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Role of Mason-Pfizer Monkey Virus (MPMV) Constitutive Transport Element (CTE) in the Propagation of MPMV Vectors by Genetic Complementation Using Homologous/Heterologous *env* Genes

TAHIR A. RIZVI,*,†,[1] KATHY A. LEW,* EDWIN C. MURPHY, JR.,‡ and RUSSELL D. SCHMIDT*

*The University of Texas M. D. Anderson Cancer Center, *Department of Veterinary Sciences, Bastrop, Texas 78602; †Department of Carcinogenesis, Science Park-Research Division, Smithville, Texas 78957; and ‡Department of Tumor Biology, 1515 Holcombe Boulevard, Houston, Texas 77030*

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To study Mason-Pfizer monkey virus (MPMV) replication over a single round, virus particles were generated that contain a replication-defective vector encoding a dominant selectable marker, the *hygromycin B phosphotransferase (hyg^r)* gene. Genetic complementation with a homologous MPMV envelope glycoprotein (Env-gp) or pseudotyping by several heterologous Env-gps from a variety of viruses resulted in infectious MPMV particles containing the replication-defective RNA. Recently, it has been shown that human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) Rev and Rev-responsive element (RRE) functions can be substituted *in vitro* by a *cis*-acting sequence, the constitutive transport element (CTE), from simian type D retroviruses like MPMV and simian retrovirus type 1 (SRV-1). To determine whether CTE of MPMV is necessary for MPMV nucleic acid propagation, an MPMV vector that lacked the terminally located CTE was generated. Propagation of this vector was completely abrogated in the absence of CTE, showing the importance of CTE in MPMV replication. Insertion of CTE back into the MPMV genome in the sense orientation rescued replication to wild-type levels. Slot-blot analysis of nuclear versus cytoplasmic RNA fractions revealed that most of the messages were sequestered in the nucleus of cells transfected with the CTE(−) vectors and very little was transported to the cytoplasm. To test whether HIV-1 or SIV RREs could complement CTE function, the HIV-1 or SIV RREs were inserted in the CTE(−) vectors. *trans* complementation of CTE(−)RRE(+) vectors with Env- and Rev- expression plasmids rescued propagation of the CTE(−) vectors. Computer analysis predicted an RNA secondary structure in MPMV CTE analogous to the HIV-1 and SIV RREs that could form three stable stem loops, the first of which contains a site similar to the Rev-binding domain in the HIV-1 RRE. The presence of a higher-order CTE structure was analyzed by mutational analysis. We conclude that CTE is important in the replication of MPMV and affects the nucleocytoplasmic transport and/or stability of viral messages similar to the Rev/RRE regulatory system of HIV-1 and SIV.

INTRODUCTION

Mason-Pfizer monkey virus (MPMV) is a prototypic type D primate retrovirus that was originally isolated from a spontaneous breast carcinoma of a female rhesus monkey (Chopra and Mason, 1970; Jensen *et al.,* 1970). Since its initial discovery, rather than being found oncogenic, it has been implicated with simian acquired immunodeficiency syndrome (SAIDS) in newborn rhesus macaques (Daniel *et al.,* 1984; Marx *et al.,* 1984; Stromberg *et al.,* 1984). MPMV closely resembles other 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). It contains three genes in the order 5′-*gag-pol-env*-3′ where *gag* encodes the structural genes, *pol* the viral aspartyl protease and the reverse transcriptase, and *env* the envelope glycoprotein (Env-gp) (Sonigo *et al.,* 1986). Unlike type C retroviruses that carry out assembly and budding simultaneously at the cell membrane, MPMV preassembles its immature capsid proteins in the cytoplasm before budding at the cell membrane, releasing infectious virions (Fine and Schochetman, 1978). Not much is known about MPMV replication and cellular targets of MPMV infection.

Recently, a small 219-bp *cis*-acting element, the constitutive transport element (CTE), present in the 3′ region of MPMV and SRV-1 was implicated in interacting with cellular pathways normally used for the transport of cellular mRNAs from the nucleus to the cytoplasm (Bray *et al.,* 1994; Zolotukhin *et al.,* 1994). In complex retroviruses like human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV), transport of spliced and unspliced messages is regulated by the virally encoded Rev protein, which interacts with a short *cis*-acting sequence, the Rev-responsive element (RRE), found in their *env* genes (reviewed by Cullen, 1992). Similarly, the MPMV and SRV-1 CTEs, once inserted before the polyadenylation sequences of expression vectors expressing either *gag/pol* or *env* genes of HIV-1 and SIV, or in the *nef* gene of

[1] To whom reprint requests should be addressed at Department of Veterinary Sciences, The University of Texas M. D. Anderson Cancer Center, Route 2, Box 151-B1, Bastrop, TX 78602. Fax: 512-332-5208. E-mail: tarfm@aol.com.

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Rev(−) and RRE(−) molecular clones of HIV-1 or SIV, cause the viral mRNA expression to become independent of the Rev/RRE regulatory system (Bray *et al.,* 1994; Zolotukhin *et al.,* 1994; Rizvi *et al.,* 1996). These Rev/RRE-dependent HIV-1 mRNAs contain several *cis*-acting repressor or negative inhibitory sequences (CRS or INS) that trap these unspliced or partially spliced messages in the nucleus, destabilize them, and/or prevent their association with the polysomes (Rosen *et al.,* 1988; Cochrane *et al.,* 1991; Maldarelli *et al.,* 1991; Schwartz *et al.,* 1992a; Nasioulas *et al.,* 1994; Brighty and Rosenberg, 1994). In the absence of Rev, these messages remain sequestered in the nucleus until they are spliced, exported out, or degraded (Emerman *et al.,* 1989; Felber *et al.,* 1989; Malim *et al.,* 1989a). Rev binds to RREs found in these messages and allows their expression by transporting them to the cytoplasm (Felber *et al.,* 1989; Hammarskjold *et al.,* 1989; for more references, see review by Cullen, 1992). Rev may accomplish this by interacting with cellular cofactors involved in the nucleocytoplasmic transport of cellular messages (Bogerd *et al.,* 1995; Stutz *et al.,* 1995; Fischer *et al.,* 1995; Fritz *et al.,* 1995). Interestingly, in hepatitis B virus (HBV), a DNA virus that replicates by using reverse transcription of an RNA intermediate, a CTE/RRE-like element has been discovered (Huang and Liang, 1993; Huang and Yen, 1994). Similar to the HIV-1 and SIV RREs, it regulates gene expression by inhibiting splicing and facilitating the nucleocytoplasmic transport and utilization of HBV messages.

We were interested in studying the role of CTE in the replication of MPMV and in examining whether its function was similar to the function of Rev/RRE in the life cycle of the virus. Since the Rev/RRE regulates the expression of viral messages by affecting splicing as well as transport and stability of messages, we wanted to explore the possibility that MPMV, being a simple retrovirus, controls splicing in a manner similar to those found in other simple retroviruses. Splicing in simple retroviruses is controlled by regulatory functions that allow the formation of incompletely spliced products (reviewed by Coffin, 1985, and Stoltzfus, 1988). Regulation of splicing may occur at the level of inefficient splice site usage or mRNA instability (Arrigo and Beemon, 1988; Katz and Skalka, 1990). In addition, it is possible that *cis*-acting sequences present in other parts of the genome interfere with the splicing machinery directly by acting as negative inhibitory elements (Stoltzfus and Fogarty, 1989) or facilitate the transport of unspliced messages by acting as CTEs (Bray *et al.,* 1994; Zolotukhin *et al.,* 1994).

To study MPMV replication, a *trans* complementation assay was developed that allows study of viral replication over a single round. This assay allowed us to study the role of CTE in MPMV replication in a clear and quantitative manner. Toward this end, a modified version of an Env(−) MPMV vector was created that lacked the terminally located CTE and contained an insertion of the "SV40-Hyg^r" cassette in the *env* gene. Propagation of this vector was completely abrogated in the absence of CTE, while insertion of the CTE back into the genome in the sense orientation rescued MPMV replication to almost wild-type levels. Further analysis of the fractionated mRNAs revealed that most of the messages were sequestered in the nucleus of cells transfected with the CTE(−) vectors and very little was transported to the cytoplasm. When HIV-1 or SIV RREs were inserted in the CTE(−) vector and *trans* complemented with Rev-expressing plasmids, vector propagation was rescued, albeit at a lower efficiency compared with MPMV vectors containing CTE. Secondary RNA structure analysis of CTE indicated that it had the potential to form three stable stem loops, one of which contained a site reminiscent to the Rev-binding sequences in the HIV-1 RRE. Deletion/insertional mutation analysis suggested the presence of an RNA secondary structure of CTE. Together, these results suggest that CTE is critical for the regulation of MPMV gene expression and functions in the viral life cycle by interacting with constitutively present cellular factors analogous to the Rev/RRE regulatory system of HIV/SIV.

MATERIALS AND METHODS

Numbering system

Nucleotide designations for MPMV, SIVmac239, and HIV-1-NL4-3 are based on GenBank Accession Nos. M12349 (Sonigo *et al.,* 1986), M33262 (Kestler *et al.,* 1990), and M19921 (Adachi *et al.,* 1986), respectively.

