CRM1 and Ran are present but a NES-CRM1-RanGTP complex is not required in Balbiani ring mRNP particles from the gene to the cytoplasm

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Summary

Messenger RNA is formed from precursors known as pre-mRNA. These precursors associate with proteins to form pre-mRNA-protein (pre-mRNP) complexes. Processing machines cap, splice and polyadenylate the pre-mRNP and in this way build the mRNP. These processing machines also affect the export of the mRNP complexes from the nucleus to the cytoplasm. Export to the cytoplasm takes place through a structure in the nuclear membrane called the nuclear pore complex (NPC). Export involves adapter proteins in the mRNP and receptor proteins that bind to the adapter proteins and to components of the NPC. We show that the export receptor chromosomal region maintenance protein 1 (CRM1), belonging to a family of proteins known as importin-β-like proteins, binds to gene-specific Balbiani ring (BR) pre-mRNP while transcription takes place. We also show that the GTPase known as Ran binds to BR pre-mRNP, and that it binds mainly in the interchromatin. However, we also show using leptomycin B treatment that a NES-CRM1-RanGTP complex is not essential for export, even though both CRM1 and Ran accompany the BR mRNP through the NPC. Our results therefore suggest that several export receptors associate with BR mRNP and that these receptors have redundant functions in the nuclear export of BR mRNP.

Key words: Gene expression, mRNA export, Export receptors

Introduction

Transport of RNA and proteins to and from the cell nucleus occurs through the nuclear pore complex (NPC) (Stutz and Rosbach, 1998; Görlich and Kutay, 1999; Kuersten et al., 2001; Conti and Izaurralde, 2001; Weis, 2002). In general, transport is mediated by soluble transport receptor proteins that bind to signals on the cargo and interact with nuclear pore complex components that contain phenylalanine/glycine (FG)-rich repeat motifs (Rout et al., 2000; Ribbeck and Görlich, 2001; Stewart et al., 2001). Three classes of transport receptors have been identified: the family of importin-β-like proteins (importins and exportins, together called karyopherins), the nuclear transport factor 2 (NTF2) and the nuclear export factor (NXF) protein family.

Karyopherins bind the small GTPase Ran, which regulates the interaction between the karyopherins and their cargoes (Azuma and Dasso, 2000; Kuersten et al., 2001). In the nucleus, Ran is present as Ran-GTP, which induces the release of cargoes from import karyopherins and assists the binding of cargoes to export karyopherins (Conti and Izaurralde, 2001). The GTP hydrolysis activity and the guanine nucleotide affinity of Ran are modulated by accessory proteins (Bischoff et al., 1994; Bischoff et al., 1995; Ohtsubo et al., 1989; Bischoff and Ponstingl, 1995). As a result, it is expected that a steep gradient in the concentration of Ran-GTP/Ran-GDP will exist across the nuclear envelope (Mattaj and Englmeier, 1998; Kalab et al., 2002). This asymmetric distribution is crucial for the directionality of nucleocytoplasmic transport (Izaurralde et al., 1997).

Export signals are not part of mRNA itself, but reside in adapter proteins that associate with the mRNA in mRNA-protein (mRNP) complexes. Each pre-mRNA associates with many different proteins (Dreyfuss et al., 1993; Daneholt, 1997; Krecic and Swansson, 1999). Processing events rebuild the pre-mRNP and contribute to form export competent mRNP (Brodsky and Silver, 2001; Maniatis and Reed, 2002; Weis, 2002). Many putative adapter proteins, containing export signals, are present in mRNP (Nakielny and Dreyfuss, 1999; Gallouzi and Steitz, 2001). Components of the exon-exon junction complex (EJC) (LeHir et al., 2000; Kataoka et al., 2000; McGarvey et al., 2000), most notably RNA and export factor (REF)/Aly, play a role in mRNA export (for references see Conti and Izaurralde, 2001). Other putative adapter proteins include hnRNP A1 (Pinol-Roma and Dreyfuss, 1992; Michael et al., 1995), which shuttles through interaction of a 38-amino acid residue M9 sequence and the receptor transportin 1 (Pollard et al., 1996; Siomi et al., 1997; Gallouzi and Steitz, 2001); hnRNP K (Görlich and Kutay, 1999); and the serine and arginine-rich (SR) protein family members SRp20, 9G8 and ASF/SF2 (Caceres et al., 1998; Huang and Steitz, 2001). The three SR proteins appear to serve as adapter proteins for the export receptor Tip-associated protein (TAP) (Huang et al., 2003). In yeast, transcriptionally coregulated transcripts use specific mRNA export factors and multiple export pathways. This may represent a mechanism for the

coordination of export of functional groups of mRNA (Hieronymus and Silver, 2003).

The role of karyopherins in mRNA export is unclear. CRM1 (chromosomal region maintenance protein 1) recognises a leucine-rich nuclear export signal (NES) (Stade et al., 1997; Fukuda et al., 1997), and the complex with the NES is stabilised by the co-operate binding of RanGTP (Askjaer et al., 1998). CRM1 employs different cofactors to support recognition of its different cargoes (Kuersten et al., 2001). In addition to mediating the export of many proteins, CRM1 is an export receptor for U snRNAs (Ohno et al., 2000), the 60S ribosomal subunit (Ho et al., 2000), 5S rRNA (Murdoch et al., 2002) and HIV-1 RNA (Pollard and Malim, 1998). CRM1 binds the cytotoxin leptomycin B (LMB). LMB modifies a critical cysteine residue and in this way prevents the formation of the trimeric NES-CRM1-RanGTP complex. Experiments using LMB have shown that CRM1 inhibition influences mRNA export (Watanabe et al., 1999), while experiments using a phenotype of a temperature-senistive crm1/xpo1 allele suggests that crm1/xpo-1 is involved in mRNA export (Stade et al., 1997). Several observations, however, have shown that mRNA export in general is not mediated by CRM1 and Ran-GTP (Izaurralde et al., 1997; Neville and Rosbach, 1999; Clouse et al., 2001; Herold et al., 2003).

The NXF1 export receptor (also called TAP in humans and Mex67p in *Saccharomyces cerevisiae*) is the dominating export receptor for mRNA (Segref et al., 1997; Grüter et al., 1998; Braun et al., 1999; Kang and Cullen, 1999; Tan et al., 2000; Braun et al., 2001). TAP binds to REF/Aly and forms a heterodimer with an NTF2-like protein, p15 (for references see Conti and Izaurralde, 2001; Braun et al., 2002). Given the complexity of a dynamic mRNA population, it is, however, not surprising that more than one mRNA export pathway exist. Higher eukaryotes have several NXF members, some of which are tissue specifically expressed (Herold et al., 2000). One member, NXF3, also lacks a C-terminal F/G binding domain and may export certain mRNAs by interacting with CRM1 (Yang et al., 2001). AU-rich elements in 3′ untranslated regions of mRNAs of early response genes interact with regulatory proteins. One such protein, HuR, serves as an adapter for c-fos mRNA export through a heat shock-sensitive interaction with karyopherin β2B (transportin 2) (Gallouzi and Steitz, 2001), and it interacts with CRM1 through two protein ligands, pp32 and APRIL (Gallouzi and Steitz, 2001). HuR may also be the export adapter for heat shock mRNAs (Gallouzi et al., 2001). Recently, it was reported that the karyopherin β2B is important for the export of a considerable portion of mRNA in HeLa cells (Shamsher et al., 2002). Karyopherin β2B is bound to TAP in a manner that depends on Ran-GTP. It is therefore at present unclear to what extent mRNA export is dependent on karyopherins and Ran-GTP, and it is unclear whether there are species differences in this respect (Izaurralde et al., 1997; Clouse et al., 2001; Shamsher et al., 2002).

Here, we ask whether CRM1 and Ran bind to individual mRNP complexes and, if so, where in the nucleus they bind in relation to the intranuclear gene expression pathway. We also ask whether this binding, if present, plays a role in nuclear export. We have taken advantage of the fact that the gene-specific Balbiani ring (BR) mRNPs in salivary gland cells of *Chironomus tentans* can be followed from their synthesis on the genes to their export through the NPCs (Daneholt, 1997). CRM1 associates with BR pre-mRNP during transcription, and Ran is recruited to the BR mRNP mainly in the interchromatin. Both CRM1 and Ran then accompany the BR mRNP to and through the NPCs. Inhibition of CRM1 function with LMB shows that a NES-CRM1-RanGTP complex is not essential for nuclear export of this mRNP, but may contribute to efficient translocation of the BR mRNP through the NPC.

Materials and Methods

Animals and cells

Chironomus tentans was raised as described by Meyer et al. (Meyer et al., 1983). *C. tentans* diploid epithelial cells were cultivated as previously described (Wyss, 1982).

Antibodies

Monoclonal antibodies against the N-terminal part of human CRM1 and human Ran were obtained from DB Transduction Laboratories. The anti-human von Willebrand factor VIII antibody (DAKO) was used as a negative control. Monoclonal antibodies specific for RNA-binding proteins in *C. tentans*, 3G1 (for hrp36) (Visa et al., 1996) and 1D3 (for hrp23) (Sun et al., 1998) were gifts from B. Daneholt (Karolinska institute). Anti-Ct-eIF4H antibodies were produced in rabbits (Björk et al., 2003). Anti-mouse immunoglobulin antibodies conjugated with 6 and 12 nm gold particles (Jackson ImmunoResearch Labs) were used as secondary antibodies for immunocytology and immunoelectron microscopy. The FITC-conjugated F(ab′) fragment of anti-rabbit immunoglobulins (DAKO) was used for immunofluorescence, and antibodies against mouse immunoglobulins (DAKO) for immunoprecipitation.

