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A Structured Retroviral RNA Element That Mediates Nucleocytoplasmic Export of Intron-Containing RNA

ROBERT K. ERNST, MOLLY BRAY, DAVID REKOSH, AND MARIE-LOUISE HAMMARSKJÖLD*

Myles H. Thaler Center for AIDS and Human Retrovirus Research and Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908

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A common feature of gene expression in all retroviruses is that unspliced, intron-containing RNA is exported to the cytoplasm despite the fact that cellular RNAs which contain introns are usually restricted to the nucleus. In complex retroviruses, the export of intron-containing RNA is mediated by specific viral regulatory proteins (e.g., human immunodeficiency virus type 1 [HIV-1] Rev) that bind to elements in the viral RNA. However, simpler retroviruses do not encode such regulatory proteins. Here we show that the genome of the simpler retrovirus Mason-Pfizer monkey virus (MPMV) contains an element that serves as an autonomous nuclear export signal for intron-containing RNA. This element is essential for MPMV replication; however, its function can be complemented by HIV-1 Rev and the Rev-responsive element. The element can also facilitate the export of cellular intron-containing RNA. These results suggest that the MPMV element mimics cellular RNA transport signals and mediates RNA export through interaction with endogenous cellular factors.

The mechanisms that govern the export of RNA from the nucleus to the cytoplasm in eukaryotic cells are to a large extent still unknown. Although some proteins have been shown to be involved in the export process, it is not at all clear how they interact or how export is regulated. Experiments performed by microinjection of oocytes do suggest, however, that RNA export is an energy-dependent, saturable process and that different pathways are utilized by different RNA species (17, 28, 29, 32). It is believed that the export pathways, in all cases, involve passage of the RNA through the nuclear pore.

Some specific export signals have been identified in RNA. In the case of spliceosomal small nuclear RNAs (snRNAs), the m^7G cap structure present in these RNAs is known to constitute an important signal (20, 27, 29, 61). This kind of cap may also play a role in mRNA export since it is also present on mRNAs. For mRNA, it has also been shown that export, in most cases, requires removal of all complete introns by splicing (6, 38). If splice sites are mutated so that intron removal is prevented or slowed, the resulting incompletely spliced mRNAs are retained in the nucleus. These types of experiments have led to the hypothesis that splicing factors serve to retain mRNA in the nucleus and that the RNA's association with the export machinery requires prior removal of all complete introns.

In accordance with this hypothesis, export of intron-containing cellular mRNAs from the nucleus to the cytoplasm seems to be a rare occurrence, although there are a few examples in the literature (41). In contrast, the export of unspliced intron-containing RNA is an imperative for the replication of all retroviruses. This is due to the fact that individual viral mRNAs are generated by alternative splicing of a single primary transcript that serves as the viral genome and as the mRNA for the *gag* and *gag-pol* gene products (8, 33, 63). This transcript is capped and polyadenylated by the normal cellular machinery.

In the case of the simpler retroviruses, the primary transcript is either spliced to generate the mRNA for the viral envelope proteins or exported directly to the cytoplasm to be translated into the viral Gag and Gag-Pol proteins (58, 63). A portion of the unspliced RNA is also exported for packaging into viral progeny particles. In the more complex retroviruses, such as human immunodeficiency virus (HIV), the situation is complicated by the fact that the primary transcript is alternatively spliced to generate many different mRNAs. The cytoplasm of HIV-infected cells contains more than 30 different mRNAs that have all been generated by alternative splicing (51–53). Many of these molecules contain complete excisable introns.

It has been well established that the export of intron-containing RNAs in HIV-infected cells requires the function of the viral Rev protein (12, 13, 21, 40). Rev is a small phosphoprotein that specifically binds to an RNA element, known as the Rev-responsive element (RRE), that is present in these RNAs (9, 36, 67). The association between Rev and the RRE directly promotes the nuclear export of the intron-containing HIV RNAs. The fact that these RNAs are retained in the nucleus in the absence of Rev has lent further support to the hypothesis that intron-containing RNAs are restricted from leaving the nucleus. This left a puzzle concerning the transport of intron-containing RNAs by the simpler retroviruses that do not encode regulatory proteins.

A clue to the puzzle was provided by the identification of a *cis*-acting RNA element from a type D simpler retrovirus (Mason-Pfizer monkey virus [MPMV]) (4). When this element was inserted in *cis* into the HIV type 1 (HIV-1) genome, it substituted for Rev and the RRE, allowing Rev-independent HIV-1 replication and structural protein expression. Since the MPMV element was able to achieve nucleocytoplasmic export of unspliced and singly spliced HIV-1 RNAs in the absence of a *trans*-acting viral protein, it was named the constitutive transport element (CTE). It has since been shown that the closely related simian retrovirus type 1 (62) contains a nearly identical CTE that allows Rev- and RRE-independent HIV-1 replication (68).

In this study we show that the CTE is a structural RNA element that plays an essential role in MPMV replication and is absolutely required for cytoplasmic accumulation of unspliced MPMV RNA. We also show that virus production from a CTE-defective MPMV provirus can be restored by the com-

* Corresponding author. Phone: (804) 982-1598. Fax: (804) 982-1590. E-mail: mh7g@virginia.edu.

bination of the HIV-1 RRE in *cis* and Rev in *trans*, strengthening the analogy between CTE and Rev and RRE function. Our results further demonstrate that the MPMV CTE can function as a discrete signal to facilitate the export of cellular intron-containing RNA.

MATERIALS AND METHODS

MPMV numbering system. The numbering system used for the MPMV sequences are those of the 6A clone present in the GenBank sequence file SIVMPCG, accession number M12349. The GenBank numbering system differs from that in the publication describing this sequence (55) due to the presence of an additional incomplete long terminal repeat (LTR) (between nucleotides [nt] 416 and 743) in the original 6A clone which was deleted in the published version.

Deletion mapping of the MPMV CTE. To map the CTE, different MPMV fragments were inserted between the *Xho*I site and a *Bgl*II site in the β-globin sequences of the previously described plasmid pSVSX5′βG (22). To do this, differently sized MPMV fragments were generated by PCR. The oligonucleotides used for the PCRs had sequences at their 5′ ends that generated a 5′ *Xho*I site and a 3′ *Bam*HI site in the amplification products.

Plasmids. pwtMPMV was derived from a plasmid containing a complete wild-type MPMV provirus genome, pSHRM15 (MPMV clone 6A) (49). pSHRM15 was obtained from Eric Hunter (University of Alabama at Birmingham). To construct pwtMPMV, an *Eco*RI-*Xba*I fragment from pSHRM15 containing the MPMV sequences was inserted into the vector pR4 (23), a poison-negative derivative of pBR322. A *Hin*dIII-*Kpn*I fragment from simian virus 40 (SV40) (SV40 nt 2533 to 294) that includes the early and late promoters and the origin of replication was also inserted into this vector. pMPMVΔCTE is a derivative of pwtMPMV with a deletion between MPMV nt 8030 and 8160. It was constructed by the splicing overlap extension (SOE)-PCR method (24). *Xho*I, *Eco*RV, and *Bgl*II sites were created at the site of the deletion.

pMPMVRRE270-r, pMPMVRRE330-r, pMPMVRRE330-w, and pMPMVRRE520-r were constructed by insertion of various-length HIV-1 fragments containing the RRE into the unique *Eco*RV restriction site at the site of the deletion in pMPMVΔCTE in either the correct (-r) or opposite (-w) orientation. The 270-nt RRE fragment (HIV-1 nt 7770 to 8039) and the 330-nt RRE fragment (HIV-1 nt 7710 to 8039) were obtained from Barbara Felber (National Cancer Institute). The 520-nt RRE contained HIV-1 nt 7611 to 8130. The numbering system corresponds to that used for HIVNL43, accession number M19921, present in GenBank.

