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The Ct-RAE1 protein interacts with Balbiani ring RNP particles at the nuclear pore

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ABSTRACT

RAE1 is an evolutionarily conserved protein that associates with both mRNPs and nucleoporins, and may bridge the interaction between mRNP export cargoes and the nuclear pore complex (NPC). However, the mechanism by which RAE1 functions in mRNA export is still unknown and the time point at which RAE1 interacts with the exported RNP has not been directly investigated. Here we have addressed this question in the Balbiani ring (BR) system of *Chironomus tentans* using immunoelectron microscopy. The RAE1 protein of *C. tentans*, Ct-RAE1, is 70% identical to human RAE1/mrnp41 (hRAE1) and is recognized by antibodies raised against hRAE1. As in vertebrate cells, Ct-RAE1 is concentrated at the nuclear envelope and also dispersed throughout the nuclear interior. Here we show that Ct-RAE1 does not bind to the BR particle either cotranscriptionally or in the nucleoplasm. Instead, the interaction between Ct-RAE1 and the exported BR particle occurs at the NPC. Moreover, the localization of Ct-RAE1 at the NPC is correlated with the presence of an exported RNP in the NPC. Finally, the anti-RAE1 antibody does not label the cytoplasmic side of BR particles in transit through the central channel, which indicates that Ct-RAE1 either remains anchored at the nuclear side of the NPC during translocation of the RNP through the central channel or becomes transiently associated with the RNP but is rapidly released into the cytoplasm.

Keywords: *Chironomus tentans*; Gle2p; immunoelectron microscopy; mRNA export; mrnp41; Rae1p

INTRODUCTION

Nuclear export of protein-coding RNAs (mRNAs) is an energy-dependent, signal-mediated process (reviewed by Görlich & Kutay, 1999; Nakielny & Dreyfuss, 1999). Virus-encoded mRNAs often contain structural elements that serve as export signals directly recognized by nuclear export receptors. The best characterized export signal of this type is the Rev response element (RRE) of the HIV-1 virus (reviewed by Pollard & Malim, 1998). The Rev protein binds cooperatively to the RRE in the HIV-1 RNA and interacts directly with the export receptor Crm1; binding of the mRNA–Rev complex to Crm1 is sufficient to target the viral mRNA to the cytoplasm (Fischer et al., 1995; Fornerod et al., 1997). In the case of cellular mRNAs, the nature of the NESs is not so well understood. Pre-mRNAs are cotranscriptionally associated with hnRNP proteins and exported to the cytoplasm as ribonucleoprotein complexes or RNPs (reviewed by Krecic & Swanson, 1999). Genetic studies in yeast have identified a number of proteins that are required for mRNP export that are highly conserved in metazoans (reviewed by Doye & Hurt, 1997; Mattaj & Englmeier, 1998; Görlich & Kutay, 1999). One of them, the DEAD-box RNA helicase Dbp5p, binds to CAN/Nup159p in the cytoplasmic fibrils of the nuclear pore complex (NPC) where it participates in late steps of mRNA export (Schmitt et al., 1999, and references therein). Two other proteins, Gle1p and Mex67p, are essential for mRNA export in *Saccharomyces cerevisiae* (Murphy & Wente, 1996; Segref et al., 1997), and their human counterparts, hGle1p and TAP, respectively, have been implicated in mRNA export as well (Grüter et al., 1998; Watkins et al., 1998; Braun et al., 1999; Katahira et al., 1999). Gle1p can interact directly with Dbp5p and with the FG-nucleoporin Rip1p at the cytoplasmic side of the NPC, and it has been proposed that Gle1p contributes to the recruitment of Dbp5 to the NPC (Strahm et al., 1999). Mex67p/TAP binds to poly(A)$^+$ RNA either directly or via RNA-binding proteins and interacts physically with multiple nucleoporins located at both sides of the NPC (Katahira et al., 1999; Bachi et al., 2000; Strasser & Hurt, 2000; Stutz et al., 2000). These features make of Mex67/TAP a good candidate for bridging the interaction between the mRNP and the NPC (Bachi et al., 2000). Another

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protein that has been implicated in mRNA export and that fulfills the requirements of an mRNA export receptor is Gle2p from *S. cerevisiae*, or its homolog Rae1p in *Schizosacharomyces pombe* (Brown et al., 1995; Murphy et al., 1996). The human counterpart of Gle2p/Rae1p, identified in two independent studies and designated mrnp41 or hRAE1, was found to be an RNA-binding protein localized to both nucleus and cytoplasm (Bharathi et al., 1997; Kraemer & Blobel, 1997). It was also shown that Gle2p, as well as hRAE1/mrnp41, can interact physically with the GLFG nucleoporin Nup116p/NUP98 via a conserved Gle2p-binding motif called GLEBS, and that this interaction is essential for the function of Gle2p in vivo (Bailer et al., 1998; Pritchard et al., 1999). Moreover, hRAE1 is a shuttling protein and its association with the NPC is dependent on ongoing mRNA synthesis (Pritchard et al., 1999). In summary, RAE1 is likely to play a direct role in mRNA through its ability to interact with both the mRNPs and the NPC. However, the mechanism of RAE1 function is totally unknown.

Thanks to the high degree of conservation of RAE1 in all eukaryotes, we have been able to identify and characterize the counterpart of RAE1 in the insect *Chironomus tentans*, and we have analyzed *in situ* the association of RAE1 with a specific pre-mRNP particle, the Balbiani ring (BR) RNP particle, during successive stages of synthesis, maturation, and export. The BRs of *C. tentans* are large puffs in the polytene chromosomes of the larval salivary gland cells that originate by active transcription of the BR genes (reviewed by Wieslander, 1994). The BR pre-mRNA has all the features of a typical pre-mRNA. The 5′ end is capped and the nuclear cap binding complex can be detected on the nascent BR pre-mRNP early after transcription initiation (Visa et al., 1996b). The BR pre-mRNA contains four introns that are spliced either cotranscriptionally or posttranscriptionally, depending on the position of the intron in the pre-mRNA (Baurén & Wieslander, 1994). After transcription termination, the BR particles are released from the chromosome, transported to the nuclear envelope and exported to the cytoplasm through the central channel of the NPC (reviewed by Daneholt, 1997).

Using transmission electron microscopy (EM), it is possible to visualize how the BR pre-mRNP particles are assembled along the BR gene, how the released BR pre-RNP particles are transported through the nucleoplasm to the nuclear envelope, and how the mature BR particles are exported through the NPC (reviewed by Mehlin & Daneholt, 1993; Daneholt, 1997). Moreover, the EM visualization of BR particles can be combined with the use of specific antibodies, and thus the association of defined proteins with BR pre-mRNP particles at different stages of gene expression can be studied *in situ*. Here we identify the RAEl protein of *C. tentans* (referred to as Ct-RAE1), we determine at what point in the nuclear export pathway Ct-RAE1 interacts with the exported BR RNP particle, and we analyze the presence of Ct-RAE1 at the NPC in the presence or absence of an export cargo.