Isolation and sequencing of cDNA

Degenerate oligodeoxynucleotide primers were based on two sets of amino acid sequences, GSISG and GVQDMA for CRM1, and VGDGG and IMFDVT for Ran**,** which are conserved in *Homo sapiens*, *Xenopus laevis*, *Drosophila melanogaster*, *Schizosaccaromyces pombe* and *Saccharomyces cerevisiae*. The primer pairs were used for touchdown PCR with *C. tentans* cDNA as template. PCR fragments were cloned into the PCR 2.1 vector (Invitrogen), sequenced and analysed by database searches. Two fragments that had a high sequence similarity to CRM1 and Ran in different species were used as probes to screen a *C. tentans* λ ZAP cDNA library. cDNA inserts were sequenced using the Dynamic ET Terminator Cycling Sequencing premix kit (Amersham Pharmacia Biotech) and analysed on a 373A Automated DNA Sequencer (Applied Biosystems). The DNA and protein sequences were analysed by the University of Wisconsin Genetics Computer Group (GCG) Sequence Analysis Programs.

Expression of the Ct-CRM1 N-terminal polypeptide and GST-Ct-Ran

The pET-15b expression vector (Novagen) was used for producing the N-terminal part (amino acid residues 1-329) of Ct-CRM1 (Ct-CRM1-N) in *Escherichia coli* BL21(DE3)LysS cells. Ct-Ran was expressed as a GST fusion protein after cloning into the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech). The His-tagged Ct-CRM1-N polypeptide was purified by Ni-NTA affinity chromatography (Qiagen). The GST-Ct-Ran protein was purified on a glutathione-Sepharose affinity column (Amersham Pharmacia Biotech).

Protein extraction and western blot analysis

Extracts from *C. tentans* tissue culture cells were prepared as

described by Zhao et al. (Zhao et al., 2002). For analyses of proteins in salivary gland cells, glands were fixed for 2 minutes at 4°C in TKM (10 mM triethanolamine-HCl, pH 7.0, 100 mM KCl, 1 mM $MgCl_2$) buffer containing 2% paraformaldehyde. The fixed glands were washed with cold TKM and the cells were manually isolated. For analysis of chromosomal proteins, approximately 1200 polytene chromosomes were isolated from salivary glands according to Zhao et al. (Zhao et al., 2002). Chromosomes or gland cells were boiled in sample buffer and, after separation by electrophoresis in 10% polyacrylamide-SDS gels, the proteins were transferred to membranes (Immobilon PVDF, Millipore). Filters were probed with the anti-CRM1 antibody (1:1000 dilution) or the anti-Ran antibody (1:5000 dilution). HRP-labelled secondary antibodies were detected by the ECL detection system (Amersham Pharmacia Biotech.).

Immunostaining of isolated polytene chromosomes

Polytene chromosomes were isolated as described above and attached to a glass surface. Immunostaining was performed as described by Zhao et al. (Zhao et al., 2002), using the anti-CRM1 antibody (1:30 dilution), the anti-Ran antibody (1:300 dilution) or the anti-von Willebrand factor VIII antibody (1:50 dilution).

Some isolated chromosomes were incubated with 100 μg/ml RNase A in TKM buffer for 60 minutes at room temperature. The chromosomes were then rinsed in TKM, fixed and incubated with primary and secondary antibodies. As a control, the RNAse-treated chromosomes were stained with anti-RNA polymerase II antibodies, which gave a signal similar to the one using untreated chromosomes (data not shown).

Immunostaining of tissue culture cells and salivary gland cells

C. tentans tissue culture cells were centrifuged onto slides (Cytospin, Shandon Astmoor, Runcorn, UK), and fixed in 4% paraformadehyde in PBS for 15 minutes at room temperature. After washing in PBS, the cells were permeabilised with 0.5% Triton X-100 in PBS for 10 minutes and washed in PBS. The cells were treated with 2% BSA in PBS and incubated for 2 hours at room temperature with the anti-CRM1 antibody (1:30 dilution), the anti-Ran antibody (1:30 dilution) or the anti-human von Willebrand factor VIII antibody (1:50 dilution) in TKM containing 2% BSA. Finally, the cells were incubated with FITC-conjugated F(ab′) fragment anti-mouse immunoglobulins (DAKO), washed with PBS, mounted in mounting medium with DAPI (Vector Laboratories) and examined in a Zeiss fluorescence microscope. Salivary glands were treated in the same way, but incubation with Triton X-100 was for 60 minutes and incubation with the primary antibody was overnight at 4°C.

Immunoprecipitation of hnRNP complexes

The hnRNP complexes were immunoprecipitated from nuclear extracts of *C. tentans* tissue culture cells using the monoclonal antibody 1D3 essentially according to Sun et al. (Sun et al., 1998) and Swanson and Dreyfuss (Swanson and Dreyfuss, 1999). In brief, affinity-purified rabbit anti-mouse antibodies (Dakopatts) were coupled to protein A-Sepharose (Amersham Pharmacia Biotech.) and cross-linked with 0.1% glutaraldehyde. In a second step, the 1D3 antibody was coupled to the anti-mouse antibodies and cross-linked with 0.1% glutaraldehyde. The protein-A-Sepharose-antibody complex was incubated with the nuclear extract for 1.5 hours at room temperature. In control experiments, the 1D3 antibody was replaced by the anti-von Willebrand factor antibody. The resin was pelleted and washed three times with 0.5 ml of PBS containing 0.1% NP-40. Proteins were eluted in SDS, precipitated with acetone, dissolved in loading buffer and subjected to western blot analysis.

Immunoelectron microscopy of ultrathin cryosections of salivary gland cells

Immunoelectron microscopy of ultrathin cryosections was performed according to Tokuyasu (Tokuyasu, 1980) as described by Visa et al. (Visa et al., 1996). The sections were incubated with the first antibody solution (anti-CRM1 antibody, 1:30 dilution, or anti-Ran antibody, 1:300 dilution, or anti-von Willebrand factor antibody, diluted 1:50, or anti-Ct-EIF4H antibody, diluted 1:10, or undiluted) for 60 minutes, and then with secondary antibody conjugated to 12 nm gold particles for 60 minutes at room temperature. The sections were stained with 2% aqueous uranyl acetate and embedded in polyvinyl alcohol (9-10 kDa, Aldrich). The specimens were examined and photographed in a Zeiss CEM 902 electron microscope at 80 kV. For analyses of the immunolabelling of the BR gene loci, all gold particles in defined areas within the active gene locus were analysed and assigned to compact chromatin, interchromatin or nascent BR mRNP particles. Only the morphologically defined BR pre-mRNP particles at the distal part of the gene (see Fig. 4B) were included. For analysis of labelling of BR mRNP particles in the interchromatin close to the nuclear membrane (within 2 μm), all BR mRNP particles were inspected and the percentage of gold labelled particles was calculated.

The anti-von Willebrand antibody gave rise to very few gold particles at all in the nucleus and no labelled BR mRNP particles could be detected. The anti-eIF4H antibody stained the interchromatin significantly above background. A total of 945 random BR mRNP particles were inspected. The antibody labelled none of these particles.

Leptomycin B treatment and electron microscopy of salivary gland cells

C. tentans larvae were treated with LMB (200 ng/ml) in culture water for 16 hours at 18°C. Control animals were kept in culture water without LMB. As a positive control for the effect of LMB, one of the two salivary glands in each treated larva was stained with an anti-Dbp5 antibody (Zhao et al., 2002). This protein accumulates in the nucleus in response to LMB treatment. The second salivary gland was either fixed, embedded and sectioned for conventional electron microscopy as previously described (Andersson et al., 1980), or used for immunoelectron micoscopy. The sections were photographed in a Zeiss CEM 902 electron microscope at 80 kV. For analysis of export through the NPCs, BR mRNP particles that were morphologically intact, connected to electron-dense fibres extending from the NPC, and within 100 nm of an NPC were considered to have docked at the NPC. BR mRNP particles that were rearranged and extending into the plane of the nuclear membrane were considered to be translocating through the NPC.

Leptomycin B treatment of tissue culture cells and in situ hybridisation

Tissue culture cells of *C. tentans* were incubated in medium containing 20 ng/ml LMB for 8 hours at 24°C. A control experiment was performed in parallel without the drug. The cells were fixed for 15 minutes in cold 3.7% formaldehyde in PBS, permeabilised for 10 minutes in 0.5% Triton X-100/PBS and equilibrated in 2× SSC for 10 minutes. The cells were incubated with hybridisation solution, 2× SSC, 1 mg/ml of tRNA, 10% dextran sulphate, 25% formamide, containing 5 ng/μl biotinylated oligo-dT(50) (SGS DNA com.), at 42°C for 12-18 hours. The cells were washed in 2× SSC for 15 minutes at 37°C or RT, in 0.5× SSC for 15 minutes and then in three changes of PBS. The cells were incubated with FITC-streptavidin for 30 minutes at RT and washed 3× PBS/0.2% Triton X-100 and twice with PBS. LMB treated and untreated cells were also stained with anti-Dbp5 antibodies as a control for the effect of LMB.

Results

Characterisation of CRM1 and Ran in *C. tentans*

The isolated CRM1 cDNA encoded a protein of 1054 amino acid residues, with a predicted molecular mass of 106 kDa. This *C. tentans* protein is closely related to CRM1 in different species throughout its entire length, including the central conserved region, which is important for stable CRM1/NES complex formation and sensitivity to LMB (Kudo et al., 1999). The *C. tentans* protein is 79% identical to the *D. melanogaster* CRM1, 73% identical to the human CRM1, 52% identical to the *S. pombe* CRM1 and 47% identical to the *S. cerevisiae* Xpo1p/exportin 1. We designated the *C. tentans* protein Ct-CRM1. The Ran cDNA encoded a protein with 215 amino acid residues and a predicted molecular mass of 25 kDa. The deduced protein was closely related to Ran in different species. For instance, it was 94% identical to *D. melanogaster* Ran, 86% identical to *H. Sapiens* and *X. laevis* Ran, 82% identical to *S. pombe* Spi1 and 80% to *S. cerevisiae* Gsp1. We therefore named the protein Ct-Ran.