Hybrid HIV-1 RRE (stem 1)-MPMV CTE constructs were synthesized by SOE-PCR using hybrid oligonucleotides complementary to both HIV-1 and MPMV sequences. In addition, these oligonucleotides contained sequences that created terminal *Xho*I or *Bam*HI sites to facilitate subsequent cloning. Two hybrid fragments were amplified by this method; the first contained HIV-1 nt 7770 to 7780 (HIV-1, isolate ARV-2/SF2; GenBank accession number, K02007), MPMV nt 8042 to 8145, and HIV-1 nt 8001 to 8011. The second fragment contained HIV-1 nt 7770 to 7780, MPMV nt 8032 to 8155 and HIV-1 nt 8001 to 8011. The PCR products were digested with *Xho*I and *Bam*HI and cloned into pBBgagpol-rre, downstream of the RRE and upstream of the β-globin poly(A) addition site. This plasmid is a derivative of the previously described pSVgagpol-rre-r (54). It lacks the β-globin intron and contains unique *Xho*I and *Bam*HI sites between the RRE and the β-globin sequences.

pβGRRE (6) contains the SV40 early promoter positioned upstream from a modified rabbit β-globin gene. In this construct, the first and second exons of the rabbit β-globin gene are fused together and are followed by a shortened version of the normal second intron containing an HIV-1 RRE. The third rabbit β-globin exon, which provides the polyadenylation signal, is 3′ of the modified intron. pb8F (6) is a derivative of pβGRRE that contains a point mutation (AG to TG) in the 3′ splice site consensus sequence.

pβGRRE(CTE-intron) and pb8F-CTE were constructed by insertion of a 334-nt fragment containing the MPMV CTE into an *Asp*718 site in the intron of pβGRRE and pb8F, respectively. This positioned the CTE just 3′ of the RRE. pβGRRE(CTE-exon) was constructed by insertion of a 334-nt fragment containing the MPMV CTE into an *Eco*RI site in the third rabbit β-globin exon of pβGRRE.

pCMVrev$^-$ and pCMVrev have been described previously (22, 54).

Cells and transfections. CMT3-COS cells express the SV40 T antigen under control of a metallothionein promoter (18, 19). They were maintained in Iscove's medium. Cells were transfected by a modified version of the DEAE-dextran method that was described previously (23).

Analysis for HIV p24 expression. Supernatants from transiently transfected CMT3-COS cells were harvested 72 h posttransfection, centrifuged for 5 min at 6,000 rpm in an Eppendorf 5415C centrifuge to remove floating cells, and stored at −20°C until assayed. p24 (HIV-1 capsid protein) expression was assayed with a commercial p24 enzyme-linked immunosorbent assay kit (Retro-Tek HIV-1 p24 antigen enzyme-linked immunosorbent assay; Cellular Products) following the manufacturer's recommendations.

RNA preparations and Northern blot analysis. The methods for total and cytoplasmic RNA extraction, poly(A) selection, and Northern blot analysis were described previously (21, 22). Northern blots were exposed to Molecular Dynamics Phosphor screens and imaged with a Molecular Dynamics PhosphorImager and ImageQuant analysis software, which allowed precise quantitation of the various RNA bands. For reprobing, blots were stripped for 1 to 2 h at 65°C in a solution containing 5 mM Tris-HCl (pH 8.0), 0.2 mM EDTA (pH 8.0), 0.05% pyrophosphate (Sigma P8010), and 0.1× Denhardt's solution.

Protein analysis. Western blot analysis was performed on extracts of cells as described previously (21) with the following modifications. After incubation with the primary antibody, the membranes were incubated in 40 ml of species-specific anti-immunoglobulin G secondary antibody (1:1,000 dilution; Amersham) conjugated with horseradish peroxidase that was diluted into 2% nonfat dry milk and 0.05% Tween 20 in phosphate-buffered saline. Incubation took place for 1 h at room temperature with gentle rocking. The membrane was then washed three times for 20 min in a solution containing 0.5% Tween 20 in phosphate-buffered saline. Finally, the membrane was developed with an enhanced-chemiluminescence kit (Amersham) according to the manufacturer's recommendations. The membrane was exposed to X-ray film for various lengths of time to detect the chemiluminescence signal.

Antisera. The HIV-1 envelope-specific (gp120) antiserum was a polyclonal rabbit antiserum made against a purified fragment of gp120 (amino acids 343 to 512) expressed in *Escherichia coli*. The antiserum was used at a 1:250 dilution for Western blot analysis.

The MPMV envelope-specific (gp70 and gPr86) polyclonal goat antiserum was a kind gift from Eric Hunter at the University of Alabama at Birmingham. The antiserum was used for Western blot analysis at a 1:500 dilution.

The MPMV Gag-specific (p27, Pr78) polyclonal rabbit antiserum was generated with a purified glutathione *S*-transferase (GST)–Gag fusion protein produced in *E. coli*. The fusion protein contained the entire MPMV Gag (p27) open reading frame (ORF) fused to the amino terminus of GST in pGEX-2T (Pharmacia). This fusion protein was affinity purified by using glutathione-Sepharose beads as specified by the manufacturer (Pharmacia) and used to immunize New Zealand White rabbits. The resulting antiserum was used at a 1:1,000 dilution.

RT activity assays. One milliliter of medium from transfected CMT3-COS cells was centrifuged for 5 min at 6,000 rpm in an Eppendorf 5415C microcentrifuge to remove floating cells and debris. The supernatant was then centrifuged at 42,000 rpm in a Beckman TLA45 rotor for 30 min at 4°C to pellet virus particles. The virus pellet was resuspended in 50 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Ten microliters of resuspended virus pellet was mixed with 50 μl of reverse transcriptase (RT) reaction mix {50 mM Tris-HCl (pH 8.0), 75 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 10 μl of poly(rA) · poly(dT)$_{12–18}$ template (1 mg/ml) per ml, 37.5 μCi of [^{32}P]dTTP per ml (3,000 mCi/mmol)}. The mixture was incubated for 1.5 h at 37°C. Five microliters of the reaction mixture was spotted onto DE81 paper (DEAE cellulose paper; Whatman) and allowed to air dry. The samples were washed three times for 5 min in 2× SSC (300 mM NaCl, 30 mM sodium citrate [pH 7.0]) and rinsed twice with 95% ethanol. The DE81 paper was blotted dry with paper towels, wrapped in plastic wrap, and exposed to a Molecular Dynamics Phosphor screen for 30 min at room temperature. RT activity was quantitated with a Molecular Dynamics PhosphorImager and ImageQuant analysis software.

Computer analysis. DNA sequences were aligned by using GAP and FASTA as part of the Wisconsin Genetics Computer Group set of programs (11). Secondary structures of the CTE were predicted by using a free-energy minimization program (MULFOLD), for Macintosh computers, based on the original Zuker program (30, 31, 69). Output from MULFOLD was viewed by using LOOP VIEWER on a Macintosh Quadra 950.