Construction of MPMV vectors

All plasmids were made by standard molecular cloning techniques. A 234-bp *Xho*I–*Bam*HI fragment from p1234 (kindly provided by Dr. Marie-Louise Hammarskjold) containing the MPMV CTE was subcloned into the *Xho*I–*Bam*HI sites of pIC19H (Marsh *et al.,* 1984) in order to acquire multiple cloning sites at both ends, resulting in pTR225. pKAL001 contains the entire MPMV genome (Fig. 1) and was constructed by removing an *Xba*I–*Eco*RI fragment from pSHRM15 (kindly provided by Dr. Eric Hunter) and cloning into the *Xba*I and *Eco*RI sites of pIC19H. Clone pKAL036, which contains a deletion in the *env* gene with a simultaneous insertion of the "SV40-Hyg^r" cassette (Fig. 1), was constructed through several steps: first, a *Hin*dIII site [nucleotide (nt) 7886] in the *env* gene of pKAL001 was blunted with the Klenow enzyme (New England Biolabs, Cambridge, MA) and ligated to an *Nhe*I linker. Next, an *Nhe*I–*Nhe*I fragment containing the "SV40-Hyg^r" cassette was inserted in the *Nhe*I site in the sense orientation with the simultaneous deletion of 705 bp in *env.*

pKAL013 was an intermediate clone constructed by ligating the 1432-bp *Nhe*I–*Sma*I fragment from pKAL001 into the *Nhe*I–*Eco*RV sites of pTR276. pKAL013 contains MPMV sequences from the *Nhe*I site (nt 7181) in the *env*

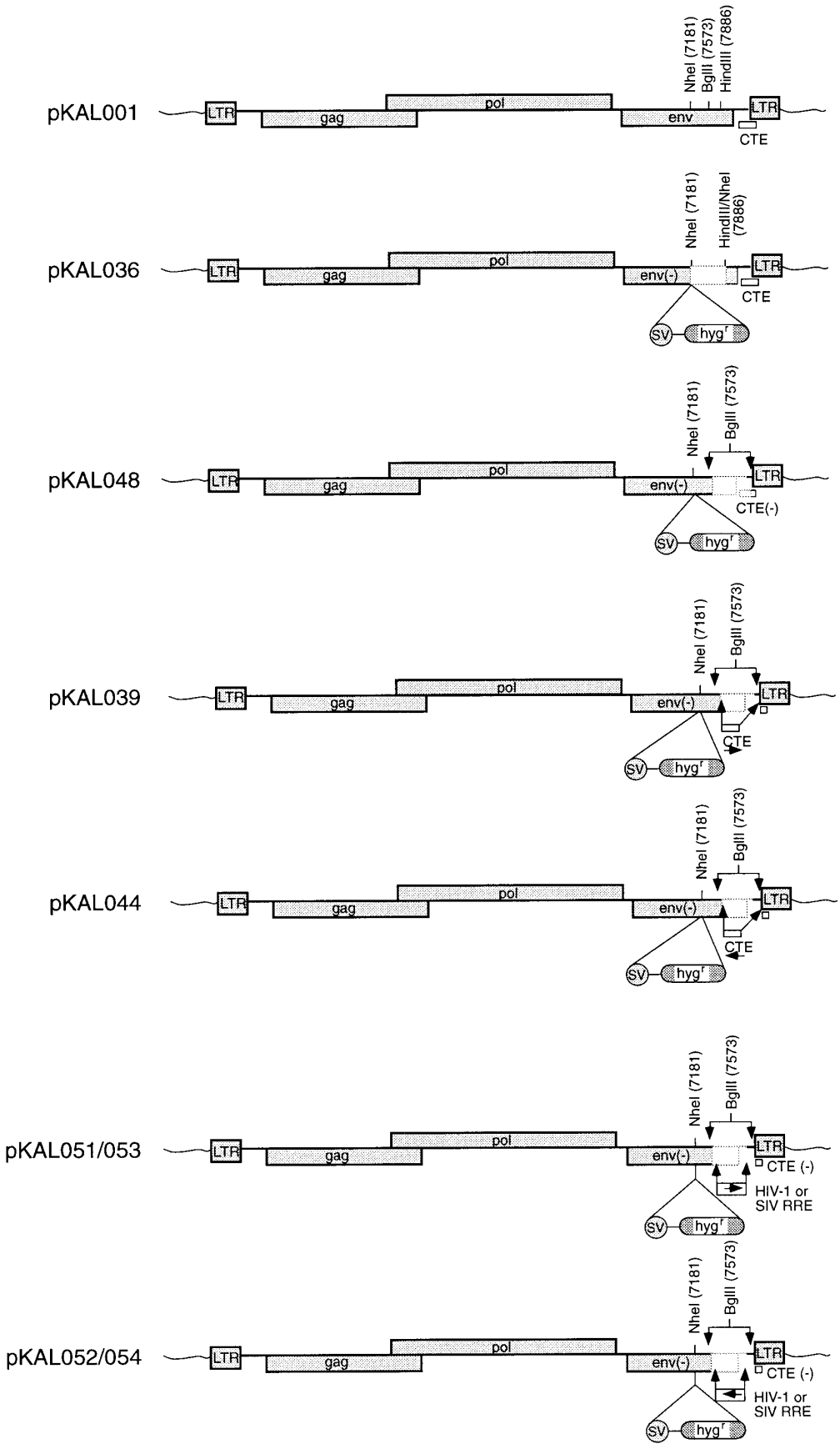


FIG. 1. Schematic representation of the MPMV genome (pKAL001) with open reading frames (ORFs) and MPMV vectors with the insertion of the "SV40 Hygr" cassette in the *env* region. Details of the origin and construction of these plasmids are presented under Materials and Methods. The dotted lines represent the extent of deletions introduced in the various vectors to abolish Env ORF and maintain or remove the MPMV CTE. RRE, Rev-responsive element; SV, simian virus 40 early promoter; *hygr*, *hygromycin B phosphotransferase* gene.

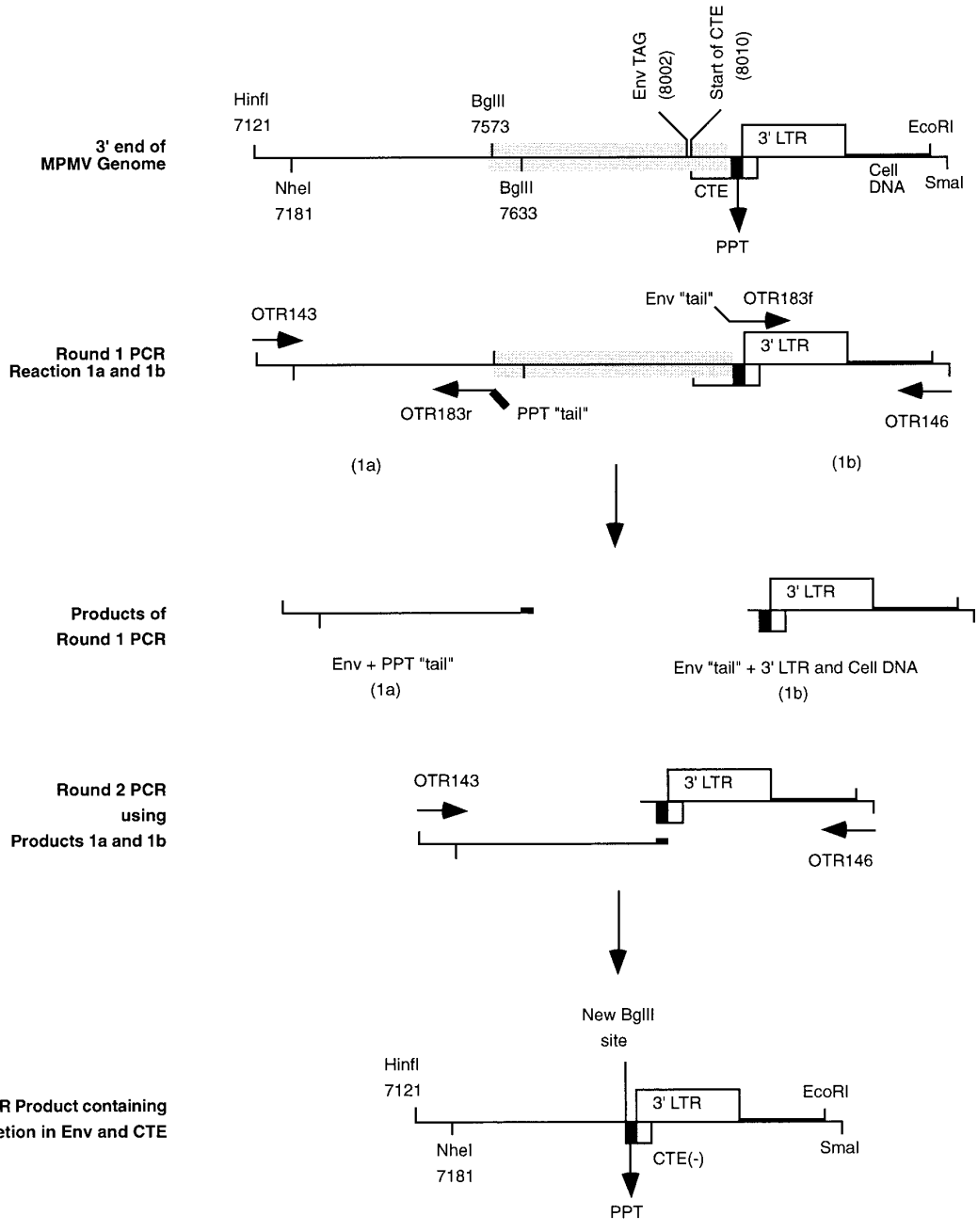


FIG. 2. Deletion introduced in the MPMV genome using splice overlap extension (SOE) PCR removing parts of *env* and CTE. The first schematic represents the 3′ end of MPMV genome followed by the PCR amplification strategy used for introducing the deletion shown in the gray shaded area. Two rounds of PCR were performed to construct the deletion with the first round of PCR representing two separate reactions (see Materials and Methods for details). The MPMV CTE is shown as a black box. Annealing sites of PCR amplification primers used are shown with respect to the 3′ LTR. Portions of hybrid primers that do not anneal are shown with offset portions. Cell DNA is shown in thick lines.