To characterise Ct-CRM1 and Ct-Ran in cells, we screened commercially available antibodies. A monoclonal antibody against amino acid residues 2-122 of human CRM1 recognised Ct-CRM1, and a monoclonal anti-human Ran antibody detected Ct-Ran. Each antibody detected a single protein with an expected relative mobility of approximately 105 kDa and 25 kDa, respectively, in extracts from both tissue culture cells and salivary gland cells (Fig. 1A,C). Fig. 1B shows that the anti-CRM1 antibody indeed recognised a recombinant Ct-CRM1 N-terminal polypeptide (amino acid residues 1-329). The anti-Ran antibody specifically recognised Ct-Ran, expressed as a GST-fusion protein in *E. coli* (Fig. 1D).

Subcellular localisation of Ct-CRM1 and Ct-Ran

In diploid tissue culture cells and salivary gland cells, Ct-CRM1 was present both in the cytoplasm and in the nucleus, and it was concentrated at the nuclear rim (Fig. 2A,B). In the salivary gland cells, immunostaining of the nuclear envelope was unequivocal but less obvious, probably an effect of the considerably larger volume of the stained cytoplasm in the gland cells. In our immunoelectron microscope experiments, Ct-CRM1 was located at the NPCs of the nuclear membrane (Fig. 2C), particularly on the cytoplasmic side of NPCs. This location agrees with previous reports that CRM1 interacts with CAN/Nup214 on the cytoplasmic side of the NPC (Fornerod et al., 1997a). In the polytene nucleus (Fig. 2B), we could detect staining in the interchromatin between the polytene chromosomes, which agrees with our immunoelectron microscopy (see Fig. 7A,B). We also detected staining of specific loci on the polytene chromsosomes. This was analysed more in detail in Fig. 3A,B.

Ct-Ran had a very similar localisation in the diploid and polytene cells (Fig. 2D,E). Ct-Ran could be found throughout the cytoplasm, but the steady-state distribution was primarily nuclear, consistent with the localisation in HeLa cells (Ren et al., 1993). Inside the nucleus, Ct-Ran was localised to the interchromatin, the polytene chromosomes and to the nucleolei (Fig. 2E), as further analysed in Fig. 5.

Ct-CRM1 is recruited to nascent pre-mRNAs in Balbiani ring gene loci and in other active chromosomal loci

If CRM1 has a direct role in mRNA export, it should be associated with mRNP at some step of the intranuclear part of gene expression. We initially asked whether Ct-CRM1 is associated with nascent transcripts on the polytene chromosomes of *C. tentans*. Polytene chromosomes were manually isolated and immunolabelled with the anti-CRM1 antibody (or with an anti-factor VIII antibody as negative control). The transcriptionally highly active Balbiani ring (BR) gene loci on chromosome IV were specifically labelled by the anti-CRM1 antibody (Fig. 3A). Many smaller chromosomal puffs on all four chromosomes, representing known sites of RNA polymerase II transcription, were also immunostained (Fig. 3B). No immunolabelling could be found in the negative control experiment (Fig. 3C,C′,D,D′). When the isolated chromosomes were digested with RNase A before immunollabeling, the staining of the gene loci was abolished (Fig. 3E,E′,F,F′). These results indicate that Ct-CRM1 is bound to nascent pre-mRNP. To further confirm that the

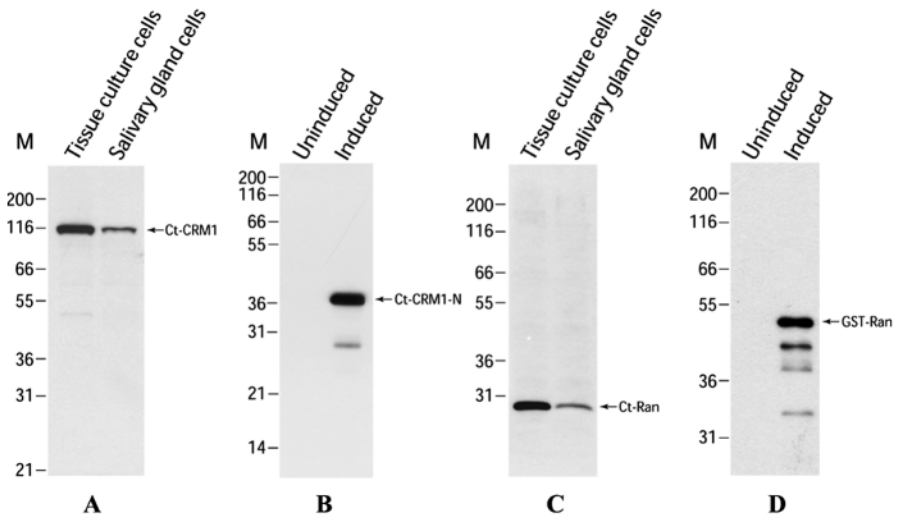

Fig. 1. Specificity of the anti-CRM1 and anti-Ran monoclonal antibodies. (A) Western blot of extracts from *C. tentans* tissue culture cells and salivary gland cells, probed with the anti-CRM1 antibody. (B) Western blot analysis of the expression of the N-terminal part of Ct-CRM1 (residues 1-329) in *E. coli*. Lane 1, before isopropyl β-D-thiogalactopyranoside (IPTG) induction of expression. In lane 2, a polypeptide migrating at approximately 36 kDa (arrow) was recorded after induction by IPTG. (C) Western blot of protein extracts from *C. tentans* tissue culture cells (lane 1) and salivary gland cells (lane 2), probed with the anti-Ran antibody. (D) Expression of a GST-Ct-Ran fusion protein in *E. coli*. In lane 1, no protein was seen before induction with IPTG. In lane 2, the fusion protein (arrow) was detected as well as smaller polypeptides, specifically reacting with the anti-Ran antibody. In A-D, M indicates the positions of size markers (in kDa).

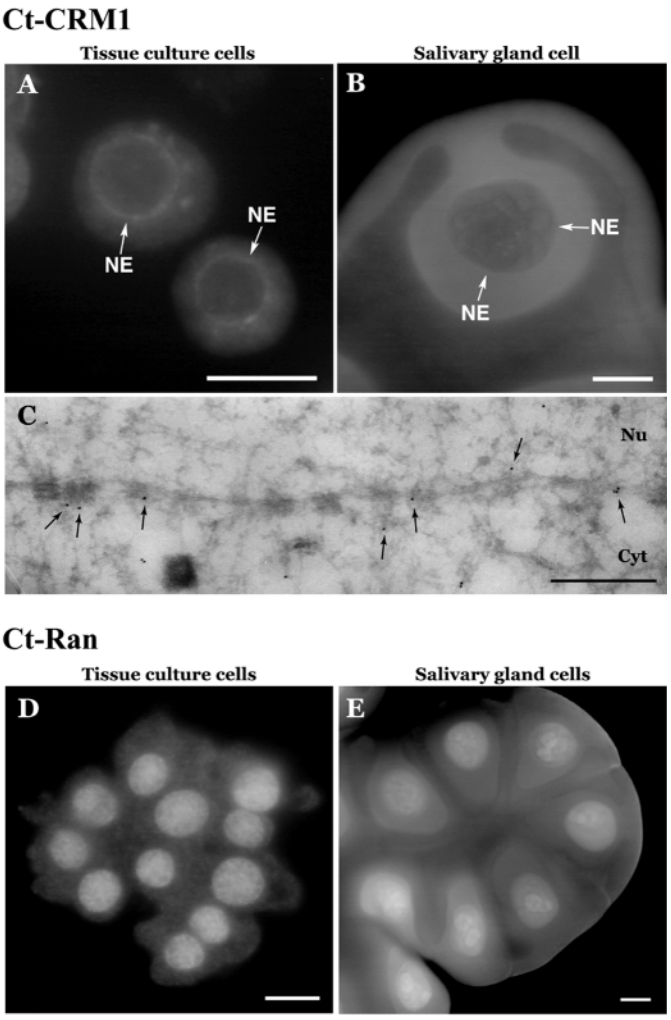

Fig. 2. Localisation of Ct-CRM1 and Ct-Ran in *C. tentans* cells. (A-C) Localisation of Ct-CRM1. Diploid tissue culture cells (A), and salivary gland cell (B). NE, nuclear envelope. The cells were immunostained with the anti-CRM1 antibody and a FITC-conjugated secondary antibody. (C) Immunoelectron microscope image of part of the nuclear membrane of a salivary gland cell. Gold particles (arrows) show the location of Ct-CRM1. Bar, 400 nm. (D-E) Localisation of Ct-Ran. (D) Tissue culture cells, showing mainly staining of the nuclei. (E) Salivary gland cells. Bars in A and C, 10 μm; bars in B and E, 20 μm.

immunostaining of nascent pre-mRNAs represented Ct-CRM1, we analysed the proteins present in isolated chromosomes by western blotting (Fig. 3). Fig. 3H (lane 2) shows that a single protein with a relative mobility characteristic of Ct-CRM1 was present in the isolated chromosomes. As a control for unspecific sticking of proteins to the chromosomes during isolation, we probed the proteins isolated from the chromosomes with an anti-Ct-eIF4H antibody. In a cell extract, this antibody detected a specific band migrating at approximately 36 kDa (Fig. 3H, lane 1), which was not present in the chromosome preparation (Fig. 3H, lane 2).

