,* 1994; Liu and Mertz, 1995; Rizvi *et al.,* 1996a,b; Ogert *et al.,* 1996; Tabernero *et al.,* 1997) may facilitate the development of retroviral vectors for gene therapy protocols. This is especially important since genes to be used as therapeutics may require nuclear export signals for their expression or harbor repressor sequences requiring positive regulatory elements like CTE for their efficient expression. The fact that CTE does not require a virally encoded transactivator like Rev for function, in addition, makes CTE-like elements indispensable for the expression of genes of interest that otherwise require Rev/RRE-like regulatory systems for their expression. Toward this end, we have already ex-

ploited this ability of CTE to express SIV *gag*/*pol* genes without the Rev/RRE regulatory system from a single vector as a retroviral-based DNA vaccine (Rizvi *et al.,* 1996a). Results presented in this report, however, caution that the use of CTE for facilitating gene expression should take into account position of CTE within the transcriptional unit.

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