TAP binds to the constitutive transport element (CTE) through a novel RNA-binding motif that is sufficient to promote CTE-dependent RNA export from the nucleus

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The constitutive transport element (CTE) of the simian type D retroviruses overcomes nuclear retention and allows nuclear export of unspliced viral RNAs by recruiting TAP, a host factor which is thought to be required for export of cellular mRNAs. In this report, we show that the first 372 amino acid residues of TAP, comprising a stretch of leucine-rich repeats, are both necessary and sufficient for binding to the CTE RNA and promoting its export to the cytoplasm. Moreover, like the full-length protein, this domain migrates to the cytoplasm upon nuclear co-injection with the CTE RNA. Together, these results indicate that the CTE-binding domain includes the signals for nuclear export. We also describe a derivative of TAP that bears a triple amino acid substitution within the CTE-binding domain and substantially reduces the export of mRNAs from the nucleus. This provides further evidence for a role for TAP in this process. Thus, the CTE-binding domain of TAP defines a novel RNA-binding motif which has dual functions, both recognizing the CTE RNA and interacting with other components of the nuclear transport machinery.
Keywords: CTE/leucine-rich repeats/mRNA export/ nuclear export/RNA–protein interactions

Introduction

Nuclear export of cellular mRNAs is highly selective, as usually only fully processed RNAs are exported. Incompletely spliced precursor mRNAs (pre-mRNAs) and excised introns are actively retained within the nucleus. Studies in yeast and in vertebrate cells suggested that pre-mRNAs are held within the nucleus by the association of spliceosomal factors (Legrain and Rosbash, 1989; Hamm and Mattaj, 1990). Although little is known about the mechanism underlying nuclear retention, the identification of a dominant nuclear retention signal (NRS) in heterogeneous nuclear ribonucleoprotein (hnRNP) C indicates that retention can also be mediated by non-spliceosomal RNA-binding proteins (Nakielny and Dreyfuss, 1996). This also implies that the mRNA export pathway may include steps at which specific retention factors are removed. Indeed, electron microscopy studies on the export of large mRNAs have shown that several proteins that are associated with the mRNA in the nucleoplasm are removed selectively, prior to or during translocation across the nuclear pore complex (NPC; reviewed by Daneholt, 1997).

Retroviral replication requires the nuclear export of unspliced forms of viral RNAs, which are necessary both as templates for the synthesis of structural proteins and as genomic RNA to be packaged in progeny virions, in the cytoplasm of the host cell (reviewed by Felber, 1997; Hope, 1997; Cullen, 1998). To circumvent the requirement for splicing prior to export, retroviruses have evolved mechanisms that allow their intron-containing RNAs to exit the nucleus. These mechanisms are all based on the presence of a *cis*-acting RNA element in the viral genome which recruits viral or cellular factors to override nuclear retention and promote nuclear export (reviewed by Cullen, 1998; Izaurralde and Adam, 1998).

In complex retroviruses such as human immunodeficiency virus type 1 (HIV-1), the *cis*-acting element, termed the Rev-responsive element (RRE), interacts with the viral Rev protein. Rev binds to the RRE through an RNA-binding domain, that also mediates multimerization of the protein and nuclear import. A second domain called the Rev activation domain functions as a nuclear export signal (NES) (for a review see Cullen, 1998 and references therein). The Rev NES is a short leucine-rich sequence shown to be present in a range of viral and cellular proteins, and is referred to as Rev-like NES or leucine-rich NES (Fischer *et al.*, 1995; Wen *et al.*, 1995; Bogerd *et al.*, 1996). Rev-like NESs interact with the nuclear export receptor CRM1/exportin 1 (Fornerod *et al.*, 1997a; Fukuda *et al.*, 1997; Stade *et al.*, 1997). CRM1 belongs to the family of Ran-binding proteins that shares similarities with the Ran-binding domain of importin-β (Fornerod *et al.*, 1997b; Gorlich *et al.*, 1997). As is the case for all members of this family, binding to their cognate substrates is regulated by the small GTPase Ran (reviewed by Mattaj and Englmeier, 1998; Stutz and Rosbash, 1998). CRM1 binds to Rev-like NES peptides in the presence of Ran-GTP, forming a trimeric complex (Fornerod *et al.*, 1997a; Bogerd *et al.*, 1998). This is likely to occur in the nucleoplasm, where Ran is thought to be present predominantly in the GTP-bound form. Dissociation of this complex requires GTP hydrolysis by Ran and is likely to occur immediately after translocation to the cytoplasm, where the Ran GTPase-activating protein (RanGAP) and the Ran-binding protein-1 (RanBP1) are localized (reviewed by Ullman *et al.*, 1997; Mattaj and Englmeier, 1998, Stutz and Rosbash, 1998). CRM1 is also involved in the export of at least one class of cellular RNA substrate, the RNA polymerase II transcribed spliceosomal U small nuclear (sn)RNAs (Fornerod *et al.*, 1997a).

Simple retroviruses do not encode a Rev-like *trans-*

acting protein, and export of their unspliced RNA relies on the interaction of *cis*-acting RNA elements with cellular factors. In simian type D retroviruses, this element is referred to as the constitutive transport element (CTE), since its interaction with cellular factors results in the constitutive export of unspliced viral RNAs (Bray *et al.*, 1994; Zolotukhin *et al.*, 1994; Tabernero *et al.*, 1996; Ernst *et al.*, 1997a,b). Microinjection experiments in *Xenopus* oocytes (Pasquinelli *et al.*, 1997; Saavedra *et al.*, 1997) and transfection experiments in mammalian cells (Zolotukhin and Felber, 1997; Bogerd *et al.*, 1998; Otero *et al.*, 1998) demonstrated that the CTE promotes export of intron-containing RNAs by a pathway distinct from that used by the RRE–Rev system. As mentioned above, while the nucleocytoplasmic export of Rev is mediated by CRM1, which is also responsible for the export of spliceosomal U snRNAs (Fornerod *et al.*, 1997a; Stade *et al.*, 1997), the CTE pathway is distinct and is shared by the cellular mRNAs (Pasquinelli *et al.*, 1997; Saavedra *et al.*, 1997; Gruter *et al.*, 1998).

The CTE folds into an extended RNA stem–loop structure comprising two identical internal loops, A and B, and an AAGA bulge adjacent to loop A (Tabernero *et al.*, 1996, 1997; Ernst *et al.*, 1997a,b). The loops are arranged in mirror symmetry on the RNA element. Analysis of the structural requirements for CTE function in mammalian cells and in *Xenopus laevis* oocytes leads to the prediction that the internal loops represent the interaction sites for cellular CTE-binding proteins (Tabernero *et al.*, 1996, 1997; Ernst *et al.*, 1997a,b; Pasquinelli *et al.*, 1997; Gruter *et al.*, 1998).

Recently, we identified TAP as the cellular factor which mediates CTE-dependent nuclear export (Gruter *et al.*, 1998). TAP binds directly to wild-type CTE RNA and stimulates the nuclear export of excised intron lariats harbouring the CTE (Gruter *et al.*, 1998). Thus TAP is the cellular factor which appears to fulfil Rev-like function for simple, CTE-containing retroviruses.

TAP is the vertebrate homologue of the yeast protein Mex67p, which is essential for mRNA export from the nucleus in yeast cells (Segref *et al.*, 1997; Santos-Rosa *et al.*, 1998). This and the observation that in *Xenopus* oocytes, titration of TAP with an excess of CTE RNA prevents cellular mRNAs from exiting the nucleus (Gruter *et al.*, 1998), suggest that TAP is an essential mediator of cellular mRNA nuclear export. However, several lines of evidence indicate that the mode of interaction of TAP with the CTE RNA is different from that with mRNAs. First, TAP clearly does not mediate cellular mRNA export by binding to RNA loops similar to those present in the CTE. Secondly, while an excess of CTE RNA saturates export of mRNAs, an excess of mRNA does not interfere with CTE export (Pasquinelli *et al.*, 1997; Saavedra *et al.*, 1997). Based on these observations, we have hypothesized that the CTE bypasses several steps in the mRNA export pathway and directly interacts with TAP, while mRNAs encounter TAP at a late stage along the export pathway and may recruit TAP via protein–protein interactions (Saavedra *et al.*, 1997; Gruter *et al.*, 1998).

In this study, we have explored further the mechanism by which TAP mediates export of CTE-containing RNAs. We show that the N-terminal 372 residues of TAP are sufficient to interact with the CTE RNA both *in vitro* and *in vivo* and to stimulate its export to the cytoplasm. The CTE-binding domain of TAP defines a novel RNA-binding motif comprising four leucine-rich repeats (LRRs) and the neighbouring N-terminal 205 amino acids. Two lines of evidence suggest that the CTE-binding domain has dual function, not only recognizing the CTE RNA but also interacting (directly or indirectly) with other components of the nuclear transport machinery. First, microinjection of the CTE-binding domain is sufficient to stimulate directly CTE-dependent nuclear export in *Xenopus* oocytes. Secondly, this domain and the full-length protein are exported to the cytoplasm upon nuclear co-injection with the CTE RNA. Taken together, these results suggest that residues essential for export are embedded within the CTE-binding domain. Finally, we show that a derivative of TAP having residues 306–308 substituted with alanines substantially reduces the export of mRNAs out of the nucleus. This provides additional evidence for a role for TAP in mediating the nuclear export of cellular mRNAs.

