

# The constitutive transport element (CTE) of Mason–Pfizer monkey virus (MPMV) accesses a cellular mRNA export pathway

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**The constitutive transport elements (CTEs) of type D retroviruses are *cis*-acting elements that promote nuclear export of incompletely spliced mRNAs. Unlike the Rev response element (RRE) of human immunodeficiency virus type 1 (HIV-1), CTEs depend entirely on factors encoded by the host cell genome. We show that an RNA comprised almost entirely of the CTE of Mason–Pfizer monkey virus (CTE RNA) is exported efficiently from *Xenopus* oocyte nuclei. The CTE RNA and an RNA containing the RRE of HIV-1 (plus Rev) have little effect on export of one another, demonstrating differences in host cell requirements of these two viral mRNA export pathways. Surprisingly, even very low amounts of CTE RNA block export of normal mRNAs, apparently through the sequestration of cellular mRNA export factors. Export of a CTE-containing lariat occurs when wild-type CTE, but not a mutant form, is inserted into the pre-mRNA. The CTE has two symmetric structures, either of which supports export and the titration of mRNA export factors, but both of which are required for maximal inhibition of mRNA export. Two host proteins bind specifically to the CTE but not to non-functional variants, making these proteins candidates for the sequestered mRNA export factors.**

**Keywords:** constitutive transport element/mRNA export/Rev protein/*Xenopus* oocytes

## Introduction

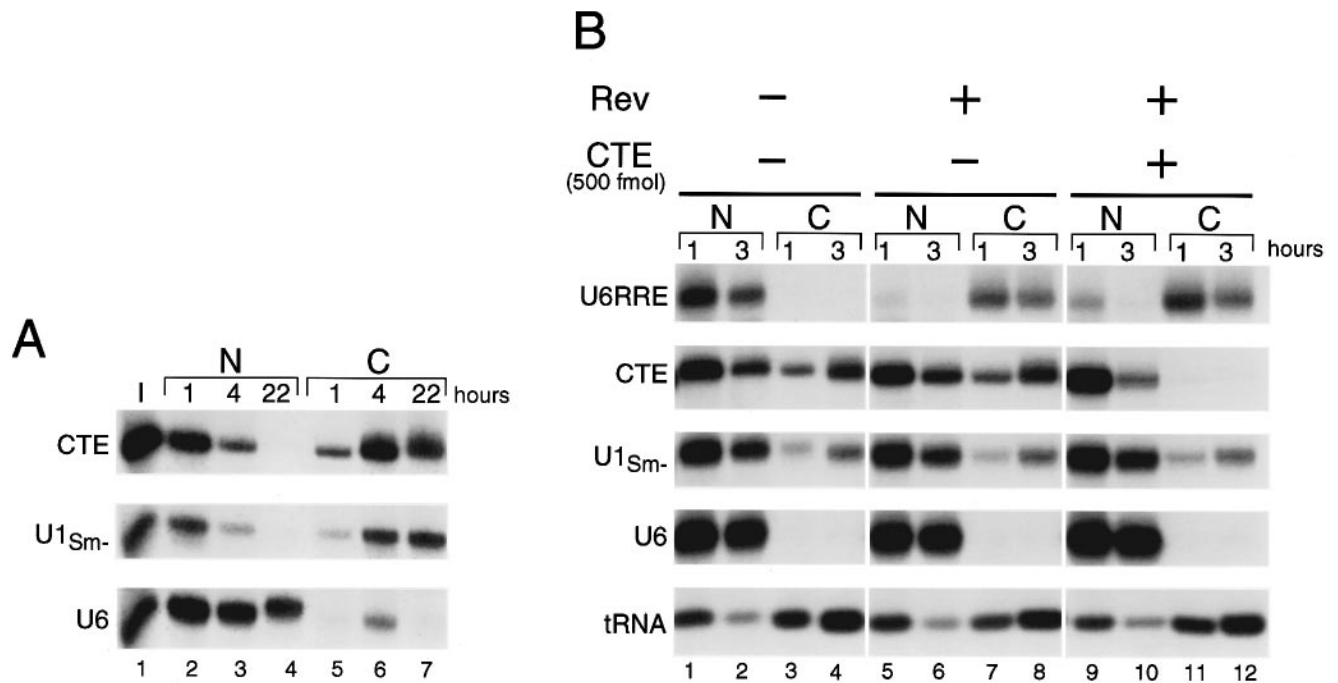
Most pre-mRNAs are spliced and polyadenylated prior to their export from the nucleus to the cytoplasm (reviewed by Izaurralde and Mattaj, 1995; Nakielnny and Dreyfuss, 1997); unspliced pre-mRNAs are retained in the nucleus, presumably because of their association with specific

splicing factors (Chang and Sharp, 1989; Legrain and Rosbash, 1989; Hamm and Mattaj, 1990). Nevertheless, replication of retroviruses depends on export of intron-containing RNAs, since the unspliced viral mRNAs are needed in the cytoplasm both for production of certain viral proteins and for incorporation into new viral particles (reviewed by Hammarskjöld, 1997). Thus, these viruses have developed ways to promote export of incompletely spliced viral RNAs.

Many complex retroviruses, like the human immunodeficiency virus type 1 (HIV-1), encode *trans*-acting factors that facilitate export of unspliced and incompletely spliced RNAs. The Rev protein of HIV-1 binds an intronic sequence in HIV-1 RNA, the Rev response element (RRE) (Daly *et al.*, 1989; Zapp and Green, 1989), and promotes export of the intron-containing HIV-1 RNAs (Emerman *et al.*, 1989; Felber *et al.*, 1989; Hammarskjöld *et al.*, 1989; Malim *et al.*, 1989a,b; Perkins *et al.*, 1989; Fischer *et al.*, 1994). This export requires a leucine-rich nuclear export signal (NES) in the Rev protein (Meyer and Malim, 1994; Fischer *et al.*, 1995; Wen *et al.*, 1995). The Rev protein has been proposed to interact with several cellular proteins such as the Rev-interacting protein (Rip or Rab) (Bogerd *et al.*, 1995; Fritz *et al.*, 1995; Stutz *et al.*, 1995), eukaryotic initiation factor 5A (eIF5A) (Ruhl *et al.*, 1993; Bevec *et al.*, 1996) and proteins of the nuclear pore complex (NPC) (Stutz *et al.*, 1995, 1996; Fritz and Green 1996).

Because simpler retroviruses do not encode *trans*-acting regulatory proteins, the mechanism by which their intron-containing mRNAs are exported to the cytoplasm has been a puzzle. A *cis*-acting RNA element located near the 3' end of one such retrovirus, Mason–Pfizer monkey virus (MPMV), has been shown to be important for export of the intron-containing viral RNA (Bray *et al.*, 1994; Ernst *et al.*, 1997a). Both the sequence and function of this element, termed the constitutive transport element (CTE), are conserved in related type D retroviruses (Zolotukhin *et al.*, 1994; Taberner *et al.*, 1996; Ernst *et al.*, 1997b). Because the CTE can substitute for RRE/Rev in promoting export of intron-containing RNAs (Bray *et al.*, 1994; Taberner *et al.*, 1996; Ernst *et al.*, 1997a), it is likely that the CTE functions by interacting with cellular export factors. The identities and functions of such factors have not yet been established; a cellular RNA helicase recently was reported to bind to the CTE, but the generality and functional significance of that interaction is unclear (Tang *et al.*, 1997; see below).

In this study, we investigated the cellular export pathway that is accessed by the CTE of MPMV. We first showed that an RNA consisting of the CTE (CTE RNA) is exported after injection into the nuclei of *Xenopus laevis* oocytes and that this export is saturable, indicating that the CTE interacts with a titratable cellular factor(s).