RESULTS

The CTE is a structured RNA element that maps between the *env* ORF and the 3′ LTR. We previously showed that a fragment from the MPMV genome could be utilized to obtain efficient Rev- and RRE-independent expression of HIV-1 structural proteins (4). Insertion of this fragment into a Rev-deficient, replication-incompetent HIV-1 provirus clone gave rise to a virus that replicated with an attenuated phenotype. These experiments also demonstrated that the MPMV fragment was a *cis*-acting element that did not require a transacting viral protein and that it acted to promote export of HIV-1 mRNA from the nucleus to the cytoplasm. Since the MPMV element appeared to be functionally equivalent to the HIV-1 RRE but acted constitutively to transport RNA, it was named the CTE.

In our original study, the CTE was mapped to a small region at the 3′ end of the MPMV genome (nt 8022 to 8240) located just 3′ of the *env* ORF. This region included the 3′ untranslated region and a portion of the 3′ LTR. Using the same reporter plasmids, further mapping experiments showed that a fragment containing MPMV nt 8022 to 8175 was fully func-

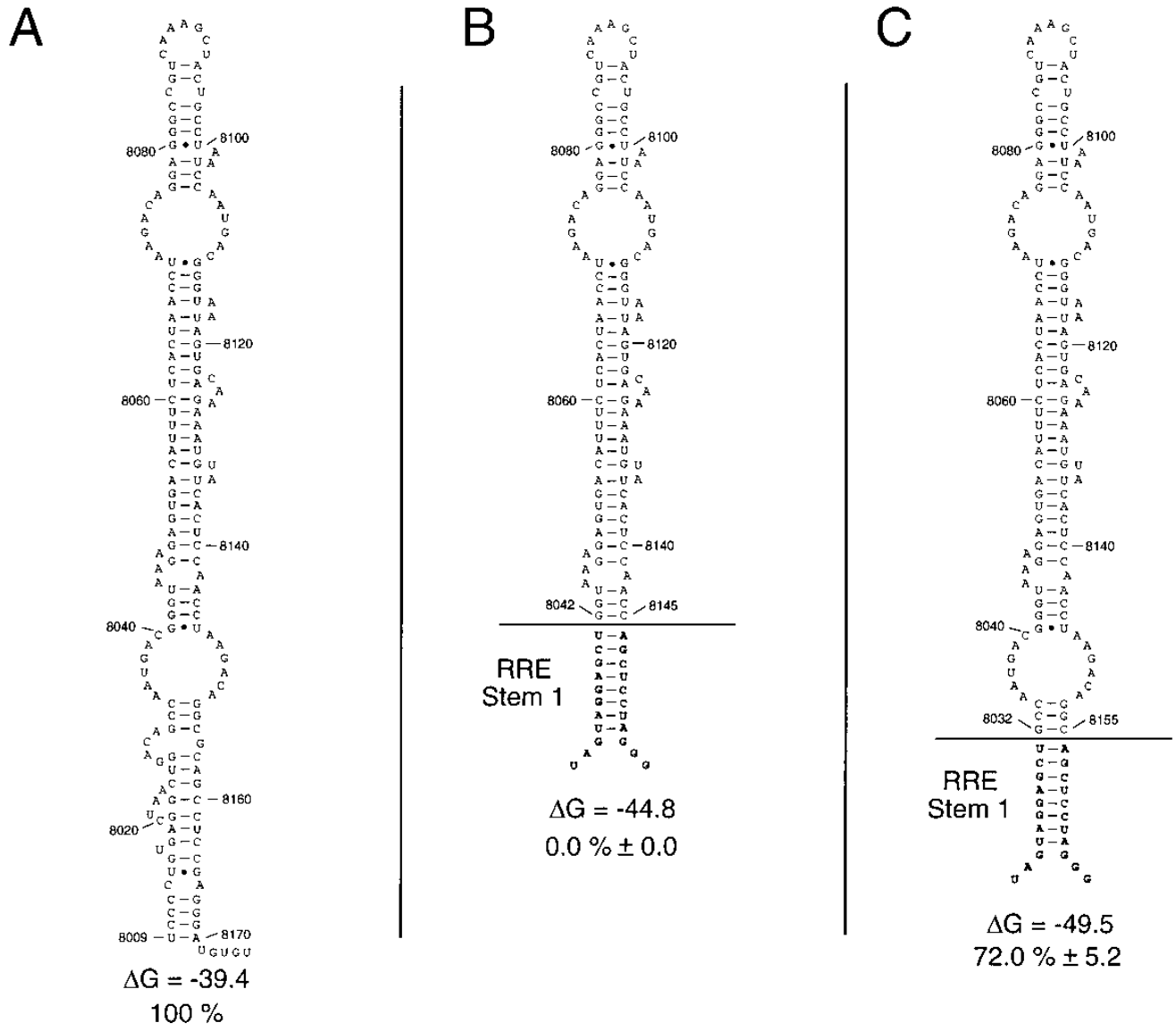

FIG. 1. Sequence and secondary structure of the MPMV CTE and chimeric CTE-RRE constructs. (A) The predicted secondary structure of the CTE. The calculated free energy for the proposed RNA structure is −39.4 kcal. Note that the sequences of the inner loops are identical but are rotated 180° relative to each other. An average of 9,250 ± 1,365 pg of p24 per ml was obtained with this element in triplicate experiments using the pBBgalpol-rre reporter plasmid described in the text. (B) The predicted secondary structure of a CTE-RRE chimeric element. This element contains MPMV nt 8042 to 8145 and HIV nt 7770 to 7780 and nt 8001 to 8011 (HIV-1, isolate ARV-2/SF2; GenBank accession number, K02007). The HIV-1 sequences are derived from stem 1 of the RRE and are shown in boldface type. The calculated free energy for the proposed RNA structure is −44.8 kcal. Note that the lower inner loop has been deleted from this structure. An average of 0 ± 0 pg of p24 per ml was obtained with this sequence in triplicate experiments using the pBBgalpol-rre reporter plasmid described in the text. (C) The predicted secondary structure of a CTE-RRE chimeric element. This element contains MPMV nt 8032 to 8155 and the same HIV-1 nucleotides described for panel B. The calculated free energy for the proposed RNA structure is −49.5 kcal. Note that the structure contains both inner loops. An average of 6,658 ± 491 pg of p24 per ml was obtained with this sequence in triplicate experiments using the pBBgalpol-rre reporter plasmid described in the text. The p24 value obtained with each construct is expressed as a percentage of the value obtained for the wild-type structure shown in part A.

tional as a CTE while plasmids that contained MPMV nt 8039 to 8175 or nt 8022 to 8140 failed to give any Rev-independent *env* or *gag-pol* expression (data not shown). Further mapping of the CTE showed that a fragment containing nt 8022 to 8160 gave some Rev-independent expression, but at reduced levels compared with that of the fragment from nt 8022 to 8175 (data not shown). Thus, the CTE overlaps neither the *env* ORF (which ends at nt 8002) nor the 3′ LTR (which starts at nt 8205) but is located entirely in an intragenic region (55).

Computer modelling of the CTE region predicted it to fold into a stable stem-loop structure as shown in Fig. 1A (30, 31, 69). The main features of this structure have been confirmed by RNase and chemical modification analyses as well as mutagenesis (12a). The structure is a hairpin stem-loop that contains two identical 16-nt inner loops (upper loop, nt 8070 to 8077 and nt 8105 to 8112; lower loop, nt 8034 to 8041 and nt 8146 to 8153) and a 9-nt terminal loop (nt 8087 to 8093). Interestingly, the inner loops are rotated 180° relative to each other.

