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Analysis of the RNA Binding Specificity of the Human Tap Protein, a Constitutive Transport Element-Specific Nuclear RNA Export Factor

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The human Tap protein has been proposed to mediate Mason Pfizer monkey virus constitutive transport element (CTE)-dependent nuclear RNA export and may also play a role in global mRNA export. Here, we have used *in vivo* assays, in both yeast and human cells, together with *in vitro* assays, to further characterize the RNA binding properties of Tap, which has been proposed to contain a novel leucine-rich RNA binding motif. Using the yeast three hybrid assay, we selected RNA molecules that retain Tap binding activity from a pool of randomized CTE sequences. The recovered RNA sequences differed only minimally from the wild-type CTE yet all displayed lower affinity for Tap both *in vivo* and *in vitro.* Analysis of the RNA export activity of the recovered CTE variants revealed that Tap affinity was highly predictive of CTE biological activity. Together, these observations provide additional evidence supporting the identification of Tap as the direct cofactor for CTE function and demonstrate that RNA binding by Tap is highly sequence specific.

INTRODUCTION

While considerable progress has recently been made in identifying and dissecting the various nuclear export pathways used by noncoding RNA molecules, the pathway utilized by cellular mRNAs remains poorly understood (reviewed by Izaurralde and Adam, 1998; Stutz and Rosbash, 1998). At present, there are at least two possible candidates as mediators of mRNA export in human cells. The first of these is a highly expressed group of proteins termed the heterogeneous nuclear ribonucleoproteins (hnRNPs), of which the prototype is hnRNPA1. The hnRNPs are known to bind to mRNAs in the nucleus and some, but not all, hnRNPs then accompany these mRNAs from the nucleus to the cytoplasm (Pinol-Roma and Dreyfuss, 1992). This protein nucleocytoplasmic shuttling is not passive, in that several hnRNPs have been shown to contain a nuclear export signal (NES) that functions independently of both RNA synthesis and RNA binding (Michael *et al.,* 1995, 1997). The NES present in hnRNPA1, termed M9, also acts as a nuclear localization signal (NLS) and these two activities cannot be mutationally segregated (Michael *et al.,* 1995; Bogerd *et al.,* 1999). Nevertheless, it appears unlikely that the transportin nuclear import factor, which is known to mediate M9 NLS function (Pollard *et al.,* 1996; Fridell *et al.,* 1997), is also the mediator of M9 NES activity (Izaurralde *et al.,* 1997b; Bogerd *et al.,* 1999). While the target of the M9 NES is therefore presently unknown, its importance for mRNA export is suggested by the finding that nuclear injection of high levels of wild-type hnRNPA1, but not of an M9 mutant, can specifically block mRNA export (Izaurralde *et al.,* 1997a).

A second candidate human mRNA export factor is Tap, a protein that has been proposed (Grüter *et al.,* 1998) to mediate the sequence-specific nuclear export of RNAs bearing the constitutive transport element (CTE) originally described in Mason–Pfizer monkey virus (MPMV) (Bray *et al.,* 1994). A role for Tap in general mRNA export is suggested by the finding that the inhibition of both CTE-dependent and general mRNA export that is induced by nuclear injection of high levels of the CTE (Pasquinelli *et al.,* 1997; Saavedre *et al.,* 1997) can be rescued by injection of recombinant Tap protein (Grüter *et al.,* 1998). A role for human Tap in nuclear mRNA export is also implied by the observation that the yeast homolog of Tap, termed Mex67p, is critical for mRNA export in yeast cells. Importantly, Mex67p has been found to be associated with bulk yeast poly(A)$^+$ RNA, thus suggesting that Mex67p might bridge the interaction between mRNAs and a nuclear export factor (Segref *et al.,* 1997; Santos-Rosa *et al.,* 1998). Consistent with this hypothesis, Tap has been shown to contain a novel leucine-rich RNA binding domain (Braun *et al.,* 1999) and also a novel type of NES that is critical for Tap function (Kang and Cullen, 1999). However, the target for this NES remains to be defined.

Although Tap has been shown to bind the CTE specifically both *in vitro* and *in vivo* (Grüter *et al.,* 1998; Braun *et al.,* 1999; Kang and Cullen, 1999), the RNA binding

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specificity of Tap has remained largely undefined and it has remained unclear whether Tap has both specific and nonspecific RNA binding properties. In this manuscript, we have attempted to shed light on the RNA binding properties of Tap by randomizing bases in the critical CTE loop sequence that serves as the Tap binding site and then using the yeast three hybrid protein:RNA interaction assay (Sengupta *et al.,* 1996) to select for RNAs that retain Tap binding. Interestingly, while we were able to identify several CTE variants that retained Tap binding, the wild-type CTE sequence clearly bound Tap with the highest affinity. Importantly, the ability of these CTE variants to bind Tap was found to closely correlate with their ability to support nuclear RNA export. Together, these data therefore shed light on the RNA binding properties of the novel leucine-rich RNA binding domain present in Tap and provide an additional line of evidence supporting the identification of Tap as the CTE-specific nuclear RNA export factor.

RESULTS

The MPMV CTE nuclear RNA export signal has been shown to form an extended RNA stem–loop structure bearing two identical 12-nucleotide (nt) internal loops, designated loop A and loop B, that are oriented at 180° relative to one another (Fig. 1) (Tabernero *et al.,* 1996; Ernst *et al.,* 1997). The integrity of both of these loops is critical for CTE function while flanking helical sequences appear to serve a primarily structural role. Both *in vitro* data and *in vivo* data obtained in mammalian cells indicate that a single CTE loop is sufficient for specific binding by Tap, a result that has yet to be reconciled with the observation that two loops are required for CTE activity in primate cells (Tabernero *et al.,* 1996; Ernst *et al.,* 1997; Grüter *et al.,* 1998; Kang and Cullen, 1999).