**Fig. 1.** CTE RNA is exported from the nuclei of *Xenopus* oocytes. **(A)** Export of CTE RNA is rapid and the RNA is stable. A mixture of RNAs containing ~5 fmol each of <sup>32</sup>P-labeled CTE RNA (see Figure 5A for the sequence and structure of the minimal CTE), U1<sub>Sm</sub>- RNA and U6 RNA was injected into nuclei of oocytes, which were dissected 1, 4 and 22 h later; RNA was extracted from the nuclear (N) and cytoplasmic (C) fractions and 0.5 oocyte equivalents of RNA were resolved on a denaturing 8% polyacrylamide gel and visualized by autoradiography. The amounts of the RNAs in the injection mixture are shown in lane 1 (I). U6 or U3 snRNAs, which normally are not exported from the nucleus (Vankan *et al.*, 1990; Terns *et al.*, 1993), served as a marker for the accuracy of nuclear injections and oocyte dissections in this case and in all other transport assays shown; hence, the apparent extent of export of U1<sub>Sm</sub>- RNA and CTE RNA at the 4 h time point (lane 5) is slightly high, as shown by the small amount of U6 snRNA present in the cytoplasm. **(B)** Excess CTE RNA saturates its own export and stability but does not affect Rev-mediated export. Mixtures of <sup>32</sup>P-labeled CTE RNA, U6RRE RNA, U1<sub>Sm</sub>- RNA, U6 RNA and tRNA<sup>Tyr</sup> were injected into oocyte nuclei alone (lanes 1–4) or together with Rev protein (lanes 5–8) or Rev protein plus 500 fmol of unlabeled CTE RNA (lanes 9–12). Oocytes were dissected 1 and 3 h after injection, and RNA export was analyzed as in (A).

However, saturation of the factor required by CTE does not affect RRE/Rev-mediated export, nor does saturation of the RRE/Rev export pathway block export of the CTE RNA. Thus, these two pathways, each of which support export of unspliced mRNAs, apparently require different saturable factors in the *Xenopus* oocyte system.

Surprisingly, CTE RNA competes very effectively for a factor(s) that is required for export of mRNAs; we have identified mutated CTE RNAs that have a diminished ability to sequester this factor(s). Moreover, wild-type CTE, but not a mutated CTE, supports export of an intron lariat from oocyte nuclei. We propose that the CTE promotes RNA export by interacting specifically with a component(s) of the cellular mRNA transport machinery that normally is needed to mobilize mRNAs for export; in the context of the isolated CTE RNA, this interaction appears to deplete the oocyte nucleus of a factor so that it no longer is available for the export of cellular mRNAs.

## Results

### **The CTE functions as an autonomous export signal in *Xenopus* oocytes**

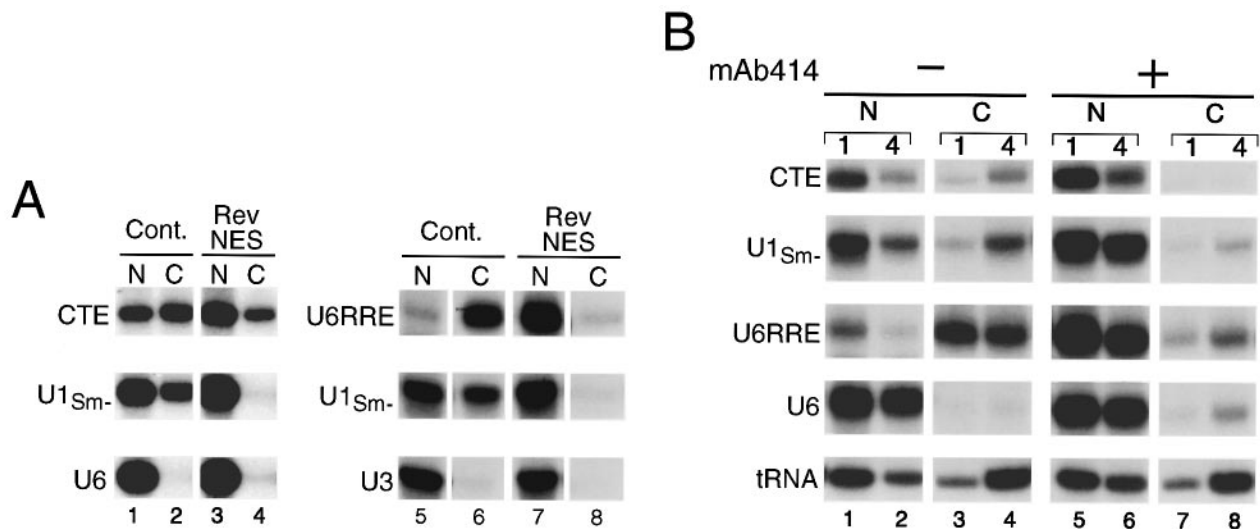
The CTE of MPMV RNA promotes export of incompletely spliced pre-mRNAs from the nucleus (Bray *et al.*, 1994; Ernst *et al.*, 1997a). Because this *cis*-acting element functions in the absence of MPMV proteins, it must use cellular factors to access an RNA export pathway. We

first asked if an RNA that contained CTE sequences but no other known export signals (see below) could be exported from the nuclei of *Xenopus* oocytes. Upon nuclear injection, ApppG-capped RNA consisting almost entirely of the CTE (CTE RNA) accumulated in the cytoplasm with time (Figure 1A). Replacement of the ApppG-cap with an m<sup>7</sup>GpppG cap, which serves as an export signal for pre-snRNAs and mRNAs (Hamm and Mattaj, 1990; Izaurralde *et al.*, 1992, 1995; Terns *et al.*, 1993), was without effect on the export of CTE RNA (data not shown). Thus, export was governed by the CTE sequences and not the cap.

In contrast, the complement of CTE RNA (anti-CTE) did not appear in the cytoplasm if it had an ApppG cap (data not shown; see Figure 5A). However, when the anti-CTE RNA contained an m<sup>7</sup>GpppG cap, it was exported (data not shown); presumably, this latter export was directed by the m<sup>7</sup>GpppG cap rather than the sequence of the RNA.

### **The limiting factor used by CTE is not required for the Rev-mediated export pathway**

The CTE originally was identified as an RNA export element that could substitute for the RRE/Rev function in facilitating export of unspliced HIV pre-mRNAs (Bray *et al.*, 1994). Because Rev-dependent export of an RRE-containing RNA can be assayed in *Xenopus* oocytes (Fischer *et al.*, 1994, 1995), we asked if the export pathway



**Fig. 2.** The CTE- and RRE/Rev-mediated export pathways are distinct. **(A)** Export of CTE RNA occurs in the presence of Rev NES-peptide conjugates. Export of CTE RNA, U6RRE RNA (+ Rev), and U1<sub>Sm-</sub> RNA was analyzed in the presence of Rev NES (Rev NES) or control peptide conjugates (Cont.) at 4 h (lanes 1–4) or 1 h (lanes 5–8) after injection. **(B)** CTE RNA- and Rev-mediated export are both inhibited by mAb414 antibodies. Export of CTE RNA, U1<sub>Sm-</sub> RNA, U6RRE RNA + Rev, U6 RNA and tRNA<sup>Tyr</sup> in the presence or absence of mAb414 antibodies was compared at 1 and 4 h after injection.

used by the CTE RNA overlaps with that used by the RRE and Rev. To do that, we first examined the effects of an excess of CTE RNA on the export of U6RRE RNA, which contains U6 RNA and a minimal RRE sequence (D.Young, E.Lund and M.Zapp, in preparation); export of this chimeric RNA was dependent on the presence of co-injected Rev protein (Figure 1B, compare lanes 1–4 with lanes 5–8). Injection of an excess of CTE RNA did not interfere with Rev-mediated export of the U6RRE RNA (lanes 5–12). Similarly, the amount of Rev protein injected (~50 fmol, or an ~10-fold molar excess over the CTE RNA in lanes 1–8) did not affect export of CTE RNA (lanes 1–8). Very little decrease in the rate of export of U1<sub>Sm-</sub> snRNA was observed upon co-injection of either the Rev protein or the CTE RNA.