Results

Human TAP has an N-terminal extension of 60 amino acids

We previously have identified TAP as the cellular factor which mediates CTE-dependent nuclear export (Gruter *et al.*, 1998). TAP is the vertebrate homologue of the yeast protein Mex67p, with which it shares 23% identity (Segref *et al.*, 1997). Recently, the sequences of rat and murine TAP cDNAs were deposited. Comparison of the amino acid sequence of human TAP with those of the murine and rat proteins shows that the three proteins are 89% identical (Figure 1A). However, both the murine and rat sequences extend beyond what Yoon *et al.* (1997) proposed to be the initiating methionine for the human protein (indicated by an asterisk in Figure 1A). Database searches reveal a list of human expressed sequence tags (ESTs) that are likely to represent TAP cDNA clones, as they are essentially identical to the cDNA reported by Yoon *et al.* (1997), except that the ESTs extend at least 180 nucleotides further into the 5′ region. Moreover, these additional sequences continue to show extensive identity with the murine and rat sequences at both the nucleotide and amino acid levels. Of the 180 nucleotides 5′ to the translation start site assigned by Yoon *et al.* (1997), 84% are identical with the murine and rat 5′ coding sequences. Consideration of the deduced amino acid sequence of this putative non-coding region indicates that 51 of the 60 potential amino acids would be identical to those found in the murine and rat protein sequences (Figure 1A). Since there is no in-frame stop codon in this 5′ region, nor is there an optimal Kozak consensus sequence surrounding the putative initiation codon assigned by Yoon *et al.* (1997), there would appear to be no particular reason for the assumption that the entire open reading frame was present in the Yoon *et al.* cDNA. Indeed, by RT–PCR, we have obtained human TAP cDNA clones that are essentially identical to that reported by Yoon *et al.* (1997), except that our clones encode the 60 additional amino acids and have, like the murine and rat proteins, a tryptophan at position 119 instead of a cysteine. Therefore, we conclude that the Yoon *et al.* cDNA represents a
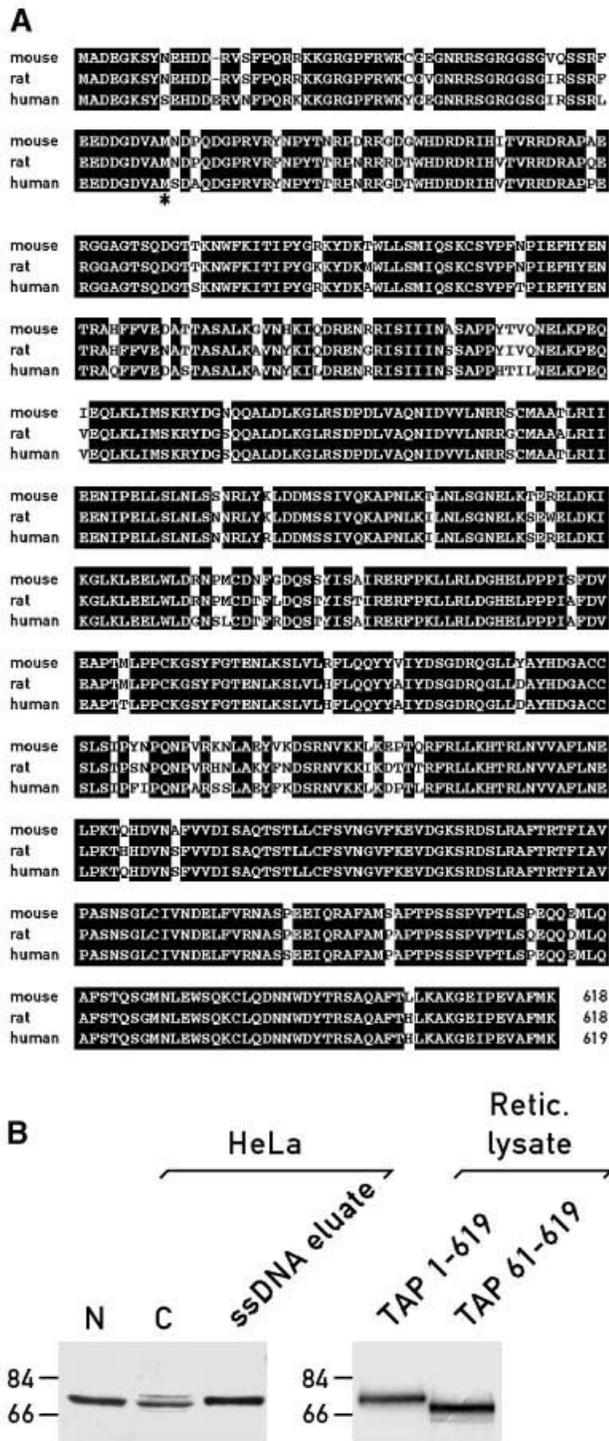
A

B

Fig. 1. TAP has an N-terminal extension of 60 amino acids.
(**A**) Complete amino acid alignment of predicted TAP protein sequences from human, mouse and rat using ClustalW1.7. Residue identity between species is highlighted in black. The position of the first methionine assigned by Yoon *et al.* (1997) is indicated by an asterisk below the human sequence. The sequence data for human TAP has been submitted to the DDBJ/EMBL/GenBank under accession No. AJ132712. The accession Nos for the mouse and rat cDNAs are AF093140 and AF093139, respectively. (**B**) Protein samples from HeLa nuclear extracts, cytoplasmic S-100 extracts and from single-stranded DNA column eluates were analysed by Western blot using a rabbit anti-TAP antibody. In parallel, ^{35}S-labelled TAP 1–619 and TAP 61–619 were analysed.

truncated clone of human TAP and that the full-length protein has an N-terminal extension of 60 amino acids.

We have noticed previously that when purified from HeLa nuclear extracts, TAP migrates with an apparent mol. wt of 73 kDa in SDS–PAGE, while the recombinant protein lacking the N-terminal 60 amino acids exhibits an apparent mol. wt of 68 kDa (Gruter *et al.*, 1998). Figure 1B shows a comparison of the migration of the full-length (1–619) and truncated (61–619) proteins synthesized *in vitro* in rabbit reticulocyte lysates. In parallel, the protein present in HeLa cells was detected by Western blotting using rabbit antibodies raised against the recombinant truncated protein. The *in vitro*-translated full-length protein migrates with the apparent molecular weight of the protein detected in HeLa nuclear extracts or purified on a single-stranded DNA column (Gruter *et al.*, 1998). In cytoplasmic S-100 extracts, the same antibody reveals an additional minor band with a slightly lower mobility. This may represent a modified form of the protein (Segref *et al.*, 1997) or a cross-reactivity of the antibody. Although we cannot distinguish between these two possibilities, we can exclude that the truncated protein (61–619) represents a major form of TAP *in vivo*.

CTE-binding and export functions of TAP are not affected by the 60 amino acid N-terminal extension

In our previous report, we have shown that when expressed in *Escherichia coli*, the truncated form of TAP (61–619) binds to the CTE RNA with the same specificity as the protein purified from HeLa nuclear extracts (Gruter *et al.*, 1998). Moreover, the recombinant protein stimulates CTE-dependent nuclear export in *Xenopus* oocytes (Gruter *et al.*, 1998). Thus, the truncated protein carries the amino acid residues essential for the binding and export functions of TAP. To investigate whether the additional 60 amino acids contribute to these functions, we compared fragment 61–619 and the full-length protein (1–619) for their ability to bind to the CTE RNA *in vitro* and to stimulate its export *in vivo* in *Xenopus* oocytes.