gene to the *Sma*I site in the cellular DNA at the 3′ end. A deletion in the *env* gene and CTE was made by using the splice overlap extension (SOE) PCR strategy (Gibbs *et al.,* 1994). This strategy required conducting two separate rounds of PCR (Fig. 2). The first round of PCR consisted of two separate reactions using pKAL001 as the template. The first reaction used sense (S) primer OTR143 (5′ GATTCCAATTGCCTTTACGC 3′; MPMV *env* nt 7121–7140) and antisense (AS) hybrid primer OTR183r (5′ tttaatAGATCTTGTATAGTG 3′; MPMV nt 8196–8191, encompassing the PPT, shown in lowercase, followed by *env* nt 7578–7564, shown in uppercase). These primers created a deletion in MPMV *env* and most of CTE. The PCR amplification generated a fragment (product 1a) containing nt 7121–7578 of the MPMV *env* gene with a 6-bp region (PPT "tail") at the 3′ end complementary to 6 bp of the CTE region starting just upstream of the PPT. The second reaction used S hybrid primer OTR183f (5′ AGATCTattaaaaagggtgac 3′; MPMV nt 7573–7578 in *env,* shown in uppercase, followed by nt 8191–8205,

shown in lowercase), creating a 424-bp deletion in *env* and a 180-bp deletion in the CTE, and AS primer OTR146 (5′ CCCGGGAATTCGCGGTACCC 3′) encompassing sequences in cell DNA downstream of the 3′ LTR of the pSHRM15 clone. This reaction produced a fragment (product 1b) containing the PPT immediately upstream from the 3′ LTR and some cellular DNA sequences immediately downstream from the 3′ LTR; the 5′ end of this fragment contains a 6-bp region (*env* "tail") corresponding to nt 7573–7578 in the MPMV *env* gene and containing a *Bgl*II site. The two PCR reactions allowed addition of complementary sequences to the appropriate ends of MPMV *env* and PPT to enable joining of these sequences in a subsequent round of PCR (Fig. 2). All PCR reactions were conducted in the Perkin–Elmer Cetus 9600 block thermocycler (Perkin–Elmer, Foster City, CA). The PCR reactions were performed in 50-μl volumes using the GeneAmp PCR reagent kit with native *Taq* polymerase (Perkin–Elmer). Conditions for PCR were as follows: an initial denaturation step at 94° for 5 min, followed by a cycling program of 94° for 45 sec, 55° for 45 sec, and 72° for 2 min for 30 cycles, followed by a final extension step at 72° for 7 min, and finally cooling to 4°. Products 1a and 1b were purified by the QIAquick PCR purification kit (Qiagen, Chatsworth, CA). A second round of PCR was performed using products 1a and 1b as templates and primers OTR143 and OTR146 under the same amplification conditions as described above but only for 10 cycles (Fig. 2). The final PCR product was isolated by agarose gel electrophoresis and purified by the Prep-A-Gene kit (Bio-Rad, Hercules, CA). The gel-purified PCR product was digested with *Nhe*I and *Sph*I and ligated into the *Nhe*I–*Sph*I sites of pKAL013, creating pKAL014, which contains a 613-bp deletion of nt 7578–8191. Sequences including the PPT and 3′ LTR, along with the flanking regions, were verified by the Promega "*fmol* DNA Cycle Sequencing System" using the end-labeled primer method (Promega, Madison, WI). pKAL014 was digested with *Nhe*I–*Eco*RI and the PCR-amplified product was cloned into the *Nhe*I–*Eco*RI sites of pKAL001. Simultaneously, an *Nhe*I–*Nhe*I fragment containing the "SV40-Hyg^r" cassette was inserted in the sense orientation into the unique *Nhe*I site in the *env* gene (Fig. 1). This clone was named pKAL048; it contains a 613-bp deletion (nt 7578–8191) from the first *Bgl*II site in *env* to the start of the PPT. In the process, 427 bp of the *env* gene and most of the CTE (186 bp) were deleted except for 50 bp consisting of 4 bp 5′ to the PPT, 11 bp of the PPT, and 35 bp of U3 sequences in the 3′ LTR which were kept to avoid disrupting sequences important in the initiation of the plus-strand DNA synthesis (Smith *et al.,* 1984).

pKAL039 was created by re-introducing the CTE into pKAL048, while maintaining the aforementioned deletion in *env* (Fig. 1). A *BamH*I fragment containing 234 bp of the MPMV CTE from pTR225 was inserted in the correct orientation at the *Bgl*II site in pKAL048, resulting in pKAL039. An identical clone containing the CTE in the opposite orientation was named pKAL044. Since pKAL048 retained 46 bp of the CTE (overlapping with PPT and the U3 region of the 3′ LTR), pKAL039 and pKAL044 contain a 46-bp duplication of the CTE (Fig. 1).

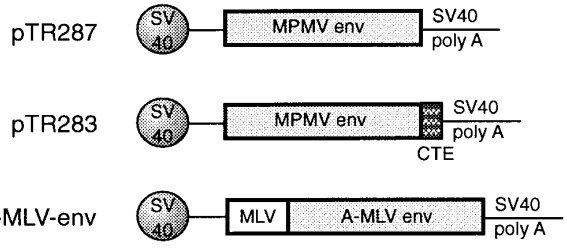


FIG. 3. Schematic representation of MPMV and A-MLV Env-gp expression constructs in the presence or the absence of MPMV CTE. pTR287 and pTR283 express the MPMV Env-gp from the SV40 early promoter and uses the SV40 poly(A) in the presence or the absence of the MPMV CTE. pSV-A-MLV-env expresses the A-MLV Env-gp from the MLV LTR and contains the SV40 enhancer promoter and SV40 poly(A). Details of the origin and construction of these plasmids are presented under Materials and Methods. MPMV CTE is shown as a stippled box. SV40, simian virus 40 early promoter; A-MLV, amphotropic murine leukemia virus; poly(A), polyadenylation sequences.

To replace the MPMV CTE with HIV-1 RRE, a fragment from pNL4-3 containing HIV-1 RRE (nt 7610–8130) was inserted at the *Bgl*II site of pKAL048. The clone containing the HIV-1 RRE in the sense orientation was named pKAL051, while pKAL052 contained the HIV-1 RRE in the antisense orientation (Fig. 1).

To replace the MPMV CTE with SIVmac239 RRE, a fragment from p239SpE3′ (Kestler *et al.,* 1990) containing SIVmac239 RRE (nt 8329–8725) was inserted at the *Bgl*II site of pKAL048. The clone containing the SIVmac239 RRE in the sense orientation was named pKAL053, while pKAL054 contained the SIVmac239 RRE in the antisense orientation.

To disrupt the predicted RNA secondary structure of MPMV CTE, PCR was used to make deletions and insertional mutations in CTE. This was achieved by creating a *Bgl*II site in stem loop III at position A (nt 8190/8191) in CTE which was cloned into the CTE(−) vector, pKAL048, resulting in pKAL074. pKAL074 was used to delete sequences in stem loop I (nt 8006–8038), stem loop III (nt 8149–8190), and stem loops I and III, simultaneously resulting in pKAL076, pKAL077, and pKAL078, respectively. Additional 9-bp insertional mutations were introduced into pKAL074 between nt 8037 and 8038 at site B, between nt 8073 and 8074 at site C, and between nt 8089 and 8090 at site D in stem loops I and II, resulting in pKAL079, pKAL080, and pKAL081, respectively. All mutations were verified by *fmol* sequencing as specified above. Details of the design and construction of these vectors can be obtained from the authors upon request.