The amount of competitor CTE RNA injected in the samples shown in lanes 9–12 of Figure 1B was sufficient to saturate completely the export of CTE RNA (compare lanes 3 and 11). The decrease in the level of CTE RNA present at 3 h indicates titration of a stabilizing factor, as is the case with other RNAs (Jarmolowski *et al.*, 1994). Because CTE RNA was stable when injected directly into the cytoplasm (data not shown), this degradation probably occurred in the nucleus. In spite of the destabilization, sufficient amounts of CTE RNA remained in the nucleus, at both 1 and 3 h, to serve as substrate for export if it had not been saturated. It is unclear if the same nuclear factor is responsible for both stabilization and export of CTE RNA.

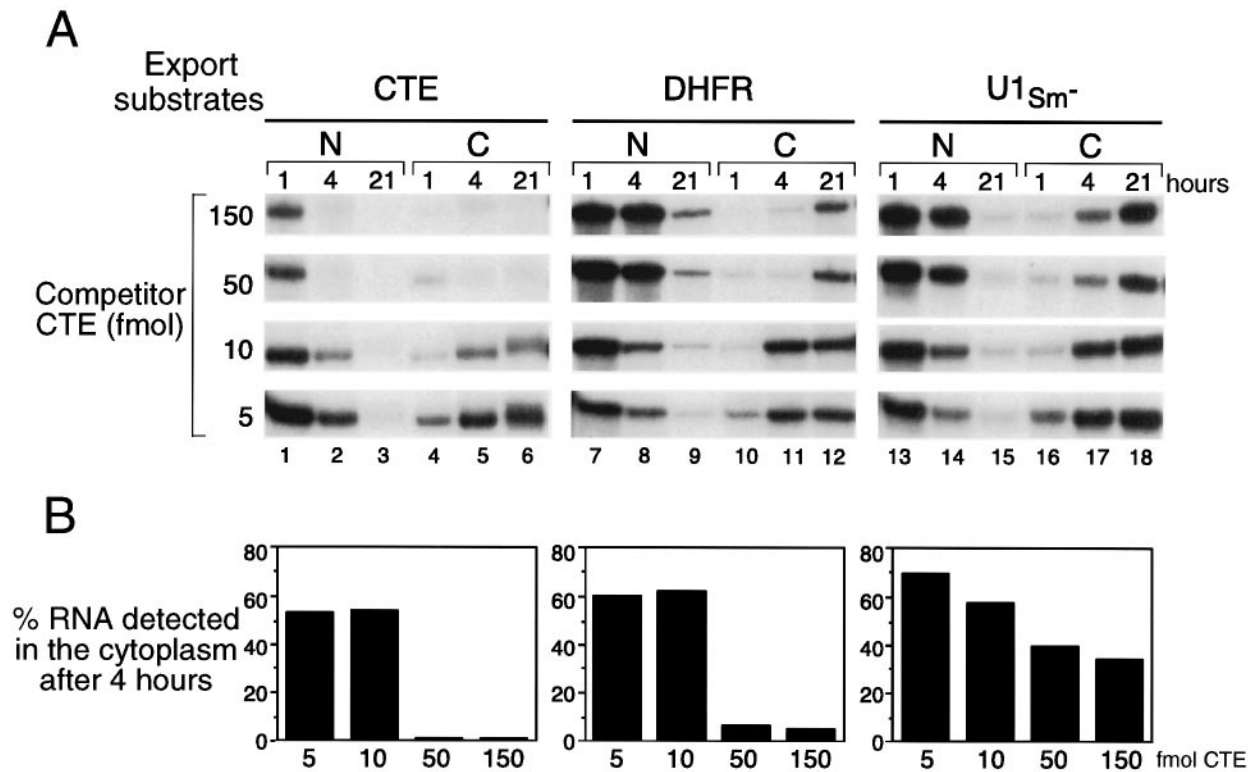
To determine if the titratable factors needed for export and stabilization of CTE RNA are the same or similar to those used by the RRE/Rev system, we examined the effects of Rev NES-peptide conjugates on the export of CTE RNA. Rev-mediated export of RRE-containing RNAs can be inhibited by conjugates consisting of multiple copies of Rev NES-peptides attached to bovine serum albumin (BSA); such peptide conjugates are thought to compete for nuclear components also needed for export

of snRNAs and 5S rRNA (Fischer *et al.*, 1995). As shown in Figure 2A, the conjugate containing the REV NES caused only a slight decrease in the export of CTE RNA (lanes 2 and 4), while strongly inhibiting export of U6RRE RNA (lanes 6 and 8) and U1<sub>Sm-</sub> snRNA (lanes 2, 4, 6 and 8); it was without effect on tRNA export (data not shown; see also Fisher *et al.*, 1995). The decrease in the rate of CTE RNA export indicates that the CTE- and Rev-mediated export pathways use some common components, but the incomplete nature of this inhibition is evidence that the two pathways are not congruent.

We tested if export of CTE RNA required nucleoporins that contain phenylalanine-glycine (FG) repeat domains; these proteins play an important role in the nuclear export of many RNAs and proteins (Bastos *et al.*, 1996; Stutz *et al.*, 1996; Dahlberg and Lund, 1997; Nakielny and Dreyfuss, 1997; Powers *et al.*, 1997). Co-injection of mAb414 antibodies, which are specific for the FXFG repeats (Davis, 1995), effectively blocked export of both the CTE RNA and the U6RRE RNA (Figure 2B), showing that the export pathways used by both of these RNAs require FXFG nucleoporins. As expected, the mAb414 antibodies inhibited export of U1<sub>Sm-</sub> snRNA but not of tRNA (Terns and Dahlberg, 1994; Dahlberg and Lund, 1997).

#### **Export of CTE RNA and specific types of cellular RNAs requires a common limiting factor(s)**

Members of various classes of RNAs, such as mRNAs, snRNAs and tRNAs, are exported by specific pathways, as determined by competition experiments (Jarmolowski *et al.*, 1994). To learn if the export pathways of CTE RNA and normal cellular mRNAs overlap, we measured the abilities of CTE RNA and mRNA to inhibit each other's export. As shown in Figure 3A, 50 fmol of CTE RNA was sufficient to inhibit its own export (lanes 1–6) as well as that of dihydrofolate reductase (DHFR) mRNA (lanes 7–12). Significantly, the excess CTE RNA appeared



**Fig. 3.** Export of mRNA is strongly inhibited by CTE RNA. (A) <sup>32</sup>P-labeled CTE, DHFR and U1<sub>Sm</sub><sup>-</sup> RNAs were co-injected with increasing amounts of unlabeled CTE RNA so that the final concentrations of CTE RNA were 5, 10, 50 or 150 fmol/oocyte. The oocytes were dissected 1, 4 and 21 h after injection, and the distributions of CTE (lanes 1–6), DHFR (lanes 7–12) and U1<sub>Sm</sub><sup>-</sup> RNAs were analyzed as in Figure 1. (B) PhosphorImager analysis was performed to quantitate the extents of RNA export at 4 h in the presence of increasing amounts of CTE RNA competitor; this analysis is expressed as the percentage of RNA detected in the cytoplasm [calculated as (C/C+N)×100%].

not to affect the nuclear stability of the DHFR mRNA, although, it destabilized labeled CTE RNA (as also seen in Figure 2B). In other experiments (see Figure 5B, below; data not shown) we found that as little as 25 fmol of CTE RNA was sufficient to block the export of both CTE RNA and mRNAs completely. Thus, CTE RNA very effectively titrates a factor(s) needed for the export but not the stability of DHFR mRNA. Export of a different mRNA (AdML) generated *in situ* by splicing of an injected pre-mRNA (AdML pre-mRNA) was also blocked by CTE RNA (see Figure 5B, below), indicating that the titrated factor is a general component of the mRNA export pathway.