Tap binds the CTE specifically in the yeast three hybrid assay

While the interaction between the CTE and Tap has been shown to be specific, the nucleotides involved in RNA recognition by Tap have remained undefined. Previously, we have used a strategy of sequence randomization followed by genetic selection in yeast cells to determine the protein sequence determinants required for the biological activity of both leucine-rich NESs and the M9 NLS/NES (Bogerd *et al.,* 1996, 1999), and we have used a similar strategy to define the DNA sequence requirements for DNA binding by the Bel-1 transcriptional activator (Kang and Cullen, 1998). We therefore decided to use a similar strategy to more clearly define the RNA sequence requirements for Tap binding and to derive a set of CTE variants that retained partial Tap binding activity. Because this strategy requires the ability to genetically select positive clones from a background

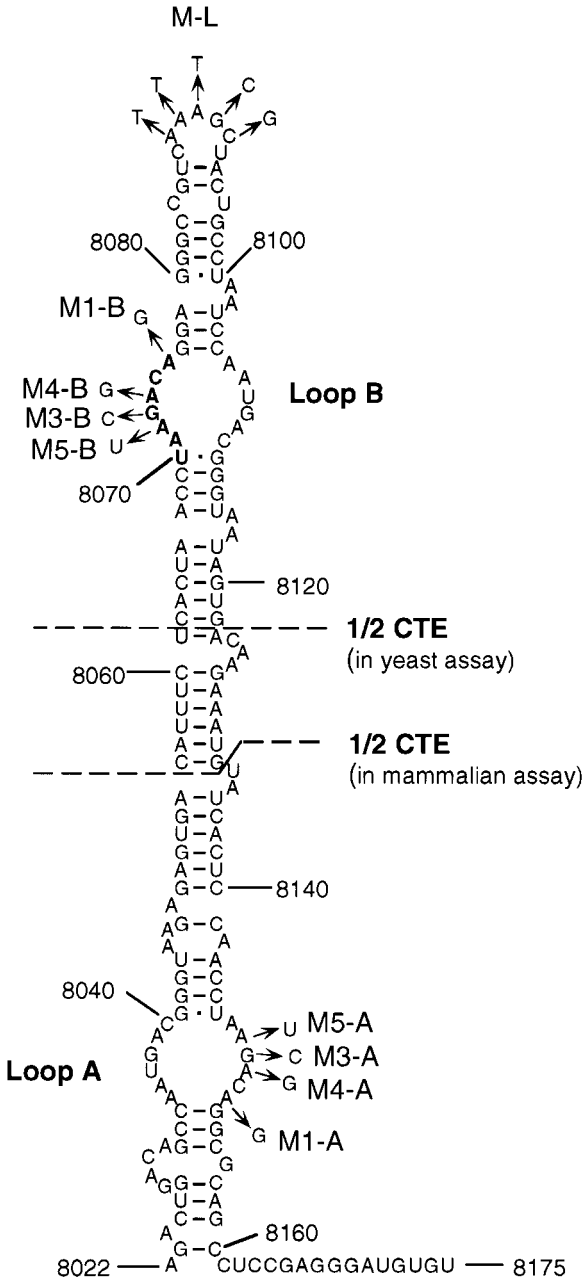

FIG. 1. Predicted structure of the MPMV CTE RNA element. The CTE has been shown to fold into a helical RNA structure that presents two identical internal RNA loops, designated loop A and loop B, that serve as specific protein binding sites. The full-length MPMV CTE used in this manuscript, and the extent of the "1/2 CTE" RNA sequences used in the yeast and mammalian RNA:protein binding assays, is shown, as is the location of selected point mutants and of the seven nucleotides in loop B that were subjected to randomization. Sequence coordinates refer to the sequence of the full-length MPMV genome and are as defined by Ernst *et al.* (1997).

of negative clones in a yeast genetic screen, we first asked whether Tap would specifically bind to the MPMV CTE in the yeast three hybrid assay. As shown in Table 1, the full-length Tap protein indeed proved able to effectively bind to the full-length CTE but not to an antisense

TABLE 1

Interaction of Tap with the MPMV CTE in Yeast Cells

	β-gal activity (mOD/ml)	
	VP16-Tap	VP16
IRE	<4	<4
TAR	<4	<4
CTE		
CTE	602	11
CTE AS	<4	<4
M1-A	406	<4
M1-B	148	<4
M1-AB	<4	<4
M-L	597	<4
1/2 CTE		
1/2 CTE	1691	21
M-L	509	15
M1	<4	<4

Note. The ability of human Tap to bind to wild-type and mutant forms of the MPMV CTE was analyzed using the yeast three hybrid RNA: protein interaction assay. The indicated levels of β-gal activity, induced upon interaction of a fusion protein consisting of the VP16 activation domain fused to the full-length Tap protein with the indicated RNA targets, represent the average of three independent experiments. Controls included the HIV-1 TAR element, a human iron response element (IRE), and the anti-sense CTE (CTE AS). The single nucleotide M1 mutation, which inactivates CTE function, is shown in Fig. 1 and was introduced into loop A (M1-A), loop B (M1-B), or both CTE loops (M1-AB). The terminal CTE loop mutation M-L, which does not block CTE function, is also shown in Fig. 1.

CTE (CTE AS) or to such irrelevant structured RNA sequences as the human immunodeficiency virus type 1 (HIV-1) TAR element and a human iron response element (IRE).