Env-gp expression plasmids

To construct an MPMV *env* expression plasmid, pTR283 (Fig. 3), a 2196-bp region (nt 6224–8420) con-

taining *env* and CTE was PCR-amplified using S primer OTR119 (5′ TTTCCCTTGTCGACAGATATGAA 3′; nt 6224–6246; this oligonucleotide creates a unique *Sal*I site upstream of the *env* initiation codon by mutating two nucleotides, shown in bold letters) and AS primer OTR120 (5′ TTATATACACAGGCAGCAAG 3′; nt 8420–8401; a primer complementary to sequences in the 3′ LTR). The resulting PCR product was digested with the artificially created *Sal*I site and the naturally occurring *Sph*I and ligated into the *Sal*I and *Sph*I sites of pIC20R (Marsh *et al.,* 1984). To avoid sequencing the entire PCR-amplified *env* region, a 1693-bp fragment internal to the PCR product was exchanged with the wild-type region using the *Spe*I and *Sph*I sites (nt 6680–8373), resulting in pTR275. The sequence of the remaining PCR-amplified *env* region was verified by sequencing. Next, a *Sal*I–*Xho*I fragment from pTR275 containing MPMV *env* and CTE was cloned into the *Xho*I site of pZeoSV expression vector (Invitrogen, San Diego, CA), resulting in pTR283. To create an MPMV *env* expression vector without the CTE, pTR287, the MPMV *env* region was PCR-amplified using S primer OTR119 and AS primer OTR147 (5′ GCTGTCCAGTCGACCTCACAGGGG 3′; nt 8033–8010; this oligonucleotide creates a unique *Sal*I site immediately downstream from the *env* stop codon by mutating two nucleotides, shown in bold letters, from the wild type). The resulting PCR product was digested with *Sal*I and cloned into the *Xho*I site of pZeoSV in the sense orientation, creating pTR287 (Fig. 3).

Most of these clones were made through several stages of cloning, details of which can be obtained from the authors upon request.

Amphotropic murine leukemia virus (A-MLV) Env-gp expression plasmid pSV-A-MLV-env (Page *et al.,* 1990), which expresses A-MLV Env-gp under the control of the MLV LTR, was obtained from the AIDS Research and Reference Reagent Program of the National Institutes of Health. pCMV-HIV-1-Env expresses the *env* gene of HIV-1-HXB-2 under the control of human cytomegalovirus (CMV) promoter and was kindly provided by Dr. Harriet Robinson, and pCMV-HIV-2-Env expresses the *env* gene of HIV-2-Rod also under the control of human CMV promoter and was kindly provided by Dr. Antonito Panganiban. The Rev-expression plasmid, pCMV-*rev* (Lewis *et al.,* 1990), expresses the HIV-1 Rev under the control of the simian CMV immediate early promoter and was obtained from the AIDS Research and Reference Reagent Program of the National Institutes of Health.

Transfections and infections of cells

Cos cells were maintained at 37° in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum from Hyclone (Logan, UT). In order to provide Env-gp in *trans,* MPMV vectors were cotransfected individually with different Env-gp expression plasmids into Cos cells by the DEAE–dextran method (Cullen, 1988). Viral stocks were harvested 72 hr posttransfection and subjected to low-speed centrifugation to remove cellular debris. A portion of the viral stock was used to infect HeLaT4 cells in the presence of 8 μg/ml DEAE–dextran (Pharmacia, Piscataway, NJ). Forty-eight hours postinfection, selection for hygromycin resistance was initiated by replacing old media with media containing 200 μg/ml hygromycin B (Calbiochem, La Jolla, CA). After 9–11 days, hygromycin-resistant (Hyg^r) colonies were either stained with 0.5% crystal violet in 50% methanol and counted or pooled and expanded to prepare genomic DNA.

Ultracentrifugation of viral particles and reverse transcriptase (RT) assay

To determine whether viral particles containing RT activity were released from cells expressing MPMV vectors, Cos cells were transfected as described above, and the medium was changed 24 hr posttransfection. After an additional 48 hr, supernatants were harvested and clarified of cellular debris by low-speed centrifugation in a table-top Sorvall centrifuge at 2500 rpm. Viral particles were pelleted by ultracentrifugation using a Sorvall TH641 swinging bucket rotor at 80,000 *g* for 2 hr at 4° with a 20% sucrose cushion. Viral pellets, obtained from 9 ml of supernatant, were resuspended in 50 μl of TNE buffer (50 m*M* Tris–Cl, pH 7.4, 100 m*M* NaCl, and 1 m*M* EDTA, pH 8.0) and stored at −80°.

The RT assay was performed by disrupting 10 μl of resuspended particles in 50 μl of RT cocktail (50 m*M* Tris–Cl, pH 7.8, 63 m*M* KCl, 4.2 m*M* $MgCl_2$, 0.08% Nonidet P-40, 0.85 m*M* EDTA, 4.2 μg/ml poly(A), and 0.13 μg/ml of oligo(dT). Eight microliters of 0.5 *M* DTT and 1 μl of 10 μCi/μl of [^{32}P]dTTP (NEN Dupont, Boston, MA) per milliliter were added to this mix immediately before use, and the reaction mix was incubated at 37° for 1.5 hr. Ten microliters of the reaction mix was spotted onto a DEAE filtermat (Wallac No. 1205-405), washed four times with 2× SSC for 5 min each and once with ethanol, dried, and counted in the Wallac 1409 liquid scintillation counter (Wallac, Gaithersburg, MD).

RNA fractionation and slot blot analysis

Seventy-two hours posttransfection, cells were trypsinized, washed in 1× phosphate-buffered saline (PBS), and processed for RNA fractionation. RNA was isolated using the Qiagen RNeasy Total RNA kit. Briefly, cytoplasmic RNA was isolated by lysing cells on ice in the lysing buffer (50 m*M* Tris–Cl, pH 8, 140 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 0.5% Nonidet P-40). β-Mercaptoethanol (β-ME) at 10 μl/ml and RNasin at 1000 U/ml were added to the buffer immediately before use. The lysates were centrifuged at 4° for 2 min at 300 *g* and the supernatant was transferred to an RNase-free tube and processed for cytoplasmic RNA isolation according to the manufacturer's recommended protocol. The resulting pellet con-

taining the nuclear RNA and cell debris was collected and processed for nuclear RNA extraction. Briefly, the nuclear pellet was resuspended in the RLT lysis buffer (Qiagen) containing β-ME at 10 μl/ml. The suspension was applied to the Qiashredder column and centrifuged at 12,000 rpm for 1 min to shear genomic DNA. The lysate contained the nuclear RNA, which was isolated according to the manufacturer's recommendations. Following RNA isolation, both cytoplasmic and nuclear RNAs were treated with RNase-free DNase to eliminate any contaminating DNA, and dilutions (1:1, 1:2, 1:4) of RNA were transferred to nitrocellulose using the slot-blot apparatus. The filter was hybridized with a 1.52-kb *pol*-specific DNA probe (nt 3148–4672) (Fig. 5A) using the Rapid-hyb buffer (Amersham Life Sciences, Arlington Heights, IL). To control for RNA amounts, further dilutions of RNA (1:5, 1:25, 1:125) were transferred onto a separate nitrocellulose filter and probed with a 2-kb human β-actin cDNA control probe from Clonetech (Palo Alto, CA). The probes were labeled using the Redivue stabilized [^{32}P]dCTP (Amersham Life Sciences) and the Rediprime DNA Labeling System (Amersham Life Sciences). The filter was washed, air-dried, and exposed to Kodak XAR-5 X-ray film with a Du Pont Cronex Lightning-Plus intensifying screen. To quantitate relative intensities of signal in each band, the radioactive counts per minute (cpm) were measured using a Betascope 603 blot analyzer (Betagen, Mountain View, CA).

RNA secondary structure analysis

The putative secondary RNA structure of the MPMV CTE (nt 8006–8240) was determined on a DEC-Alphaserver 2100 5/250 computer using the MFold program of the University of Wisconsin Genetics Computer Group (GCG) software package. MFold predicts both optimal and suboptimal secondary RNA structures with minimal free energy using published criteria (Zuker, 1989; Jaeger *et al.,* 1989).

RESULTS

Experimental approach

To study the role of CTE in the replication of MPMV, a genetic complementation assay was developed that allowed the study of MPMV replication over a single round. This entailed the construction of several replication-defective MPMV vectors that contained a deletion and a simultaneous insertion of a *hyg*^{r} gene "cassette" expressed from an SV40 promoter/enhancer in their *env* genes. Propagation of such vectors depended upon *trans* complementation by the *env* gene. Genetic complementation between the *env*-defective vector and *env*-expressing plasmid generated virus particles able to infect susceptible cells. Infected cells were selected in medium containing hygromycin B. Only cells with successful integration of vector DNA into the host genome and expressing the *hyg*^{r} gene gave rise to Hyg^r colonies. The number of Hyg^r colonies obtained was directly proportional to the amount of infectious virus produced. The virus particles produced were infectious for only one round of replication since they lacked the *env* gene for further propagation. This assay was designed based on other *trans* complementation assays that have been used successfully to study replication of HIV-1 (Page *et al.,* 1990; Delwart *et al.,* 1992; Geraghty and Panganiban, 1993) and SIV (Rizvi and Panganiban, 1992a,b).