Reversal of the block to mRNA export was very slow. A significant amount of DHFR mRNA was still present in the nuclear fractions 21 h after injection of 50 or 150 fmol CTE RNA (Figure 3A, lanes 7–12) even though very little CTE RNA was detectable in either the nucleus or cytoplasm 4 h after injection. The continued inhibition of mRNA export even in the absence of detectable amounts of full-length CTE RNA indicates that a cellular factor(s) needed for the mRNA export pathway has been sequestered or inactivated. It is unclear whether this factor is the one that is required for export and/or stabilization of CTE RNA itself.

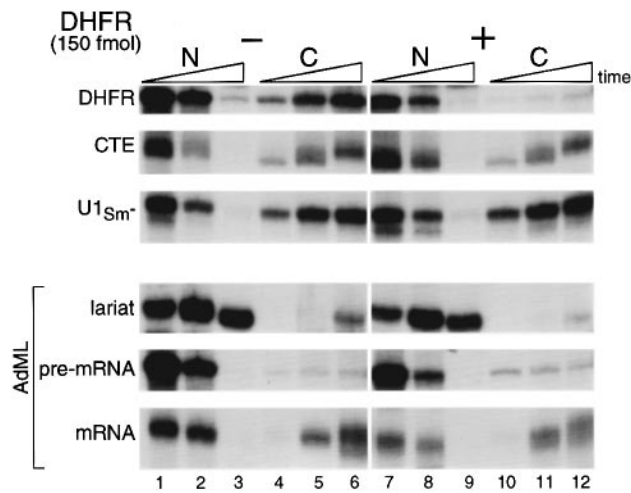
The excess CTE RNA had much less of an effect on the rate of export of U1<sub>Sm</sub><sup>-</sup> snRNA than on that of mRNA (lanes 13–18); likewise, it slowed but did not block export of U5 RNA (data not shown). The results of these competition experiments, quantified in Figure 3B, indicate

that CTE RNA functionally depletes oocyte nuclei of a factor(s) that is needed primarily for mRNA export.

#### **A saturating amount of DHFR mRNA does not interfere with the export of CTE RNA**

Because CTE RNA efficiently titrates a factor(s) required for mRNA export (Figure 3), we asked, conversely, if excess DHFR mRNA could compete for export of CTE RNA. Injection of 150 fmol of DHFR mRNA left export of CTE RNA virtually unaffected and did not interfere with export of U1<sub>Sm</sub><sup>-</sup> snRNA (Figure 4, top three panels) but, consistent with previous reports (Fischer *et al.*, 1995), it led to saturation of its own export. Thus, excess DHFR mRNA titrates a saturable factor required for its own export and stability but not for that of CTE RNA. If this factor is the one that is sequestered by excess CTE, the lack of reciprocity in competition might reflect a difference in binding affinities for the two RNAs; alternatively the factor could bind to CTE RNA but not be needed for the export of that RNA.

Surprisingly, saturation of DHFR mRNA export did not inhibit export of all mRNAs. As shown in the bottom three panels of Figure 4, AdML mRNA that was generated *in situ* by splicing was exported normally in the presence of excess DHFR mRNA (bottom panel, lanes 7–12). This difference may indicate that the factor(s) titrated by DHFR mRNA is required only for the export of mRNAs that are not spliced or that a variety of specific factors are used for export of different mRNAs. Thus, mRNAs can enter the export pathway at several different points.



**Fig. 4.** Excess DHFR mRNA does not interfere with export of CTE RNA or AdML mRNA.  $^{32}$ P-labeled DHFR, CTE, pre-AdML and U1<sub>Smr</sub> RNAs were co-injected into oocytes alone (lanes 1–6) or together with 150 fmol of unlabeled DHFR mRNA (lanes 7–12). Oocytes were dissected 1, 3.5 and 21 h after injection, and the RNAs in the nuclear and cytoplasmic fractions were analyzed as in Figure 1.

#### **Mutated CTE RNAs are exported but are weak inhibitors of mRNA export**

The assays in injected oocytes described above show that the CTE RNA can both direct its own export and compete for a titratable factor(s) needed for export of mRNAs. We therefore analyzed several mutated CTE RNAs that are unable to promote expression of unspliced mRNAs in transiently transfected mammalian cells (Ernst *et al.*, 1997b; our unpublished data). The sequence changes in these mutated RNAs (M1, M2, M2/M6, M2/M11, M4, M7 and M9) and a variant missing about half of the CTE ( $\Delta$ ) are shown in Figure 5A, along with a summary of their abilities to function in the expression assay.

The wild-type CTE has two regions that are almost identical in sequence and structure, and one of these regions remains intact in each case, except for the double mutant M2/M11. When tested directly for their ability to be exported from oocyte nuclei, all of the ApppG-capped mutated CTE RNAs except for M2/M11 and anti-CTE were exported with kinetics similar to those of wild-type CTE RNA (Figure 5A, right). These results indicate that one copy of the repeat suffices to promote export from oocyte nuclei. We note that the doubly mutated M2/M11 and the anti-CTE RNAs were unstable in the nucleus (but not in the cytoplasm; data not shown). Nevertheless, sufficient amounts of these RNAs were present in the nucleus to have served as a source of export substrate within the first hour after injection. Thus, RNAs lacking both copies of the repeat are apparently unable to bind one or more proteins that participate in the stabilization and export of CTE RNA.

The mutated CTE RNAs that are exported from oocyte nuclei differ from the wild-type RNA in their abilities to inhibit export of themselves or of mRNAs. For example, co-injection of 25 fmol of M2 (Figure 5B) or M7 CTE RNAs (not shown) was without significant effect on the export of DHFR and AdML mRNAs (compare lanes 1–6 with 7–12); likewise, 25 fmol of the mutated CTE RNAs were almost without effect on their own stability and export (lanes 7–12). In contrast, the same amount of wild-

type CTE RNA severely affected export of both mRNAs, caused instability of itself and saturated its own export (compare lanes 13–18 with 19–24). However, when the amount of mutated CTE RNAs injected was increased from 25 to 100 fmol (Figure 5C), the mutated RNAs exhibited about the same degree of mRNA export inhibition and self-destabilization as the wild-type CTE RNA, indicating that they interact with the limiting factor(s), but do so with lower efficiency.