Since the primary sequences in the two inner loops in the CTE structure are identical, we hypothesized that these loops might be crucial for CTE function. Also, since an MPMV

fragment that contained a much shorter bottom stem (nt 8022 to 8160) showed some CTE activity, we reasoned that the primary function of this stem might be to provide stability to the structure. To test these hypotheses, we made two HIV-1 RRE-CTE hybrid constructs. In these constructs, we fused the bottom part of stem-loop 1 of the HIV-1 RRE to a CTE either containing or lacking the sequences of the bottom inner loop. These constructs and their predicted secondary structures and ΔG values are shown in Fig. 1B and C. Since the HIV-1 RRE sequences form a perfect helix, the resulting hybrid structures are predicted to have more favorable ΔG values than the wild-type CTE.

To measure function, the wild-type and hybrid CTEs were inserted into the reporter plasmid pBBgagpol-rre and assayed for CTE activity after transfection of CMT3-COS cells. In the presence of a functional CTE, this reporter plasmid produces noninfectious HIV-1 particles, which can be scored by quantitation of p24 in the medium of the transfected cells. The results of these experiments showed that the hybrid construct containing only one inner loop failed to display any CTE function, whereas the construct containing both inner loops gave 72% of the activity of the wild-type CTE. These results strengthen the hypothesis that the inner loops are important for CTE function and indicate that all of the primary sequence information necessary for function is contained between nt 8032 and 8155.

Deletion of the CTE abolishes MPMV particle production. To analyze the role played by the CTE in the MPMV replication cycle, we made a 131-nt deletion (nt 8030 to 8160) in an infectious MPMV proviral clone (49) and introduced restriction enzyme cleavage sites at the point of the deletion by SOE-PCR mutagenesis (24). These manipulations removed the bulk of the CTE. Virus production from this construct (pMPMVΔCTE) was then compared to that of the wild-type proviral clone (pwtMPMV). To do this, CMT3-COS cells were transfected individually with the two constructs and viral particle release was determined by assaying the level of RT in particles released into the medium. Medium was harvested at 24, 48, and 72 h posttransfection, and RT activity was measured after the virions were concentrated by ultracentrifugation (Fig. 2A).

The results of this experiment showed increasing levels of RT activity throughout the assay period in cells transfected with pwtMPMV. In contrast, no significant RT activity was detected in the medium from cells transfected with the pMPMVΔCTE construct. Thus, the CTE is essential for efficient MPMV particle production. Since particle production is an essential step in the replication cycle, our data demonstrate that the CTE is required for viral replication.

Insertion of an HIV-1 RRE restores virus production from pMPMVΔCTE only if the Rev protein is provided in *trans*. Since we previously showed that the MPMV CTE could substitute for Rev and the RRE in the context of an HIV-1 proviral clone (4), it was of interest to determine if Rev and the RRE could complement the defect in pMPMVΔCTE. To do this, a 330-nt fragment containing the HIV-1 RRE was cloned into an *Eco*RV restriction enzyme site located at the site of the deletion in pMPMVΔCTE. The RRE was inserted in both the right and wrong orientations relative to its orientation within the HIV genome. The resulting constructs (pMPMVRRE330-r and pMPMVRRE330-w) were then transfected into CMT3-COS cells either alone or in cotransfections with pCMVrev. An RT analysis was performed as described above on particles secreted into the medium (Fig. 2B).

Insertion of the RRE in the correct orientation partially restored MPMV particle production, but only in the presence of the Rev protein. Levels of RT obtained with pMPMVRRE330-r in the presence of Rev were 20 to 25% of those obtained with the wild-type proviral clone. No RT activities greater than background values were obtained with the RRE330-r construct alone or with pMPMVRRE330-w with or without Rev. Similar results were also obtained when 270- and 520-nt fragments containing the HIV-1 RRE were inserted into pMPMVΔCTE (Fig. 2C). Although virus particle production from the Rev-dependent MPMV constructs was not as efficient as that of the wild-type clone, perhaps due to the fact that Rev had to be supplied in *trans* from a second plasmid, these experiments clearly show that MPMV can be converted into a Rev-dependent virus. This further strengthens the hypothesis that the CTE performs a function analogous to that of Rev and the RRE.

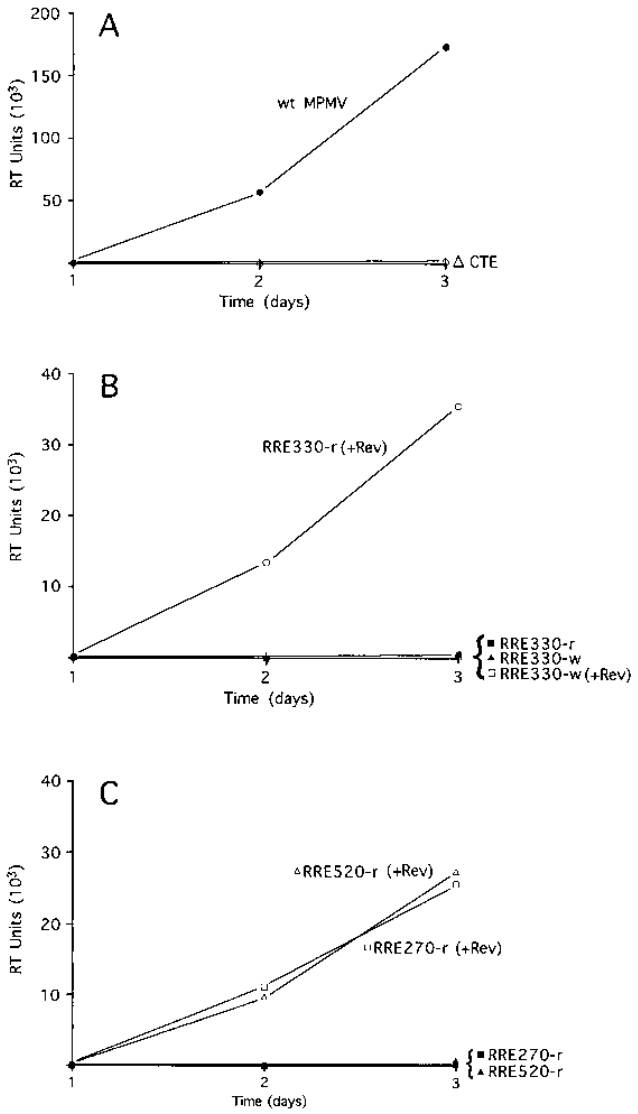

FIG. 2. Virus production from MPMV proviral constructs in CMT3-COS cells. Proviral constructs were transfected into CMT3-COS cells and virion-associated RT activity in the culture medium was analyzed at the indicated days posttransfection as described in Materials and Methods. Note that the *y*-axis scale in panels B and C differs from that in panel A. Analyses of medium from cells transfected with pwtMPMV and pMPMVΔCTE (A), pMPMVRRE330-r or pMPMVRRE330-w either alone or together with pCMVrev (+Rev) (B), and with pMPMVRRE270-r or pMPMVRRE520-r either alone or together with pCMVrev (+Rev) (C) are shown.