Binding to the CTE RNA was tested by an electrophoretic gel mobility retardation assay. To avoid interference resulting from the presence of tags or of additional sequences, both proteins were produced by *in vitro* translation in rabbit reticulocyte lysates. A ^{32}P-labelled CTE RNA probe was incubated with the *in vitro*-translated proteins, and the resulting complexes were resolved in a native polyacrylamide gel and visualized by autoradiography (Figure 2A). To assess the specificity of the interaction, binding reactions were supplemented with increasing amounts of unlabelled CTE RNA or dihydrofolate reductase (DHFR) mRNA. As a non-specific competitor, we used the M36 RNA, a CTE derivative which has the same predicted secondary structure as the wild-type CTE but which carries several nucleotide changes in the loop sequences, the binding sites for TAP (Gruter *et al.*, 1998). Formation of TAP–CTE complexes was competed efficiently by increasing amounts of competitor CTE RNA (Figure 2A, lanes 5–8 and 15–18), while the M36 RNA added in a 1000-fold molar excess relative to the CTE RNA had no effect (Figure 2A, lanes 4 and 14). An excess of DHFR mRNA decreased the formation of TAP–CTE complexes, but only at the
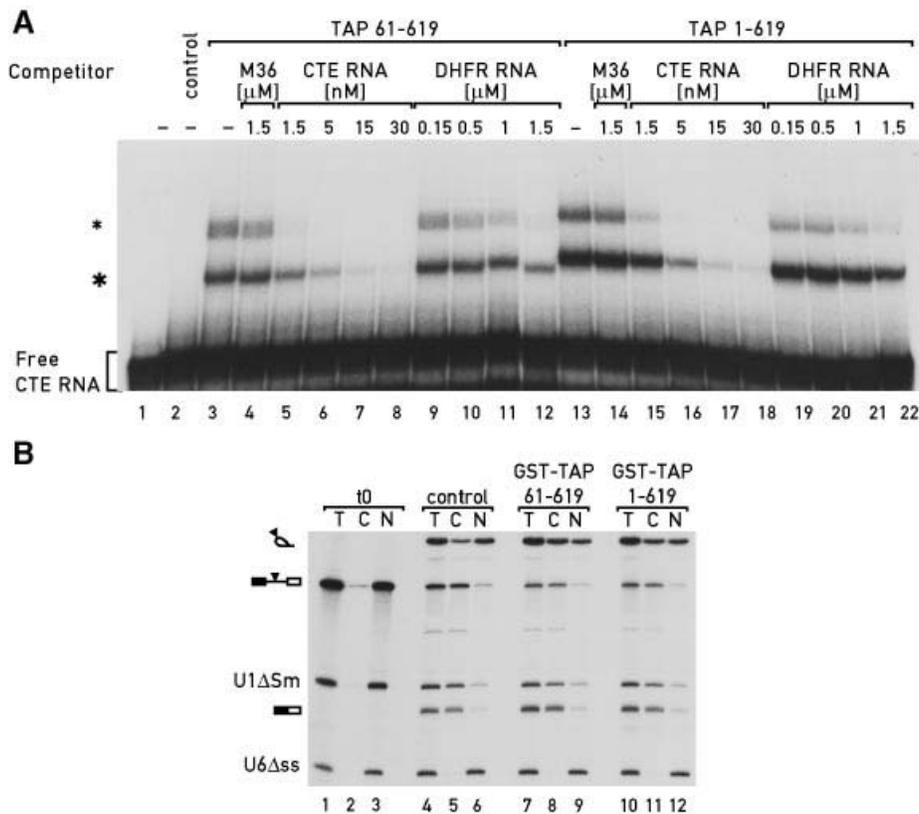
Fig. 2. The N-terminal extension does not affect the CTE-binding and export functions of TAP. (**A**) A gel mobility retardation assay was performed with reticulocyte lysates programmed with cDNAs encoding TAP 61–619 (lanes 3–12) or 1–619 (lanes 13–22). In lanes 4 and 14, unlabelled M36 competitor RNA was added. In lanes 5–8 and 15–18, CTE competitor RNA was included in the reaction mixtures, while in lanes 9–12 and 19–22, DHFR mRNA was used as a competitor. The concentrations of the competitor RNAs are indicated above the lanes. The positions of the free RNA probe and of the TAP–CTE RNA complex are indicated on the left. The upper complex may represent two molecules of TAP bound to the CTE RNA. (**B**) *Xenopus* oocyte nuclei were injected with a mixture of *in vitro*-transcribed ^{32}P-labelled U1ΔSm RNA, U6Δss RNA and a precursor RNA containing the SRV-1 CTE inserted at the intron (Ad-CTE). Purified recombinant GST–TAP 61–619 or 1–619 was included in the injection mixtures as indicated. The concentration of the protein in the injected samples was 8 μM. RNA samples from total oocytes (T), cytoplasmic (C) and nuclear (N) fractions were collected immediately after injection (lanes 1–3) or 2.5 h after injection (lanes 4–12). Products of the splicing reaction were resolved on 10% acrylamide–7 M urea denaturing gels. One oocyte equivalent of RNA, from a pool of 10 oocytes, was loaded per lane. The mature products and intermediates of the splicing reaction are indicated diagrammatically on the left of the panels. The filled triangle represents the CTE.

maximal concentration tested (Figure 2A, lanes 9–12 and 19–22). Quantification of the data shown in Figure 2A, and of data obtained in independent experiments using different protein preparations (data not shown), demonstrated that TAP–CTE complexes formed with either TAP 61–619 or the full-length protein respond identically to the CTE and DHFR RNA competitors. It is noteworthy that the affinity of both proteins for the CTE RNA is about three orders of magnitude higher than for the DHFR mRNA.

We next tested the effect of both proteins on CTE-dependent export. Purified recombinant proteins were injected into *Xenopus* oocyte nuclei together with a mixture of three labelled RNAs. This mixture consisted of an adenovirus-derived precursor mRNA bearing the CTE in the intron (Saavedra *et al.*, 1997), U6Δss RNA and U1ΔSm RNA. U6Δss RNA is neither imported nor exported from the nucleus and serves as a control for accurate nuclear injection (Vankan *et al.*, 1992). U1ΔSm is exported by a different pathway than the CTE RNA (Pasquinelli *et al.*, 1997; Saavedra *et al.*, 1997) and serves as a control for the specificity of the recombinant proteins. Immediately after injection, all RNAs were found in the nuclear fraction

(Figure 2B, lanes 1–3). Following 2.5 h of incubation, splicing of the precursor RNA was complete, and the resulting mRNA, ~30% of the intron lariat bearing the CTE and U1ΔSm RNA were found in the cytoplasmic fraction (Figure 2B, lanes 4–6). In oocytes co-injected with similar amounts of GST–TAP 61–619 or 1–619, export of the intron lariat was stimulated and ~50% reached the cytoplasm (Figure 2B, lanes 7–9 and 10–12, respectively). Export of U1ΔSm RNA and of the spliced Ad mRNA was not affected (Figure 2B, lanes 7–12 versus 4–6). These results, together with data obtained using different protein preparations (data not shown), indicate that the additional 60 amino acids do not contribute to the stimulation of CTE-dependent export observed in *Xenopus* oocytes. Note that the modest stimulatory effect of TAP 1–619 on CTE-mediated export is due to the fact that the protein could not be assayed at higher concentrations; a stronger stimulatory effect was obtained when.TAP 61–619 was injected in higher amounts (Figure 5; Gruter *et al.*, 1998).

In the present report, most experiments were performed utilizing the full-length TAP (1–619). However, in some of the experiments described below, we utilized

Table I. Domain mapping of TAP

	CTE-binding *in vitro*	Stimulation of CTE-mediated export
1-619	+	+
61-619	+	+
61-372	+	+
80-372	+*	+*
102-372	+/–*	+/–*
146-372	–	n.d.[1]
195-372	–	–
61-341	+/–	n.d.
61-329	–	n.d.
61-265	–	–
540-619	–	–

* when fused to GST
[1] n.d. non determined

TAP 61–619, as the CTE-binding and export functions of this truncated protein cannot be distinguished from those of TAP 1–619 and higher yields are obtained when expressed in *E.coli*.

The CTE-binding domain of TAP defines a novel RNA-binding motif including a stretch of LRRs

To map its CTE-binding domain, various TAP deletion derivatives were expressed in *E.coli* as GST fusions. The ability of the truncated proteins to bind to the CTE RNA was tested by the electrophoretic gel mobility retardation assay described above. The specificity of the interaction was assessed by adding an excess of either competitor CTE RNA or M36 RNA in the binding reactions. A summary of the results is presented in Table I.

A truncated protein consisting of amino acids 61–372 from TAP bound the CTE RNA as efficiently as the fragment 61–619 (Figure 3, lanes 5–7 and 2–4, respectively). Formation of this RNA–protein complex was prevented by including unlabelled CTE RNA, but not M36 RNA, in the reaction mixtures (lane 6 versus 7). These findings suggest that this fragment is sufficient to bind specifically to the CTE RNA. This fragment includes four LRRs, which span amino acids 265–372 (Segref *et al.*, 1997). Deletion of additional amino acids from the C-terminus of truncation 61–372 compromised or abrogated binding. A truncated protein including amino acids 61–341 was still able to bind specifically to the CTE RNA, albeit with a reduced efficiency (lanes 8–10), while the truncation 61–329 no longer interacted with the CTE RNA (lanes 11–13). Thus, the C-terminus of the CTE-binding domain appears to correspond to the C-terminal boundary of the LRRs.