#### **CTE directs export of chimeric RNAs from oocyte nuclei**

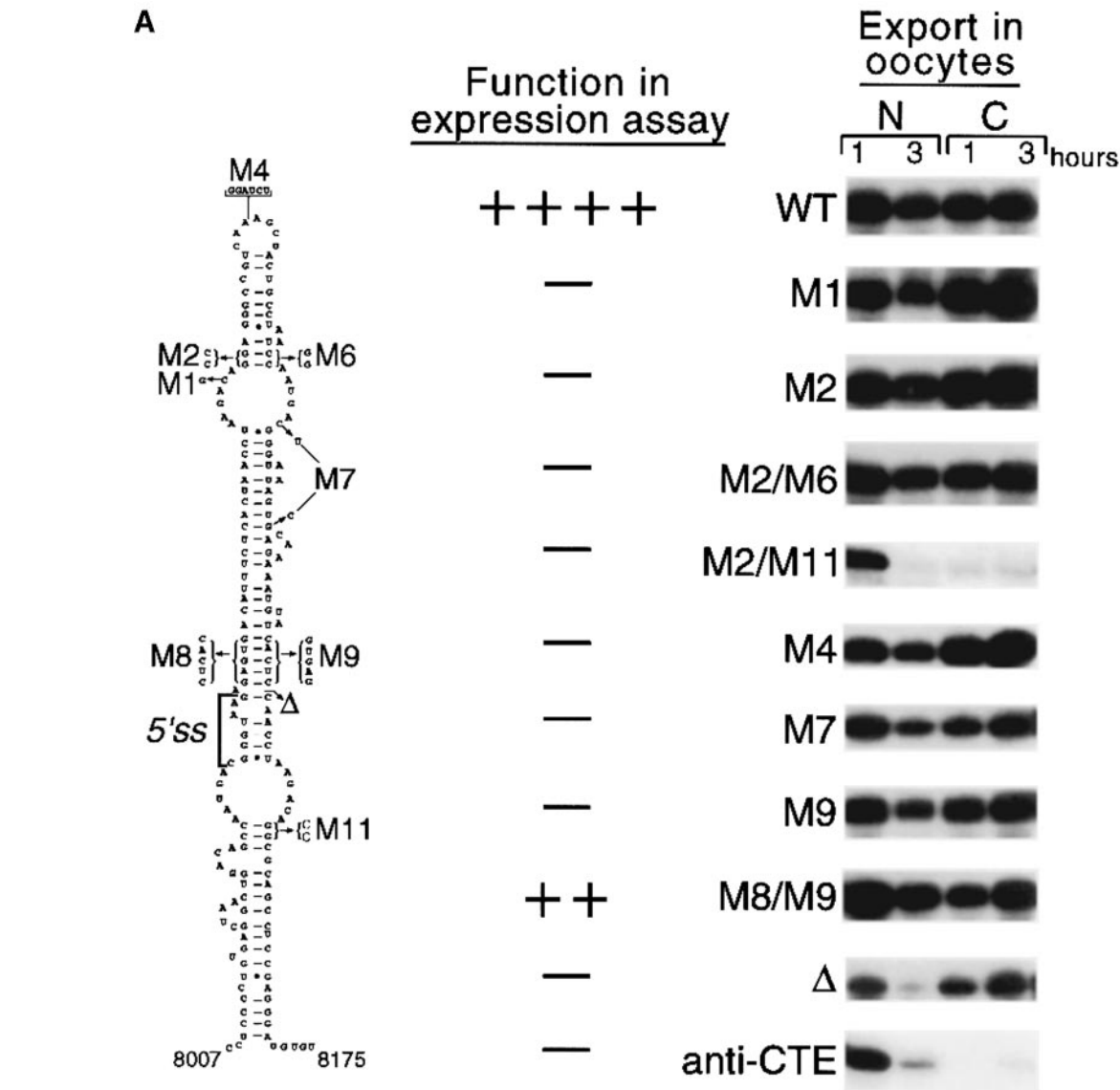
During MPMV infection, both spliced and intron-containing mRNAs must be exported (Hammarskjöld, 1997). The CTE supports export of pre-mRNA that accumulates due to suboptimal splice sites (Ernst *et al.*, 1997a). To determine if the CTE could promote export of unspliced mRNAs from oocyte nuclei, we used a variant AdML pre-mRNA lacking a 5' splice site (AdML $\Delta$ 5'ss, Figure 6A); this RNA normally is retained in partially assembled spliceosomes (Vankan *et al.*, 1992) and is not exported (Hamm and Mattaj, 1990). We made m<sup>7</sup>GpppG-capped chimeric RNAs containing either the CTE or, as a control, the M2/M11 doubly mutated form of the CTE (Figure 5A), inserted near the 5' end of the AdML $\Delta$ 5'ss transcript (Figure 6A).

Unexpectedly, both of the m<sup>7</sup>GpppG-capped chimeric RNAs were spliced efficiently, producing stable intron-containing lariats (Figure 6B). Apparently, a sequence in the CTE, probably (C)-G-G-G-U-A-A-G- denoted as 'cryptic 5'ss' in Figure 5A, was used as a 5' splice site, substituting for the site that had been deleted from the AdML $\Delta$ 5'ss pre-mRNA (see Figure 6A). Utilization of this sequence as a splice site would result in inclusion of the top half of the CTE or mutated CTE within the stable lariats. The excised lariat containing the wild-type CTE was exported efficiently, with kinetics resembling those of the CTE RNA alone (Figure 6B, lanes 6–9); however, the lariat containing the doubly mutated M2/M11 version of the CTE remained in the nucleus (lanes 15–18). Export of the CTE-containing lariat was by the CTE-mediated pathway, since it was inhibited by co-injection of 40 fmol of non-labeled CTE (Figure 6C, lanes 14–17). This lack of export of the stable CTE-containing lariat shows that an export factor was being sequestered by the non-labeled CTE RNA competitor.

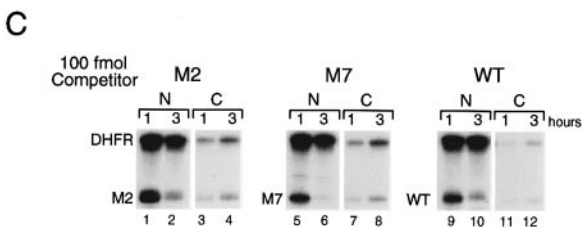
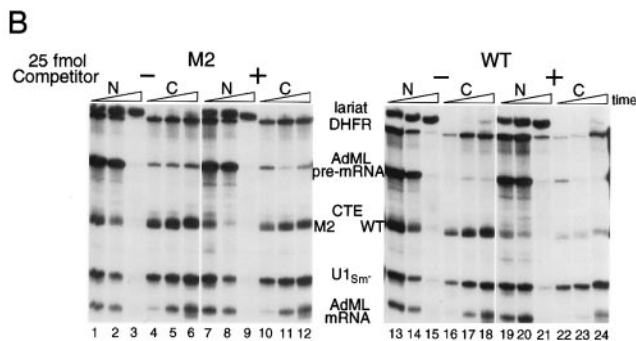
Nuclear injection of the m<sup>7</sup>GpppG-capped chimeric RNAs also resulted in the appearance of a small amount of non-spliced transcript in the cytoplasm in a time-dependent manner (Figure 6B, lanes 6–9 versus 15–18). This cytoplasmic RNA was detected only when the RNA contained the wild-type CTE; however, the efficient splicing to the cryptic site, particularly in the mutated CTE (see Discussion), reduced the pool of intranuclear pre-mRNAs, making it difficult to assess the significance of this observation.

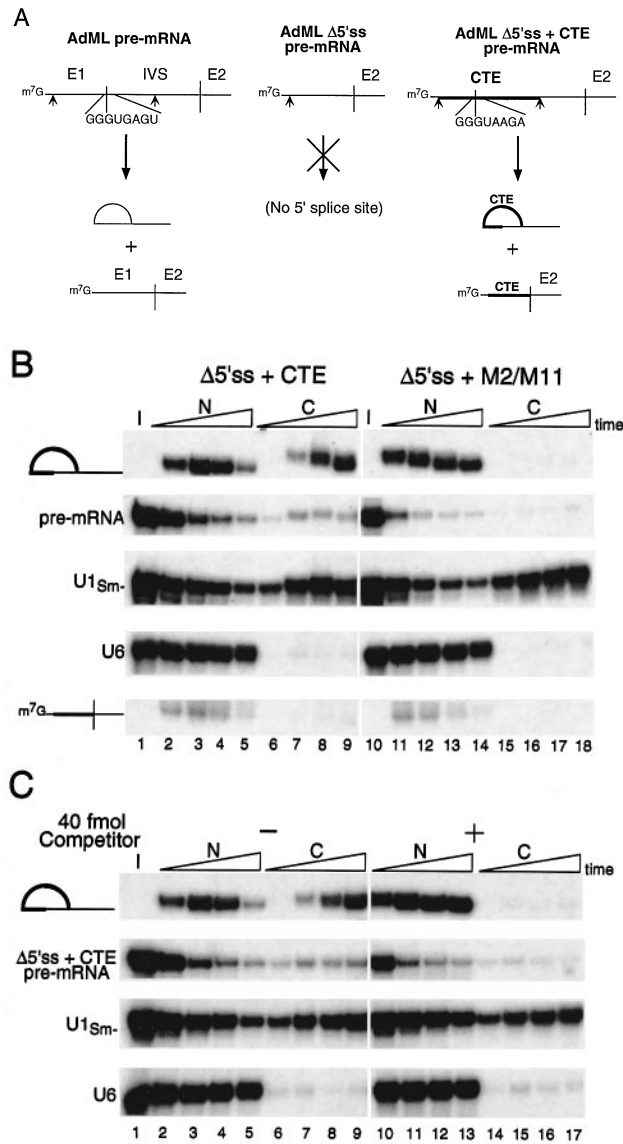
#### **The CTE selectively binds two nuclear proteins**