RESULTS

Cloning and sequencing of a cDNA encoding *C. tentans* RAE1

We followed a semi-nested PCR approach using degenerated oligonucleotide primers to clone a cDNA encoding the RAE1 homolog in *C. tentans*. The amino acid sequences of RAE1 in humans, *Caenorhabditis elegans*, *S. cerevisiae*, *S. pombe*, and *Drosophila melanogaster* (EST AA141856) were compared, and three degenerated primers corresponding to highly conserved regions were designed. Using genomic DNA of *C. tentans* as a template, two consecutive PCR reactions were carried out as described in Materials and Methods, and a PCR product 650-nt-long was obtained. This initial PCR fragment was sequenced and found to be highly homologous to various RAE1 proteins in the databases. To get the full sequence of RAE1, the PCR product was used as a probe to screen a *C. tentans* cDNA library. One of the isolated clones, λ-RAE1-1, was fully sequenced and analyzed. The clone λ-RAE1-1 was found to contain a 800-nt-long 5′ UTR, an open reading frame (ORF) encoding 349 amino acids, and approximately 700 nt of 3′ UTR. The protein encoded by λ-RAE1-1 had a predicted molecular mass of 39.3 kDa and a predicted pI of 7.8. NetBlast and Fasta searches confirmed that the protein encoded by λ-RAE1-1, referred to as Ct-RAE1, was indeed related to RAE1 from human (70% identity), *C. elegans* (57% identity), *S. pombe* (48% identity), and *S. cerevisiae* (43% identity). The highest homology was with the product of the CG9862 gene of *D. melanogaster* (FBgn0034646), which was 76% identical to Ct-RAE1. All these proteins are characterized by four conserved WD-repeats that are also highly conserved in Ct-RAE1. The similarities, however, extended beyond the conserved WD repeat residues (Fig. 1).

In addition to the overall sequence conservation, several residues shown by genetic studies to be functionally important were also conserved in Ct-RAE1. One such region comprised amino acids 214–226 in Ct-RAE1 and was located between the third and fourth WD repeats. In yeast, point mutations at any of the two glycine residues in this region (mutations rae1-1 and gle2-222G in *S. pombe* and *S. cerevisiae*, respectively) caused defects in polyA$^+$ RNA export at a nonpermissive temperature (Brown et al., 1995; Murphy et al., 1996). This region was identical in 12 out of 13 residues between *C. tentans* and human. Also the arginine residue in position 286, shown to have a suppressor effect over the rae1-1 mutation in *S. pombe* (Bharathi

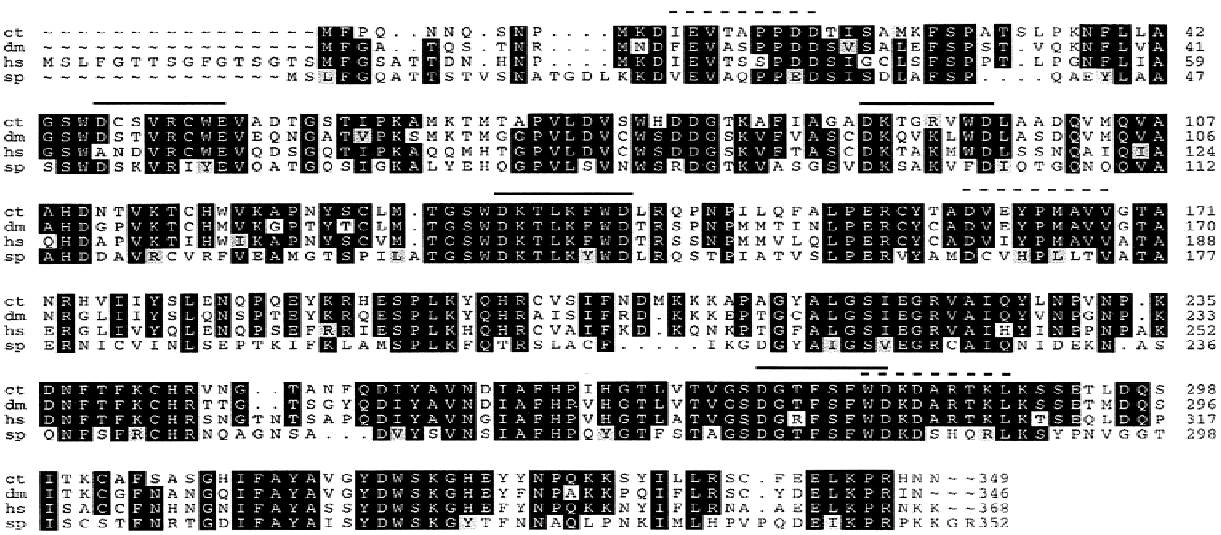

FIGURE 1. Amino acid sequence of RAE1 in *C. tentans* (ct), *D. melanogaster* (dm), human (hs), and *S. pombe* (sp). The sequences were compared and aligned using the PileUp and PrettyBox programs of the GCG sequence analysis software package. Identical residues in at least 3 out of 4 sequences are boxed. Solid lines on the Ct-RAE1 sequence indicate conserved residues at the end of each WD repeat. The dashed lines show the sequences used for the design of primers for nested PCR. The sequences presented in the figure are available under accession numbers AJ277787, Fbgn0034646, U84720, and P41838 for *C. tentans*, *D. melanogaster*, human, and *S. pombe*, respectively.

et al., 1997), was present in Ct-RAE1 and in most of the known Gle2p/RAE1 proteins.

Based on the features of the primary structure, the high percentage of similarity over the whole sequence, and the conservation of residues known to be functionally relevant, we concluded that Ct-RAE1 was the *C. tentans* homolog of RAE1.

Antibodies against hRAE1 recognize Ct-RAE1

A polyclonal antibody against recombinant hRAE1 was raised in rabbits (see Materials and Methods). To determine whether this antibody was useful for immunocytochemical studies in *C. tentans*, we tested its reactivity against Ct-RAE1. For this purpose, we expressed Ct-RAE1 in *Escherichia coli* and the recombinant protein, with a T7-tag at the N-terminus, was probed by western blotting. Antibodies against the T7-tag detected a main band of the expected size, 40 kDa, in crude extracts of bacteria expressing Ct-RAE1 (arrow in Fig. 2A). The same band was recognized by the anti-RAE1 antibody, but not by the preimmune serum (Fig. 2A). This 40-kDa polypeptide was not detected by any of the antibodies in noninduced bacterial extracts. We concluded that the anti-RAE1 antibody cross-reacts with recombinant Ct-RAE1.