To define the N-terminus of the CTE-binding domain,

N-terminal deletions of the 61–372 fragment were performed. Deletion derivatives consisting of amino acids 80–372 and 102–372 specifically bound the CTE RNA (Table I; Figure 3, lanes 14–16), while the truncations containing amino acids 146–372 (lanes 17–19) or 195–372 (lane 20) were no longer able to interact with the CTE RNA. Binding of fragment 102–372 to the CTE RNA probe was specific, as evidenced by its competition with the CTE RNA. However, the complex formed was also partially competed by the M36 RNA and migrated with an unexpectedly low mobility (lanes 14–16). While the partial competition by the M36 RNA is likely to reflect a reduced affinity and/or specificity of the truncated protein for the CTE RNA, the basis of the anomalous migration of the formed complexes is unclear. Moreover, while fragment 61–372 without a GST tag binds to the CTE RNA when synthesized *in vitro* in rabbit reticulocyte lysates, fragments 80–372 and 102–372 bind the CTE RNA more efficiently when fused to GST (data not shown). These results suggest that amino acids 61–102 are likely to be required for the proper folding of fragment 102–372 rather than specifically contributing to the interaction.

In conclusion, the stretch of four LRRs plus its flanking N-terminal 205 amino acids (fragment 61–372) constitute the CTE-binding domain of TAP. Within this domain, amino acids 102–372 define the minimal CTE-binding domain, as this fragment can still interact specifically with the CTE RNA when fused to GST. Thus, the minimal CTE-binding domain appears to contain the RNA recognition determinants. These conclusions were confirmed further by the alanine scan analysis described below.

The β-strand region, but not the α-helices, of the LRRs may contact the CTE RNA directly

To gain more insight into how TAP interacts with the CTE RNA and to identify amino acid residues important for this function, we performed an alanine scan on the full-length protein. Three consecutive residues were substituted with alanines at the positions indicated in Figure 4A. To test their binding to the CTE RNA, mutant proteins were synthesized by coupled transcription–translation reactions *in vitro* (Figure 4B) and assayed by the electrophoretic gel mobility retardation assay described above (Figure 4C). The binding properties of the mutant proteins are summarized in Figure 4D.

Alanine substitutions between residues 1–106 and 437–619 did not affect CTE binding (Figure 4C, lanes 2–7 and 18–22, respectively). This result was expected as these mutations are located either outside or at the N-terminal boundary of the minimal CTE-binding domain (residues 102–372). Unexpectedly, substitution of residues 402–405 with alanines reduced CTE binding to ~15% (Figure 4C, lane 17). Because these residues are located downstream of the CTE-binding domain, we conclude that, in the context of the full-length protein, this mutation indirectly affects binding to the CTE RNA, probably by altering the folding of the neighbouring residues.

Within the minimal CTE-binding domain, substitution of amino acids 183–185 (TAP183) did not affect binding (Figure 4C, lane 9), while substitution of residues YDK at position 130–132 (TAP130) and of residues NRR at position 248–250 (TAP248) abolished or reduced binding
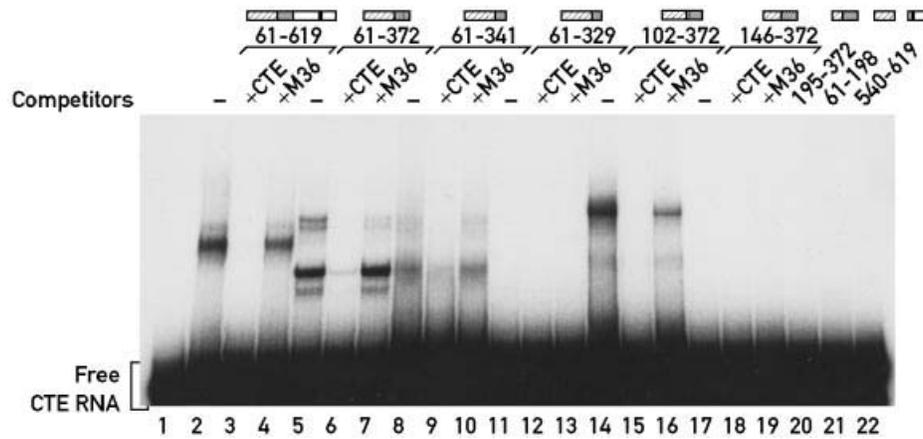
Fig. 3. The N-terminal domain of TAP including the LRRs is sufficient to interact with the CTE RNA. An electrophoretic mobility retardation assay was performed with a labelled CTE RNA probe and the purified recombinant proteins indicated above the lanes. For each protein, binding reactions were performed in the absence of competitor or in the presence of either CTE or M36 competitor RNAs as indicated. The concentration of the competitors was 15 µg/ml for the CTE and 50 µg/ml for the M36 RNA. The concentration of the recombinant proteins in the binding reactions was 10 µg/ml, with the exception of TAP 61–619 and 61–372, which were tested at 4 µg/ml, and TAP 102–372, 61–198 and 540–619, which were added at 8 µg/ml. The position of the free RNA probe is shown on the left. The additional complexes in lanes 5–7 were not characterized further.

to ~35%, respectively (Figure 4C, lanes 8 and 10). Further competition experiments revealed that TAP183 has a reduced affinity for the CTE RNA compared with the wild-type protein (data not shown). Interestingly, alanine substitutions in the predicted β-strand regions of the LRRs (mutants 269, 295, 319 and 349) abolished CTE binding (Figure 4C, lanes 11, 12, 14 and 16, respectively). In contrast, alanine substitutions within the predicted α-helices (mutants 306 and 332, Figure 4C, lanes 13 and 15, respectively) did not affect the interaction with the CTE RNA. Thus, residues located within the β-strand region of the LRRs may contact the CTE RNA directly. However, we cannot rule out that the absence of binding is due to misfolding of the mutant proteins.

In summary, analysis of the results obtained with the mutants generated by alanine substitution reveals that residues important for CTE binding are included within the minimal CTE-binding domain, with the exception of residues 402–405. None of the substitutions located between residues 1 and 104 impaired CTE binding (Figure 4C, lanes 2–7). As three of these mutations were located between amino acids 61 and 106, and because fragment 102–372 specifically binds the CTE RNA when fused to GST, we conclude that residues between positions 61 and 102 do not contribute specifically to the interaction with the CTE RNA. However, these residues appear to contribute substantially to the proper folding of the minimal CTE-binding domain, as this domain can hardly interact with the CTE RNA on its own. These results confirm the conclusions of the deletion analysis described above. Furthermore, our results point to an essential role for the β-strand region of the LRRs in contacting the CTE RNA.

The CTE-binding domain of TAP is sufficient to mediate CTE-dependent export

To define the domains of TAP required to promote CTE export, we analysed fragments 61–372 and 102–372 for their ability to overcome saturation and to stimulate CTE-dependent export directly in *Xenopus* oocytes. Fragment 61–372 was chosen in order to determine whether residues located C-terminal to the LRRs are required to mediate

CTE-dependent export. Fragment 102–372 was chosen as it represents the smallest fragment of TAP that can interact with the CTE RNA when fused to GST.

Purified recombinant proteins (shown in Figure 5C) were injected into oocyte nuclei together with the mixture of labelled RNAs used in Figure 2B. Saturation of CTE-dependent export was achieved by injecting an excess of unlabelled competitor CTE RNA along with the labelled RNAs. Immediately after injection, all RNAs were found in the nuclear fraction (Figure 5A, lanes 1–3). Following a 3.5 h incubation, 30% of the CTE-containing intron lariat was exported in the absence of competitor, while in the presence of competitor this export was reduced to ~10% (Figure 5A, lanes 4–6 versus lanes 7–9). The presence of the competitor destabilizes CTE-containing RNAs, as indicated by the increased intensity of the degradation products labelled by asterisks in Figure 5A. In oocytes co-injected with similar molar amounts of recombinant TAP 61–619 or truncation 61–372, saturation of the intron lariat export was not only overcome, but export was stimulated compared with the control without competitor (Figure 5A, lanes 10–15; 56% export for 61–619 and 62% export for 61–372). These results demonstrate that residues located C-terminal to the LRRs are not required to overcome saturation or to stimulate CTE-dependent export. Co-injection of truncation 102–372 also overcame saturation and re-established export, but only up to the level obtained in the absence of competitor, even though this protein was 2-fold more concentrated than fragment 61–372 (Figure 5A, lanes 16–18). This indicates that fragment 102–372 can at least bind and titrate the competitor RNA *in vivo*. Truncations 195–372 and 540–619, which did not interact with the CTE *in vitro* (Figure 3), had no effect (lanes 19–24). Note that, as expected, the three proteins that can overcome saturation also decrease the appearance of the degradation products (Figure 5A, lanes 10–18).