Introduction of the M1 point mutant (Fig. 1) into either loop A (M1-A) or loop B (M1-B), either of which is sufficient to block CTE function (Kang and Cullen, 1999), reduced but did not prevent CTE binding by Tap (Table 1). However, introduction of the M1 mutation into both internal loops (M1-AB) blocked CTE binding by Tap. Mutation of the terminal CTE loop (M-L), which does not prevent CTE function (Tabernero *et al.,* 1996 and see below), had little effect on Tap binding to the complete CTE (Table 1). To confirm that Tap could indeed bind a single CTE loop, we next tested the apical half of the CTE (Fig. 1) in the three hybrid assay. As shown in Table 1, the 1/2 CTE indeed bound Tap effectively. Introduction of the M1 mutation into the single remaining internal CTE loop blocked Tap binding while mutation of the terminal loop (M-L) had only a modest effect. We therefore conclude that Tap can bind the CTE specifically in the yeast three hybrid assay and that this interaction is dependent on the presence of at least one intact CTE internal loop.

Selection of CTE variants that retain tap binding

Having shown that the 1/2 CTE indeed interacted avidly and specifically with Tap in the yeast three hybrid assay, we next randomized the seven nucleotides that form the 5′ part of CTE loop B (Fig. 1) in the 1/2 CTE context and then selected for CTE variants that retained the ability to bind Tap in the yeast three hybrid assay, as shown by detection of β-galactosidase (β-gal) indicator enzyme expressing yeast colonies by *in situ* analysis.

As shown in Table 2, this randomization led to the recovery of five CTE sequences that proved able to specifically interact with Tap, all of which were recovered two or more times. In addition to the wild-type CTE sequence, we recovered four CTE variants (V1 to V4, Table 2) that showed a level of Tap binding that varied between 69 and 25% of that seen with the wild-type CTE, thus strongly suggesting that the wild-type CTE has the optimal RNA sequence for Tap binding. Variants V1 to V3 were found to differ from the wild-type sequence by only one nucleotide, while V4 differed at four of seven positions (Table 2). In addition to the CTE variants that bound Tap, we also repeatedly (13 times) recovered a strongly β-gal-positive variant of the CTE that, however, was equally positive both in the absence and presence of the

TABLE 2

Interaction of Tap and CTE Mutants and Selected CTE Variants in Yeast Cells

		Relative β-gal activity	
Clone	Sequence	+VP16-Tap	+VP16
WT(×8)	UAAGACA	100	<1
V1(×6)	UAA**A**ACA	33	<1
V2(×5)	UAAG**C**CA	69	<1
V3(×2)	UA**G**GACA	25	<1
V4(×2)	**G**G**A**A**A**A	50	≤1
V5(×13)	**G**A**U**G**G**CA	241	273
M2	UAA**AGU**A	<1	≤1
M3	UAA**C**ACA	<1	≤1
M4	UAAG**G**CA	16	<1
M5	UA**U**GACA	9	<1

Note. Seven nucleotides that form the 5′ side of CTE loop B were randomized in the context of a 1/2 CTE bearing the inactivating M2 mutation. CTE variants that displayed significant β-gal activity in the presence of VP16-Tap were then selected using the three hybrid screen in yeast cells. The six recovered sequences, together with the number of times each was recovered, are given at the top of the table as WT and the variants V1 to V5. Several CTE mutants (M2 to M5) were also constructed and are given in the lower part of the table. Relative binding of these 1/2 CTE variants and mutants to full-length human Tap was determined in yeast, as described in the legend of Table 1, and is given as a percentage of the activity of the wild-type 1/2 CTE averaged over several experiments. Variant V1 has previously been independently reported as mutant #30, and mutant M4 as mutant #31, by Tabernero *et al.* (1996), who reported both as partially active CTE mutants.

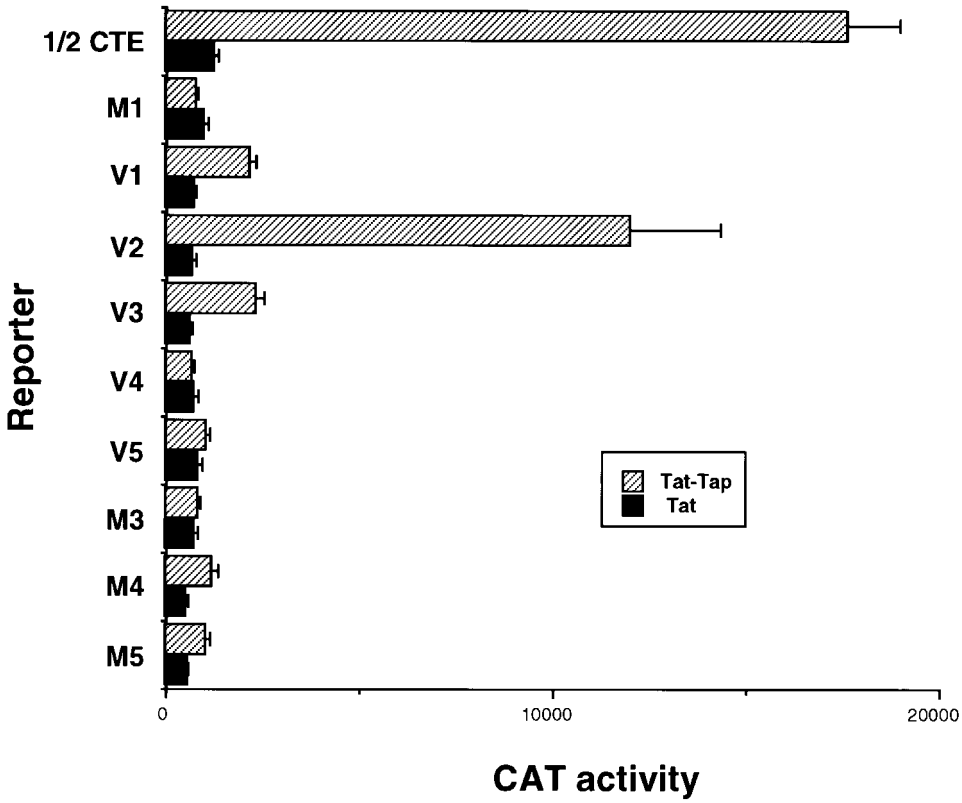

FIG. 2. Analysis of Tap binding to CTE variants in human cells. RNA binding data were obtained using the Tat-based RNA:protein interaction assay. 293T cells were transfected with 500 ng of an HIV-1 LTR-based indicator plasmid, bearing the indicated wild-type or mutant form of the 1/2 CTE substituted in place of TAR, together with 50 ng of a Tat or Tat–Tap fusion protein expression plasmid plus 1 μg of pBC12/CMV. Transfected cells were analyzed for HIV-1 LTR driven CAT expression levels at 48 h posttransfection. Average of three independent transfections plus or minus the standard deviation.