TABLE 1

Generation of MPMV Particles by *trans* Complementation of pKAL036 with Homologous and Heterologous Env-gps[a]

Env-gp-expressing plasmids	Description of Env-gp	Titer[b] (CFU/ml ± SD)
pTR283	MPMV Env; CTE(+)	404 ± 9
pTR287	MPMV Env; CTE(−)	0
pSV-A-MLV-env	Murine amphotropic Env	2712 ± 17
pCMV-HIV-1 Env	HIV-1-HXB-2 Env	56 ± 4
pCMV-HIV-2 Env	HIV-2-Rod Env	7 ± 1

[a] No Hyg^r colonies were observed for any of the Env-gp-expressing plasmids, pKAL036 by itself, or mock transfected.

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

Successful propagation of MPMV vectors by homologous and heterologous Env-gps

To test the functionality of the *trans* complementation assay, the ability to propagate *env*-defective MPMV vectors was studied using homologous and heterologous Env-gps. The Env-gp-expressing plasmids used included the homologous MPMV Env-gp with CTE (pTR283) and without CTE (pTR287) (Fig. 3). In addition, several heterologous Env-gp-expressing plasmids were used: pCMV-HIV-1 ENV from HIV-1, pCMV-HIV-2 ENV from HIV-2, and an amphotropic Env-gp from murine leukemia virus (pSV-A-MLV-env). A *rev*-expression plasmid, pCMV-*rev,* was provided in *trans* for the expression of RRE-containing HIV-1 and HIV-2 *env*-expression plasmids. Vectors propagated following *trans* complementation by Env-gps were tested for infectivity using HeLaT4 cells.

Table 1 shows results of several independent experiments following genetic complementation between the *env*-defective MPMV vector, pKAL036, and a variety of homologous and heterologous Env-gps. pTR283, encoding MPMV Env-gp with CTE, could *trans*-complement pKAL036, giving rise to 404 colony-forming units (CFU)/ml, whereas *env* expression plasmid pTR287, lacking CTE, could not, suggesting that MPMV Env-gp expression is dependent upon the presence of CTE in *cis.*

pKAL036 could also be propagated using heterologous Env-gps. The amphotropic Env-gp was the best in its ability to pseudotype MPMV, giving rise to high titers of pseudotyped virus (2712 CFU/ml; Table 1), while the

TABLE 2

Role of MPMV CTE and HIV-1 and SIV RREs in the Propagation of MPMV Vectors[a]

Vectors	Presence or absence of *cis*-acting elements	Mutation(s) in MPMV genome	Titer (CFU/ml ± SD)[b]	
			Rev(+)	Rev(−)
pKAL036	CTE(+)	SV-Hyg^r insertion and a 705-bp deletion in *env*	2448 ± 24	2712 ± 17
pKAL048	CTE(−)	SV-Hyg^r insertion, a 424-bp deletion in *env,* and 180-bp deletion of CTE up to the start of PPT	ND	0
pKAL039	CTE(+) (S)	Same as in pKAL048 with a reinsertion of CTE in the sense orientation	ND	2064 ± 20
pKAL044	CTE(+) (AS)	Same as in pKAL048 with a reinsertion of CTE in the antisense orientation	ND	0
pKAL051	CTE(−); HIV-1 RRE (S)	Same as in pKAL048 with an insertion of HIV-1 RRE in the sense orientation	468 ± 14	0
pKAL052	CTE(−); HIV-1 RRE (AS)	Same as in pKAL048 with an insertion of HIV-1 RRE in the antisense orientation	0	0
pKAL053	CTE(−); SIV RRE (S)	Same as in pKAL048 with an insertion of SIV RRE in the sense orientation	384 ± 9	0
pKAL054	CTE(−); SIV RRE (AS)	Same as in pKAL048 with an insertion of SIV RRE in the antisense orientation	0	0

Note. ND, not done; S, sense orientation; AS, anti-sense orientation.

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

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

HIV-2 Env-gp was the least efficient in its ability to pseudotype pKAL036, giving rise to very low titers of pseudotyped virus (7 CFU/ml). HIV-1 Env-gp was intermediate in its ability to pseudotype pKAL036 (56 CFU/ml). Interestingly, the homologous Env-gp expression plasmid, pTR283, was sevenfold less efficient in its ability to rescue virus replication than the A-MLV Env-gp (404 CFU/ml vs 2712 CFU/ml). Based on these results, we decided to use the heterologous A-MLV Env-gp in the *trans* complementation experiments to study the role of CTE in MPMV replication.

The Hyg^r colonies observed in the *trans* complementation assay were the result of successful infection events by the fully complemented MPMV genome. However, some colonies could also have arisen due to breakthrough in drug selection or spurious transfection with plasmid DNA carried over from Cos cell transfection. To eliminate these possibilities, Hyg^r colonies were expanded, DNA was isolated, and Southern blot hybridization performed to detect the presence of plasmid DNA versus MPMV viral DNA. Such analyses revealed that the Hyg^r colonies were a result of retrovirus-mediated integration of viral DNA; no evidence of integration of plasmid DNA was found (data not shown).

Since the strength of our genetic complementation assay lay with its ability to carry out a single round of replication, we wanted to test for the generation, if any, of replication-competent viruses using both the homologous and heterologous Env-gps. Thus, supernatants from Hyg^r colonies generated by viruses propagated using the homologous MPMV Env-gp (pTR283) and the heterologous A-MLV Env-gp (Fig. 3) were harvested and used to infect fresh HeLaT4 cells in the presence of DEAE–dextran; infected cells were selected for hygromycin B resistance. In both cases, no Hyg^r colonies were obtained, indicating that vector propagation was limited to a single round and no replication-competent viral genomes were generated by recombination using either the homologous or the heterologous Env-gp. Therefore, we concluded that the level of recombination yielding replication-competent viruses was below the level of detection in our system and we could use this assay to study the role of CTE in the replication of MPMV in an unambiguous and quantitative manner.

CTE is critical for the ability of MPMV to replicate

To study the role of CTE in the replication of MPMV, a set of molecular clones were generated that contained a deletion and an insertion of the "SV40-Hyg^r" cassette

in their *env* genes in the presence or absence of CTE. Table 2 reveals the results of several *trans* complementation assays performed using the CTE(+) and CTE(−) MPMV molecular clones. The CTE(+) vector, pKAL036, containing the entire MPMV genome except for the deletion and insertion mutations in the *env* gene, propagated well, giving rise to 2712 CFU/ml of virus stock. When CTE was deleted, as in the case of pKAL048, virus replication was completely abrogated and no Hyg^r colonies were observed. However, when sequences encompassing the CTE were reinserted in the sense orientation, generating pKAL039, virus replication was restored to nearly wild-type levels (2064 CFU/ml). The somewhat lower titers observed in case of pKAL039 may reflect the effect of duplication of the PPT and U3 region of the 3′ LTR of the viral genome in which the CTE was reinserted. Insertion of CTE in the antisense orientation, as in pKAL044, could not rescue virus replication (Table 2). Thus, CTE was critical in the ability of MPMV to replicate and produce infectious virions and functioned only in the sense orientation, as reported for HIV-1 (Bray *et al.,* 1994) and SIV (Rizvi *et al.,* 1996).

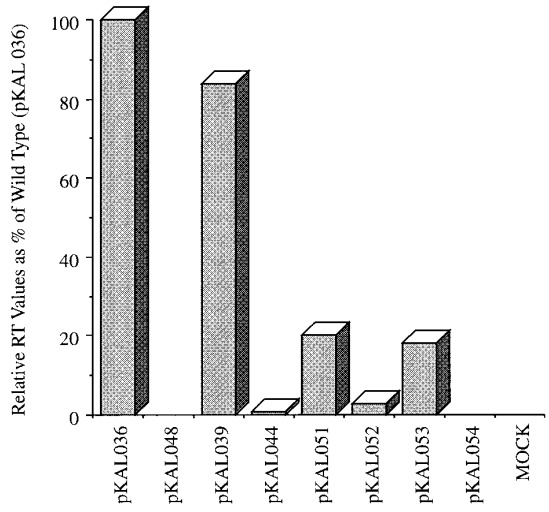


FIG. 4. Quantitation of RT-containing viral particles released from Cos cells transfected with MPMV vectors as measured by counts per minute (see Materials and Methods for details). HIV-1 and SIV RRE-containing plasmids (pKAL051, pKAL052, pKAL053, and pKAL054) were cotransfected with a HIV-1 Rev-expressing plasmid, pCMV-*rev.* The amount of reverse transcriptase activity observed in pelleted particles is presented as a percentage of the wild-type CTE-containing vector, pKAL036, with the background counts subtracted from the values shown. Mock, mock-transfected cells.