We examined directly if specific proteins could be isolated based on their abilities to bind CTE. When mixed with extracts of Cos cell nuclei, CTE RNA bound several proteins (Figure 7A). To distinguish between specific and non-specific binding, we also used as target RNA a random



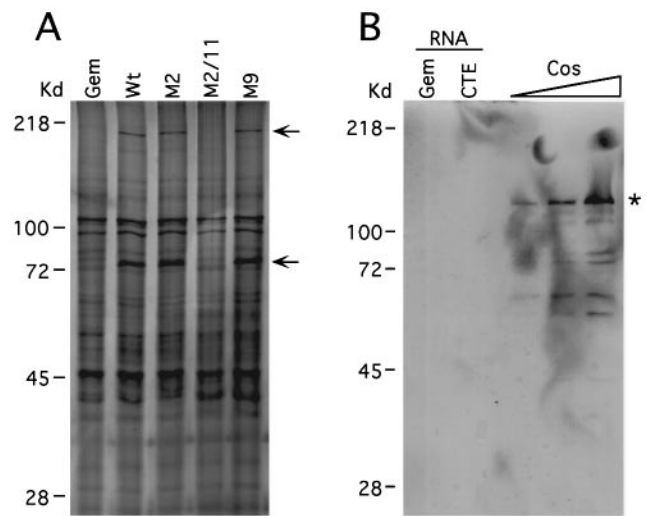
**Fig. 5.** Many mutated CTE RNAs are exported but unable to compete efficiently for limiting factors. (A) Structure and function of wild-type and mutated CTEs. The sequence and structure of the MPMV CTE (positions 8007–8175) and the sites of mutations are shown on the left (Ernst *et al.*, 1997b). The putative 5' splice site (cf. Figure 6A) is indicated by a bracket and denoted 5'ss. Double mutants contain both of the indicated sequence alterations. M4 contains the indicated insertion, the Δ CTE RNA lacks sequences on the 3' side of the arrow and anti-CTE contains the complement of the CTE sequence. The middle column summarizes the abilities of the wild-type and mutated CTE sequences to substitute for RRE/Rev-mediated expression from an unspliced construct in mammalian cells (Ernst *et al.*, 1997b; our unpublished data). Analyses of the export of the <sup>32</sup>P-labeled CTE RNAs are shown on the right. Export was assayed at 1 and 3 h after RNA injection, as in Figure 1A. (B) Mutated CTE RNAs are inefficient competitors of mRNA export. <sup>32</sup>P-labeled DHFR, pre-AdML and U1<sub>Sm</sub> RNAs were co-injected into oocytes together with labeled M2 CTE RNA (lanes 1–6), labeled M2 CTE RNA plus 25 fmol of unlabeled M2 CTE RNA (lanes 7–12), labeled wild-type CTE RNA (lanes 13–18) or labeled wild-type CTE RNA plus 25 fmol of unlabeled wild-type CTE (lanes 19–24). Export was analyzed at 1, 3.5 and 24 h following injection. In all cases, the labeled CTE RNAs contributed ~3.5 fmol per oocyte. (C) High levels of mutated CTE RNAs compete for mRNA export. <sup>32</sup>P-labeled DHFR mRNA was co-injected with labeled M2 CTE RNA, M7 CTE RNA or wild-type CTE RNA plus 100 fmol of the individual unlabeled RNAs; oocytes were dissected 1 and 3 h after injection.





**Fig. 6.** CTE RNA directs export of chimeric RNAs. (A) Structure of AdML- and CTE-containing pre-mRNAs, and their splicing products. The splice sites (thin vertical lines) and splicing products of AdML (Hamm and Mattaj, 1990) and AdML $\Delta$ 5'ss + CTE pre-mRNAs are shown. Upward pointing arrows indicate the *Hind*III cleavage sites used to generate the AdML $\Delta$ 5'ss and AdML $\Delta$ 5'ss + CTE DNA templates. Both AdML and CTE 5' splice site sequences conform to the consensus sequences -A-G<sup>2</sup>-G-U-R-A-G-U- (Senapathy *et al.*, 1990). (B) Splicing and export of RNAs containing CTE sequences. <sup>32</sup>P-labeled  $\Delta$ 5'ss + CTE or  $\Delta$ 5'ss + M2/M11 RNAs were co-injected with U1<sub>Sm</sub>- and U6 RNAs into oocyte nuclei. Oocytes were dissected 1, 2.5, 4 or 7 h after injection, and distributions of the RNAs were analyzed as described in Figure 1. The RNAs in the injected samples (I) are shown in lanes 1 and 10. The CTE-containing lariat introns and spliced exons are indicated by the symbols used in (A). (C) Titration of a factor required for CTE-directed export of chimeric RNA. <sup>32</sup>P-labeled  $\Delta$ 5'ss + CTE, U1<sub>Sm</sub>- and U6 RNAs were co-injected into oocyte nuclei in the absence (-) or presence (+) of 40 fmol of unlabeled CTE RNA, and RNA export was assayed as in (B).

transcript made from the pGEM vector (Gem). Proteins of apparent mol. wts of 190 and 85 kDa bound specifically to wild-type CTE RNA and to the exportable M2 and M9 CTE RNAs but not to the non-specific control RNA. Significantly, neither of these proteins bound to the non-exportable M2/M11 CTE RNA. The specificity of binding



**Fig. 7.** Specific proteins bind CTE RNA. (A) CTE RNA selectively binds two proteins from Cos cell nuclear extract. Nuclear extract (250  $\mu$ g) was incubated with RNA-beads containing the indicated wild-type, mutated CTE (M2, M2/M11, M9) or control (Gem) RNAs. The bound proteins were eluted as described in Materials and methods and analyzed on a 12% SDS-polyacrylamide gel which was silver stained. The arrows indicate CTE-specific binding proteins of ~85 and 190 kDa. (B) CTE RNA does not bind RNA helicase A. Proteins eluted from control (Gem, lane 1) or wild-type CTE (lane 2) RNAs (as in A) were assayed for the presence of RNA helicase A by immunoblotting with the same antibody that was used by Tang *et al.* (1997). As a source of marker for RNA helicase A (\*), 5, 10 or 20  $\mu$ g of Cos cell nuclear extract was also analyzed.

with functional CTE RNAs leads us to propose that these two proteins are important in the export of CTE RNA. None of the bound proteins had the size or immunological reactivity of RNA helicase A (Lee and Hurwitz, 1993; Figure 7B), a protein that had been reported to bind to CTE-containing RNA (Tang *et al.*, 1997).

## Discussion

The CTE of MPMV is a *cis*-acting RNA element that promotes export of incompletely spliced mRNAs from the nucleus (Bray *et al.*, 1994; Ernst *et al.*, 1997a). Here, we studied the role of the CTE in export, by injecting a short RNA, whose only known export element was the CTE, into the nuclei of *Xenopus* oocytes. Our results indicate that the isolated CTE binds a limiting cellular factor(s), thereby removing the factor(s) from the pool that is available for the export of mRNAs. We propose that in the context of an intron-containing viral pre-mRNA, the CTE recruits a cellular factor(s) specifically needed for release of mRNAs from the splicing machinery and, hence, for their export to the cytoplasm.