VP16–Tap fusion protein (Table 2). Because the MS2-1/2 CTE RNA is not capable of encoding a protein, this selected RNA sequence, which differs from the 1/2 CTE at only three locations, must presumably act by recruiting an endogenously expressed yeast RNA binding protein that also contains a potent transcriptional activation domain.

Because this randomization/selection screen led to the recovery of only four CTE variants that retained the ability to bind Tap, we generated three additional single nucleotide mutants of the CTE, termed M3, M4, and M5 (Fig. 1, Table 2), in order to confirm that other CTE point mutants would indeed show little or no Tap-binding activity. This was particularly important for the M4 CTE mutant, as this mutant has been previously reported (Tabernero *et al.,* 1996) to retain partial CTE function. In fact, as shown in Table 2, both the M4 and, to a lesser extent, the M5 CTE mutant did display a detectable level of Tap binding that was, however, significantly lower than the level of binding seen with any of the four recovered CTE variants. In contrast, the M3 point mutant, like the more extreme M2 CTE mutant that served as the template for the randomization/selection screen, proved unable to bind Tap.

Analysis of CTE binding by tap in human cells and *in vitro*

To confirm the relevance of these yeast RNA binding data to the mammalian system, we next measured the level of Tap binding to each of these CTE mutants, in the 1/2 CTE context, using a previously described mammalian RNA:protein binding assay (Blair *et al.,* 1998; Kang and Cullen, 1999). This assay is based on the ability of the HIV-1 Tat protein to transcriptionally activate the HIV-1 long terminal repeat (LTR) promoter element when recruited either to the TAR element normally located in the LTR or to a heterologous RNA target sequence substituted in place of TAR (Selby and Peterlin, 1990). We have previously shown (Kang and Cullen, 1999) that the Tat:Tap fusion protein can potently activate an HIV-1 LTR bearing the 1/2 CTE sequence in place of TAR, and this result is reproduced in Fig. 2. As further demonstrated in Fig. 2, data obtained using this mammalian RNA:protein binding assay was in excellent agreement with the yeast three hybrid assay for all CTE variants and mutants tested with the exception of V4. Specifically, CTE variant V2 gave strong Tap binding, variants V1 and V3 gave modest binding, M4, and possibly M5, gave weak bind-

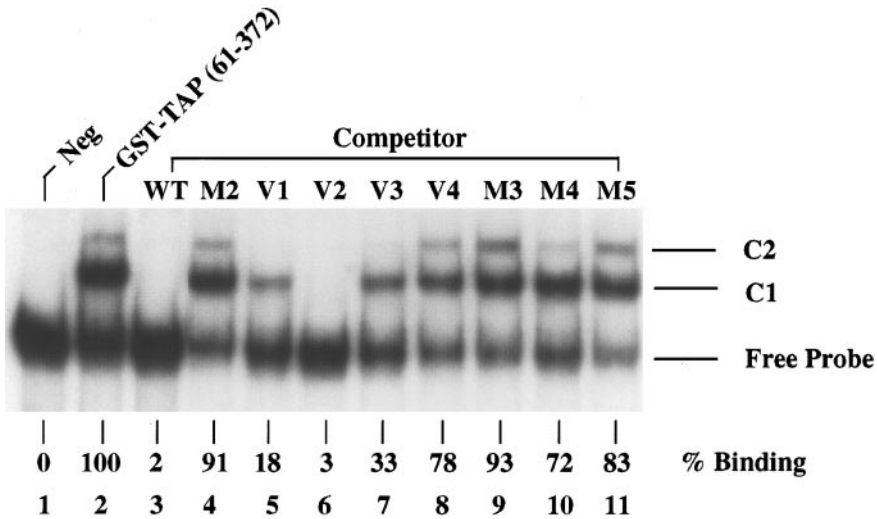

FIG. 3. Tap binds the CTE specifically *in vitro*. The GST-Tap (61–372) fusion protein was expressed in bacteria and purified by affinity chromatography. A full-length wild-type CTE probe was synthesized by *in vitro* transcription in the presence of [α-^{32}P]CTP and ~10^4 cpm (~0.1 ng) then mixed with ~25 ng of the GST–Tap fusion protein in a 20-μl reaction mix containing 5 μg of nonspecific competitor RNA. Competitor RNAs, consisting of the indicated CTE mutations introduced into the full-length CTE, were synthesized *in vitro* and added at an ~200-fold molar excess to the Tap:CTE binding reaction 10 min prior to addition of the labeled CTE RNA probe. After incubation at 4°C for 20 min, the reaction products were resolved on a 5% native polyacrylamide gel and visualized by autoradiography. C1 and C2 refers to the two distinct retarded complexes observed when using the full-length wild-type CTE probe. The relative level of binding was quantitated by Phosphorimager and is given as percentage residual binding, with binding in the absence of specific competitor set at 100%.

ing, while mutants M2 and M3, as well as variants V4 and V5, gave no detectable binding.