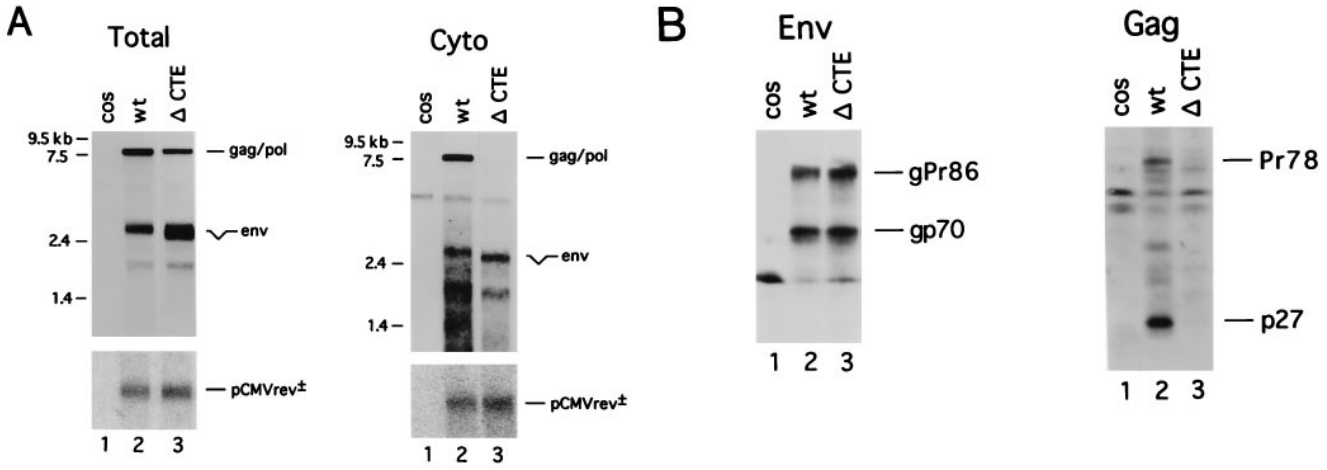

FIG. 3. Northern (A) and Western (B) blot analysis of CMT3-COS cells transfected with wild-type pwtMPMV or pMPMVΔCTE proviral constructs. (A) Total and cytoplasmic (Cyto) poly(A)$^+$-selected RNA was isolated 48 h posttransfection from cells transfected with pwtMPMV (wt) or pMPMVΔCTE (ΔCTE). RNA from cells that were not transfected (cos) was also analyzed. The RNA was separated on 1% denaturing agarose gels and transferred to nitrocellulose membranes. The blots were initially probed with a 5′ end-labelled panel of five oligonucleotide probes complementary to RNA from the *gag*, *pol*, and *env* regions of the MPMV genome (upper panels). The positions of the unspliced *gag-pol* and spliced *env* MPMV RNAs are indicated. As a control for loading, the blots were then stripped and reprobed with an oligonucleotide specific for RNAs expressed from pCMVrev and pCMVrev$^-$ (lower panels). Positions of commercial molecular size markers (RNA ladders; Bethesda Research Laboratories, Inc.) are indicated. (B) Cell lysates were made at 72 h posttransfection from cells transfected with pwtMPMV (wt) or pMPMVΔCTE (ΔCTE). Lysates from cells that were not transfected (cos) were also made. The lysates were separated on sodium dodecyl sulfate–12% polyacrylamide gels which were then blotted onto Immobilon membranes. The blots were developed with an enhanced chemiluminescence kit (Amersham), after incubation with MPMV Env- or Gag-specific antibodies. Env proteins were detected with a rabbit antiserum raised against the MPMV envelope surface protein, gp70. This serum also reacts with the envelope protein precursor, gPr86. The bands corresponding to these proteins are indicated. Gag proteins were detected with a rabbit antiserum raised against a fusion protein between GST and the MPMV capsid protein. This antiserum reacts with both the capsid protein, p27, and its precursor, Pr78. The bands corresponding to these proteins are indicated.

The CTE is required for nuclear export of unspliced MPMV RNA. To further analyze the nature of the expression defect caused by deletion of the CTE from the proviral clones, we performed an analysis of RNA from transfected cells. To do this, cells were transfected with pwtMPMV and pMPMVΔCTE and either pCMVrev or a similar plasmid that expressed a frameshift, inactive form of the Rev protein (pCMVrev$^-$). Total or cytoplasmic poly(A)$^+$ RNA isolated at 48 h posttransfection was then analyzed by Northern blot analysis using MPMV-specific probes (Fig. 3A). These blots were later reprobed with a *rev*-specific oligonucleotide that hybridized to both the wild-type and mutant *rev* mRNAs (Fig. 3A, lower panel). These RNAs served as internal controls for transfection efficiency and gel loading.

As expected, in the total RNA preparation (Fig. 3A, left panel), two major species of MPMV RNA were detected. The larger RNA species (8.0 kb) was the unspliced *gag-pol* mRNA, whereas the second, smaller RNA species (3.0 kb) represented the spliced *env* mRNA. In the ΔCTE construct, the ratios between unspliced and spliced RNA were shifted in favor of the spliced RNA. When cytoplasmic RNA was analyzed (Fig. 3A, right panel), both species of RNA were again detected in cells transfected with pwtMPMV. However, only spliced RNA was observed with the ΔCTE construct.

Taken together, the results of the RNA analysis demonstrated that the MPMV CTE was absolutely required for the accumulation of unspliced MPMV RNA in the cytoplasm. Deletion of the CTE also appeared to increase the levels of spliced RNA in total, but not cytoplasmic, RNA preparations. Whether this is due to a direct effect of the CTE on splicing or an indirect effect due to retention of the unspliced RNA in the nucleus cannot be determined by these experiments. It is also possible that these RNA species display differential stability in the absence and presence of a CTE.

A minor, third species of RNA (around 1.8 kb) was also observed in these blots. This RNA appears to be specific for transfected cells but does not correspond to a known species of MPMV RNA. Its accumulation in the cytoplasm was not affected by the CTE. We are presently analyzing whether this RNA represents an alternatively spliced MPMV RNA. A fourth RNA species (around 6 kb) was also detected in cytoplasmic RNA and was weakly detected in total RNA. Since this species was present in the RNA from mock-transfected cells, it seems likely that it represents a cellular mRNA that cross-hybridized with the MPMV probe.

Expression of MPMV Gag, but not Env, requires the CTE. We next analyzed the pattern of MPMV protein expression in cells transfected with pwtMPMV and pMPMVΔCTE. Transfected cells were harvested 72 h posttransfection and analyzed on Western blots which were developed with antisera specific for either the MPMV Env or Gag proteins (Fig. 3B).

This analysis showed that both pwtMPMV and pMPMV ΔCTE expressed equivalent levels of the MPMV Env protein, gp70, and its precursor, gPr86. As expected, pwtMPMV also produced the MPMV capsid protein, p27, and the Gag precursor, Pr78. In contrast, pMPMVΔCTE failed to produce any Gag-related gene products.

The results of this experiment showed that expression of the MPMV Env proteins from proviral constructs did not require the CTE, while expression of the Gag-related proteins was absolutely dependent on the presence of this element. There was a good correlation between cytoplasmic RNA levels and protein expression in these experiments. This indicated that the CTE had no major effect on translation.