To analyze the specificity of the anti-RAE1 antibody in *C. tentans*, we analyzed nuclear and cytoplasmic protein extracts prepared from tissue culture cells by western blotting (Fig. 2B). The antibody reacted with only one main band of ~40 kDa in the nuclear fraction, whereas the preimmune serum was completely negative in the same conditions. In the cytoplasmic fraction, several faint bands, including the 40-kDa one, were recognized by the anti-RAE1 antibody.

Ct-RAE1 is concentrated at the nuclear envelope but also diffusely distributed inside the nucleus

To determine the intracellular localization of Ct-RAE1 in salivary gland cells, semithin cryosections were stained with the anti-RAE1 antibody using a gold-conjugated secondary antibody. The labeling was visualized after silver enhancement under bright field light microscopy (LM). As illustrated in Figure 3A, the cell nucleus was the most intensely stained structure in the cell. Thanks to the large dimensions of the salivary gland cells and to their highly organized polytene nucleus, three distinct nuclear compartments could be identified in the preparations, apart from the nuclear envelope: the polytene chromosomes, the nucleolus, and the nucleoplasm or interchromosome space. The nucleolus was not stained, the chromosomes displayed a weak banded staining, and the most intensely labeled compartment inside the nucleus was the nucleoplasm. The nuclear periphery was sharply delineated, which was indicative of intense labeling at the nuclear envelope. Only a faint diffuse staining was observed in the nucleus when the sections were incubated with preimmune serum (Fig. 3B), which supported the specificity of the nuclear labeling obtained with the anti-RAE1 antibody.

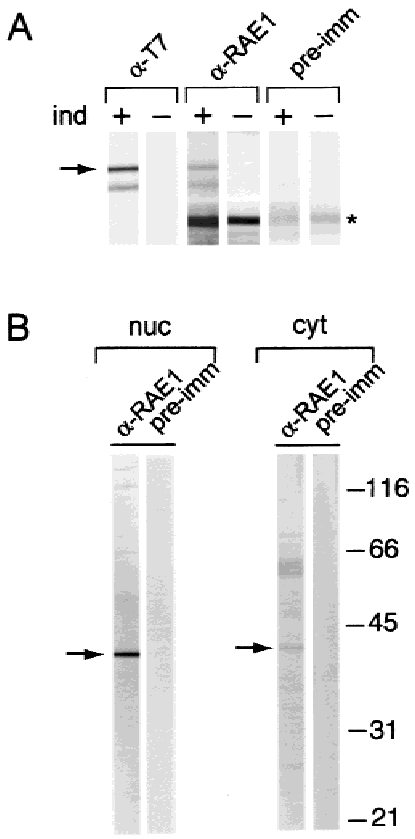

FIGURE 2. Specificity of the anti-RAE1 antibody. **A**: The ORF of Ct-RAE1 was expressed in *E. coli* with a T7-tag at the N-terminus. Bacterial extracts expressing recombinant Ct-RAE1 were analyzed by western blot with antibodies against T7 and hRAE1, and with preimmune serum. In all cases, extracts prepared before (ind −) and after (ind +) induction of expression were analyzed in parallel. The anti-T7 and anti-RAE1 antibodies were positive after induction of recombinant protein expression; both antibodies labeled a main band at 40 kDa (arrow). A second band of lower molecular mass was also observed with anti-T7 and anti-RAE1; this band was likely to be a degradation product of Ct-RAE1 as it was not observed in non-induced extracts. Additional proteins in the bacterial extract were recognized by both anti-RAE1 and preimmune serum (asterisk and not shown). **B**: Proteins from *C. tentans* nuclear and cytoplasmic extracts were analyzed by western blot with anti-RAE and pre-immune sera. A strong band of approx 40 kDa was seen in nuclear extracts incubated with anti-RAE1 antibody (arrow). In cytoplasmic extracts, a number of faint bands including the 40 kDa one were observed. The mobility of molecular mass standards is indicated on the right (in kilodaltons).

The anti-RAE1 antibody also gave some staining in cytoplasm and gland lumen, but this was not considered specific because it was also found in sections incubated with preimmune serum (Fig. 3B). This observation, together with the western blot results reported above, indicated that the anti-RAE1 antibody was not suitable for *in situ* studies in the cytoplasm. However, the antibody was highly specific in the nucleus.

To analyze the nuclear localization of Ct-RAE1 at higher resolution, we performed immuno-EM on thin sections of whole-mount salivary glands. The glands were fixed, cryoprotected, frozen, and cryosectioned as described in Materials and Methods. The sections were incubated with either anti-RAE1 or preimmune serum, and with a secondary antibody conjugated to colloidal gold. A quantitative analysis was carried out to assess the intensity of labeling in the various nuclear compartments. For this purpose, the immunolabeled sections were photographed and the density of labeling on chromosomes, nucleoli, nucleoplasm, and nuclear envelope was calculated by counting the number of gold markers per square micrometer on the micrographs. The results of the immuno-EM assays are summarized in Table 1. The highest density of labeling was at the nuclear envelope. As shown in Figure 4A, the nuclear envelope was decorated by gold markers at both nucleoplasmic and cytoplasmic sides. The nucleoplasm and the polytene chromosomes (Figs. 4B and 4C, respectively) were also labeled, although the densities of labeling in these two compartments were three to four times lower than in the nuclear envelope (Table 1). The lowest labeling density was found in the nucleolus, in agreement with the light microscopy results reported above.

In summary, our data indicated that the concentration of Ct-RAE1 was highest at the nuclear envelope but that a significant fraction of Ct-RAE1 was diffusely distributed inside the nucleus. Similar results had been previously reported for hRAE1 in mammalian cells (Kraemer & Blobel, 1997; Pritchard et al., 1999).