While the inability of the V5 CTE variant to bind Tap in the mammalian nucleus was expected, given that the biological activity of V5 in the yeast three-hybrid assay was entirely independent of Tap (Table 2), the clear discrepancy between the yeast and mammalian binding assays in the case of V4 was surprising given the close similarity in the data recovered for all other CTE variants. After confirming the sequence integrity of the relevant clones, we hypothesized that this difference might reflect either the presence of a facilitating yeast gene product or the presence, in mammalian cells, of a gene product that blocks Tap binding to V4. To distinguish between these two possibilities, we used a competitive RNA gel shift analysis to compare binding by these various CTE variants *in vitro.* As shown in Fig. 3, and also previously reported (Kang and Cullen, 1999), a recombinant protein consisting of residues 61 to 372 of Tap, which include the entire Tap RNA binding domain, fused to glutathione *S*-transferase (GST) is able to specifically bind to an RNA probe consisting of the full-length CTE. To compare the ability of the different CTE mutants and variants (Table 2) to bind to Tap *in vitro,* we engineered each mutant or variant into both loops of the CTE and then transcribed these RNAs *in vitro* to generate unlabeled competitor RNAs. These were then added to the RNA:protein binding reaction at an ~200-fold molar excess over the labeled wild-type CTE probe. Binding of Tap by the added unlabeled wild-type CTE competitor can specifically inhibit binding by the labeled probe, and the level of residual binding of the CTE probe is therefore a measure of the relative affinity for Tap of each tested mutant CTE competitor.

As shown in Fig. 3, addition of wild-type CTE competitor entirely blocked probe binding (lane 3), while addition of a similar level of the inactive M2 CTE mutant had little, if any, effect (lane 4). In addition, this *in vitro* assay revealed strong competition for Tap binding by the V2 CTE variant, significant competition by V1 and V3, and little or no competition by V4 or by the CTE mutants M3, M4, and M5. We therefore conclude that the interaction of V4 with Tap in yeast cells is likely to be an artifact that is not reproduced either *in vitro* or in a mammalian cell-based RNA binding assay. In contrast, binding of Tap to the V1, V2, and V3 CTE variants and to the M2, M3, M4, and M5 CTE mutants was equivalent in all three assay formats.

The level of tap binding by CTE variants predicts biological activity

A major rationale for generating the CTE variants shown in Table 2 was to ask whether Tap-binding activity indeed correlated with CTE function in primate cells. The pDM128/CTE indicator plasmid, which encodes the *cat* indicator gene and wild-type CTE located between functional 5′ and 3′ splice sites, has been previously described (Hope *et al.,* 1990; Bogerd *et al.,* 1998). Because splicing removes the *cat* open reading frame, efficient CAT expression is dependent on the nuclear export of the unspliced *cat* mRNA, i.e., on CTE function. As shown