Insertion of the RRE into the ΔCTE provirus restores cytoplasmic accumulation of *gag-pol* mRNA and protein expression when Rev is supplied in *trans*. We also performed an RNA and protein analysis of cells transfected with the RRE-containing MPMV proviral constructs with and without Rev present in *trans*. As controls, cells were also transfected with

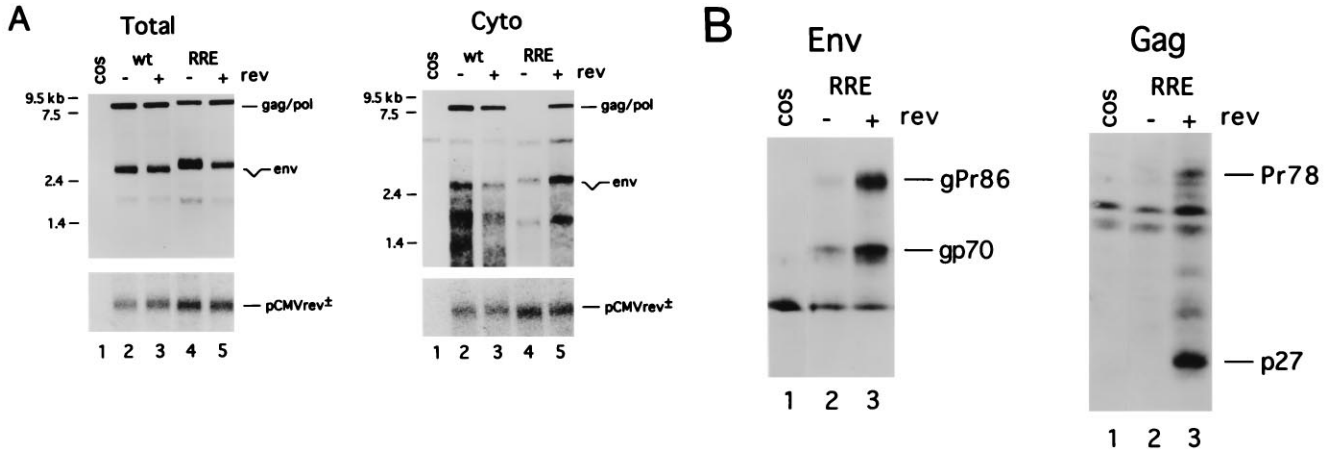

FIG. 4. Northern (A) and Western (B) blot analysis of RNA from CMT3-COS cells transfected with wild-type pwtMPMV or pMPMVRRE330-r in the absence or presence of functional Rev. (A) Total and cytoplasmic (Cyto) poly(A)$^+$-selected RNA was isolated 48 h posttransfection from cells transfected with pwtMPMV (wt) or pMPMVRRE330-r (RRE) and pCMVrev (+) or pCMVrev (−). RNA from cells that were not transfected (cos) was also analyzed. The RNA was separated, blotted, and analyzed as described in the legend to Fig. 3A. (B) Cell lysates were made at 72 h posttransfection from cells transfected with pMPMVRRE330-r (RRE) and pCMVrev (+) or pCMVrev (−). Lysates from cells that were not transfected (cos) were also made. The lysates were separated, blotted, and analyzed as described in the legend to Fig. 3B.

the wild-type proviral clone. The results of the experiments with the construct containing the 330-nt RRE are shown in Fig. 4. Similar results were obtained with the other RRE constructs (data not shown).

Northern blot analysis of total RNA from cells transfected with the RRE-containing construct (Fig. 4A, left panel, lanes 4 and 5) showed that both unspliced and spliced MPMV RNAs were present with or without Rev. As in the case of the ΔCTE construct (Fig. 3A, left panel), more spliced RNA was observed from the RRE-containing construct in the absence of Rev than was observed from the wild-type clone. However, cotransfection with Rev restored the ratio.

Analysis of cytoplasmic RNA from cells transfected with the RRE construct (Fig. 4, right panel, lanes 4 and 5) showed the accumulation of unspliced *gag-pol* mRNA in the presence of Rev only. In contrast, spliced *env* mRNA was observed both with and without Rev, although the levels were significantly higher when Rev was present.

Western blot analysis of the transfected cells, performed as described above, showed that the RRE-containing construct expressed MPMV *gag* proteins only when Rev was present (Fig. 4B). In contrast, *env* proteins were expressed even when Rev was absent. However, the levels of *env* expression were significantly higher in the presence of Rev, parallelling the results of the cytoplasmic RNA analysis.

These experiments indicate that Rev and the RRE can substitute for the CTE to achieve cytoplasmic accumulation of unspliced MPMV RNA. Interestingly, insertion of the RRE into the MPMV genome appeared to lead to significant nuclear retention of the spliced *env* mRNA in the absence of Rev (cf. Fig. 4A, lanes 4). This is consistent with the previously reported observations that the RRE can act as a negative element to prevent nuclear export of RNA when Rev is not present (5, 45).

The CTE can mediate nuclear export of intron-containing cellular mRNAs but does not directly inhibit splicing. Mutation of individual splice sites frequently leads to accumulation of intron-containing RNA that is usually retained in the nucleus (6, 38). Chang and Sharp showed that mutation of either the 5′ or 3′ splice site in a construct containing a complete rabbit β-globin intron led to retention of unspliced RNA in the nucleus. However, this RNA was exported from the nucleus if the HIV-1 RRE was inserted into the intron of the mutated constructs and the Rev protein was provided in *trans.*

To determine if the MPMV CTE could facilitate the export of intron-containing cellular RNAs in a fashion similar to Rev and the RRE, we first inserted a 334-nt fragment containing the CTE (nt 8007 to 8340) into the intron of pb8F, one of the β-globin gene constructs utilized in the study by Chang and Sharp (6). pb8F contains a β-globin intron with an inserted HIV-1 RRE. The intron retains a consensus 5′ splice site, but the 3′ splice site contains an AG-to-TG mutation, which leads to significant accumulation of the intron-containing RNA. The MPMV CTE was inserted immediately downstream of the RRE in this construct.

The original pb8F construct and the construct containing the CTE (pb8F-CTE) were transfected into CMT3-COS cells with either pCMVrev or pCMVrev$^-$. Total and cytoplasmic poly(A)$^+$ RNA was isolated from the transfected cells 48 h after transfection and analyzed on Northern blots with an oligonucleotide probe complementary to sequences in the 3′ exon of the β-globin gene. This probe also hybridizes to sequences in the RNA expressed from the pCMVrev constructs, since the 3′ end of this RNA contains rabbit β-globin sequences.

The results of these experiments are shown in Fig. 5. Total RNA from cells transfected with pb8F and pCMVrev or pCMVrev$^-$ showed two major RNA species of about 1.0 and 1.6 kb, respectively (Fig. 5A, lanes 1 and 2). The lower band represented RNA expressed from the Rev constructs, whereas the upper band represented unspliced RNA expressed from pb8F. Similar amounts of pb8F RNA were observed in both lanes. Analogous RNAs were detected in total RNA from cells transfected with pb8F-CTE and the two Rev constructs (Fig. 5A, lanes 3 and 4). In this case, the unspliced RNA expressed from the globin construct was 1.9 kb, due to the insertion of the 330-nt MPMV CTE. Similar amounts of this RNA were seen whether or not a functional Rev protein was present.

With the probe used in our experiments, no spliced RNA was observed. This probe maps to a region just 3′ of the mutated 3′ splice site. These results indicate that the mutated 3′ splice site was not utilized. In the original study using this

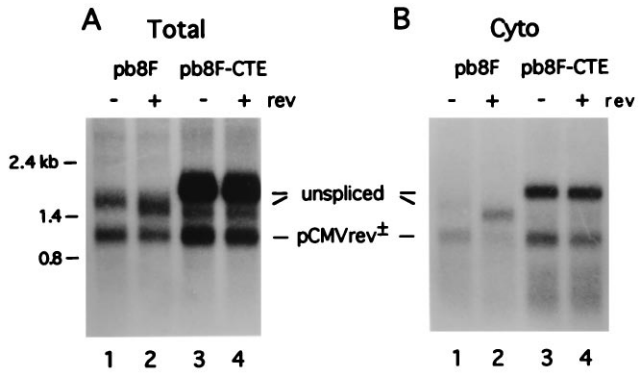

FIG. 5. Northern blot analysis of total (A) and cytoplasmic (B) poly(A)$^+$ RNA from CMT3-COS cells transfected with pb8F and pb8F-CTE. Total and cytoplasmic poly(A)$^+$-selected RNAs were extracted from cells transfected with pb8F or pb8F-CTE and either pCMVrev (+) or pCMVrev$^-$ (−). The RNA was separated on a 1.5% denaturing agarose gel and subjected to Northern blot analysis using the probe described in the text. The positions of RNAs expressed from pb8F and pb8F-CTE (unspliced) and the pCMVrev plasmids (pCMVrev$^\pm$) are indicated.

construct, Chang and Sharp observed cleavage at the 5′ splice site during an RNase protection assay. The two sets of results can be reconciled if a 3′ splice site downstream of our probe is utilized or if a splicing intermediate, cleaved at the 5′ splice site, accumulates with this construct.