Immunoelectron microscopy of salivary gland cells was used to characterise Ct-CRM1 in relation to the assembly and transport of the gene-specific BR pre-mRNP particles. The active BR genes form large transcription loops with numerous short thick fibres and granules representing nascent pre-mRNP particles (Fig. 4A,B). Each BR locus contains about 8000 active genes and each cryosection represents a slice with a thickness that is approximately 1/200 of the thickness of one BR locus. Therefore, only short pieces of many transcriptionally active genes representing different parts of the BR genes (proximal, middle and distal) are present in each section. The nascent BR pre-mRNP particles were decorated by gold markers specific for Ct-CRM1 (Fig. 4A, arrows), whereas the negative control sections were essentially avoid of gold particles (data not shown). In all, 87% of a total of 234 analysed gold particles were located within or touching nascent BR mRNP particles. The remaining 13% were located in interchromatin regions between the BR gene loops. Few, if any, gold particles were present in compact, inactive chromatin regions, present in the central part of the BR gene locus. The gold particles were present on nascent BR pre-mRNPs on the proximal (Fig. 4C), middle (Fig. 4D) and distal (Fig. 4E,F) portions of the active BR genes. We conclude that Ct-CRM1 is associated with growing pre-mRNP particles. No obvious increase in the number of gold particles per pre-mRNP could be recorded along the gene, and it is likely that Ct-CRM1 is recruited to the nascent transcripts at an early stage of transcription. In a few instances, we detected more than one gold particle closely associated with BR mRNP particles (Fig. 4E,F). This was also occasionally recorded in BR mRNP particles in the interchromatin (see Fig. 7A). This indicates that more than one CRM1 molecule can be associated with a single BR mRNP.

Ct-Ran is mainly associated with chromatin in polytene chromosomes

The polytene chromosomes were generally immunostained with the anti-Ran antibody (Fig. 5A,B). No immunolabelling could be found in the negative control experiment (data not shown). Transcriptionally active chromosomal puffs, including the BR1, 2 and 3 gene loci on chromosome IV, were weakly labelled (Fig. 5A). The immunolabelling of the BR gene loci after RNase treatment was clearly reduced, but not completely removed (Fig. 5D). This suggests that Ct-Ran is bound to the nascent BR mRNPs to some extent, and that it also is associated with chromatin in the BR gene loci. The overall staining pattern of the chromosomes with the anti-Ran antibody was similar to the distribution of DNA (Fig. 5C). RNAse treatment reduced the staining of the chromosomes only slightly (Fig. 5D). Ct-Ran is therefore mainly associated with chromatin. It is also associated with nascent pre-mRNP in transcriptionally active gene loci, but considerably less so.

The gold labelling of Ct-Ran had a different distribution within the BR gene loci than that of Ct-CRM1 (Fig. 5E). Out of 428 analysed gold particles, 53% were located over interchromatin areas between transcribing gene loops, 27% were associated with compact chromatin within the BR gene loci and only 20% were touching or overlapping the BR pre-mRNP particles. This localisation in the EM sections of the cells is largely consistent with immunostaining of the isolated chromosome IV (Fig. 5A,C). In the sections, Ct-Ran in interchromatin between active gene loops was likely to be

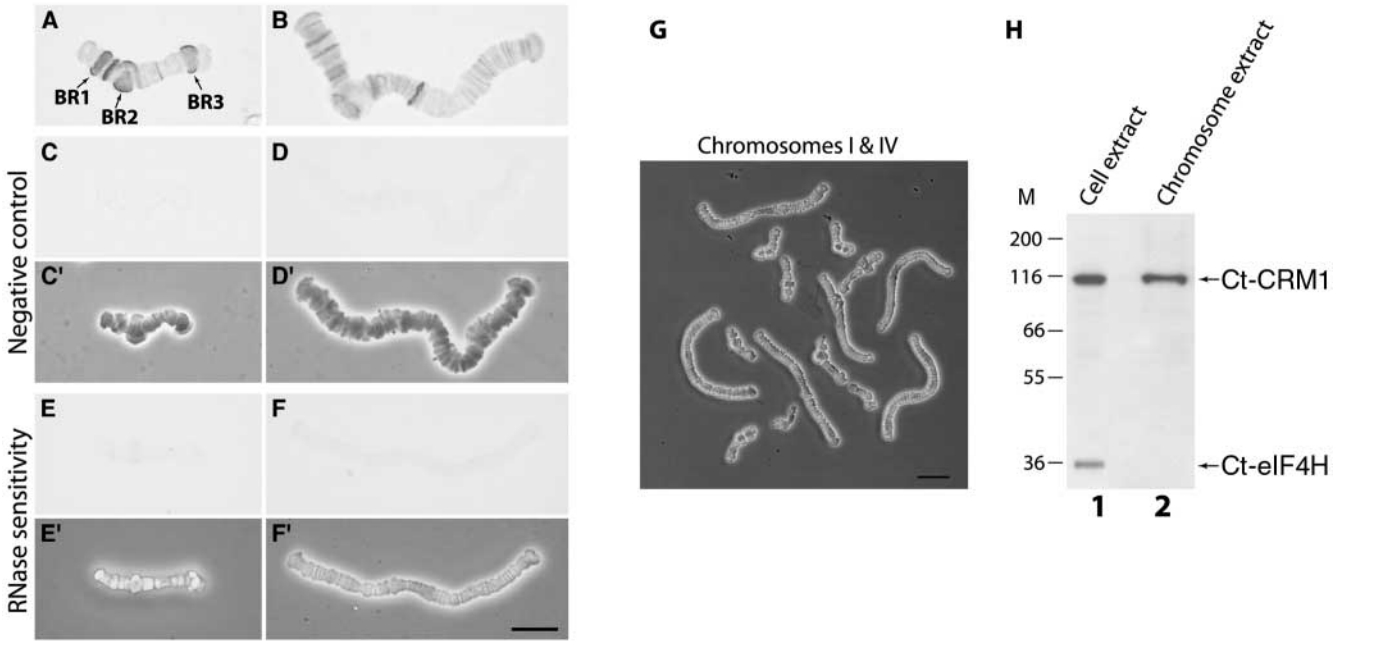

Fig. 3. Ct-CRM1 is associated with pre-mRNA at active gene loci in polytene chromosomes. (A) The three active BR gene loci on chromosome IV (arrows), and a few additional gene loci on the chromosome, were stained with the anti-CRM1 antibody. (B) Many gene loci on chromosome I were also stained by the antibody. (C, D) No staining of the gene loci was seen when a control antibody (anti-von Willebrand factor antibody) was used. (C′,D′) The chromosomes (in C and D) in phase contrast. (E,F) RNase treatment of the chromosomes before antibody staining abolished the staining of the gene loci. (E′,F′) Phase contrast images of the same chromosomes stained with the antibody in E and F; Bar, 10 μm. (G) Isolated chromosomes (chromosomes I and IV). Bar, 10 μm. (H) Western blot analysis of protein extract from tissue culture cells (lane 1) and the isolated chromosomes (seen in G) (lane 2). Both lanes were probed with anti-CRM1 and anti-Ct-eIF4H antibodies. Ct-eIF4H is known to be present mainly in the cytoplasm, but also in the interchromatin of the nucleus (Björk et al., 2003).

washed away from the isolated chromosomes, and the chromatin-associated Ct-Ran agrees with chromatin staining of the isolated chromosomes. The gold particles associated with nascent BR mRNP probably correspond to the weak staining of the transcribing loops in the isolated chromosomes. The results from the isolated chromosomes and the ultrathin sections indicate that Ct-Ran binds mainly to chromatin, and that it binds to nascent BR mRNP. The chromatin association of Ct-Ran could be direct (Bilbao-Cortes et al., 2002), or it could be related to its guanine nucleotide exchange factor on the chromatin (Nemergut et al., 2001).

Ct-CRM1 and Ct-Ran are co-immunoprecipitated with hnRNP complexes

We next asked whether Ct-CRM1 and Ct-Ran are present in hnRNP complexes. We purified hnRNP complexes located mainly in the interchromatin from *C. tentans* tissue culture cells by immunoprecipitation of nuclear extracts with the mAb 1D3. This antibody is specific for the hnRNP protein hrp23, which is known to be associated with pre-mRNP and mRNP exclusively in the nucleus (Sun et al., 1998). The immunoprecipitated hnRNP complexes were shown to contain the major hnRNP protein hrp36, which is known to be present in mRNP (Visa et al., 1996), using western blot analysis (Fig. 6). Ct-CRM1 and Ct-Ran were specifically detected in the immunoprecipitated hnRNP complexes, in addition to hrp23 and hrp36. This suggests that Ct-CRM1 and Ct-Ran are bound to a substantial part of the hnRNP in the interchromatin of tissue culture cells.

Ct-CRM1 and Ct-Ran are associated with BR mRNP particles in the interchromatin

After transcription, BR mRNP particles are released into the interchromatin for transport to the NPCs. These BR mRNP particles have a well-defined morphology and size and can be identified in the electron microscope (Daneholt, 2001). Immunoelectron microscope labelling of Ct-CRM1 and Ct-Ran in the interchromatin of cell nuclei of salivary gland cells showed that gold particles were specifically associated with BR mRNP particles, both for Ct-CRM1 and for Ct-Ran.