HIV-1 and SIV RREs can complement the function of CTE in the life cycle of MPMV

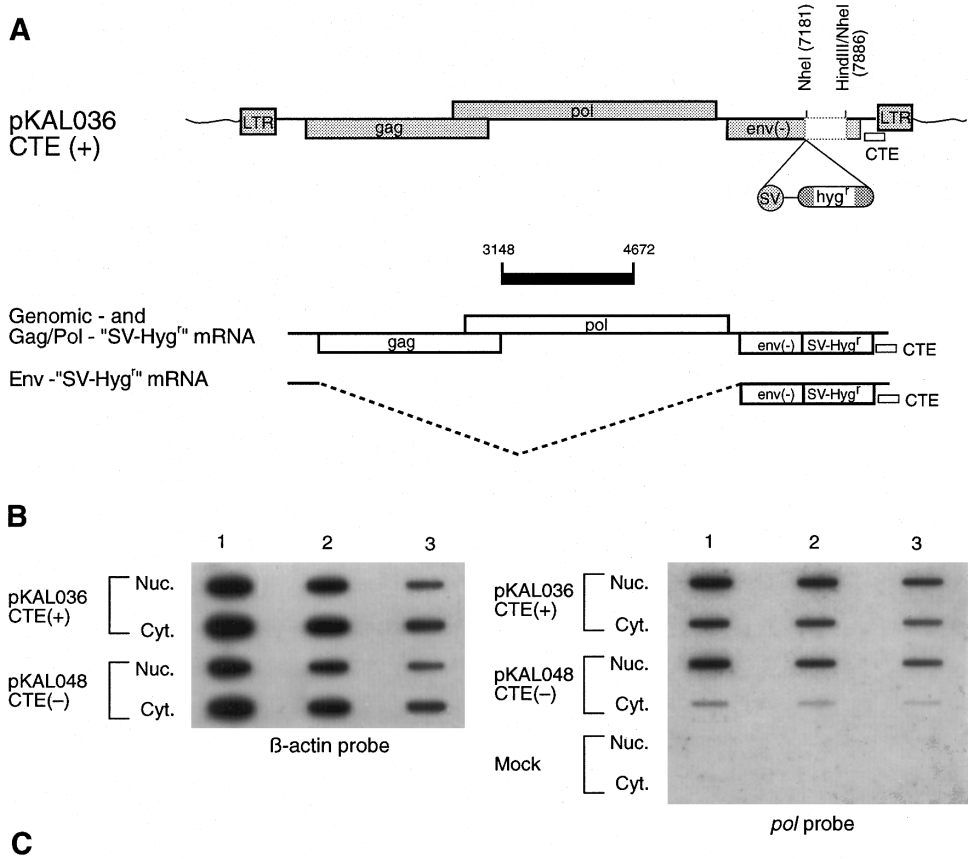
We next attempted to test if the HIV-1 and SIV Rev/RRE regulatory system could complement the role of CTE in the replication of MPMV. Therefore, HIV-1 and the SIV RREs were inserted in the sense or antisense orientation in the MPMV CTE(−) vector, pKAL048 (Table 2). Insertion of HIV-1 RRE (pKAL051) or SIV RRE (pKAL053) in the sense orientation and provision of Rev in *trans* resulted in the restoration of viral replication, albeit at a five- to sixfold lower level than observed with CTE(+) vector, pKAL036 (Table 2). In the presence of Rev, pKAL051 and pKAL053 yielded 468 and 384 CFU/ml of virus, respectively, compared with 2712 CFU/ml obtained using pKAL036 (Table 2). The slightly lower titers observed in the case of pKAL053, which contained the SIV RRE, compared with pKAL051, which contained the HIV-1 RRE, may partly be explained by the use of HIV-1 Rev instead of SIV Rev in the *trans* complementation assay. As expected, rescue of virus replication did not occur in the absence of Rev. MPMV replication could also not be rescued when the HIV-1 RRE (pKAL052) or SIV RRE (pKAL054) were inserted in the antisense orientation irrespective of the presence or absence of Rev (Table 2). These findings are consistent with earlier published reports documenting that HIV-1 RRE functions only in the sense orientation (Malim *et al.,* 1989a).

Since there seemed to be functional similarity between CTE and RRE, we tried to detect any possible Rev/CTE interactions by supplying HIV-1 Rev in *trans* to the CTE(+) vector, pKAL036. If such an interaction existed, we should have observed either higher or lower titers of virus in the presence of Rev depending upon the nature of the interaction. Indeed, 10% lower titers of virus were produced by pKAL036 in the presence of Rev (2448 CFU/ml) than in its absence (2712 CFU/ml), suggesting a possible competition of Rev with a Rev-like cellular factor that binds CTE or a cellular factor that interacts with the activation domain of Rev. Overexpression of Rev, therefore, could inhibit processing of CTE-dependent mRNAs.

In addition to virus expression at the RNA level, we also determined whether the inappropriate regulation of viral messages lead to inappropriate release of progeny virions from transfected cells. Toward this end, the CTE(+), CTE(−), and HIV-1/SIV RRE(+) MPMV molecular clones were transfected into Cos cells and supernatants harvested 72 hr posttransfection were tested for the release of virus particles by the RT assay (see Materials and Methods). Results obtained with the RT assay agreed with those obtained with the single round of replication assay (Fig. 4 and Table 2). MPMV clones defective for CTE produced little or no RT compared to clones containing CTE, or clones containing HIV-1/SIV RRE when cotransfected with Rev in *trans.* These results confirm the importance of CTE in the MPMV life cycle and suggest that CTE may function in a manner similar to the Rev/RRE regulatory system of HIV-1/SIV.

Role of CTE in the transport of messages from the nucleus to the cytoplasm

Since the Rev/RRE regulatory system of HIV-1/SIV has been shown to be important for the nucleocytoplasmic transport of unspliced and partially spliced messages, we investigated the transport of MPMV mRNAs from the



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VECTOR	Cytoplasmic/Nuclear RNA Fractions	
	Percent cpm	Relative Ratio
pKAL036 CTE(+)	32/68	1:2
pKAL048 CTE(-)	14/86	1:6

FIG. 5. Slot blot analysis of fractionated RNAs isolated from Cos cells transfected with CTE(+) and CTE(−) MPMV vectors. (A) Schematic representation of the wild-type vector, pKAL036, the two forms of mRNAs that it encodes, and the location of the ^{32}P-labeled 1.52-kb *pol*-specific DNA probe (shown as a black bar) used to detect the genomic and Gag/Pol message excluding the spliced Env mRNA. (B) Nuclear and cytoplasmic RNA fractions were transferred onto a nitrocellulose filter using the slot-blot apparatus. Left panel shows an X-ray film of a filter probed with a human β-actin cDNA control probe. Lanes 1, 2, and 3 refer to fivefold sequential dilutions of the nuclear and cytoplasmic RNAs. The right panel shows an X-ray film of a filter probed with the *pol*-specific probe shown in above in part A. Lanes 1, 2, and 3 refer to twofold sequential dilutions of the nuclear and cytoplasmic RNAs. (C) Betascope analysis of the filter hybridized with the *pol*-specific probe with the results presented as: the proportion of counts per minute (cpms) observed in the cytoplasmic versus the nuclear fractions shown as a percentage of the total cpms, and the relative ratio of the cpms observed in the cytoplasmic and nuclear fractions. Nuc., nuclear, and Cyt., cytoplasmic, RNA fractions; Mock, mock-transfected cells.

nucleus to the cytoplasm to see if any disruption occurred in the absence of CTE. Cos cells were transfected with CTE(+) (pKAL036) and CTE(−) (pKAL048) MPMV molecular clones, respectively. Nuclear and cytoplasmic RNAs were fractionated 72 hr posttransfection and transferred onto a nitrocellulose filters using the slot-blot apparatus. One filter was probed with a 1.52-kb *pol*-specific DNA probe (nt 3148–4672) to detect the presence of genomic and Gag/Pol messages (Fig. 5A), and a second filter was probed with the human β-actin-specific cDNA probe to control for RNA amounts.

Slot-blot analysis of the nuclear and cytoplasmic RNAs revealed reduced amounts of MPMV-specific RNA in the absence of CTE (Fig. 5B). Cells transfected with the CTE(+) vector, pKAL036, contained twofold more genomic and Gag/Pol RNA in the nucleus than the cytoplasm. In comparison, sixfold more MPMV RNA was found in the nucleus of cells transfected with the CTE(−) vector, pKAL048, than the cytoplasm (Figs. 5B and 5C). This is despite similar amounts of RNAs transferred onto nitrocellulose as shown by the detection of the β-actin message (Fig. 5B), suggesting aberrant nucleocytoplasmic transport or instability of the genomic and the Gag/Pol messages in the absence of CTE. When CTE was rein-