Ct-RAE1 co-localizes with BR RNP particles at the NPC

The observation that Ct-RAE1 was located not only at the nuclear envelope but also in chromosomes and nucleoplasm suggested that RAE1 could bind to the exported RNP in early steps of intranuclear transport. To determine if this was the case, we analyzed the association of Ct-RAE1 with a specific pre-mRNP particle, the BR particle, at different stages of synthesis, processing, and export. In cryosections of whole-mount salivary glands, we could analyze *in situ* three different groups of BR RNP particles: chromosomal BR particles being synthesized at the BR transcription units, nucleoplasmic BR particles in transit from the transcription site to the nuclear envelope, and BR particles at the nuclear envelope (Fig. 5A). To determine when the RAE1 protein interacts with the BR particle during the synthesis/processing/export pathway, we used immuno-EM with the anti-RAE1 antibody and determined the percentage of labeled BR particles in the three different locations mentioned above (Table 2). BR particles located at more than 150 nm from the nuclear envelope were classified as nucleoplasmic particles. BR particles located at 150 nm or less from the central plane of the nuclear envelope were classified as particles at the nuclear envelope. The results of this study

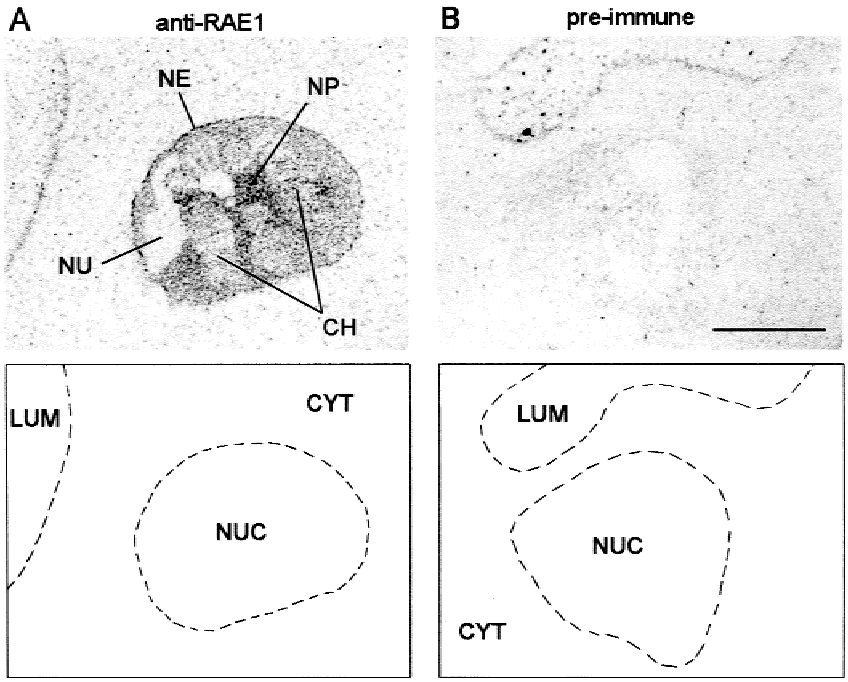

FIGURE 3. Localization of Ct-RAE1 in *C. tentans* salivary gland cells. Semithin cryosections of salivary glands were stained with either the anti-RAE1 (**A**) or the preimmune (**B**) sera. The antibody-binding sites were detected with a gold-conjugated secondary antibody and visualized after silver enhancement. The results were analyzed and photographed under bright field microscopy. Schematic interpretations of the micrographs are provided under the pictures. CH: chromosome; CYT: cytoplasm; LUM: gland lumen; NE: nuclear envelope; NP: nucleoplasm; NU: nucleolus; NUC: nucleus. The bar represents 25 μm.

are summarized in Figure 5 and Table 2. In transcription sites and nucleoplasm, the percentages of labeled BR particles were very low (1.1% and 1.6%, respectively). Instead, as many as 15.2% of the particles located at the nuclear envelope were found to be labeled. It was hard to judge to what an extent the low percentages found at transcription sites and nucleoplasm were significant, but the 10-fold increase observed at the nuclear envelope was highly significant and indicated that Ct-RAE1 interacts with BR particles in the proximity of the nuclear envelope.

Due to the existence of close contacts between the exported BR particles and the constituents of the NPC during docking and translocation (Mehlin et al., 1995; Kiseleva et al., 1996), and considering the resolution limits of our immuno-EM assays (see Materials and Methods), in most cases we could not tell whether the labeling was due to Ct-RAE1 molecules associated with the BR RNP, with the NPC, or with both. In some cases, however, we found basket filaments decorated by gold markers located at more than 25 nm from the BR particle (arrows in Fig. 5D, panels 1 and 2), which strongly suggested that at least in some cases, Ct-RAE1 was present in the basket and not in contact with the BR particle itself. In many other cases, the gold markers were located much closer to the BR particle or on the BR particle itself. BR particles were found to be labeled both during docking (i.e., Fig. 5D, panels 3 and 4) and translocation through the central channel (Fig. 5D, panels 6–8). It is interesting to note that in all cases the

TABLE 1. Intranuclear distribution of Ct-RAE1 analyzed by immunoelectron microscopy.[a]

Nuclear compartment	α-RAE1[b]	Preimmune[c]	Net density[d]
Nuclear envelope[e]	8.2 ± 3.5	1.3 ± 0.7	6.9
Nucleoplasm	3.1 ± 0.7	0.8 ± 0.6	2.3
Chromosome	2.8 ± 0.8	0.8 ± 0.5	2.0
Nucleolus	1.9 ± 1.4	0.7 ± 0.6	1.2

[a]Density of labeling as number of gold particles per μm^2 (mean ± standard deviation).
[b]Density of labeling with anti-RAE1 serum (3,003 gold markers analyzed).
[c]Density of labeling with preimmune serum (568 gold markers analyzed).
[d]Net density calculated as density of labeling with anti-RAE1 serum minus density of labeling with preimmune serum.
[e]Defined as a 400-nm-wide region extending ±200 nm from the central plane of the nuclear envelope.

TABLE 2. Association of Ct-RAE1 with BR RNP particles located at different nuclear compartments.[a]

Location	α-RAE1[b]	Preimmune[c]	Net (%)[d]
Transcription site	3.6 ± 2.6	2.5 ± 0.3	1.1
Nucleoplasm	2.3 ± 1.0	0.7 ± 0.7	1.6
Nuclear envelope	16.0 ± 7.3	0.8 ± 1.1	15.2

[a]Percentage of labeled BR RNP particles in each location expressed as mean value ± standard deviation.
[b]Percentage of BR RNP particles labeled with anti-RAE1 serum (9,231 BR RNP particles analyzed).
[c]Background level in negative control experiments expressed as percentage of BR RNP particles labeled with preimmune serum (3,559 BR RNP particles analyzed).
[d]Net percentage calculated as percentage of BR particles labeled with anti-RAE1 minus background level.