Examples of BR mRNP particles labelled with immunogold specific for Ct-CRM1 and Ct-Ran are shown in Fig. 7A,B and Fig. 7E,F. For BR mRNP particles located within 2 μm from the nuclear membrane (the diameter of the nucleus is approximately 70 μm), we compared the proportion of labelled BR mRNP particles with the proportion of labelled nascent BR pre-mRNP particles in the BR gene loci. In the BR gene loci, we included only the distal nascent BR pre-mRNP particles (see Fig. 4B), because these could be clearly morphologically identified. The labelling of the BR mRNP particles was generally low. Compared with control antibodies (see Materials and Methods), the labelling was, however, significantly above background and it was specific. For Ct-Ran, 3.1% (20 out of

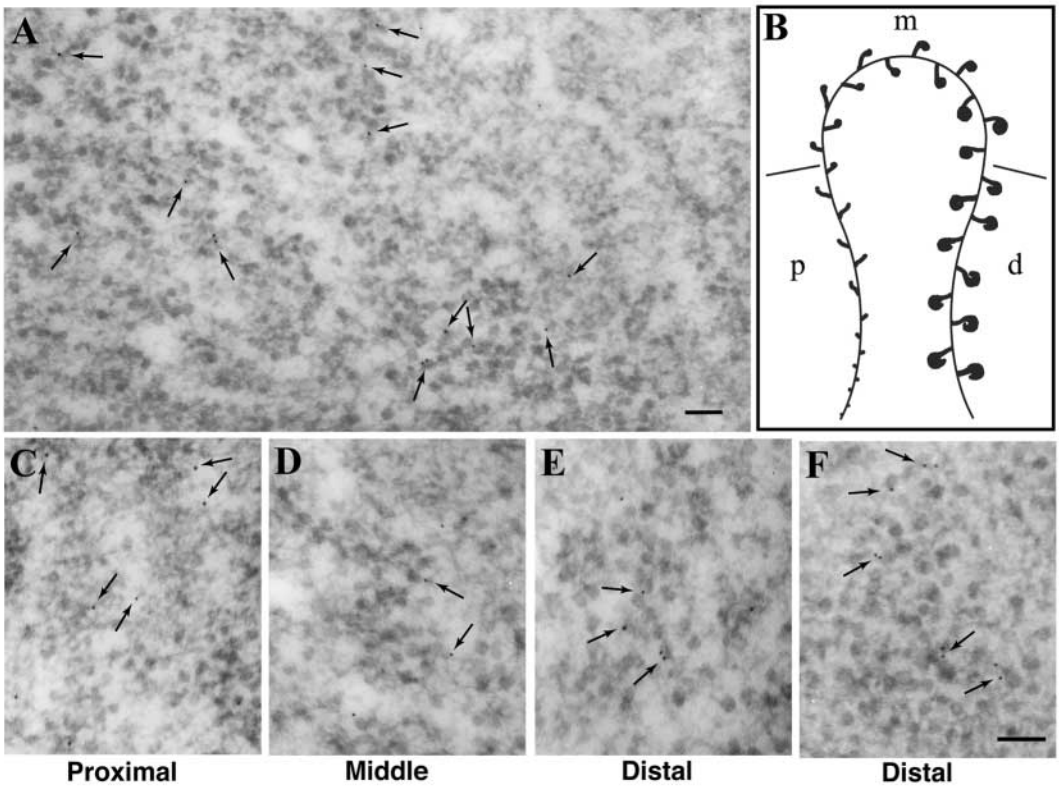

Fig. 4. Immunoelectron microscopy localisation of Ct-CRM1 in salivary gland cell nuclei. (A) View of a region of an active BR gene locus. Short stretches of many different transcribing BR genes and of different parts of the BR gene are seen. Nascent BR pre-mRNP particles are labelled by the secondary, gold-conjugated antibody. The gold particles are approximately 6 nm in diameter. (B) A complete active BR gene is shown schematically with the growing BR pre-mRNP particles. At the proximal (p) part of the gene, the BR pre-mRNPs form short fibres. These fold into larger and larger spherical pre-mRNP particles seen in the middle (m) and distal (d) parts of the active gene. (C-F) sections highlight areas containing essentially proximal, middle and distal parts of the gene, respectively (the sizes of the nascent mRNPs are different according to the drawing in B.) Gold particles can be seen labelling the BR pre-mRNP in all three cases (arrows). Bars, 200 nm.

653) of the nascent distal BR pre-mRNP particles were labelled and 10.2% (30 out of 295) of the BR mRNP particles close to the nuclear membrane were labelled. For Ct-CRM1 about 3.8% (23 out of 613) of the nascent BR pre-mRNP and about 2.1% (10 out of 466) of the interchromatin BR mRNPs were labelled. These numbers suggest not only that Ct-Ran is added to nascent BR pre-mRNP, but also that it becomes, to a large extent, associated with BR mRNP particles in the interchromatin. The situation appears different for Ct-CRM1. In this case, our results suggest that Ct-CRM1 is added co-transcriptionally and that little, if any, Ct-CRM1 is added in the interchromatin. Inherent properties in the immunoelectron microscopy methodology make it very difficult to interpret our data in terms of the relative number of Ct-CRM1 and Ct-Ran per BR mRNP particle. Furthermore, we have no indication that the labelled BR mRNP particles represent a subset of the population of BR mRNP particles. Each antibody gives a certain efficiency of labelling, and it is probable that both CRM1 and Ran are present in all BR mRNP particles. Because both our antibodies are mouse monoclonal antibodies, we could not either perform double-labelling experiments to investigate if the same BR mRNP particle contained both Ct-CRM1 and Ct-Ran. Nor could we determine whether, if that were the case, how close the two proteins were to each other in the particle.

Ct-CRM1 and Ct-Ran accompany BR mRNP particles through the NPC

The BR mRNP particles are known to interact with the basket structure of the NPC extending into the nucleus (Mehlin et al., 1992). These ‘docked’ BR mRNPs were labelled with the Ct-CRM1 and Ct-Ran gold markers (Fig. 7C,D and G,H respectively). The BR mRNP particles are subsequently fed through the NPC with the 5′ end first in a structurally modified mRNP particle. These translocating BR mRNPs were also labelled by the Ct-CRM1 and Ct-Ran gold markers (Fig. 8A,B and E,F). The gold markers could further be detected in association with the BR mRNP at the cytoplasmic side of the NPC (Fig. 8C,D and G,H), indicating that Ct-CRM1 and Ct-Ran are exported to the cytoplasm together with the BR mRNP. It should be noted that all the gold particles in all cases were within or were touching the BR mRNP particles, strongly suggesting that the antigen was located within the BR mRNP.

A NES-Ct-CRM1-Ct-RanGTP complex is not essential for export of BR mRNP particles but may contribute to translocation through the NPC

Ct-CRM1 and Ct-Ran are associated with many different mRNPs in *C. tentans* (Fig. 3A,B and Fig. 6). We therefore

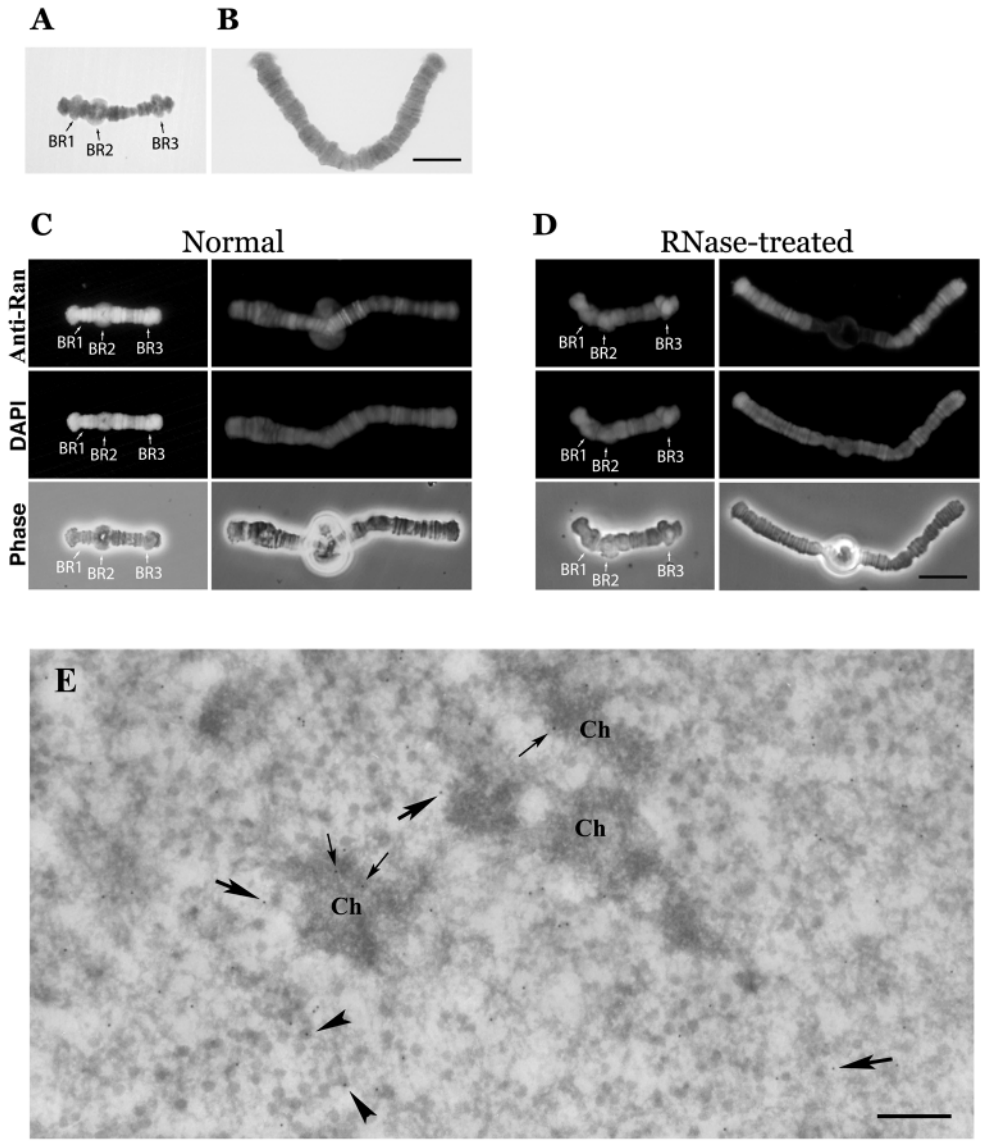

Fig. 5. Ct-Ran is bound to chromatin, but also to nascent BR pre-mRNP. Anti-Ran staining of isolated chromosome IV (A) and chromosome I (B); Bar, 10 μm. In chromosome IV, the decondensed BR gene loci are only weakly stained (arrows). (C) Chromosome IV and III staining with the anti-Ran antibody is similar to staining of the chromosomal DNA with DAPI. In addition, the nucleolus on chromosome III was labelled. The two chromosomes are also shown in phase contrast. (D) RNase treatment before staining with the anti-Ran antibody did not significantly influence the staining pattern (except in the nucleolus). Bar, 10 μm. (E) Immunoelectron microscope analysis of Ct-Ran in a section through a BR gene. Gold labelling is seen of compact chromatin (Ch, thin arrows), of interchromatin regions between segments of transcribing BR genes (thick arrows), and of nascent BR pre-mRNP particles (arrowheads). Bar, 300 nm.