To test whether fragment 102–372 could stimulate CTE-dependent export directly, the recombinant proteins were injected into oocyte nuclei together with the mixture of labelled RNAs described above, but in the absence of
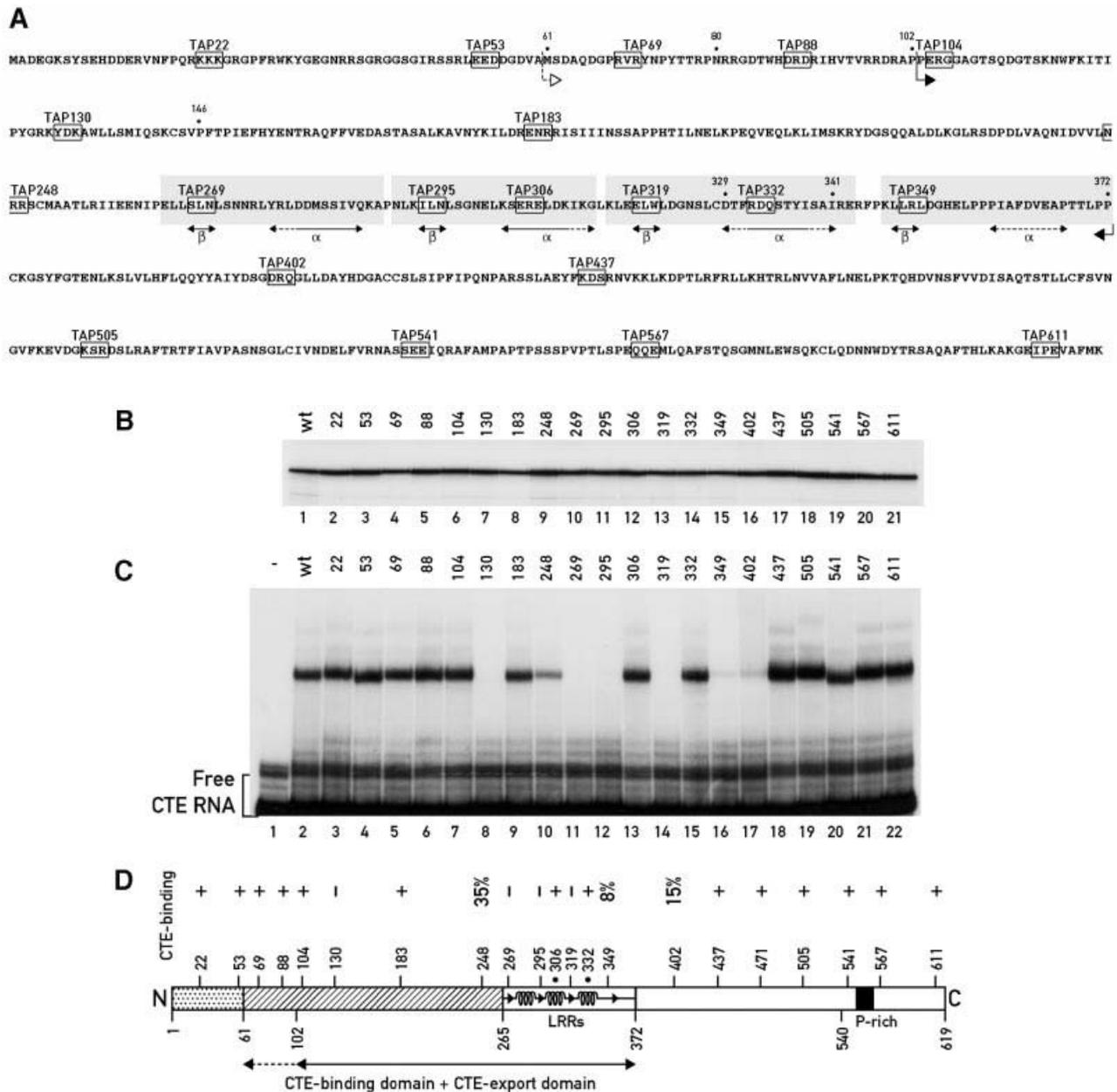
Fig. 4. The β-strand region, but not the α-helices, of the LRRs may contact the CTE RNA directly. (**A**) The positions of the alanine scan mutants are shown on the primary amino acid sequence of human TAP. Mutants are designated according to the position of the first substituted residue. The predicted folding of the LRRs is indicated below the sequence. The black dots above the sequence indicate the N- and C-termini of the truncations described in Figures 3 and 5. The open arrowhead below the sequence shows the N-terminus of the CTE-binding domain, while the filled arrowheads indicate the boundaries of the minimal CTE-binding domain. (**B**) Alanine scan mutants, indicated above the lanes, were *in vitro* translated in the presence of [35S]methionine. Proteins were analysed by SDS–PAGE followed by fluorography. (**C**) An electrophoretic mobility retardation assay was performed with a labelled CTE RNA probe and the *in vitro*-translated proteins indicated above the lanes. Symbols are as in Figure 2A. (**D**) Domain organization of human TAP protein. TAP domains are highlighted with different patterns. The predicted folding within the LRRs is indicated diagrammatically. The position of alanine substitutions and the binding properties of the mutants are shown. Binding activities were defined relative to the wild-type protein. +, binding at the wild-type level; –, binding activity below 5% relative to the wild-type protein. Note that although under these conditions mutant 183 was scored +, this mutant was impaired in binding in the presence of higher concentrations of competitor.

competitor RNA. Following a 3.5 h incubation, ~50% of the intron lariat bearing the CTE was found in the cytoplasmic fraction (Figure 5B, lanes 4–6). In oocytes co-injected with the same molar amounts of TAP 61–619 or fragment 61–372, export of the intron lariat was stimulated directly, and ~75 or 70%, respectively, reached the cytoplasm (Figure 5B, lanes 7–12). Fragment 102–372 stimulated CTE-dependent export up to 60% when

injected 2.5-fold more concentrated than fragment 61–372 (lanes 13–15), while it had no effect when injected at the same concentration (data not shown). Truncated proteins including amino acids 540–619 (lanes 16–18), 195–372 and GST alone (data not shown; see Table I), which did not bind to the CTE RNA *in vitro*, neither stimulated nor inhibited CTE-dependent export at the concentration tested. Thus, the minimal CTE-binding domain, when
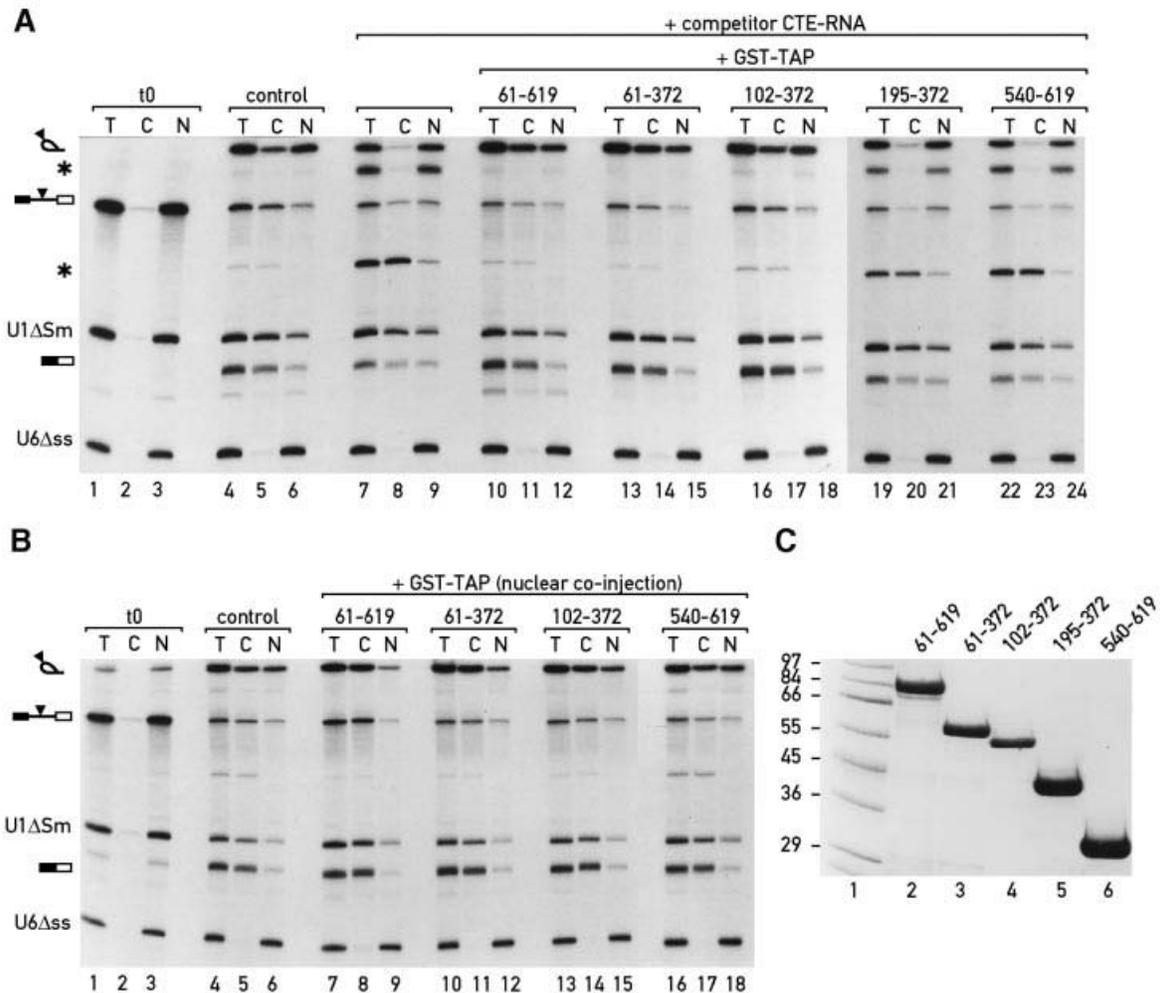
Fig. 5. The CTE-binding domain is sufficient to mediate CTE-dependent export. *Xenopus* oocyte nuclei were injected with the recombinant proteins indicated above the lanes and a mixture of radiolabelled Ad-CTE pre-mRNA, U1ΔSm RNA and U6Δss RNA. RNA samples from total oocytes (T), cytoplasmic (C) and nuclear (N) fractions were collected 3.5 h after injection, or immediately after injection [t_0: lanes 1–3 in (A) and (B)]. Products of the splicing reaction were resolved on 10% acrylamide–7 M urea denaturing gels. One oocyte equivalent of RNA, from a pool of 10 oocytes, was loaded per lane. The products of the splicing reaction are indicated diagrammatically on the left of the panel. The asterisks on the left of (A) indicate the positions of RNA molecules that are likely to be due to degradation of the intron lariat and of the precursor RNA. (**A**) In lanes 7–24, unlabelled competitor CTE RNA was included in the injection mixtures at 0.8 µM. The concentration of the proteins in the injected samples was as follows: 61–619, 14 µM; 61–372, 18 µM; 102–372, 195–372 and 540–619, 36 µM. (**B**) The concentration of the proteins in the injected samples was as follows: TAP 61–619 and 61–372, 14 µM; 102–372 and 540–619, 36 µM. (**C**) SDS–PAGE of the purified recombinant proteins used in (A) and (B).