In the cytoplasmic preparations, only a trace of unspliced RNA was detected in RNA from cells transfected with pb8F in the absence of a functional Rev protein (Fig. 5B, lane 1). In contrast, significant amounts of unspliced pb8F RNA were seen when a functional Rev protein was present (Fig. 5B, lane 2). In the cells transfected with pb8F-CTE, large amounts of unspliced RNA were observed in the cytoplasm both in the absence and in the presence of a functional Rev protein (Fig. 5B, lanes 3 and 4). These results demonstrated that the CTE enabled the nuclear export of intron-containing cellular RNA in a manner similar to that observed with Rev and the RRE.

To accurately measure the relative levels of the unspliced globin RNAs in total and cytoplasmic RNA, the different RNA bands in the Northern blots were quantitated by image analysis with a PhosphorImager. The results of this analysis are shown in Table 1. The values for the unspliced RNA expressed from the β-globin constructs were normalized for the intensity of the pCMVrev and pCMVrev$^-$ bands in each lane to correct for variation in transfection efficiency and/or loading. The last column of the table shows the ratio of the normalized amounts of unspliced β-globin RNA detected in the presence and absence of a functional Rev protein for both pb8F and pb8F-CTE.

As can be seen in this table, the presence of a functional Rev protein did not change the relative amount of unspliced RNA in the total RNA preparations from either construct. However, the presence of a functional Rev protein increased the cytoplasmic levels of unspliced pb8F RNA more than eightfold. In contrast, in the case of pb8F-CTE, the cytoplasmic levels of unspliced β-globin RNA remained the same whether or not a functional Rev protein was present.

It should be noted that in cells transfected with pb8F, the unspliced RNA was slightly smaller in the presence of the Rev protein (cf. Fig. 5A, lanes 1 and 2). This is due to a trimming of the poly(A) tail of the RNA in the presence of Rev (data not shown), similar to what has been observed with other Rev-dependent RNAs (22, 39).

We also performed experiments in which the CTE was inserted into the downstream exon of pb8F. These experiments yielded results similar to the ones described above (data not shown), showing that the CTE, like the RRE, is able to function in both an intron and an exon position.

The study by Chang and Sharp also utilized a plasmid similar to pb8F that contained wild-type rabbit β-globin 5′ and 3′ splice sites. The RNA from this construct (pβGRRE) was spliced efficiently, and the presence of the Rev protein did not lead to increased levels of unspliced RNA or nuclear export of such RNA. These results demonstrated the inability of Rev to inhibit splicing of an efficiently spliced intron in vivo or to achieve export of unspliced RNA if the RNA was rapidly spliced.

To determine whether the CTE was able to inhibit splicing and/or mediate export of unspliced RNA from β-globin constructs containing wild-type splice sites, we inserted the CTE into either the intron or the exon of pβGRRE to create the plasmids pβGRRE(CTE-intron) and pβGRRE(CTE-exon). Cells were then transfected with these constructs in either the absence or presence of a functional Rev protein. Total and cytoplasmic poly(A)$^+$ RNA was prepared, and a Northern blot analysis was performed as described above.

In the case of pβGRRE(CTE-exon), the analysis showed a single 1.1-kb band in all RNA preparations (Fig. 6, lanes 1 and 2), whereas a single 0.8-kb band was seen in all preparations in the case of pβGRRE(CTE-intron) (Fig. 6, lanes 3 and 4). These RNAs were of the sizes expected for spliced RNAs from the respective constructs. No unspliced RNA was observed in any of the samples, and the spliced RNA was efficiently exported in both cases. These experiments showed that the CTE was incapable of inhibiting splicing of an efficiently spliced intron. These results thus further strengthen the analogy between CTE and Rev and RRE function.

DISCUSSION

The results presented in this study demonstrate that the MPMV genome contains a discrete *cis*-acting element, the CTE, that interacts directly with the cellular machinery to

TABLE 1. Quantitation of the effect of the CTE and Rev and the RRE on the transport of an unspliced cellular mRNA[a]

RNA preparation and plasmid	Rev	Intensity of band		Normalization factor[b]	Normalized intensity of unspliced band[c]	Ratio[d]
		Unspliced RNA	Rev			
Total RNA (Fig. 5A)						
pb8F	−	2,057	2,116	1.61	3,311	
pb8F	+	2,288	1,741	1.95	4,470	1.35
pb8F-CTE	−	7,805	3,402	1.00	7,805	
pb8F-CTE	+	7,953	2,892	1.18	9,385	1.20
Cytoplasmic RNA (Fig. 5B)						
pb8F	−	186	524	2.29	426	
pb8F	+	739	246	4.89	3,614	8.48
pb8F-CTE	−	1,962	1,202	1.00	1,962	
pb8F-CTE	+	1,747	914	1.32	2,306	1.18

[a] Analysis of Fig. 5.
[b] The normalization factor for total RNA was calculated as 3,402/intensity of Rev band, and that for cytoplasmic RNA was calculated as 1,202/intensity of Rev band.
[c] Normalized intensity was calculated as follows: measured intensity of unspliced RNA band × normalization factor.
[d] Ratio = normalized intensity of unspliced band with Rev/normalized intensity of unspliced band without Rev.

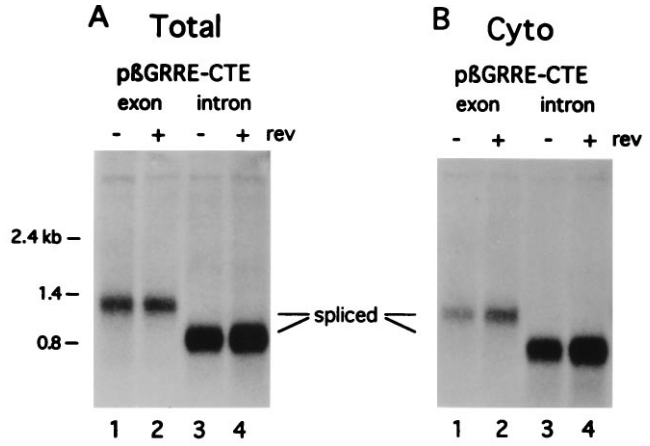

FIG. 6. Northern blot analysis of total (A) and cytoplasmic (B) poly(A)$^+$ RNA from CMT3-COS cells transfected with pβGRRE(CTE-intron) and pβGRRE(CTE-exon). Total and cytoplasmic poly(A)$^+$-selected RNAs were extracted from cells transfected with pβGRRE(CTE-intron) and pβGRRE(CTE-exon) in the presence (+) or absence (−) of Rev and subjected to Northern blot analysis as described in the legend to Fig. 5. The positions of the spliced RNAs expressed from these plasmids are indicated.

achieve export of full-length unspliced MPMV RNA from the nucleus to the cytoplasm. The CTE functions in a fashion analogous to that of the HIV-1 RRE and Rev as demonstrated in several different experiments. This report shows that Rev and the RRE can substitute for the CTE in MPMV expression and virus production, while a previous article demonstrated that the CTE could complement Rev and the RRE in the context of HIV gene expression and replication (4). Our present results also show that the CTE can serve as a signal for the nuclear export of cellular intron-containing RNAs previously shown to be exported with the help of Rev and the RRE (6). However, a crucial difference between the two transport mechanisms is that Rev allows HIV to exert control over the expression of intron-containing RNAs, while MPMV CTE-mediated RNA transport is totally dependent on endogenous cellular processes.