asked whether Ct-CRM1 is important for the export of mRNAs in general. *C. tentans* tissue culture cells were treated with LMB (20 ng/ml) for 8 hours. Compared with control cells, LMB treatment resulted in nuclear accumulation of the protein Dbp5, indicating that the treatment was effective. Dbp5 shuttles between the nucleus and the cytoplasm in an Xpo1p-dependent manner in yeast (Hodge et al., 1999). LMB

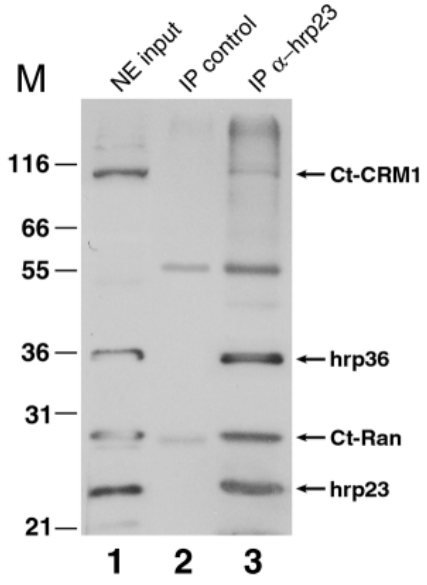

Fig. 6. Immunoprecipitation of hnRNP complexes. Western blot of *C. tentans* nuclear proteins immunoprecipitated with the anti-hrp23 antibody. The blot was probed with a mixture of antibodies directed against hrp36 (similar to the mammalian hnRNP A/B proteins) (Visa et al., 1996), hrp23, a homologue of the *Drosophila* RSF1 protein (Labourier et al., 1999), CRM1 and Ran. Ct-CRM1, Ct-Ran, hrp36 and hrp23 were specifically detected (lane 3). A nuclear extract incubated with Protein A-Sepharose beads coupled to anti-mouse antibodies and anti-von Willebrand antibodies did not result in any precipitation of the proteins (lane 2). 1/80 of the nuclear extract used for immunoprecipitation is shown in lane 1. The bands migrating at approximately 55 kDa and 24 kDa were present in the negative control. In lane 3, the 24 kDa band is partly hidden by the Ct-Ran band.

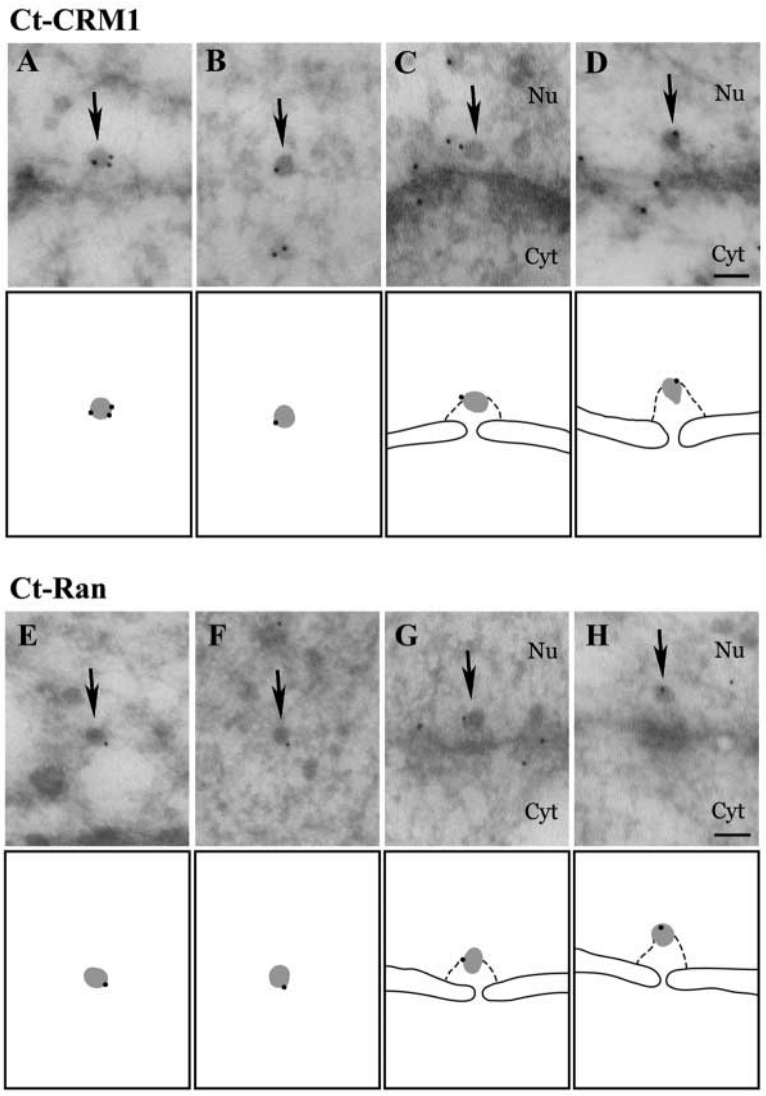

Fig. 7. Ct-CRM1 and Ct-Ran are associated with the BR mRNP particles during transport through the interchromatin. (A,B) BR mRNP particles (arrows) in the interchromatin labelled by Ct-CRM1-specific gold particles. (C,D) Ct-CRM1-labelled BR mRNP particles (arrows) docked at the NPCs. (E, F) BR mRNP particles (arrows) in the interchromatin, labelled by the anti-Ran antibody. (G,H) BR mRNP particles (arrows) docked at the NPCs, labelled by the anti-Ran antibody. To assist interpretation, schematic representations are shown below each electron micrograph. The labelled BR mRNP is shown in grey and the gold label in black. The nuclear membrane and the NPCs with their basket structure are outlined. Cyt, cytoplasm; Nu, nucleus. Bars, 100 nm.

treatment did not result in any significant nuclear retention of total poly(A)+ RNA as judged by in situ hybridisation using a biotinylated oligo-dT probe (Fig. 9A,B). This result shows that Ct-CRM1 is not a major export factor for mRNA in *C. tentans*, but the result does not rule out the possibility that it is important for subsets of the mRNA population.

Ct-CRM1 and Ct-Ran are part of the BR mRNP particles as these dock and translocate through the NPC. Using LMB, we tried to demonstrate a function for a NES-Ct-CRM1-Ct-RanGTP complex in BR mRNP export. Larvae of *C. tentans* were treated with LMB, and the salivary glands were isolated and analysed. One of the salivary glands in each animal was stained with anti-Dbp5 antibodies (Fig. 10). With LMB-treatment, Dbp5 accumulated to a large extent in the nucleus, showing that LMB extensively inhibited CRM1-mediated nucleocytoplasmic export. We investigated if the LMB-treatment influenced the association of Ct-CRM1 and Ct-Ran with BR mRNP. Using immunolabelling, we found that LMB drastically reduced the anti-Ran antibody labelling of BR mRNP particles in the interchromatin. Five out of 353 BR mRNP particles were labelled (1.4%). This is approximately seven times lower than the value we obtained in the absence of LMB (10.2%). This supports our conclusion that the LMB-treatment was efficient. In contrast to Ct-Ran, Ct-CRM1 was still associated with BR mRNP particles in the presence of LMB. Staining of nascent BR mRNPs in isolated chromosomes was essentially the same as in the absence of LMB (data not shown). When we analysed BR mRNP particles in the interchromatin, we found that 3.6% (13 out of 362) of the particles were labelled with the anti-CRM1 antibody (compare with 2.1% in the absence of LMB). These results suggest that the binding of Ct-CRM1 to the BR mRNP particles is not dependent on an intact NES-Ct-CRM1-Ct-RanGTP complex.

We then analysed whether LMB treatment influenced the export of the BR mRNP particles. Control animals were kept without LMB and analysed in parallel. Using immunoelectron microscopy, we detected approximately the same number of anti-CRM1-labelled BR mRNP particles being translocated through NPCs in the presence and absence of LMB (data not shown). By contrast, we detected no anti-Ran-labelled BR mRNP particles in transit through NPCs during LMB treatment. This suggests that BR mRNP particles that were influenced by the LMB treatment were still exported. No morphological changes were seen in nascent BR pre-mRNPs or in BR mRNPs during transport in the interchromatin after LMB treatment (data not shown). Neither could we detect any accumulation of BR mRNP particles close to the nuclear membrane. Finally, we analysed the effect of LMB on the export of BR mRNP particles through the NPCs in detail. LMB did not block the export of BR mRNPs. Docking of BR mRNP particles to the NPC basket (Fig. 11, compare A and E) and translocation of BR mRNP particles through the NPC (Fig. 11, compare B-D and F-H), still appeared morphologically normal following LMB treatment.