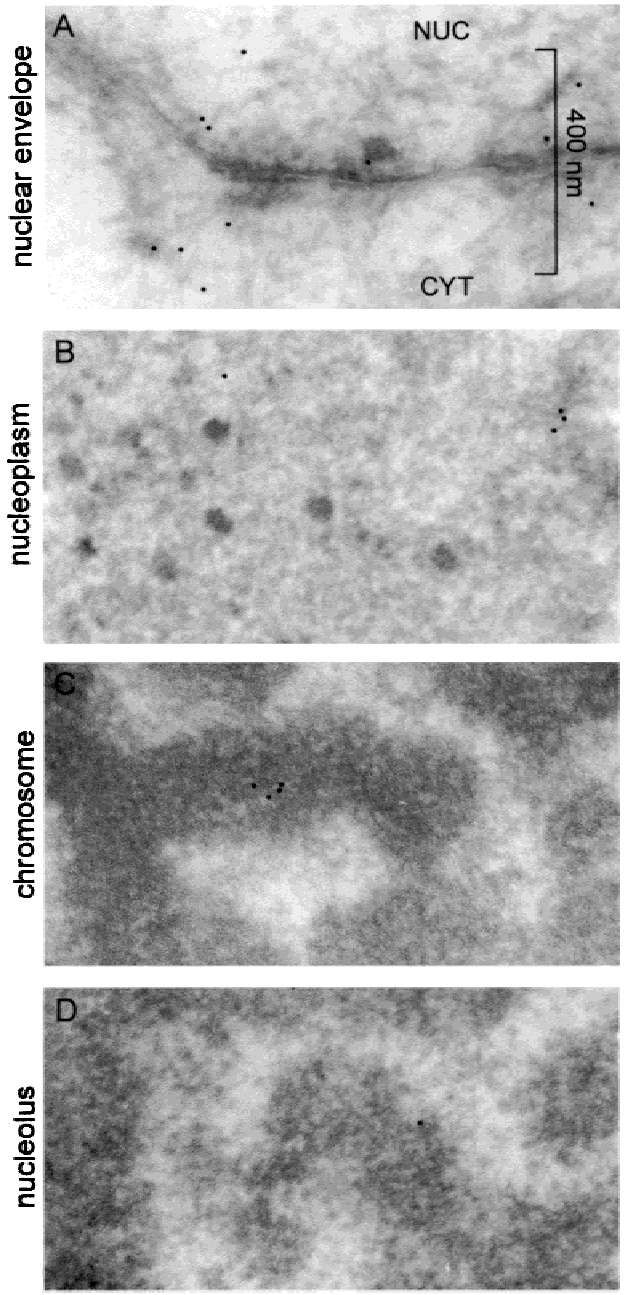

FIGURE 4. Distribution of Ct-RAE1 in the nucleus of the salivary gland cells analyzed by immuno-EM. Thin cryosections of salivary glands were incubated with anti-RAE1 serum, followed by a secondary antibody conjugated to 6 nm gold markers. Random areas were photographed and used for quantitative measurements of Ct-RAE1 localization as described in Materials and Methods. The figure shows examples of anti-RAE1 labeling in nuclear envelope (**A**), nucleoplasm (**B**), polytene chromosome (**C**), and nucleolus (**D**). In the case of the nuclear envelope, the area analyzed in the quantitative study was a 400-nm-wide region extending ±200 nm from the central plane of the nuclear envelope, as shown in (**A**). In all photographs, a black dot has been pasted on each of the original 6-nm gold markers to facilitate the visualization of the results.

labeling was located exclusively at the nuclear side of the NPC, even in late stages of translocation.

In the quantitative study reported above, the group of BR particles at the nuclear envelope constituted a heterogeneous population from the functional point of view. Although all the BR particles included in this group were located within 150 nm of the central plane of the nuclear envelope, the individual NPCs were not visible in many cases, due to the thickness of the specimen and to the high density of the nuclear envelope. In those cases where individual NPCs could not be visualized, it was not possible to decide whether the BR particles were really docked at the NPC or simply close to the nuclear membrane. To be able to study the association of Ct-RAE1 with BR particles located unambiguously at the NPC, we looked for tangential sections of NPCs. Approximately one-third (37 out of 110) of the NPCs observed in tangential sections were found to be engaged in the export of BR particles, and as many as 31 out of 37 (84%) NPCs engaged in export of BR particles were labeled with anti-RAE1 antibody (Table 3, Fig. 6). In some cases, the gold markers were far enough from the BR particle to support the conclusion that the immunolabeled Ct-RAE1 protein was not associated with the BR particle. In most cases, however, the labeling could be associated with the BR particle, the NPC, or both.

As an alternative approach to test whether RAE1 was associated with pre-mRNP particles in the nucleoplasm, we immunoprecipitated pre-mRNP complexes from nucleoplasmic extracts of *C. tentans* tissue culture cells and checked whether Ct-RAE1 was coimmunoprecipitated together with the pre-mRNPs. The immunoprecipitations were performed using mAb 1D3 against Ct-hpr23, an abundant pre-mRNA-binding protein of *C. tentans*, under conditions that allow the immunoprecipitation of pre-mRNP complexes (Wurtz et al., 1996; Sun et al., 1998). The eluted proteins were separated by SDS-PAGE and either stained with Coomassie or analyzed by western blot. As shown in Figure 7A, the Coomassie staining revealed bands of ~23, 36, 65, and 120 kDa corresponding to some of the major hrp proteins in *C. tentans*, as previously described (Wurtz et al., 1996). As expected, the anti-RAE1 antibody recognized a band of ~40 kDa in the nucleoplasmic extract used as input for immunoprecipitation, but did not react with any of the eluted proteins (Fig. 7B). As a positive control, the eluted polypeptides were probed in parallel with mAb 3G1 against Ct-hrp36, an hnRNP protein known to coimmunoprecipitate with Ct-hrp23 in pre-mRNP complexes (Sun et al., 1998). As shown in Figure 7B, the anti-hrp36 antibody was positive in both nucleoplasmic extract and eluate. We could not strictly rule out the possibility that the interaction between Ct-RAE1 and pre-mRNPs could be too weak to be detected in the conditions of our experiment, but the data indicated that Ct-RAE1 was not present in pre-mRNP complexes in conditions that did not disrupt the association of other hnRNP proteins with the pre-mRNA.