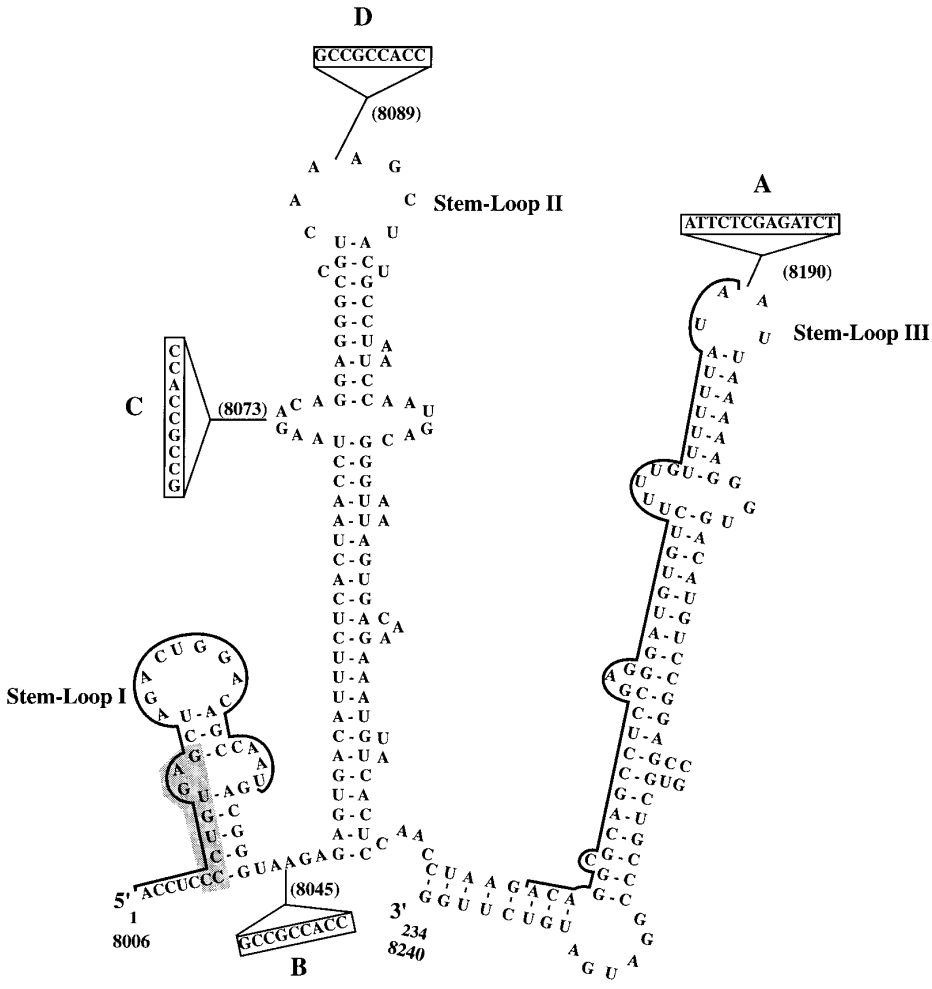

FIG. 6. Secondary RNA structure of MPMV CTE predicted by the University of Wisconsin GCG MFold analysis of optimal/suboptimal and minimal free-energy folding. The region shown encompasses 234 nucleotides (nt 8006 to 8240) of the MPMV CTE. The gray area highlights the 9-bp region of the CTE with 67% homology to the Rev-binding domain of HIV-1 RRE. Solid lines wrapping around stem loops I and III illustrate the extent of deletions made in CTE for the mutational analysis. The boxed areas at sites A, B, C, and D show the points of insertional mutations into stem and bulge areas of CTE in stem loops I, II, and III. Numbers in parentheses reflect the nucleotide numbers at the point of insertional mutations.

serted in the sense orientation in pKAL048, generating pKAL039, transport of the genomic and Gag/Pol messages was restored as in the case of pKAL036 (data not shown). These data suggest that CTE affects the nucleocytoplasmic transport and/or stability of the MPMV genomic and Gag/Pol messages.

MPMV CTE has the potential to form a stable secondary RNA structure

The putative RNA secondary structure of the MPMV CTE was predicted using the MFold program of the University of Wisconsin GCG sequence analysis software. Figure 6 reveals the predicted RNA secondary structure for MPMV CTE (nt 8006–8240) that displayed three stable stem loops each of which contained 2–4 bulge regions. Interestingly, a 9-bp tract at the 5′ end of the CTE (5′ CCCUGUGAG 3′; nt 8011–8019), which extends into stem loop I, was a 6/9 match for the Rev-binding sequence (5′ CACUAUGGG 3′) in the HIV-1 RRE (Holland *et al.,* 1990, 1992; Kjems *et al.,* 1991). Hence, substitution of the MPMV CTE function by the HIV-1 and SIV Rev/RRE regulatory system may be evidence for a similar protein/CTE interaction that stabilizes MPMV mRNAs or facilitates their export from the nucleus to the cytoplasm.

To determine the relevance of the predicted RNA secondary structure to CTE function, a genetic approach was employed to disrupt the predicted higher-order structure of CTE using two types of mutations. The first category of mutations were designed to disrupt both the primary sequences and the predicted secondary structure by deleting different regions of CTE. These deletions removed sequences in stem loop 1 only (a 29-bp deletion removing the putative Rev-like binding domain), in stem loop III only (a 41-bp deletion), and in both stem loops I and III (a total 70-bp deletion) (see Fig. 6 and Materials and Methods). The second category of mutations were designed to destabilize the predicted RNA secondary structure by introducing 9-bp insertions of heterologous sequences at sites B, C, and D (in stem loops I and II)

TABLE 3

Effect of Deletion and Insertional Mutations in CTE on the Propagation of MPMV Vectors[a]

Vectors	Mutations in the predicted RNA secondary structural elements of MPMV CTE	Titer[b] (CFU/ml ± SD)
pKAL036	CTE(+)	2695 ± 11
pKAL048	CTE(−)	0
pKAL074	Insertion at site A	713 ± 1
pKAL076	Deletion in stem loop I + site A insertion	3.0 ± 4.2
pKAL077	Deletion in stem loop III + site A insertion	1.5 ± 0.7
pKAL078	Deletion in stem loops I & III + site A insertion	4.0 ± 4.2
pKAL079	Insertions at sites A + B	1.5 ± 2.1
pKAL080	Insertions at sites A + C	8.0 ± 4.2
pKAL081	Insertions at sites A + D	167 ± 13

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

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

of the predicted higher-order structure using SOE PCR (Fig. 6). To allow testing of these mutations in the single round of replication assay, a plasmid was constructed that made it possible to clone the mutated CTEs into the CTE(−) vector, pKAL048, without altering the genomic position of CTE or duplicating any of its terminal sequences. This was achieved by inserting a *Bgl*II site in stem loop III at position A, and the mutated CTE containing the *Bgl*II insertion was cloned into pKAL048, resulting in pKAL074. Testing of pKAL074 in the single round of replication assay resulted in ~75% reduction in vector propagation (713 CFU/ml versus 2695 CFU/ml observed for pKAL036; Table 3) suggesting the role of higher-order structure for CTE function. To study the effects of other mutations on the predicted structural elements of CTE, CTE fragments containing deletion and insertional mutations were substituted for the homologous fragment in pKAL074. The resulting clones not only contained the deletion or insertional mutations, but also the *Bgl*II insertion at site A. The mutated CTEs were tested for effects on vector propagation in the single round of replication assay.

Table 3 reveals the results of several *trans* complementation assays using MPMV vectors containing the mutant CTEs. Deletion of sequences in stem loop I containing the Rev-like binding domain, or in stem loop III, or in both stem loops I and III simultaneously resulted in the abrogation of CTE function. These deletions could have removed primary sequences important for CTE function and/or destabilized putative RNA structural elements. Similarly, testing of mutant CTEs containing insertions designed to disrupt the predicted CTE RNA secondary structure revealed that CTE was unable to tolerate most of the mutations introduced. The 9-bp insertions at site B (at the base of stem loop I and II) and at site C (bulge in stem loop II) were the most disruptive, rendering CTE nonfunctional (Table 3). The insertion at the apical bulge in stem loop II (site D) was the least disruptive, giving rise to 167 CFU/ml compared to 713 CFU/ml observed for pKAL074. The fact that CTE could not tolerate most of the mutations introduced suggests the importance of the predicted higher-order structure for CTE function.

DISCUSSION

In this study, we developed a genetic complementation assay to analyze MPMV replication over a single round. The *env*-defective MPMV particles produced after the first round of replication could not initiate another round of infection without the presence of Env-gp. Replication-defective vectors have been used previously to study recombination (Hu and Temin, 1990a), characterization of frequency of mutation (Dougherty and Temin, 1988; Pathak and Temin, 1990a,b), RNA packaging (Rizvi *et al.,* 1993; Mansky *et al.,* 1995), and the nature of strand switching during reverse transcription (Hu and Temin, 1990b; Panganiban and Fiore, 1988). This *trans* complementation assay allowed us to measure the differing abilities of the homologous Env-gp and a variety of heterologous Env-gps to pseudotype MPMV. In addition, we could directly study the effects of the lack of CTE on the replication of MPMV in a quantitative and reproducible manner revealing that MPMV CTE was critical for the replication of the virus. In the absence of CTE, sixfold more MPMV RNA was found to be sequestered in the nucleus than in the cytoplasm. The function of CTE could be substituted by HIV-1 and SIV Rev/RRE regulatory systems. Computer analysis of sequences encompassing CTE predicted a stable RNA secondary structure containing three stem loop structures the first of which contained a sequence motif similar to the Rev-binding domain of HIV-1 RRE (Holland *et al.,* 1990, 1992; Kjems *et al.,* 1991). Mutations designed to disrupt structural elements of the predicted RNA secondary structure drastically affected CTE function, suggesting the presence of higher-order structure in CTE.