However, LMB affected the number of translocating BR mRNPs per unit length of nuclear membrane. We separately recorded the number of BR mRNPs docking at the NPC (Fig. 11E) and BR mRNPs being translocated through the NPC (Fig. 11F,G). We compared the number of BR mRNP particles docked at the NPC and translocating through the NPC in 11 cells treated with LMB and in 11 control cells (Table 1). We did not find a significant effect of LMB treatment on the docked BR mRNP particles (average 50.1 docked BR particles/100 μm of nuclear membrane) compared with the control group (average 49.9 docked BR particles/100 μm of

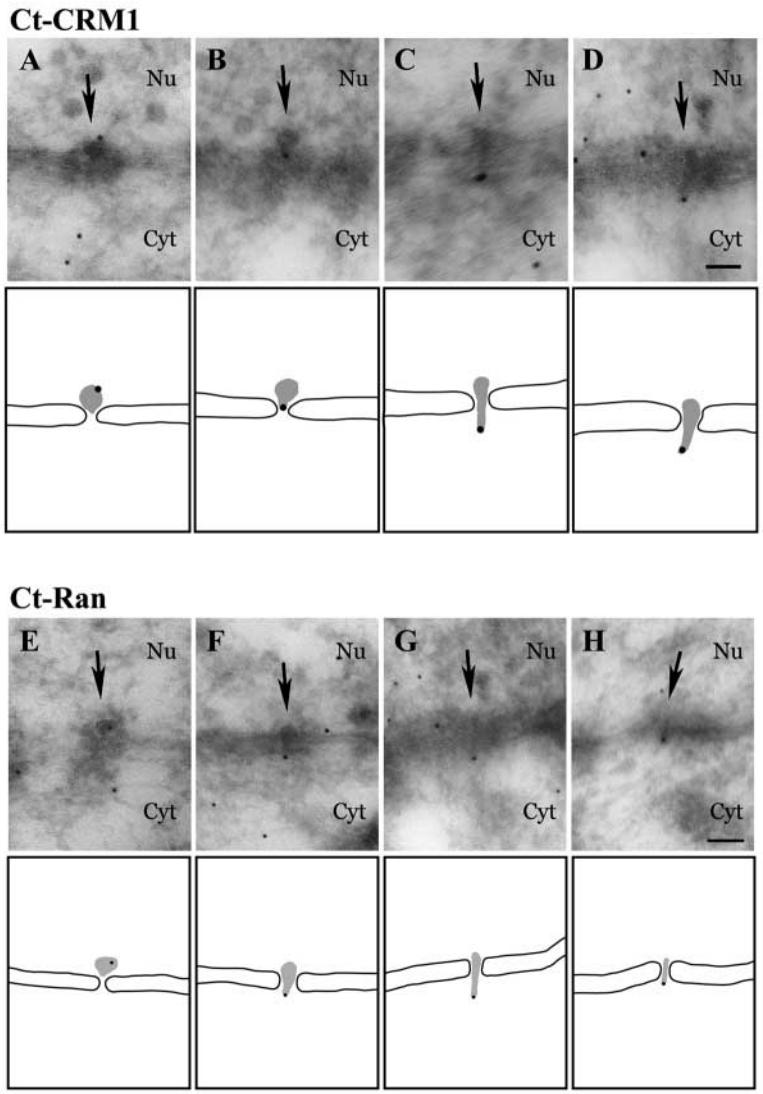

Fig. 8. Ct-CRM1 and Ct-Ran are translocated through the NPCs with the BR mRNP particles. Immunoelectron microscope labelling of sections through the nuclear membrane of salivary gland cells. (A-D) Anti-CRM1 labelling of BR mRNP particles (arrows) during progressive translocation through NPCs. (E-F) Anti-Ran labelling of BR mRNP particles (arrows) during translocation through NPCs. In the drawing below each micrograph, the BR mRNP is depicted in grey and the gold labelling in black. Cyt, cytoplasm; Nu, nucleus. Bars, 400 nm.

nuclear membrane). However, LMB treatment resulted in a significant effect (P<0.02) on the number of translocating BR mRNP particles (average 40.8 translocating BR particles/100 μm of nuclear membrane) as compared with the control (average 30.7 translocating BR particles/100 μm of nuclear membrane). This suggests that the BR mRNP particles reach and dock at the NPCs normally in the presence of LMB, but that the transition through the NPC is extended in time. We cannot exclude that this effect is indirect. These data however show that, although Ct-CRM1 and Ct-Ran are present in the BR mRNP particles, a NES-Ct-CRM1-Ct-RanGTP complex is not essential for export. At most, it contributes to translocation through the NPCs.

Discussion

We have shown that Ct-CRM1 and Ct-Ran are present in specific mRNP complexes, the BR mRNP particles, as these are transported from the genes to and through the NPCs. CRM1 is a well-characterised export receptor (reviewed by Görlich and Kutay, 1999). It is difficult to determine whether CRM1 palys a role in mRNA export. Inhibition experiments with LMB (Fornerod et al., 1997b; Neville and Rosbach, 1999), competition by NES substrates (Paraskeva et al., 1999) and nuclear depletion of RanGTP (Izaurralde et al., 1997; Clouse et al., 2001) have shown that CRM1 is not responsible for bulk mRNA export. A large-scale analysis of mRNA export in *Drosophila* has shown that CRM1 can be an important export receptor for only a minority of all mRNAs (Herold et al., 2003). At the same time, clear evidence has been obtained for CRM1-mediated export of subsets of mRNA, such as short-lived mRNAs containing AU-rich sequences in their 3′ UTR, and at least some heat-shock mRNAs (Gallouzi and Steitz, 2001).

Ct-CRM1 is loaded onto the BR pre-mRNP while transcription takes place. Our results suggest that this occurs early during transcription. In snRNPs, CRM1 binds through the CBC (cap binding complex) and the phosphoprotein PHAX (Fornerod et al., 1997; Ohno et al., 2000), but it has been suggested that CRM1 and PHAX do not associate with mRNA in *Xenopus* oocytes (Ohno et al., 2002). Our immunoelectron

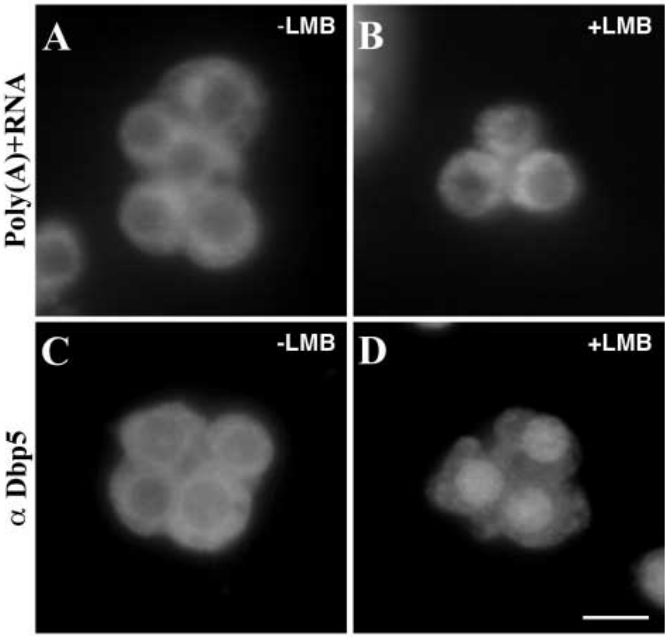

Fig. 9. LMB treatment of *C. tentans* tissue culture cells does not influence the distribution of poly(A)+ RNA. Tissue culture cells, treated (B) or not treated (A) with LMB, were hybridised with an oligodT probe. No difference in the nuclear-cytoplasmic distribution of poly(A)+ RNA was seen. As a control for the effect of LMB, cells were stained for Dbp5 in the absence of LMB (C) and in the presence of LMB (D). Bar, 10 μm.

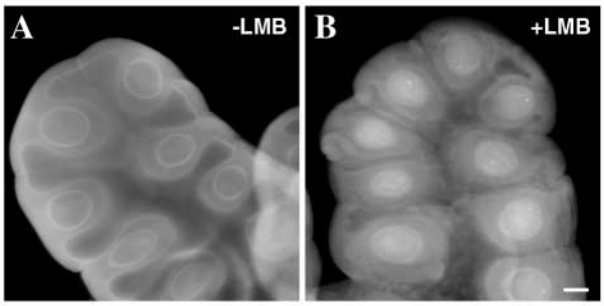

Fig. 10. Effect of LMB treatment on salivary gland cells. *C. tentans* larvae were treated with LMB in water for 16 hours. Control animals were kept in water in parallel. Salivary glands were isolated, fixed and stained with anti-Dbp5 antibodies. In control glands (A, –LMB), Dbp5 is mainly seen in the cytoplasm and at the nuclear envelope. In LMB-treated glands (B, +LMB), Dbp5 is to a large extent present in the cell nuclei. Bar, 20 μm.

microscopy labelling experiments do not show where in the BR pre-mRNP complex Ct-CRM1 binds. It is known that a single nascent BR pre-mRNP associates with a number of different proteins. Some of these proteins remain with the BR mRNP to the NPCs or all the way into the cytoplasm, and they may influence BR mRNP export. The hnRNP protein hrp36 coupled to actin (Visa et al., 1996; Percipale et al., 2001) is associated with BR mRNA from the gene loci to the polysomes. The hnRNP protein hrp23 binds to BR pre-mRNP at the gene and leaves the BR mRNP on the nuclear side of the NPC (Sun et al., 1999). The hrp23 protein inhibits splicing in vitro (P. Björk, I. Wetterberg, G. Baurén and L. W., unpublished), as does the RSF1 protein in *D. melanogaster* (Labourier et al., 1999). The SR proteins hrp45 (Alzhanova-Ericsson et al., 1996), SC35 and 9G8 (S.-B. Jin, J. Zhao, U. Hellman and L. W., unpublished) all associate with the BR mRNP at the gene locus. While hrp45 leaves the BR mRNP at the NPC, SC35 and 9G8 remain with the BR mRNP into the cytoplasm.