fused to GST, can interact with the CTE both *in vitro* and *in vivo* and stimulate its export to the cytoplasm, although with a reduced efficiency compared with fragments 61–619 and 61–372. In contrast, fragment 61–372 appears to be as efficient as the full-length protein in stimulating CTE-mediated export *in vivo*, indicating that residues required for export are comprised within the CTE-binding domain. These results suggest that the CTE-binding domain not only recognizes the CTE RNA but interacts with other components of the nuclear transport machinery.

The CTE-binding domain includes the signals for nuclear export

The reduced efficiency of fragment 102–372 in stimulating CTE-dependent export may reflect its reduced affinity for the CTE RNA rather than an impaired export function. To distinguish between these two possibilities and to investigate further how TAP and this domain promote CTE export, we analysed their interaction with the CTE

RNA during the export process by co-immunoprecipitation. Recombinant TAP 61–619 or the fragment 102–372, fused to GST, were co-injected into the oocyte nucleus together with labelled Ad-CTE pre-mRNA, U6Δss RNA and U1ΔSm RNA. Export was allowed to take place for 3 h. After this period, total oocytes or cytoplasmic and nuclear fractions were collected and subjected to immunoprecipitation using anti-GST antibodies. RNAs in the input fractions, supernatants and immunoprecipitates were analysed on denaturing polyacrylamide gels.

Figure 6A shows that the injected proteins co-immunoprecipitate CTE-containing RNAs from both the nuclear and cytoplasmic fractions (lanes 9–11 and 12–14, respectively). Similar results were obtained with fragment 61–372 (data not shown). The co-immunoprecipitations were specific for the following reasons. First, only CTE-harbouring RNAs were precipitated above the background levels; the spliced mRNA, U1ΔSm and U6Δss RNAs remained in the supernatant fractions (Figure 6A, lanes
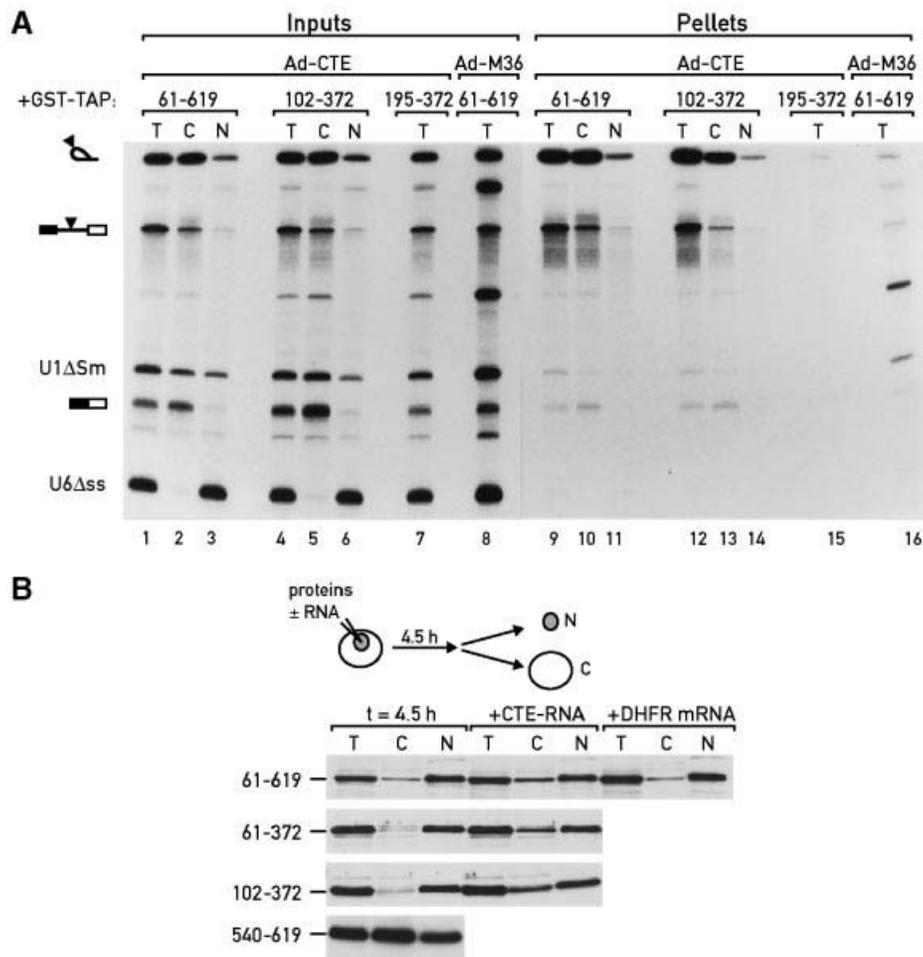
Fig. 6. TAP and the minimal CTE-binding domain enter the cytoplasm upon nuclear injection. (**A**) Purified recombinant TAP (61–619) or the truncations 102–372 or 195–372 were injected into oocyte nuclei together with a mixture of radiolabelled U1ΔSm, U6Δss and Ad-CTE RNAs. In lanes 8 and 16, Ad-CTE was replaced by Ad-M36 RNA. RNA samples were collected 3 h after injection and analysed directly (Inputs), or subjected to immunoprecipitation using anti-GST antibodies (Pellets). The concentration of the proteins in the injected samples was 14 μM for GST–TAP 61–619 and 36 μM for 102–372 and 195–372. RNAs in the input or the precipitates were analysed as described in Figure 2B. Symbols are as in Figure 2B. (**B**) Purified recombinant TAP and the truncated proteins indicated on the left of the panels were injected into *Xenopus* oocyte nuclei in either the absence or the presence of CTE RNA or DHFR mRNA as indicated above the lanes. Protein samples from total oocytes (T), cytoplasmic (C) and nuclear (N) fractions were collected 4.5 h after injection, and analysed by Western blotting using a rabbit anti-GST antibody. One oocyte equivalent of proteins, from a pool of 10 oocytes, was loaded per lane. The concentration of TAP fragments in the injected samples was ~18 μM, and of the CTE and DHFR RNAs 9 and 6 μM, respectively.

9–16; data not shown). Secondly, when GST–TAP was co-injected with an RNA mixture in which Ad-CTE was replaced by Ad-M36, the M36-bearing substrates were not co-immunoprecipitated (Figure 6A, lane 16). Thirdly, no co-immunoprecipitation of CTE-bearing RNAs was observed when the truncation 195–372, which does not bind to the CTE, was co-injected with the labelled RNAs (Figure 6A, lane 15). As the recombinant proteins initially were injected into the nucleus, the observation that they co-immunoprecipitate CTE-containing RNAs from the cytoplasmic fractions suggests that both TAP 61–619 and the CTE-binding domain entered the cytoplasm upon nuclear injection.