Several recent reports in the Rev/RRE system reveal

that the HIV-1 Rev activation domain acts as a nuclear export signal interacting with a nucleopore-associated cofactor to facilitate the export of unspliced and partially spliced messages from the nucleus to the cytoplasm (Bogerd *et al.,* 1995; Fischer *et al.,* 1995; Fritz *et al.,* 1995; Stutz *et al.,* 1995). The functional and structural similarities seen between MPMV CTE and HIV-1/SIV Rev/RRE regulatory systems and the fact that MPMV does not contain any accessory genes analogous to Rev to carry out its function suggests that CTE may exploit cellular host factors to carry out nucleocytoplasmic mRNA transport and/or stabilize viral messages. Whatever the cellular factor(s) interacting with CTE are, they must be widespread like the nucleopore-associated proteins, since we were able to get the same phenomenon in a variety of cell lines including Cos, HOS (human osteosarcoma cells), HeLa, and RD (rhabdomyosarcoma) (data not shown).

Several considerations dictated our selection of a heterologous Env-gp to study the role of CTE in MPMV replication using the *trans* complementation assay. First, use of a heterologous Env-gp reduced chances of the *env*-defective vector reacquiring the deleted *env* sequences via homologous recombination with the homologous Env-gp. Second, we have shown that MPMV Env-gp expression is dependent on CTE. Therefore, it was necessary to avoid any possible homologous recombination between the CTE-containing sequences carried by the *env* expression plasmid and sequences in the vector to be packaged that could have resulted in the regeneration of CTE in our CTE(−) molecular clones. Finally, in the case of HIV-1, pseudotyping with the murine amphotropic Env-gp has been shown to produce higher titers of virus than *trans* complementation with the homologous Env-gp (Page *et al.,* 1990; Buchschacher and Panganiban, 1992; Delwart *et al.,* 1992; Geraghty and Panganiban, 1993). We found this to be true in the case of MPMV also; the murine amphotropic Env-gp turned out to be the best (sevenfold better) in its ability to pseudotype MPMV than the homologous Env-gp. This may be due to the ubiquitous and plentiful nature of the cellular receptor with which the amphotropic Env-gp interacts. It is not known what the receptor for MPMV is, how good its distribution on HeLaT4 cells is, and how permissive HeLaT4 cells are compared with other cells to MPMV infection.

Studies conducted in this paper provide direct evidence that CTE is important for the nucleocytoplasmic transport and/or stability of full-length genomic and unspliced Gag/Pol messages (Fig. 5) . Threefold more full-length RNA was found to be sequestered in the nucleus of cells transfected with the CTE(−) vector, pKAL048, compared with messages found in the nucleus of cells transfected with the CTE(+) vector, pKAL036 (Figs. 5B and 5C). We purposely excluded the detection of the spliced Env-gp mRNA by selecting a *pol*-specific DNA probe since MPMV *env* had been disrupted by a significant deletion and a simultaneous insertion of the "SV40-Hyg^r" cassette. These mutations could have affected *env* splice site and/or any regulatory sequences that may have been present in the Env ORF altering not only the expression of Env-gp message, but also its potential regulation by the CTE. However, indirectly we were able to show that MPMV CTE is important for the expression of Env-gp. We were able to *trans*-complement the *env*-defective MPMV vector using only the MPMV Env-gp expression plasmid containing the CTE, but not the same plasmid without the CTE, revealing that CTE is important for the expression of Env-gp from a heterologous promoter (Table 1). We have made similar observations regarding the expression of MPMV *gag/pol,* which is CTE-dependent even when under the control of a heterologous promoter (unpublished observations).

Analogous to the HIV-1/SIV Rev/RRE feedback loop, it is possible that MPMV contains *cis*-acting repressor sequences (CRS) that are counteracted by interaction of CTE with cellular factors. The Rev/RRE regulatory system has been shown to counteract the presence of several CRS scattered throughout the HIV-1 genome (Cochrane *et al.,* 1991; Maldarelli *et al.,* 1991; Schwartz *et al.,* 1992a). The HIV-1 RRE itself has been shown to act as a CRS (Nasioulas *et al.,* 1994; Brighty and Rosenberg, 1994). The CRS retain viral messages containing the RRE sequestered within the nucleus until Rev can overcome the inhibition. Removal of the CRS results in Rev/RRE-independent expression of Gag/Pol proteins and replication of the virus (Schwartz *et al.,* 1992a,b). Using a *secreted alkaline phosphatase (SEAP)* gene reporter assay, our ongoing experiments suggests the presence of such CRS in the MPMV genome (unpublished observations).

cis-acting repressor sequences have been shown to be rich in adenylate (A) and uridylate (U) residues (Schwartz *et al.,* 1992a; Tan and Schwartz, 1995; Tan *et al.,* 1995). AU-rich elements (AREs) may be a general feature of cellular and viral sequences (Caput *et al.,* 1986; Shaw and Kamen, 1986; Schwartz *et al.,* 1992a; Tan and Schwartz, 1995; Tan *et al.,* 1995) and have been associated with mRNA instability (Shaw and Kamen, 1986; Savant-Bhonsale and Cleveland, 1992; Chen and Shyu, 1994; Chen *et al.,* 1994) and translatability (Tan and Schwartz, 1995; Tan *et al.,* 1995). Two AU-rich motifs, a pentanucleotide, AUUUA (Caput *et al.,* 1986), and an octanucleotide, UUUUUAUA (Kruys *et al.,* 1989), have been found to be a common feature of the 3′ untranslated regions (UTRs) of many cellular mRNAs that are under posttranscriptional regulation, such as c-*fos* (Treisman, 1985; Wilson and Treisman, 1988; Shyu *et al.,* 1989), c-*myc* (Rabbitts *et al.,* 1985; Jones and Cole, 1987), and granulocyte-macrophage colony stimulating factor (Shaw and Kamen, 1986). They are also found in the late 3′ untranslated region of viruses such as human papillomavirus (HPV) type 1 (Tan and Schwartz, 1995), as well as in the coding sequences of viral genes such as HIV-1-*gag* (Schwartz *et al.,* 1992a) and HPV-16 L1 (Tan *et al.,* 1995).

Computer analysis of the MPMV genome has revealed that the entire viral genome is unusually AU rich (59%). This is in contrast to most cellular mRNAs, which maintain a 50% AU content (Schwartz *et al.,* 1992a). We have found multiple copies of the AU-rich motifs in the MPMV *gag* and *pol* genes. Specifically, the Gag ORF contains 1 copy of AUUUA, while Pol contains 10 copies of AUUUA and 1 copy of UUUUUAUA. At present, the role of AREs in CTE dependence of MPMV mRNA transport, stability, and/or translation is not known. While we have demonstrated that CTE plays a role in the nucleocytoplasmic transport and/or stability of viral mRNAs, we cannot discount the role of CTE in mRNA translational efficiency. We find that in the absence of CTE, some mRNA does get to the cytoplasm, yet it does not seem to be translated, as evidenced by the lack of RT and vector propagation via the genetic complementation assay (Figs. 4 and 5, and Table 2). Similar effects of certain AU-rich repressor sequences have been observed on HPV mRNA transport and translational efficiency, though not to the same magnitude. These repressor AU-rich motifs have been mapped to the 3′ untranslated region of HPV-1 (Tan and Schwartz, 1995), as well as the L1 coding region of HPV-16 (Tan *et al.,* 1995), and it has been shown that the HIV-1 Rev/RRE as well as SRV-1 CTE can overcome the effects of these repressor sequences. Mutational analysis of AU-rich sequences in the MPMV Gag and Pol ORFs is currently under way and should reveal any role of these motifs in the regulation of MPMV gene expression.

Using a genetic approach, our data suggests the importance of a higher-order structure for CTE function. The fact that insertional mutation at site A, by itself, had a significant effect on CTE function, while it took double insertional mutations to completely abrogate CTE function (mutations at sites A + B and A + C) suggests that CTE may have a multipartite secondary structure that contains more than one functional element important for optimal CTE function. In addition, the predicted Rev-like binding domain in stem loop I may be a potential site for interaction with a Rev-like cellular factor. However, we cannot rule out the possibility that primary sequences within CTE may also be important. Experiments are under way to carry out a more detailed mutational analysis of the CTE RNA secondary structure which should reveal the role of primary sequences and secondary structural elements essential for CTE function.

The importance of CTE in the replication of MPMV may be a common feature of all simple retroviruses that do not contain accessory regulatory systems like Rev/RRE. Simple retroviruses like Rous sarcoma viruses (RSVs) and murine leukemia viruses (MLVs) accomplish basic replication functions like transcription, splicing, and polyadenylation by using cellular factors (Temin, 1992 and 1993). Recently, an element similar to MPMV CTE has been discovered in RSV that facilitates the nucleocytoplasmic transport and stability of full-length RSV mRNAs (Ogert *et al.,* 1996). This finding provides another window of understanding into how simple retroviruses replicate and interact with cellular machinery to carry out their life cycle and/or cause disease. The fact that a similar element has been found in HBV important for the expression of its late gene products (Huang and Liang, 1993; Huang and Yen, 1994), while retroviral CTE and Rev/RRE regulatory systems can overcome inhibition of repressor sequences in HPV-1 and 16, suggests a common evolutionary pathway used by both DNA and RNA viruses to regulate expression of their late structural genes via *cis*-acting elements that interact with *trans*-acting viral or cellular factors.

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