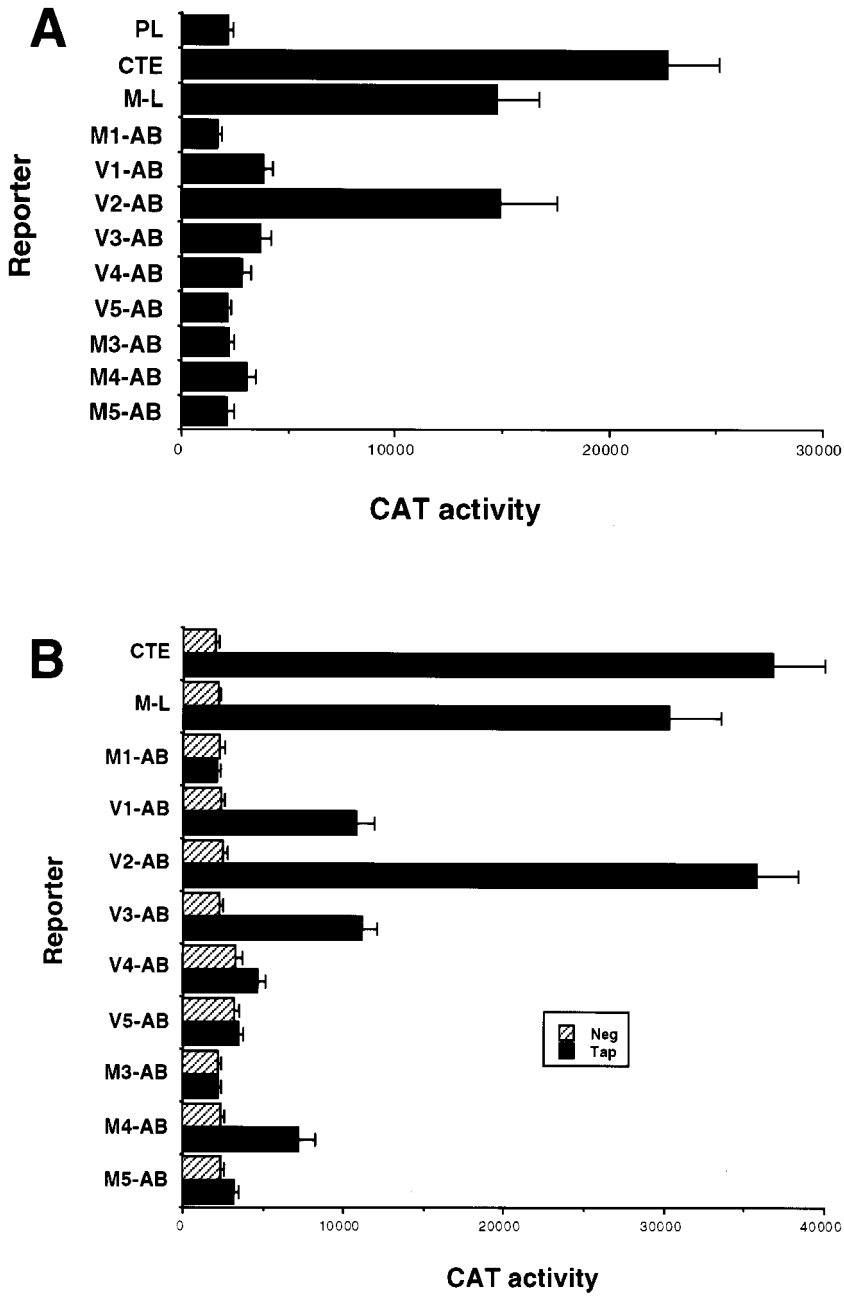

FIG. 4. RNA export activity of the indicated CTE variants in human and quail cells. The pDM128/CTE indicator construct contains the *cat* gene and CTE located between 5′ and 3′ splice sites. CAT expression is therefore dependent on the nuclear export of an unspliced *cat* mRNA, a process that is inefficient in the absence of a functional *cis*-acting RNA export signal, such as the CTE. pDM128/CTE variants, containing the indicated CTE mutants, were analyzed in comparison to the wild-type CTE or to a form of DM128 (pDM128/PL) that contains a poly-linker in place of the CTE and that therefore serves as a negative control. (A) Human 293T cells (35 mm culture) were transfected with 25 ng of the indicated pDM128 derivative, 25 ng of pBC12/CMV/β-gal, and 1 μg of pBC12/CMV as a carrier. Induced CAT and β-gal activities were determined at ~48 h after transfection. (B) Quail QCI-3 cells (35 mm culture) were transfected with 25 ng of pBC12/CMV/β-gal, 25 ng of the indicated pDM128/CTE based reported plasmid, and 100 ng of either the parental pBC12/CMV plasmid or pcTap plus 400 ng of pBC12/CMV as filler. Cells were harvested at ~48 h after transfection and CAT activities determined. Both panels show the average of three independent transfection experiments plus or minus the indicated standard deviation.

in Fig. 4A and previously described elsewhere (Bogerd *et al.,* 1998; Kang and Cullen, 1999), the wild-type CTE induces a significant increase in the level of CAT expression in transfected human 293T cells relative to a plasmid, termed pDM128/PL, that is identical except for the lack of a CTE. We therefore next constructed a set of pDM128/CTE derivatives bearing the CTE mutations described in Table 2, introduced into both CTE loops, and then measured their biological activity in transfected human cells. As shown in Fig. 4A, there was indeed a

close correlation between Tap-binding activity and CTE biological activity. Specifically, we observed that the V2 and M-L mutants displayed strong partial activity, while the V1, V3, and possibly M4 CTE mutants displayed weak activity. All other CTE mutants, including V4, proved to be essentially inactive.

Previously, we have demonstrated that the quail cell line QCI-3 is refractory to CTE function but is rendered fully permissive upon transfection of a plasmid expressing the human Tap protein (Kang and Cullen, 1999). We therefore next tested the biological activity of this same set of CTE mutants and variants by transfection into QCI-3 cells in the presence and absence of a cotransfected Tap expression plasmid. As shown in Fig. 4B, the quail cells, in the presence of Tap, gave a pattern of biological activity that was qualitatively similar to, but somewhat higher than, the level seen in human cells in the absence of any cotransfected Tap (Fig. 4A). Specifically, CTE variant V2 and the M-L mutant were highly active, variants V1 and V3 moderately active, and mutant M4 weakly active, while all other CTE mutants were essentially inactive.

DISCUSSION

The hypothesis that MPMV CTE-dependent nuclear RNA export is dependent on the specific and direct recruitment of the cellular Tap protein to the CTE is supported by several observations. Thus, Tap has been shown to bind the CTE specifically both *in vitro* and *in vivo* and expression of human Tap has been shown to enhance CTE function in Xenopus oocytes and to render the otherwise nonpermissive quail cell line QCI-3 permissive for CTE function (Grüter *et al.,* 1998; Braun *et al.,* 1999; Kang and Cullen, 1999). Finally, Tap has recently been shown to contain a novel NES sequence that is critical for the activation of CTE function in QCI-3 cells (Kang and Cullen, 1999).

While the evidence supporting a role for Tap in mediating CTE function is therefore increasingly persuasive, the evidence supporting a role for Tap in mediating global mRNA export is more circumstantial. Specifically, it has been reported that injection of a high level of the CTE into the nucleus of Xenopus oocytes selectively inhibits mRNA export but not other nuclear RNA export pathways (Pasquinelli *et al.,* 1997; Saavedra *et al.,* 1997). This inhibition can be rescued by injection of recombinant human Tap protein (Grüter *et al.,* 1998). In addition, in the yeast system, the Mex67p protein, which appears to be the yeast homolog of human Tap, has been shown to be essential for mRNA export from the nucleus and to be associated with bulk poly(A)$^+$ RNA (Segref *et al.,* 1997). Together, these observations suggest that Tap might play a sequence-specific role in the export of a small number of mRNAs that contain CTE-like elements and a sequence nonspecific role in global mRNA export. How Tap would be recruited to mRNAs lacking a CTE is not presently apparent. In addition, direct recruitment of Tap to the CTE results in the nuclear export of unspliced mRNAs that are otherwise retained in the nucleus. Therefore, the nonspecific recruitment of Tap to mRNAs, if it occurs, must either result in a different consequence than specific recruitment or, alternately, may not normally occur until after the completion of RNA splicing.

In this manuscript, we have used a series of assays for RNA:protein binding in an attempt to characterize the RNA binding specificity of Tap, which contains a novel leucine-rich RNA binding motif (Braun *et al.,* 1999; Kang and Cullen, 1999). More specifically, we have used a strategy of CTE RNA sequence randomization, followed by selection for Tap binding in the yeast three hybrid RNA binding assay, to derive a set of CTE variants that retain Tap binding ability. The purpose of this selection was, first, to shed light on the target flexibility of the RNA binding domain of Tap and, second, to derive a set of CTE variants with different affinities for Tap so that we could test whether this binding affinity is predictive of CTE function *in vivo,* as would be expected if Tap is indeed the Tap cofactor.