To confirm these observations further, we followed the fate of the injected proteins. Recombinant proteins were injected into the nucleus with or without the CTE RNA. Following 4.5 h incubation, their final location was analysed by Western blot using anti-GST antibodies. In the absence of the CTE RNA, TAP 61–619 as well as fragments 61–372 and 102–372 remained predominantly

in the nuclear compartment (Figure 6B). However, when the CTE RNA was co-injected, ~40% of the injected samples entered the cytoplasm. Co-injection of similar amounts of DHFR mRNA (Figure 6B) or M36 RNA (data not shown) did not result in the cytoplasmic accumulation of the recombinant proteins. Although we cannot distinguish whether the proteins were exported as a complex with the CTE RNA, or whether they were exported on their own and bound to the CTE RNA in the cytoplasm, these results clearly establish that TAP and the minimal CTE-binding domain are exported from the nucleus. Therefore, this domain includes the signals for nuclear export. Fortuitously, we observed that a fragment comprising amino acids 540–619 from TAP migrated to the cytoplasm upon nuclear injection, suggesting that TAP may have an additional NES at its C-terminus. However, this putative NES was not characterized further as it was not required to stimulate CTE-dependent export in *Xenopus* oocytes.

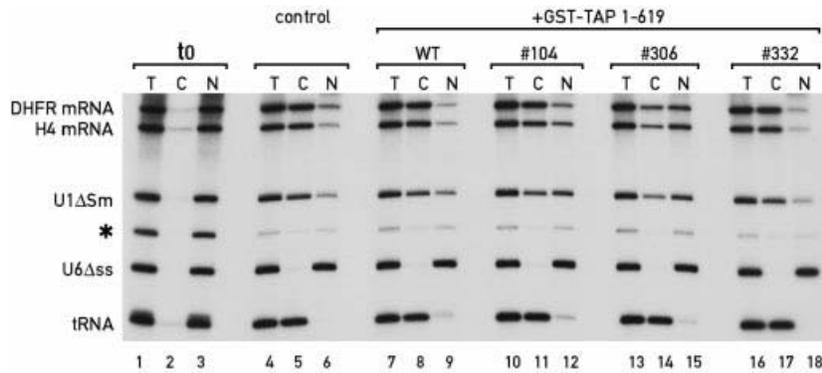The observation that DHFR mRNA has no effect on

Fig. 7. Alanine substitutions within the CTE-binding domain interfere with mRNA export from the nucleus. Purified recombinant TAP 1–619 and the mutant proteins indicated above the lanes were injected into the oocyte cytoplasm. After 1 h incubation, a second microinjection was performed into the oocyte nuclei with a mixture of radiolabelled RNAs. This mixture consisted of DHFR mRNA, histone H4 mRNA, U1ΔSm, U6Δss and human initiator methionyl tRNA. RNA samples from total oocytes (T), cytoplasmic (C) and nuclear (N) fractions were collected 140 min after injection, and analysed on 8% acrylamide–7 M urea denaturing gels. One oocyte equivalent of RNA, from a pool of 10 oocytes, was loaded per lane. The concentration of the proteins in the injected samples was ~10 μM. The asterisk indicates the position of a degradation product derived from U1 snRNA which was not stable in the oocytes.

TAP export, and that under the conditions in which GST– TAP co-immunoprecipitates the CTE RNA neither the spliced mRNA nor DHFR mRNA were precipitated (Figure 6; data not shown), provides support for the hypothesis that the mode of interaction of TAP with the CTE RNA must be different from that with cellular mRNAs (see Introduction and Discussion).

The CTE-binding domain is also implicated in mediating mRNA nuclear export

To identify essential residues required for the export function of TAP, the alanine substitution mutants described in Figure 4A were expressed in *E.coli* as GST fusions. The recombinant proteins were purified on glutathione– agarose beads, and tested for their effects on CTE- dependent and mRNA nuclear export by microinjection in *Xenopus* oocytes. TAP derivatives that could no longer interact with the CTE RNA at the concentration tested, had no effect on CTE-dependent export. In contrast, mutants that interacted with the CTE RNA *in vitro* stimulated its export to the cytoplasm (data not shown). Thus, we have been unable to uncouple the CTE-binding and export functions of TAP.

TAP has also been implicated in mediating mRNA export from the nucleus (see Introduction). To investigate whether the mutants we had generated had an effect on this process, we injected the full-length protein and the alanine-scanning mutants located in the CTE-binding domain into *Xenopus* oocytes and analysed their effect on mRNA export. One hour after injection of the recombinant proteins, a mixture of DHFR mRNA, histone H4 mRNA, U1ΔSm, U6Δss and tRNA was injected into the nucleus and their export was analysed 140 min later. Figure 7 shows the results obtained when mutants 104, 306 and 332 were pre-injected in the oocyte cytoplasm. Similar results were obtained when the recombinant proteins were injected into oocyte nuclei (data not shown). In control oocytes and in oocytes injected with the full-length protein or mutant 332, ~70% of the DHFR mRNA, 72% of histone H4 mRNA and 60% of the U1 snRNA moved to the cytoplasm (Figure 7, lanes 4–9 and 16–18). tRNA export was complete (Figure 7, lanes 4–18). In oocytes pre- injected with mutant 306, export of both DHFR and H4

mRNAs was reduced, and only 47 or 38%, respectively, reached the cytoplasm (Figure 7, lanes 13–15). Although the inhibitory effect of TAP306 was not complete, it was obtained reproducibly in several independent experiments (data not shown). U snRNA export was reduced down to 66% relative to the control oocytes. However, this inhibi- tion did not increase with increasing concentrations of the recombinant protein (data not shown), and was also observed with mutant 104 (Figure 7, lanes 10–12). This inhibition is reminiscent of the small inhibitory effect that an excess of the CTE RNA has on U snRNA export (Pasquinelli *et al.*, 1997; Saavedra *et al.*, 1997). Binding of mutant 306 to the CTE RNA *in vitro* is almost indistinguishable from binding of the wild-type protein, and *in vivo* this mutant did not affect the export of tRNA (Figure 7) nor of the intron lariat bearing the CTE (data not shown). Thus, we conclude that mutant 306 interferes *in vivo* with the function of the wild-type protein in mediating the export of mRNAs. This observation consti- tutes direct evidence for a role for TAP in this process.

Discussion

Previously we have shown that TAP directly binds to the CTE RNA and stimulates its export to the cytoplasm (Gruter *et al.*, 1998). Here we show that binding of TAP to the CTE RNA is mediated by a novel RNA-binding motif including a stretch of LRRs. We also show that the CTE-binding domain is sufficient to stimulate CTE- dependent export *in vivo*, suggesting that this domain includes sequences essential for export. Finally, we show that a TAP derivative, having three residues substituted with alanines within the CTE-binding domain, reduces the export of mRNAs.

The CTE-binding domain of TAP defines a novel RNA-binding motif

The experiments reported here show that the LLRs plus an N-terminal extension of 205 amino acids mediate specific CTE binding. This domain bears no obvious similarity to other RNA-binding proteins (reviewed by Burd and Dreyfuss, 1994) and thus appears to constitute a novel RNA-binding motif. LRRs are found in a number

of otherwise unrelated proteins and are thought to be involved in protein–protein interactions (reviewed by Kobe and Deisenhofer, 1994, 1995). Although some LRR-containing proteins can bind non-protein ligands, to our knowledge LRRs have not yet been reported to be implicated directly in RNA binding.

TAP is present in HeLa nuclear extracts, co-purifies with general RNA-binding proteins on single-stranded DNA columns (Gruter *et al.*, 1998) and exhibits a low, non-specific affinity for RNAs (unpublished results). Thus, TAP may normally act as an RNA-binding protein. Alternatively, TAP may not contact cellular RNAs directly (see below), and the CTE-binding domain may act as a protein–protein interaction domain when TAP performs its cellular function(s).

The crystal structure of porcine ribonuclease inhibitor, which is almost completely composed of LRRs, demonstrated that the repeats correspond to β-strand/α-helix structural units (Kobe and Deisenhofer, 1994, 1995). Due to the high conservation of residues at consensus positions throughout the LRR superfamily, it has been proposed that the structure of LRRs in other proteins will closely resemble that of LRRs in ribonuclease inhibitor (Kobe and Deisenhofer, 1994, 1995). Based on the proposed role of the β-strand region of LRRs in protein–protein interaction and on the observation that the β-strand region of the LRRs appears to contact the CTE RNA directly, it is tempting to speculate that the CTE may have evolved in order to mimic a protein interaction(s) and directly contacts TAP, thereby subverting the protein from its normal cellular function. However, because we cannot rule out the possibility that mutants impaired in binding exhibit folding defects, structural studies on CTE–TAP complexes and the identification of the cellular ligand(s) of this 'orphan' LRR domain will be required in order to confirm this hypothesis.