We concluded that the interaction between Ct-RAE1 and the BR RNP particle was not an early nucleoplas-

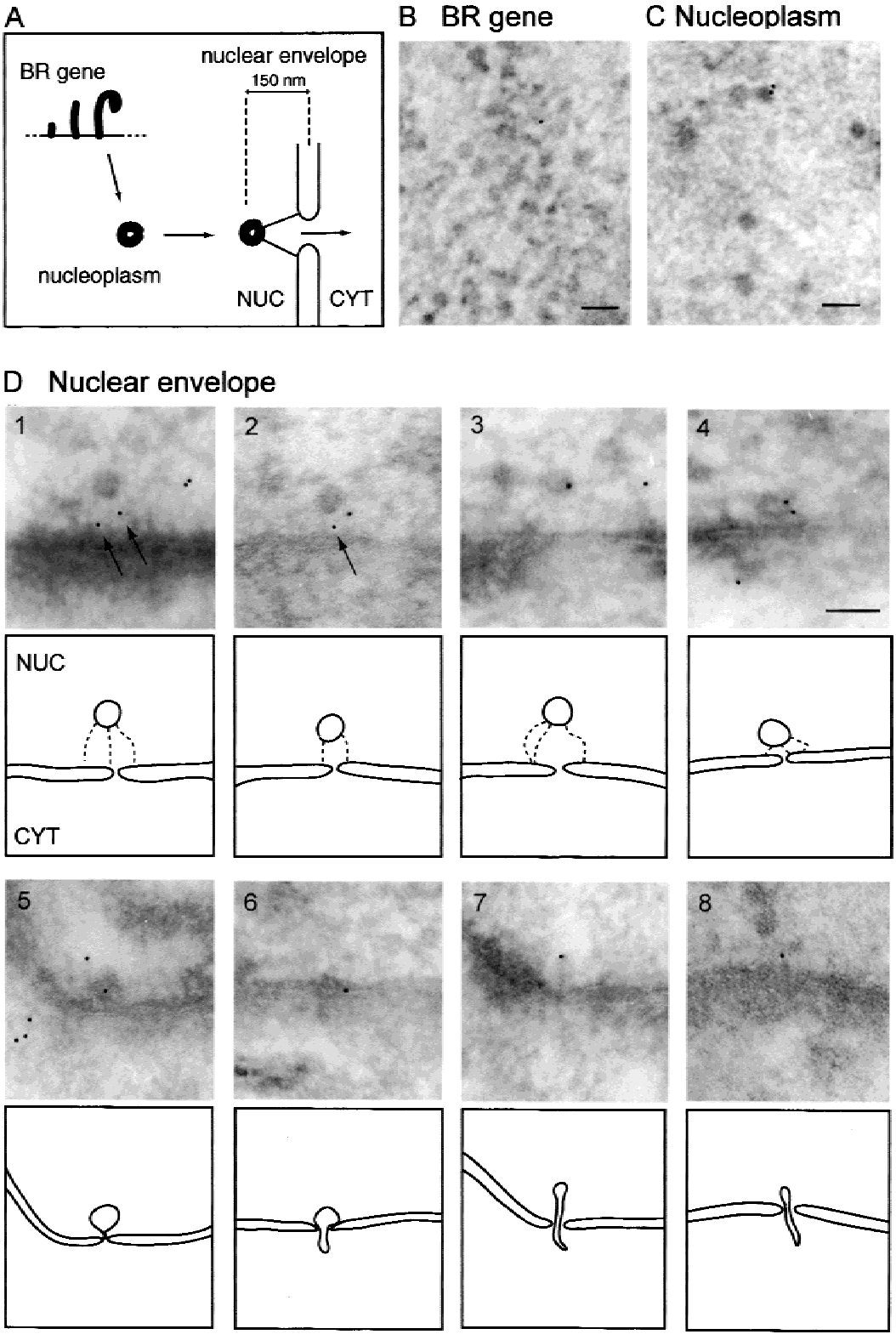

FIGURE 5. Immuno-EM analysis of Ct-RAE1 in BR RNP particles at different stages of maturation and nucleocytoplasmic transport. **A**: Schematic representation of BR pre-mRNP particles during assembly at the BR gene, transport through the nucleoplasm, and export through the NPC. Thin cryosections of salivary glands were incubated with anti-RAE1 antibody and with a secondary antibody conjugated to 6 nm gold markers. The figure shows examples of labeled BR particles located in the BR gene (**B**), in the nucleoplasm (**C**), and in successive stages of export through the NPC (**D**). In **D**, all pictures are oriented with nucleus (NUC) in the top part of the image and cytoplasm (CYT) in the bottom, and schematic interpretations of the images are provided under the micrographs. Arrows in **D**, panels 1 and 2, point at gold markers that are located more than 25 nm from the BR particle. To facilitate the visualization of the results, each gold marker in the original micrographs has been covered with a black dot. The bars represent 100 nm.

TABLE 3. Anti-RAE1 labeling in tangentially sectioned NPCs.[a]

	NPCs with BR particle	NPCs without BR particle
Labeled	31 (84%)	22 (30%)
Unlabeled	6 (16%)	51 (70%)
Total	37 (100%)	73 (100%)

[a]Expressed as number of observed NPCs in each class. Percentages of labeled versus unlabeled NPCs are given in parenthesis.

mic event, as suggested by the presence of Ct-RAE1 in the nucleoplasm. Instead, our results revealed that the interaction occurs at the NPC.

Localization of Ct-RAE1 at the NPC is correlated with the presence of an export cargo

The analysis of tangential sections of NPCs also provided relevant information as to whether Ct-RAE1 was permanently located at the NPC or, alternatively, associated transiently with the NPC in a more dynamic manner. If Ct-RAE1 was stably bound to the NPCs, all NPCs would be equally labeled by the anti-RAE1 antibody, regardless of whether they were transporting mRNPs or not. On the other hand, if Ct-RAE1 was dynamically recruited to the NPC concomitant with an export event, the percentage of labeled NPCs would be high for NPCs engaged in the export of BR particles and low for NPCs devoid of export cargoes. The observed data was compatible with the latter prediction: 84% of the NPCs engaged in the export of BR particles were labeled by the anti-RAE1 antibody, whereas only 30% of the NPCs devoid of BR particles were labeled by the same antibody. Thus the results suggested that the Ct-RAE1 was preferentially located in NPCs engaged in mRNA export.

The observation that Ct-RAE1 was present in 30% of the NPCs without BR particles could be taken as an indication that Ct-RAE1 was indeed attached to some NPC even in the absence of exported mRNP. However,