The strategy used was to randomize the seven nucleotides that form the critical 5′ half of the loop B internal CTE loop (Fig. 1) and then to select RNAs from the resultant library of 16,384 CTE variants, using the yeast three hybrid assay, that displayed readily detectable levels of β-gal activity in the presence of the VP16–Tap fusion protein. Perhaps surprisingly, this screen resulted in the recovery of only five CTE sequence variants, in addition to the wild-type sequence, from this fairly extensive library, and one of these sequences proved able to induce β-gal indicator enzyme entirely independently of the VP16–Tap fusion and is therefore merely an interesting artifact (Table 2).

To confirm that the CTE variants recovered in this randomization/selection screen were indeed able to bind to Tap, we also analyzed binding in mammalian cells and *in vitro.* Unexpectedly, while the Tap binding ability of the CTE variants V1, V2, and V3, as well as of several additional CTE mutants, was similar in all three assays, the V4 CTE variant bound the Tap protein in yeast (Table 1) but not in either mammalian cells (Fig. 2) or *in vitro* (Fig. 3). The reason for this discrepancy is unknown but must presumably reflect the presence of a gene product in yeast cells, that can mediate the specific recruitment of VP16-Tap to the V4 CTE variant, that is not present in the other two assay systems. Therefore, neither of the two CTE variants V4 and V5, that differ substantially in sequence from the CTE wild-type (Table 2) are, in fact, genuine Tap binding sequences.

The remaining authentic Tap-binding CTE variants V1, V2, and V3, which each differ from the wild-type CTE sequence by only a single nucleotide, were next combined with a small number of synthetic CTE mutants

(M-L, M1, M3, M4, and M5) and then analyzed for biological activity by transfection into the CTE-permissive human cell line 293T (Fig. 4A) or into QCI-3 cells in the presence of a cotransfected human Tap expression plasmid (Fig. 4B). These data demonstrate that (A) the Tap-binding activity of these CTE variants and mutants measured in either mammalian cells (Fig. 2) or *in vitro* (Fig. 3) is highly predictive of their biological activity *in vivo* and (B) the observed CTE activity in 293T cells in the absence of cotransfected Tap is very similar to the biological activity of these same CTE variants in QCI-3 cells in the presence of cotransfected human Tap.

The data shown in Table 2 demonstrate that RNA sequence randomization followed by selection, in the yeast three hybrid assay, of variants that retain protein binding can indeed generate interesting information about the sequence specificity of an RNA:protein interaction, and this technique may therefore have general utility. Nevertheless, this approach did give rise to two problems. Specifically, while the V5 variant of the CTE, which gave strong β-gal activity even in the absence of the VP16–Tap fusion protein, could be readily detected and eliminated from further consideration, the V4 variant is disconcerting in that it specifically interacted with VP16–Tap in yeast cells (Table 2) yet was unable to bind Tap either *in vitro* (Fig. 3) or in mammalian cells (Fig. 2). This observation suggests that RNA binding data obtained in the yeast three hybrid system, like data obtained in any other system, should preferably be confirmed using another assay. A second concern was that the approach of identifying β-gal expressing yeast colonies using an *in situ* assay for β-gal expression clearly selected preferentially for high expressors (Table 2). Thus, the CTE wild type and the V5 variant, which each express high levels of β-gal, were recovered 8 and 13 times, respectively. In contrast, the V3 variant, which is ~25% as active as wild type, was recovered only twice while mutant M4, which gives ~16% of the β-gal activity of wild type, was not recovered at all. Therefore, while the screen summarized in Table 2 almost certainly recovered all highly active CTE variants, additional CTE variants that retain only weak Tap binding are likely to have been missed. We note, however, that weak Tap binding in the yeast three hybrid assay correlated with poor CTE function in transfected vertebrate cells (Fig. 4).

In conclusion, we have demonstrated that the Tap RNA binding domain is highly sequence specific and that CTE variants that do retain the ability to bind Tap do so with reduced affinity when compared to wild type (Table 2; Figs. 2 and 3). Thus, the CTE wild-type sequence is, in fact, the optimal RNA binding sequence for the human Tap protein. Second, we have demonstrated that Tap binding affinity is highly predictive of the ability of CTE variants to support nuclear RNA export in not only human cells but also in a quail cell line that only supports CTE function when cotransfected with a human Tap expression plasmid (Fig. 4). Together, these data therefore further define the RNA sequence specificity of the novel leucine-rich RNA binding domain in Tap and provide additional data supporting the identification of Tap as the cofactor for MPMV CTE-dependent nuclear RNA export.

MATERIALS AND METHODS

Plasmid construction

The following expression plasmids have been previously described: the mammalian expression plasmid pBC12/CMV, pcTat, pBC12/CMV/β-gal (Tiley *et al.,* 1992); pcTat-Tap (Kang and Cullen, 1999); indicator constructs containing the MPMV CTE (pDM128/CTE), or a polylinker (pDM128/PL) (Bogerd *et al.,* 1998), in place of the CTE; a reporter plasmid containing the *cat* indicator gene under the control of the wild-type HIV-1 LTR (pTAR/CAT) (Tiley *et al.,* 1992) and an equivalent construct bearing the 1/2 CTE (Fig. 1) in place of TAR (Kang and Cullen, 1999).