Several proteins known to be involved in the nucleocytoplasmic export of mRNA are added to the nascent BR pre-mRNP. In addition to Ct-CRM1, these include RNA helicase Dbp5 (Zhao et al., 2002), which is believed to function mainly at the NPC (Tseng et al., 1998; Snay-Hodge et al., 1998; Schmitt et al., 1999), and Ct-HEL/UAP56 and REF/Aly (Kiesler et al., 2002). The four introns in the BR pre-mRNA are excised essentially while transcription is taking place (Baurén and Wieslander, 1994), suggesting that EJC components are deposited onto the nascent BR mRNP. Both adapter and receptor proteins are thus already present in the BR mRNP at the gene locus, and in this sense make the BR mRNP export competent at the gene locus. Export proteins are, in addition, added to the BR mRNP after transcription. Ct-RAE1 associates with BR mRNP close to the NPC (Sabri and Visa, 2000), and our results suggest that even if Ct-Ran starts to bind to BR mRNP at the gene, it binds mainly in the interchromatin.

After transcription, the BR mRNPs move in all directions away from the BR genes. This behaviour is compatible with the hypothesis that diffusion drives their motion (Singh et al., 1999), although it has also been observed that BR mRNPs transiently interact with fibres in the interchromatin. This interaction involves the hrp65 protein (Miralles et al., 2000). LMB did not influence transport of BR mRNP through the interchromatin and there was no accumulation of BR mRNPs at the NPCs. The number of docked BR mRNPs at the NPCs was not changed (Table I). A NES-CRM1-RanGTP complex is therefore is not essential for intranuclear transport to the NPCs.

Individual BR mRNP particles may be exported by multiple adapter-export receptor pathways

The TAP export receptor plays a dominant role in mRNA export through a Ran-independent pathway in such organisms as yeast, *Xenopus* (for references see Conti and Izaurralde, 2001), and dipteran insects (Herold et al., 2001). In mammalian cells, a Ran-dependent pathway involving TAP and

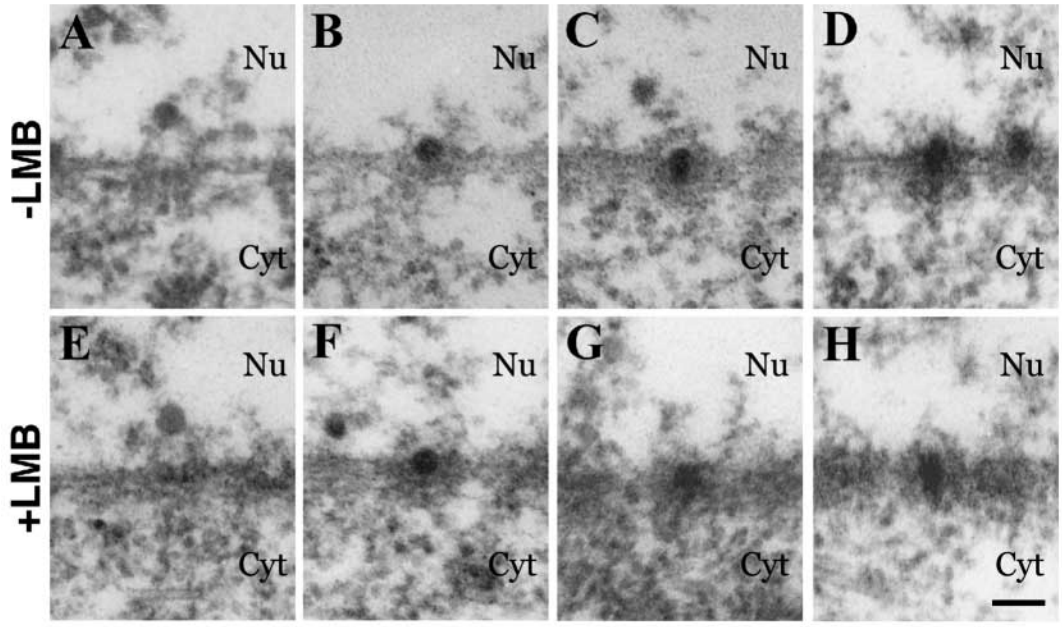

Fig. 11. Treatment with LMB leads to an increase in the number of BR mRNP particles that are in transit through the NPCs. *C. tentans* larvae were treated with LMB and the salivary gland cells were analysed by electron microscopy. (A-D) Control cells without LMB. (A) BR mRNP particle docking at the NPC. (B-D) BR mRNP particles translocating through the NPC. (E-H) LMB-treated cells. (E) BR mRNP particle docking at the NPC. (F-H) BR mRNP particles translocating through the NPCs. Cyt, cytoplasm; Nu, nucleus. Bar, 100 nm.

Table I. LMB treatment results in an increase in BR mRNP particles being translocated through the NPC

	LMB treatment			Control		
Cell	Measured NE length (μm)	Docked BR particles	Translocating BR particles	Measured NE length (μm)	Docked BR particles	Translocating BR particles
1	39	19	16	173	101	57
2	45	20	23	86	34	27
3	184	109	92	99	23	25
4	110	55	66	59	38	24
5	83	30	26	86	35	26
6	88	31	24	42	8	15
7	83	40	39	144	55	36
8	150	81	65	185	105	64
9	227	120	78	233	141	74
10	186	93	82	234	143	69
11	220	111	66	190	96	53
Total	1415	709	577	1531	779	470
BR particles/100 μm		50.1	40.8		49.9	30.7

BR mRNP particles docking at the NPC (see Fig. 11A,E) and translocating through the NPCs (see Fig. 11B-D,F-H) were separately analysed in *C. tentans* salivary gland cells treated or not treated with LMB. 11 cells from treated salivary glands and 11 cells from untreated salivary glands were analysed. For each cell, the number of BR mRNP particles docked at the NPCs and the number being translocated through the NPCs were recorded for the indicated length of the nuclear membrane. The numbers of BR mRNP particles per 100 μm of nuclear membrane were compared for the untreated and treated cells. There was no statistical difference for the docked BR mRNPs, but for the translocating BR mRNP particles the difference was statistically significant (Mann Whitney nonparametric test, P=0.02, z=2.98).

karyopherin β2B also appears to be important (Shamsher et al., 2002). It is unclear to what extent subsets of mRNA are exported to the cytoplasm by different adapter-receptor pathways. Some individual mRNAs are exported by M9-Transportin 1 (DHFR mRNA), by the HNS (HuR nucleocytoplasmic shuttling)-karyopherin β2B (c-fos mRNA) and the NES-CRM1 pathway (hsp70 mRNA) (Gallouzi and Steitz, 2001), and this allows us to conclude that mRNA export is diversified. The shift in HuR association from karyopherin β2B to CRM1 via APRIL and pp32 upon heat shock (Galouzzi and Steitz, 2001) shows that physiological situations can influence which export pathway is used. These examples have emphasised that individual mRNAs depend on one or the other export pathway, but apparently only one at a time.

Both Ct-CRM1 and Ct-Ran are present in the BR-mRNP particles during export, but a trimeric NES-CRM1-RanGTP complex is not necessary for the export of BR mRNP. The fact that Ct-CRM1 binds cotranscriptionally to BR mRNPs, while Ct-Ran association increases in the interchromatin, raises the possibility that Ct-CRM1 binding to the BR mRNP does not depend on Ct-Ran. Ct-CRM1 binding to BR mRNP did not either decrease upon LMB treatment, while the binding of Ct-Ran did decrease. It has previously been shown that CRM1 can interact directly with the human immunodeficiency virus type 1 encoded protein Rev, independent of a functional NES (Askjaer et al., 1998). In this case, RanGTP induces a LMB-sensitive RNA-Rev-CRM1-RanGTP complex. Our results similarly suggest that Ct-CRM1 interacts not only with Ct-Ran; it appears to also bind to some component of the BR mRNP particles independent of Ct-Ran. This may reflect a two-step formation of an export competent complex. It may also indicate that Ct-CRM1 has other functions in the BR mRNP particle. Still, our results suggest that Ct-CRM1 may contribute to the export of BR mRNP. Translocation of BR mRNP particles through the NPC was influenced by LMB (Table 1). This could be due to inhibition of Ct-CRM1 function, although we cannot exclude the possibility that translocation through the NPC was influenced as the result of an indirect effect. It is clear, however, that a single BR mRNP contains several potential adapter proteins, and our results show that individual BR mRNPs simultaneously use multiple export adapter-receptor pathways. Because Ct-CRM1 is part of the BR mRNP but a NES-CRM1-RanGTP complex is not essential for BR mRNP export, additional adapter-export receptor partners are important. The BR mRNP contains REF/Aly (Kiesler et al., 2002), and this strongly suggests that TAP is involved in export. Other adapter-receptor pathways may also operate, for example SR proteins and TAP (Huang et al., 2003). The BR mRNA is exceptionally long, approximately 35 kb (Wieslander, 1994), and the BR mRNP particle is substantial, with a diameter of 500 nm. During translocation through the NPC, the BR mRNP undergoes a conformational change and is translocated as an extended particle with a leading 5′ end (Mehlin et al., 1992; Mehlin et al., 1995). BR mRNPs may exemplify the export conditions of huge mRNPs, and such mRNPs may need multiple export receptors for efficient transport through the NPC, perhaps providing more possibilities for interaction with the F/G repeats in the NPC channel. A complete description of the factors involved in mRNA export and their importance will require an analysis of individual mRNAs and individual adapter-receptor pathways under different physiological conditions.

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