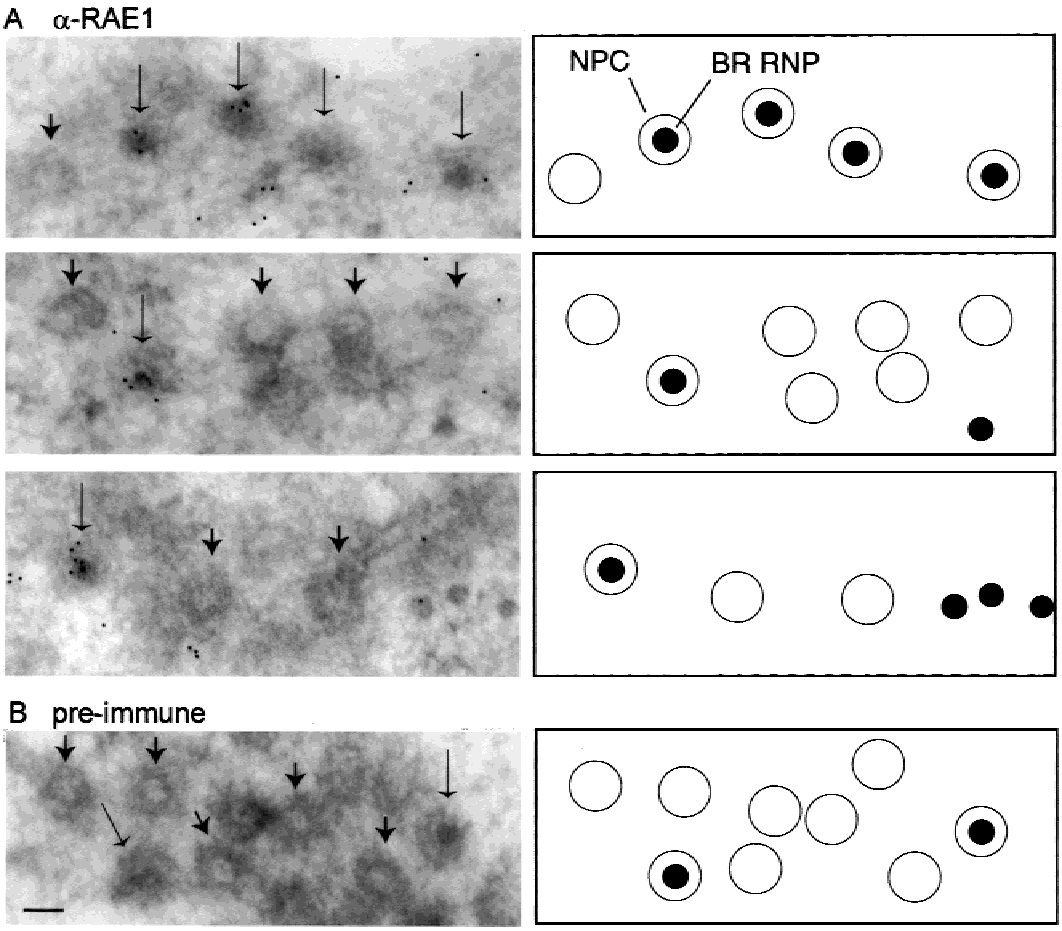

FIGURE 6. Immuno-EM analysis of Ct-RAE1 in tangential sections of NPCs. **A**: Three examples of tangentially sectioned NPCs labeled with anti-RAE1 antibody. Sections incubated in parallel with preimmune serum did not show any significant labeling (**B**). Long arrows point at NPCs engaged in the export of a BR particle. Short arrows point at NPCs devoid of BR particle. Schematic interpretations of the images are provided beside each micrograph. A black dot has been pasted on each of the original gold markers to facilitate the visualization of the results. The bar represents 100 nm.

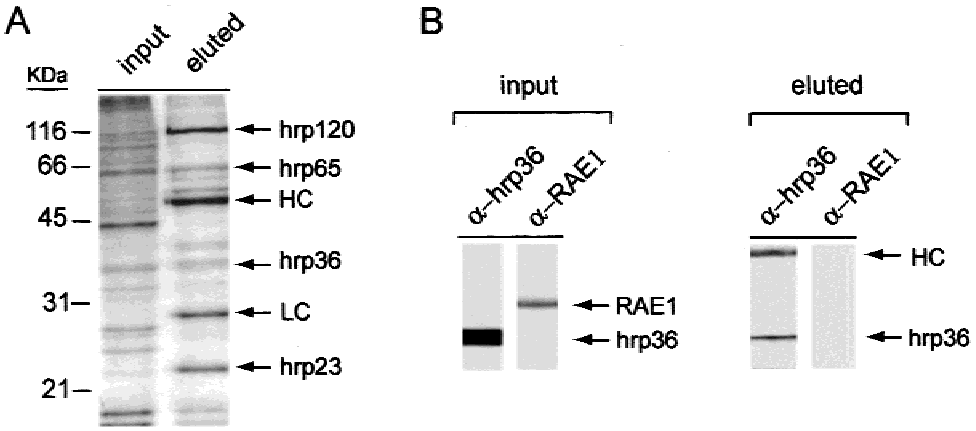

FIGURE 7. Western blot analysis of Ct-RAE1 in pre-mRNP complexes isolated by immunoprecipitation. Pre-mRNP complexes were immunoprecipitated from RNP extracts of *C. tentans* using mAB 1D3 against Ct-hrp23. **A**: The RNP extract used as input for the immunoprecipitation (input) and the immunoprecipitated proteins (eluted) were resolved by SDS-PAGE and stained with Coomassie blue. The position of molecular mass standards is shown to the left in kilodaltons. The arrows point at some of the known immunoprecipitated proteins, such as hrp23, hrp36, hrp65, and hrp120. HC and LC correspond to the heavy and light chains, respectively, of mAb 1D3. **B**: Both the input extract and the eluted proteins were analyzed by western blot with the anti-RAE1 antibody, using mAb 3G1 against hrp36 as a positive control. Ct-RAE1 was detected only in the input extract but not in the preparation of eluted proteins, whereas hrp36 was detected in both.

we want to point out that this 30% value is likely to be overestimated for two main reasons. First, NPCs with BR particles at late stages of translocation, such as the ones shown in Figure 5D, panels 7 and 8, would appear almost "empty" and would be classified as NPCs without BR particle. Second, at any given time there must be a considerable fraction of NPCs engaged in the export of mRNPs from other genes. Because most of the non-BR mRNPs are much smaller and probably exported much faster than the BR particles, these NPCs would also be classified as NPCs without BR particle. Thus, many of the NPCs included in this 30% are indeed engaged in mRNA export.

In summary, our results indicate that Ct-RAE1 is preferentially located at NPCs that are actively involved in export of BR particles and that the association of Ct-RAE1 with the NPC is dynamic and related to the presence of an export cargo in the NPC.

DISCUSSION

RAE1 is a protein associated with the NE that can interact with both pre-mRNPs and nucleoporins. Thus RAE1 exhibits many of the properties expected for an mRNA export receptor (reviewed by Görlich & Kutay, 1999; Bachi et al., 2000). However, the mechanism by which RAE1 functions in mRNA export is still unknown and the time point at which RAE1 interacts with the exported RNP has not previously been investigated. Here we have addressed this question in the BR system of *C. tentans* and concluded that the interaction between Ct-RAE1 and the exported BR particle occurs at the NPC, and that localization of Ct-RAE1 at the NPC is dynamic and correlated with the presence of an export cargo.