Although both the CTE of MPMV and the RRE of HIV-1 facilitate export of unspliced pre-mRNAs (reviewed by Hammarskjöld, 1997), these two retroviral RNA elements must use distinct mechanisms, since the CTE depends exclusively on endogenous cellular factors, whereas the RRE also requires the viral-encoded Rev protein (Emerman *et al.*, 1989; Felber *et al.*, 1989; Hammarskjöld *et al.*, 1989; Malim *et al.*, 1989a,b; Fischer *et al.*, 1994). Also, as we show here, the two export pathways do not appear to compete for a limiting cellular factor; excess CTE RNA has no appreciable effect on the

Rev-dependent export of the chimeric U6RRE RNA (Figure 1B), and a conjugate containing multiple copies of the NES of Rev (Fischer *et al.*, 1995) does not block export of CTE RNA effectively (Figure 2A). Recently, similar results were obtained using a homologous CTE (93% identical to MPMV) of simian retrovirus type-1 (SRV-1) (Saavedra *et al.*, 1997). However, the CTE and RRE/Rev export pathways do merge at some point since they are both inhibited by mAb414 (Figure 2B), which is specific for FXFG repeats present in multiple proteins of the NPC (Davis, 1995). We note that the inhibitory effect of mAb414 extends to most nuclear export pathways, including those for snRNAs, mRNAs and rRNAs, but not tRNAs (Terns and Dahlberg, 1994; Dahlberg and Lund, 1997).

Additional evidence for independent export pathways used by the CTE and RRE/Rev systems has been presented by Zolotukhin and Felber (1997), who observed that overexpression of Ran-binding protein 1 (RanBP1), both with and without its NES (Richards *et al.*, 1996), interfered with Rev but not CTE function. However, this difference may also reflect a need for import of Rev protein into the nucleus, which could be blocked due to interference with the Ran GTPase system.

The CTE is a very effective competitor for export of mRNAs, regardless of whether or not they are subject to splicing (Figures 3 and 5B; data not shown). Surprisingly, inhibition of mRNA export persists even after CTE RNA is no longer detectable in the nucleus, indicating that a factor(s) essential for export of mRNAs has been sequestered or inactivated by the CTE. Because the factor can be titrated by relatively low amounts of CTE RNA (Figures 3 and 5B), it may normally interact only transiently with mRNAs. Differences in affinities of the factor for CTE RNA versus cellular mRNAs could account for the lack of reciprocity in the competition between CTE RNA and DHFR mRNA (Figure 4). We note that excess DHFR mRNA also does not compete for export of spliced AdML mRNA, indicating that mRNAs can enter the export pathway at different points.

Unexpectedly, most of the mutated CTEs are exported very efficiently from oocyte nuclei (Figure 5A) even though they are unable to support expression of unspliced mRNAs in cultured cells (Ernst *et al.*, 1997b). Because these mutated CTEs are less effective inhibitors of mRNA export (Figure 5B), it is likely that the mutations diminish but do not abolish binding of the titratable factor to the RNA. As with cellular mRNAs, the lowered affinity of the factor for the mutated RNAs would destabilize the interaction, and thereby reduce the ability of the RNA to sequester the factor. Consequently, the mutated CTE RNAs would be able to prevent mRNA export only when present at relatively high levels (Figure 5C).

The structure of the wild-type CTE has a pseudo 2-fold axis of symmetry (Figure 5A; Taberero *et al.*, 1996; Ernst *et al.*, 1997b), which could support binding of two molecules of a factor (individually or as a dimer) to the duplicated binding sites, thereby allowing for cooperative RNA-protein and protein-protein interactions. Similar interactions have been shown to be important in the stabilization of other RNA-protein interactions (Witherell *et al.*, 1990; van Gelder *et al.*, 1993). Because all of the mutated CTEs that were exported still had one of these

sites intact, they could still bind one molecule of the factor but cooperativity would be lost.

The CTE, like the RRE of HIV-1, promotes export of incompletely spliced mRNAs (Ernst *et al.*, 1997a), a process that requires release of the pre-mRNA from the splicing machinery (Chang and Sharp, 1989; Lu *et al.*, 1990; Kjems *et al.*, 1991; Hammarskjöld *et al.*, 1994). In the case of the RRE, this process is dependent on the binding of multiple copies of the Rev protein (Olsen *et al.*, 1990; Huang *et al.*, 1991; Malim and Cullen, 1991; Zapp *et al.*, 1991). Likewise, binding of the titratable cellular factor to the CTE may lead to release of intron-containing pre-mRNAs into an export pathway and, as with Rev, efficient release from spliceosomes may require a tight association between the CTE and the factor, promoted through the cooperative binding discussed above.

In the oocyte system used here, the CTE appeared to direct export of an unspliced pre-mRNA, but with low efficiency (Figure 6B). Unexpectedly, we discovered that the CTE contains a cryptic 5' splice site (Figure 6A) that can substitute for the deleted site in pre-AdML $\Delta$ 5' ss mRNA. It is unclear if this site contributes to the function of the CTE during release of pre-mRNAs from spliceosomes; however, we note that extensive complementarity with U1 snRNA exists in this region. It may also be significant that the rate of splicing was reduced with wild-type CTE alone, when compared with the mutated CTE or the wild-type CTE in the presence of competitor CTE (compare Figure 6B lanes 2 and 3 with lanes 11 and 12, or Figure 6C lanes 2 and 3 with lanes 10 and 11). These differences in efficiency of splicing indicate that the binding of CTE-specific factors may interfere with splicing; again, this effect may contribute to the role of the CTE in inhibition of splicing and the export of non-spliced pre-mRNAs.

The excised lariat generated from the cryptic splice site contained sufficient CTE sequences to support nuclear export, using a pathway normally accessed by this element (Figure 6). However, the doubly mutated form of the CTE, which supplied the same cryptic splice site, was unable to direct export of the resulting lariat. Because the CTE cryptic splice site overlaps one of the presumptive export factor-binding sites (discussed above), the excised lariat RNA should contain only the site located at the top of the stem (see Figure 5A), showing that a single site is sufficient to support export of this chimeric RNA.

Most of the mutated CTEs tested here have been shown not to support expression of unspliced RNAs in transfected mammalian cells (Ernst *et al.*, 1997b; our unpublished data). Nevertheless, those that contained at least one presumptive factor-binding site could be exported from oocyte nuclei and were able to titrate a factor needed for export of cellular mRNAs (Figure 5). Thus, there is an apparent contradiction between the activities of the mutated CTEs in the two assays. Several possible explanations can be considered. First, the expression assay in mammalian cells may measure a step in addition to export; second, oocytes might have a higher level of CTE-binding factors which could support productive interaction with mutated CTEs; and third, the RNA export substrates used in the transfected mammalian cells contained an RRE of HIV-1 which, in the absence of the Rev protein, may act as a nuclear retention element (Brighty and Rosenberg, 1994)



and, thus, require a stronger release signal. Experiments to differentiate between these alternatives are in progress.

The nature and function of the factor(s) recruited and sequestered by CTE RNA remain unclear. Although export of both spliced and non-spliceable mRNAs is inhibited by an excess of CTE RNA, the stabilities of the mRNAs are not affected appreciably (Figures 3A and 5B). Thus, CTE RNA does not sequester a factor that is required for stabilization of the mRNAs. Rather, the factor may interact transiently with the mRNAs, for example during their mobilization for export (Grimm *et al.*, 1997b).

We have detected specific binding of at least two proteins to the wild-type CTE and to mutated CTEs M2 and M9, which support export, but not to a mutated form (M2/M11) that is not exported (Figure 7A). The identities of these proteins and their possible roles in CTE RNA export are under investigation. Perhaps one of these is an RNA helicase akin to those implicated in the release of spliced mRNAs from the splicing machinery (Company *et al.*, 1991; Liang *et al.*, 1996; Ohno and Shimura, 1996). However, Western blotting of the specifically bound proteins described here indicates that neither is RNA helicase A, which has been implicated as a CTE-binding factor by Tang *et al.* (1997).