The yeast expression vector pVP16 has been described (Bogerd *et al.,* 1993). A triple HA tag was inserted in frame to the VP16 coding sequence, into the *Bgl*II and *Eco*RI sites of the polylinker sequence in pVP16, to generate the cloning vector pVP16-HA. Sequences encoding full-length Tap (Kang and Cullen, 1999) were inserted into the *Eco*RI and *Xho*I sites of pVP16-HA to generated plasmids expressing Tap proteins fused to the VP16 transcription activation domain and the HA Tag. Full length and half CTE fragment were amplified by PCR and then inserted as blunt-ended DNA fragments into a *Sma*I site in the yeast plasmid pIII/MS2 (Sengupta *et al.,* 1996) to generated yeast three-hybrid plasmids that express hybrid MS2-CTE RNAs under the control of the RNase P1 promoter. Mutations were introduced into pIII/MS2 derivatives containing the complete or half CTE using the Quick change cite-directed mutagenesis kit (Stratagene). pDM128/CTE-based mutant/variant CTE indicator constructs were generated by sequential mutagenesis of both internal loops of the CTE using the Quick change site-directed mutagenesis kit. Mutant or variant forms of the CTE were PCR amplified from these pDM128/CTE-based plasmids and inserted into the *Bam*HI site present in the *in vitro* transcription plasmid pGEM-3fZ. 1/2 CTE fragments were PCR amplified from pDM128/PL-based CTE constructs using primers containing unique *Bgl*II and *Sac*I sites and inserted into pTAR/CAT digested with the same enzymes to generate mutant/variant 1/2 CTE constructs for mammalian RNA-binding assay. The sequence of plasmids bearing mutant or variant CTEs was confirmed by DNA sequencing.

Yeast three-hybrid assays

Saccharomyces cerevisiae L40-coat cells (Sengupta *et al.,* 1996) were transformed with a pIII/MS2-based hybrid RNA expression plasmid expressing wild-type or mutant

forms of the CTE and with pVP16-HA-Tap. Transformants were selected on media lacking uracil and leucine. Overnight cultures of pooled transformants were suspended in β-gal assay buffer, normalized to optical density, and β-gal activities assayed as previously described (Bogerd *et al.,* 1993).

In vivo randomization/selection of tap binding sequences

The randomized half CTE library was generated by large scale site-directed mutagenesis of the mutant half-CTE yeast three-hybrid construct pIII/MS2/1/2CTE-M2 using the following primers with randomized sequence from nucleotide 8070 to 8076 of the MPMV CTE: 5′-CCCCCGGATCCACTAACCNNNNNNNGGAGGGC-CGTCAAAGCTA-3′ and 5′-TAGCTTTGACGGCCCTCC-NNNNNNNGGTTAGTGGATCCGGGGG-3′. The mutagenesis products were transformed into *Escherichia coli* DH5α. After selection for ampicillin-resistant transformants, the randomized library was found to consist of about 50,000 independent clones. DNA from the library was pooled and cotransformed with pVP16-HA-Tap into yeast L40 coat cells. The transformed yeast cells were plated on uracil- and leucine-deficient (Ura^-Leu^-) plates covered with a Hybond-N nylon membrane (Amersham). An *in situ* β-gal assay was carried out essentially as previously described (Kang and Cullen, 1998). After 3 days of growth, the nylon membrane with yeast colonies was lifted from the plate, frozen at 140°C for 10 min and then thawed at room temperature. The nylon membrane was placed on filter papers soaked in 0.5 × Z buffer (Blair *et al.,* 1994) with 0.3 mg of chlorophenolred-D-galactopyranoside (CPRG; Boehringer-Mannheim) per milliliter and 0.1% (vol/vol) 2-mercaptoethanol. After 1 h of incubation at room temperature, colonies that turned blue were picked and recovered on Leu^- Ura^- plates, and the yeast indicator plasmids harboring candidate Tap binding site variant sequences rescued after overnight culture. A second yeast transformation and β-gal assay were then performed with the selected indicator plasmids, together with either pVP16-HA-Tap or the parental plasmid pVP16, to quantify the level of transactivation by Tap and to identify any false-positive clones. The CTE sequences in the true-positive clones were then obtained by ABI automatic cycle sequencing (PE Applied Biosystems).

Cell culture and transfection

Human 293T and quail QCI-3 were maintained as previously described (Cullen *et al.,* 1983; Bogerd *et al.,* 1998) and transfected using lipofectamine (Life Technologies) or DEAE-Dextran (Cullen *et al.,* 1983), respectively. All transfections were performed on cell cultures in 35-mm plates. Levels of DNA used in each transfection experiment are given in the relevant figure legend, with pBC12/CMV/β-gal included as an internal control. In all transfection experiments, CAT enzyme levels were determined 48 h after transfection, as previously described, and normalized to the level of β-gal activity present in the cell lysate (Bogerd *et al.,* 1998).

Gel shift analysis

A GST-fusion protein, containing the RNA binding domain (aa 61–372) of Tap, was expressed and purified on glutathione affinity resin as previously described (Kang and Cullen, 1999). The wild-type CTE RNA probe was labeled with [α-^{32}P]CTP using the Riboprobe *in vitro* transcription system (Promega) and the total isotope incorporation determined by scintillation counting after column purification. Unlabeled competitor RNAs were synthesized using the RiboMax large scale RNA production system (Promega). The binding reaction was carried out with ~10^4 cpm (~0.1 ng) of the probe and 25 ng of GST–Tap fusion protein in 20 μl of binding buffer (150 mM KCl, 10 mM Hepes (pH 7.6), 0.5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, and 10% glycerol) containing 4 μg of rRNA and 1 μg of yeast tRNA. Competitor RNAs were added at a 200-fold molar excess over the labeled probe fragment and incubated with GST–Tap (61–372) for 10 min before addition of the probe. Binding was allowed to proceed for 20 min at 4°C and the reaction products resolved on a 5% (40:1) native polyacrylamide gel and visualized by autoradiography. The results of competition experiments were quantitated with a PhosporImager and Image QuaNT software (Molecular Dynamics).

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