The minimal CTE-binding domain contains the signals for nuclear export

In spite of the anomalous migration of the complexes formed between the CTE RNA and the minimal CTE-binding domain fused to GST, this domain can stimulate CTE-dependent export, although with a reduced efficiency compared with the wild-type protein or fragment 61–372. Moreover, our data show that TAP and the CTE-binding domain are exported to the cytoplasm upon nuclear injection. Although we cannot distinguish whether the proteins are exported as a complex with the CTE RNA or independently of the RNA, the results shown in Figure 6 indicate that the minimal CTE-binding domain contains not only RNA recognition determinants but also the signals for nuclear export. Thus, by deletion analysis, we were unable to identify independent regions required only for CTE binding or for export. Similarly, analysis of the binding and export activities of the alanine scan mutants did not allow us to uncouple binding from export activity. This would be expected if both functions were carried out by the same residues. It is also possible that residues important for stimulating CTE-dependent export were not targeted in our alanine scan.

It is of note that export of TAP is only observed in the presence of the CTE RNA; in the absence of the CTE, the protein remains nuclear. Thus, if TAP is shuttling, its re-import is probably an extremely efficient process, and further studies will be required to define the NLSs on the protein. The CTE-binding domain constitutes only 50% of the wild-type protein, thus it is likely that residues located in its C-terminal half will be involved in the shuttling and recycling of the protein or in the function of TAP as an mRNA nuclear export mediator.

Role of TAP in mRNA nuclear export
A role for TAP in mRNA export was supported by the following observations. First, in yeast, the gene encoding Mex67p is essential for mRNA export (Segref *et al.*, 1997). Secondly, injection of saturating amounts of CTE RNA into *Xenopus* oocytes prevents DHFR mRNA and the spliced Ad mRNA from exiting the nucleus (Pasquinelli *et al.*, 1997; Saavedra *et al.*, 1997), and this export block is released by co-injection of TAP (Gruter *et al.*, 1998). In this study, we provide direct evidence for a role for TAP in mediating mRNA nuclear export. Among the mutations we have created and analysed, TAP306 substantially reduces the export of mRNAs from the nucleus. As TAP306 does not interfere with the nuclear exit of CTE-bearing RNAs, it is likely that the mRNA export defect of this mutant is attributable to its inability to interact with some components of the nuclear export machinery which are required for mRNA nuclear export but bypassed by the CTE. These observations provide further support for the hypothesis that the mode of interaction of TAP with cellular mRNAs appears to be different from that with the CTE RNA (see Introduction). *In vitro*, this is indeed the case, as binding of TAP to DHFR mRNA is two to three orders of magnitude lower than for the CTE RNA, even in the presence of HeLa nuclear extracts (unpublished observations). Moreover, under the conditions in which the CTE RNA was co-immunoprecipitated with TAP, we could not observe co-immunoprecipitation of DHFR mRNA nor of the spliced Ad mRNA (Figure 6; data not shown). One interpretation of these results is that TAP–mRNA complexes cannot be recovered in significant amounts because they are not stable under our experimental conditions. This may be due to the fact that the affinity of TAP for mRNAs is lower than for the CTE RNA, and/or to the fact that TAP associates with mRNAs via protein–protein interactions. Independently of whether the interaction of TAP with the mRNA is direct or not, it is also conceivable that there is an efficient cellular mechanism that triggers dissociation of TAP–mRNA complexes upon translocation through the NPC, and we mimic those conditions by homogenizing oocyte nuclei. Thus, whether TAP directly contacts the mRNA during the export process, and how this interaction is regulated, remains to be defined. Studies of the mechanism by which TAP mediates CTE-dependent export and the identification of its interacting partners are likely to lead to additional major insights into the steps governing the nuclear export of cellular mRNAs.

Materials and methods

Cloning of full-length TAP cDNA
Full-length human TAP cDNA was obtained by PCR using KlenTaq enzyme, human HeLa cDNA as a template (Clontech) and primers containing the appropriate restriction sites. Primers were designed based

on the human ESTs and on the homology with the rat and mouse cDNAs. The GenBank accession Nos of human TAP ESTs are AA173362, R14280, AA307086, T17341 and T33563. PCR fragments were cloned into the *Nco*I–*Eco*RI sites of a derivative of pBSSK+ (Stratagene) having the β-globin 5′-untranslated region inserted between the *Hin*dIII and *Eco*RI sites. TAP cDNA was also cloned into the *Nco*I–*Bam*HI sites of pGEXCS (Parks *et al.*, 1994). Plasmid pBSSK-TAP was used as a template in the *in vitro* transcription–translation coupled system.

DNA templates for in vitro RNA synthesis
DNA templates for *in vitro* RNA synthesis of CTE and M36 RNAs, Ad-CTE, Ad-M36, DHFR mRNA, histone H4 mRNA, U1ΔSm and U6Δss RNAs, and human methionyl tRNA have been described previously (Jarmolowski *et al.*, 1994; Saavedra *et al.*, 1997). Synthesis of Ad-CTE, Ad-M36, DHFR mRNA, histone H4 mRNA and U1ΔSm were primed with the m⁷GpppG cap dinucleotide, whereas synthesis of U6Δss RNAs was primed with γ-mGTP.

In vitro translation
For generation of ³⁵S-labelled *in vitro* translated proteins, the combined *in vitro* transcription–translation (TnT) kit from Promega was used. Reactions were carried out at 30°C for 2 h. Translation was checked by SDS–PAGE and subsequent fluorography using intensify solutions from Amersham (Amplify). *In vitro*-translated proteins (1 μl) were assayed directly for binding to the CTE RNA without further purification.

In vitro CTE binding assay
For native gel assays, a 224 nucleotide CTE RNA probe was employed. Synthesis and purification of the RNA probe and of unlabelled competitor RNAs was as described previously (Gruter *et al.*, 1998). Yeast RNA (0.2 mg/ml), poly(C) and single-stranded DNA (0.02 mg/ml each) were used as unlabelled competitors. Reactions were carried out in binding buffer (10 mM HEPES pH 7.9, 50 mM KCl, 5 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol and 0.025% NP-40). Final sample volumes were 10 μl. Recombinant proteins were added to the reaction mixtures from a diluted stock solution in the same buffer. After 30 min at room temperature, 1 μl of a solution containing 0.2 mg/ml of heparin and 0.05% bromophenol blue was added to the reaction mixtures, and incubation was continued for another 10 min at room temperature. Samples were applied to a 5% non-denaturing polyacrylamide gel (19:1 acryl:bisacryl ratio). Electrophoresis was carried out at a constant voltage of 17 V/cm at 4°C in 0.5× TBE buffer. Complexes were visualized by autoradiography. The amounts of recombinant proteins and of competitor RNA used are indicated on the figure legends.

TAP truncations and expression of recombinant proteins
All TAP truncations were generated by PCR and confirmed by sequencing. 5′ Oligos were designed with an *Nco*I site and 3′ oligos had a stop codon followed by a *Bam*HI site; thus all truncations were cloned as *Nco*I–*Bam*HI fragments into the *Nco*I–*Bam*HI sites of pGEXCS (Parks *et al.*, 1994). Proteins were expressed in *E.coli* as GST fusions using these pGEXCS constructs. Recombinant proteins were prepared as described by Gruter *et al.* (1998).

Mutagenesis
All mutations were introduced using an oligonucleotide-directed *in vitro* mutagenesis system from Stratagene (Quick-change site-directed mutagenesis) following the instructions of the manufacturer. Most mutants were generated on pBSSK-TAP 1–619 and subsequently subcloned into pGEXCS-TAP 1–619. Mutations were confirmed by restriction mapping and, in some cases, by sequencing. Sequences of the oligodeoxyribonucleotides are available upon request.

Xenopus laevis oocyte microinjections and RNA analysis
Oocyte injections and analysis of microinjected RNA by denaturing gel electrophoresis and autoradiography analysis were performed as previously described (Jarmolowski *et al.*, 1994; Saavedra *et al.*, 1997). Quantification was performed with a phosphoimager (Bio-Rad). The concentrations of RNAs and recombinant proteins in the injected samples are indicated in the figure legends. Isolation of protein from oocytes, SDS–PAGE and Western blot analysis were carried out as described previously (Kambach and Mattaj, 1992).

For immunoprecipitation, 20 injected oocytes or 20 nuclear and cytoplasmic fractions were homogenized in 200 μl of TNE buffer (10 mM Tris–HCl pH 8.0, 50 mM NaCl and 1 mM EDTA). The insoluble fraction (yolk and pigment) was removed by centrifugation for 5 min in a microfuge. Soluble fractions were added to 20 μl of protein A–

Sepharose CL4B beads (Pharmacia) pre-coated with anti-GST antibodies. Binding was performed for 1 h at 4°C. Beads were washed four times with 500 μl of ice-cold IPP buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl) and incubated with 300 μl of homo medium (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 5 mM EDTA, 1.5% SDS and 1.5 mg/ml of proteinase K) for 1 h at 50°C. One-tenth of the supernatants and of the inputs were also incubated with 300 μl of homo medium. RNAs were extracted twice with the same volume of equilibrated phenol, and precipitated with ethanol. Resuspension was in 50 μl of RNA dye (95% formamide, 0.05% SDS, 0.05% bromophenol blue and 0.05% xylene cyanol), and 25 μl were analysed on denaturing polyacrylamide gels.

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