If the CTE in viral mRNAs has the capacity to sequester factors needed for export of mRNAs, infection by type D retroviruses should rapidly lead to cell death. However, that does not occur, leading us to question how such sequestration is avoided. One plausible explanation is that sequences in a CTE-containing mRNA, which are not present in the CTE RNA alone, could destabilize this interaction. That could occur either through direct competition with additional nuclear RNA-binding proteins or through displacement by ribosomes during translation. In either case, release of the factor(s) would allow for its continued participation in the export of cellular or additional viral mRNAs.

## Materials and methods

### DNA templates for *in vitro* RNA synthesis

Templates for *in vitro* transcription of wild-type CTE RNAs (CTE<sub>566</sub>, MPMV nucleotides 8022–8205; and CTE<sub>250</sub>, MPMV nucleotides 8007–8240; both of these wild-type CTE RNAs behave identically in export and inhibition experiments) and mutated (M1, M2, M2/M6, M2/M11, M9, M8/M9, nucleotides 8022–8205; M4, nucleotides 8022–8175; and M7, nucleotides 8007–8240) or truncated sequence CTE RNAs ( $\Delta$ , 8007–8140) are described elsewhere (Ernst *et al.*, 1997b). The template for transcription of the antisense CTE sequence (anti-CTE) was generated by digestion of the pGEM-CTE566 (Ernst *et al.*, 1997b) plasmid with *Xba*I. The templates for transcription of AdML pre-mRNA (Hamm and Mattaj, 1990; Powers *et al.*, 1997) and DHFR mRNA (Kambach and Mattaj, 1992) were generated by linearization of previously described plasmids. Other templates were DNA fragments generated by PCR amplification as described for U1<sub>Sm</sub>- and U6 snRNAs (Terns *et al.*, 1993), U3 snRNA (Terns and Dahlberg, 1994), U5 snRNA (Pasquinelli *et al.*, 1995), mU6RRE 2.8 (U6RRE) (D.Young, E.Lund and M.Zapp, in preparation) and *X.laavis* tRNA<sup>Tyr</sup> (Gouilloud and Clarkson, 1986; E.Lund and J.E.Dahlberg, in preparation). The AdML $\Delta$ 5'ss pre-mRNA template was created from the SP6-AdML pre-mRNA template (Powers *et al.*, 1997) as described previously (Hamm and Mattaj, 1990) by deletion of a *Hind*III fragment, which eliminated most of the first exon and the 5' splice site. The AdML $\Delta$ 5'ss + CTE and AdML $\Delta$ 5'ss + M2/M11 pre-mRNA templates were generated by insertion of PCR-amplified wild-type or M2/M11 CTE sequences 8022–8175 (Ernst *et al.*, 1997b) flanked by *Hind*III sites into the *Hind*III site at the 5' end of the AdML $\Delta$ 5'ss sequences (see Figure 6A). The orientation of the inserted

fragments was verified by digestion with *Fok*I. Templates for transcription of the various AdML $\Delta$ 5'ss constructs were generated by linearization with *Sac*I.

### *In vitro* transcription

*In vitro* synthesis of RNAs labeled with [ $\alpha$ -<sup>32</sup>P]GTP was performed as described (Melton *et al.*, 1984; Pasquinelli *et al.*, 1995) using T7 RNA polymerase (for wild-type and mutated CTE, U6RRE, U6 and DHFR RNAs) or SP6 RNA polymerase (for tRNA<sup>Tyr</sup>, U1<sub>Sm</sub>-, U5, anti-CTE, pre-AdML, AdML $\Delta$ 5'ss + CTE and AdML $\Delta$ 5'ss + M2/M11 RNAs) according to the manufacturer's conditions (Promega). The CTE and anti-CTE RNAs were synthesized with ApppG or m<sup>7</sup>GpppG caps; the U6 and U6RRE RNAs were made with  $\gamma$ -mpppG caps; the DHFR, U1<sub>Sm</sub>-, U5, pre-AdML, AdML $\Delta$ 5'ss + CTE and AdML $\Delta$ 5'ss + anti-CTE RNAs were made with m<sup>7</sup>GpppG caps (Terns *et al.*, 1993). Unlabeled ApppG-capped CTE RNA and m<sup>7</sup>GpppG-capped DHFR RNAs were synthesized *in vitro* as described (Grimm *et al.*, 1997a) according to Maxiprep conditions (Promega).

### Analysis of RNA export in *Xenopus* oocytes

Preparation and injection of *X.laavis* stage V and VI oocytes were performed as previously described (Terns *et al.*, 1993; Pasquinelli *et al.*, 1995). Mixtures (15 nl) containing 1–5 fmol of individual <sup>32</sup>P-labeled RNAs along with unlabeled RNAs at the concentrations indicated in the figure legends were injected into oocyte nuclei or cytoplasm. U6 or U3 snRNA, which normally are not exported to the cytoplasm (Vankan *et al.*, 1990; Terns *et al.*, 1993; Terns and Dahlberg, 1994), and blue dextran (Jarnolowski *et al.*, 1994) were included in all injection mixtures as controls for the accuracy of nuclear injections and oocyte dissections. At the time points indicated in the figures, the oocytes were dissected under oil (Lund and Paine, 1990) and the nucleocytoplasmic distributions of the RNAs were analyzed by polyacrylamide gel electrophoresis as previously described (Pasquinelli *et al.*, 1995).

In experiments that included co-injection of recombinant Rev protein, RNA mixtures in Rev binding buffer (50 mM Tris pH 7.9, 50 mM KCl) were incubated on ice at a molar ratio of 10:1 Rev protein to U6RRE RNA prior to injection (D.Young, E.Lund and M.Zapp, in preparation). Rev NES-peptide conjugates were similar to those used by Fischer *et al.* (1995) (CLPLERLTL for co-injection with CTE RNA; CLPLERLTL for co-injection with U6RRE + Rev protein; the amino acid sequence of the control peptides was CTKNILDGALKLALNE) and were injected at concentrations of ~130 ng/oocyte nucleus. mAb414 antibodies (Davis, 1995; a gift from D.Goldfarb) were co-injected with RNA-Rev protein mixtures at a concentration of 150 ng/per oocyte as described (Terns and Dahlberg, 1994).

### Preparation of nuclear extracts

The nuclear extracts of Cos cells used in Figure 7A and B were prepared essentially as described by Dignam *et al.* (1983).

### Immunoblot analysis

Western blot analysis was performed as described previously (Hammarskjöld *et al.*, 1989) with the following modifications. An RNA helicase A-specific polyclonal rabbit antiserum was used as primary antibody at a dilution of 1:1000 (a kind gift from Dr Jerard Hurwitz of the Memorial Sloan-Kettering Cancer Center, New York). Anti-rabbit IgG conjugated with alkaline phosphatase was used as a secondary antibody (1:1000 dilution, Sigma, A-8025). The membrane was washed and developed using enhanced chemifluorescence (ECF) substrate (Amersham, RPN 5785) according to the manufacturer's recommendations and was analyzed using a Molecular Dynamics FluorImager 595 with ImageQuant analysis software.

### Protein purification by RNA affinity

This procedure was based on the methods of Smith *et al.* (1991) and Yeakly *et al.* (1996) with some modifications. *In vitro* transcribed wild-type or mutated CTE RNAs (nucleotides 8022–8205) or a control RNA (283 nucleotides) prepared from pGEM7Zf(-) (Promega) digested with *Hha*I were attached to ultralinked immobilized streptavidin beads (Pierce 53117) via a 5'-biotinylated 2'-O-methyl oligoribonucleotide complementary to the 3' end of the *in vitro* transcribed RNA. Cos cell nuclear extract (250  $\mu$ g) was added to the bead-RNA complex. The beads were pelleted and washed extensively with a buffer consisting of 20 mM HEPES-NaOH, pH 7.6, 3 mM MgCl<sub>2</sub>, 5% glycerol, 1 mM dithiothreitol and 300 mM KCl. A final wash with buffer lacking KCl was performed before the bound proteins were eluted by boiling in 1 $\times$  SDS-polyacrylamide gel sample buffer.

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