The identification of the CTE as crucial for transport of unspliced MPMV RNA and the analogy between the CTE and HIV-1 Rev and RRE suggest that all retroviruses may have to utilize specific mechanisms to achieve export of their intron-containing RNAs. Thus, it seems likely that many, if not all, of the simpler retroviruses contain functional equivalents of the CTE. In fact, an RNA element from Rous sarcoma virus that promotes the accumulation of cytoplasmic RNA has recently been described (46). This type of transport signal could also play a role in the replication of other viruses which have to export unspliced RNA, since an element with properties similar to those of the CTE has been identified in the hepatitis B virus genome (25, 26).

The HIV Rev protein has been shown to shuttle between the nucleus and cytoplasm (34, 43, 60). Mutational analysis of Rev has defined two domains responsible for the shuttling mechanism. The first domain is a nuclear localization signal rich in basic amino acids that is also required for RNA binding (7, 37, 40, 64). The second is an activation and/or effector domain that contains a leucine-rich motif and functions as a nuclear export signal (NES) (14, 17, 66). An NES with significant homology to the leucine-rich domain in Rev is found in the cellular protein kinase inhibitor, which serves as an inhibitor of cyclic AMP-dependent protein kinase (66). Interestingly, this protein is not an RNA binding protein but has been shown to mediate export of the active subunit of the protein kinase from the nucleus to the cytoplasm. The heterogeneous nuclear ribonucleoprotein particle (hnRNP) A1 protein has also recently been shown to contain an NES (44). However, this NES shows no sequence similarity with the NES in Rev. The hnRNP A1 protein binds to poly(A)$^+$ RNA in both the nucleus and the cytoplasm and may be transported to the cytoplasm in a complex with RNA (47, 48). It has also been recently shown that the *Chironomus tentans* hrp36 protein, which demonstrates 40% identity and 64% similarity to the amino acid sequence of hnRNP A1, is incorporated into hnRNPs and is transported through the nuclear pore in a complex with RNPs (65). In view of these results, it seems possible that the MPMV CTE interacts either directly or indirectly with a cellular protein that contains an NES domain.

The NES domain of Rev interacts with several cellular proteins: eukaryotic initiation factor 5A, hRIP, Rab, and Rip1p (3, 16, 50, 59). With the exception of eukaryotic initiation factor 5A all of these proteins are nucleoporin-like proteins that contain numerous XXFG repeats present in several nucleoporins. The function of the nucleoporin XXFG repeats is currently unknown, but the highly repeated motif is predicted to assume a β-sheet conformation possibly involved in protein-protein interactions (10). The hRIP and Rab proteins, isolated from human cDNA libraries, are identical, whereas the Rip1p protein, isolated from a *Saccharomyces cerevisiae* genomic library, exhibits 40% sequence identity to the hRIP/Rab protein. It will be of obvious interest to determine whether any of these proteins are involved in CTE-mediated export.

It seems likely that the cellular factor with which the CTE interacts is involved in a pathway that mediates the export of cellular intron-containing RNA. Since the export of such RNAs seems to be a relatively rare occurrence (41), these RNAs would be expected to contain specific export signals functionally similar to the CTE. It is not yet clear how this type of transport relates to the export of other kinds of RNAs. Recent results from microinjection studies with *Xenopus* oocytes showed that export mediated by Rev and the RRE competed with transport of 5S rRNA and spliceosomal U snRNAs. In contrast, no competition between Rev- and RRE-mediated export and the transport of a fully spliced cellular mRNA was observed (14).

Our experiments showed that large amounts of unspliced MPMV RNA were observed in total RNA preparations from transfected cells both in the absence and in the presence of the CTE. However, no unspliced RNA was detected in the cytoplasm when the CTE was deleted. Thus, cytoplasmic accumulation of unspliced RNA required the presence of a functional CTE. Furthermore, since a substantial amount of unspliced MPMV RNA was retained in the nucleus when the CTE was deleted, our results suggest that the CTE is required for the export of this RNA. This conclusion is also substantiated by the fact that the CTE induced cytoplasmic accumulation of cellular intron-containing RNA that was otherwise retained in the nucleus. The fact that the amounts of unspliced RNA present in total RNA preparations were somewhat reduced in the absence of the CTE could be a secondary effect due to the reduced stability of the RNA when it is retained in the nucleus or due to increased splicing.

Deletion of the CTE from the MPMV genome led to an increase in the amount of spliced *env* mRNA relative to unspliced RNA in total RNA preparations. However, substantial amounts of unspliced RNA were still detected. This fact suggests that the MPMV genome contains additional sequences that inhibit splicing in *cis*, similar to the ones that have been found in HIV and Rous sarcoma virus (1, 2, 35, 42, 56, 57). It

should also be noted that the CTE did not inhibit splicing when inserted into an efficiently spliced β-globin construct. These results argue against a major direct effect of the CTE on splicing but rather suggest that the observed effects are secondary to the retention of the unspliced RNA in the nucleus, resulting in a larger pool of RNA available for splicing.

Since the CTE functions in its natural context within the MPMV genome to promote export of a completely unspliced RNA (the full-length MPMV transcript), it could conceivably do so by preventing a portion of this RNA from ever associating with the splicing machinery by redirecting it for direct export. However, the CTE can also substitute for Rev and the RRE in HIV replication (4). In this case, the CTE promoted the export of singly spliced RNA as well as unspliced RNA. This means that the CTE, like Rev and the RRE, can trigger the export of an RNA that has already been spliced. Furthermore, neither Rev and the RRE nor the CTE are capable of triggering export of unspliced RNA when inserted into an efficiently spliced intron. These results suggest that CTE and Rev and the RRE are likely to act downstream of association of the RNA with the splicing machinery. We have previously shown that export of HIV *env* mRNA in a subgenomic construct required binding to U1 snRNA in addition to Rev and the RRE (39). The same requirement exists for CTE-mediated export of HIV *env* mRNA (unpublished observations). In addition, preliminary experiments in our laboratory show that the presence of the major 5′ splice site in the full-length MPMV RNA and complex formation with U1 snRNA are required for CTE mediated export of this RNA.

In contrast to these results, microinjection experiments with *Xenopus* oocytes showed that RRE-containing RNA could be exported in the presence of the Rev protein independently of association with the splicing machinery (15). These investigators also performed microinjection experiments with RNAs that contained efficiently spliced introns (14, 15). It was shown that insertion of the RRE into the intron and coinjection of Rev resulted in export of large amounts of unspliced RNA as well as lariat intron. Thus, Rev and the RRE were apparently able to promote an association with the export machinery in competition with splicing components and splicing. The discrepancy between these results and our results with Rev and the RRE and the CTE may reflect differences in RNA export between oocytes and mammalian cells. Alternatively, microinjected RNA might be able to access a compartment in the nucleus which is normally not available to newly transcribed mRNA. Further experimentation is necessary to address these possibilities. It will also be of obvious interest to study the effects of the CTE on RNA transport in the *Xenopus* oocyte system.

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