RAE1 in *C. tentans*

Making use of the high degree of conservation of RAE1 in eukaryotes, we have isolated a cDNA encoding Ct-RAE1, a *C. tentans* protein of 349 amino acids. Several observations support the conclusion that Ct-RAE1 is the counterpart of RAE1 in *C. tentans*. First, the overall sequence of Ct-RAE1 is very similar to human RAE1, *S. pombe* Rae1p, and *S. cerevisiae* Gle2p, and residues known to be functionally important in these proteins are conserved in Ct-RAE1. Second, an antibody raised against hRAE1 was able to recognize recombinant Ct-RAE1 expressed in *E. coli*. Third, in *C. tentans* the anti-RAE1 antibody recognized a protein of approximately 40 kDa that localized to nucleus, cytoplasm, and nuclear envelope, as expected for RAE1. Based on these observations, we conclude that Ct-RAE1 is the homolog of RAE1 and that the antibody raised against hRAE1 is a specific tool that can be used for immunochemical purposes in *C. tentans*.

Association of Ct-RAE1 with the BR particle

UV crosslinking experiments showed that hRAE1/mrnp41 binds directly to mRNA in HeLa cells (Kraemer & Blobel, 1997). It is not known whether the RAE1 counterparts in yeast and insects also can bind to mRNA, or whether the interaction of RAE1 with the RNP complex is mediated by protein–protein contacts.

Our immuno-EM study has indicated that Ct-RAE1 does not bind to the BR particle either cotranscriptionally or in the nucleoplasm. Instead, we have found that Ct-RAE1 and the BR particle colocalize only in the proximity of the NPC. Considering that a fraction of Ct-RAE1 is dispersed throughout the nucleoplasm, the

absence of binding suggests that the interaction of Ct-RAE1 with the BR particles requires prior modification of the BR RNP, either removal of proteins that prevent binding or addition of factors that bridge the RAE1-RNP interaction. The hnRNP proteins that leave the BR particle in the proximity of the NPC, such as Ct-hrp23 (Sun et al., 1996) or hrp45 (Alzhanova-Ericsson et al., 1996), could play such a role.

It is remarkable that in our immuno-EM analysis we have not detected Ct-RAE1 at the cytoplasmic side of BR particles in transit through the NPC. We have found examples of late translocating particles, and in all of them the labeling was clearly located at the nuclear side (e.g., Fig. 5D, panels 7 and 8). Although there appears to be a significant fraction of Ct-RAE1 located in the cytoplasm, our finding suggests that the function of RAE1 is at the nuclear side of the NPC and that RAE1 is not exported to the cytoplasm as a stable component of the mRNP complex. During translocation of the BR particle through the central channel, Ct-RAE1 might interact with the RNP while remaining anchored at the NPC. Alternatively, Ct-RAE1 could become transiently associated with the RNP and quickly released at the cytoplasmic side. This latter possibility would be more consistent with the presence of RAE1 in the cytoplasm, with the observation that binding of RAE1 to the NPC is dynamic, and with the proposed shuttling ability of hRAE1 (Pritchard et al., 1999).

In summary, our results show that Ct-RAE1 does not become a stable component of the BR pre-mRNP particle. Our data is compatible with the proposal that RAE1 mediates the interaction between the RNP and the NPC, and we conclude that the function of RAE1 in mRNA export is at the level of the NPC.

RAE1 and export of BR particles

Upon transcription termination, the newly assembled BR particles are released from the transcription site and transported to the NE through a combination of free diffusion (Singh et al., 1999) and transient interactions with nucleoplasmic structures (Miralles et al., 2000). Whether such interactions are engaged in guided delivery of mature BR particles to the NPC or in nuclear retention of unprocessed transcripts remains to the elucidated. Once the BR particles reach the NE, initial contacts are established between the BR RNP and the basket of the NPC, followed by rotation of the RNP in front of the central channel and subsequent translocation of the extended RNP fiber to the cytoplasm (Mehlin et al., 1995; Kiseleva et al., 1996). In spite of the available structural information, little is known about the molecules responsible for each step of the process. The results from our immuno-EM and immunoprecipitation experiments suggest that RAE1 is not involved in any of the early events, such as release from the chromosome or interaction with nucleoplasmic structures.

To understand the function of RAE1 in mRNA export, it is important to determine the series of events that leads to the interaction between the RNP and the NPC. Does the RNP particle establish contact with the NPC before or after binding to RAE1? Our immuno-EM study has shown that at least some of the RAE1 molecules located at the NPC during the export of the BR particle are not in direct contact with the BR RNP as shown by the distance observed between the BR RNP and the anti-RAE1 labeling; such RAE1 molecules might be associated with smaller mRNPs in transit through the same NPC. Moreover, the interaction between RAE1 and the NPC is dynamic, and RAE1 colocalizes with the BR RNP only in the proximity of the NPC. At least two mechanistic models compatible with these findings can be conceived. In the first one, the BR particle would be modified in the proximity of the NPC, perhaps as a result of initial contacts with the terminal filaments of the basket. Such modifications, maybe involving release of hrp proteins, would then allow binding of RAE1 as a secondary event. This model would explain why the localization of RAE1 at the NE is dependent on ongoing transcription (Pritchard et al., 1999). In a second model, binding of RAE1 to the NPC could be the primary event that would render the NPC competent to rapidly recruit a mature RNP and initiate the translocation process. In this case, the transcription dependence of RAE1 localization at the NE could be due to indirect recycling effects and not to the direct availability of export substrates. Although we cannot rule out either of the models with our present results, the latter alternative is favored by a previous report showing that the interaction between RAE1 and the GLEBS-like motif of NUP98 is direct and mRNA independent (Pritchard et al., 1999). A better definition of the physical interactions between the molecules involved in the process is required to further understand the function of RAE1 in mRNA export. In particular, the molecular characterization of the interaction between RAE1 and the exported RNP particle is an important goal for the future.

MATERIALS AND METHODS

Animal and cell culture

C. tentans larvae were raised under conditions described by Lezzi et al. (1981). *C. tentans* cells were cultivated as described (Wyss, 1982).

Antibodies

A cDNA sequence encoding hRAE1 was amplified by PCR and cloned as a *Nhe*I-*Bam*HI fragment into the pRSETA vector (Invitrogen). His-tagged recombinant hRAE1 was produced in *E. coli*, purified under denaturing conditions on Ni-NTA agarose beads (Qiagen, Inc.), and injected into rabbits. Preimmune serum was used in all the experiments as

negative control. The anti-RAE1 antibody was affinity purified on recombinant hRAE1 immobilized to Ni-NTA agarose, eluted with glycine, and neutralized following standard procedures. The monoclonal antibodies 3G1 and 1D3, which are specific for Ct-hrp36 and Ct-hrp23, respectively, were kindly provided by B. Daneholt and are described elsewhere (Kiseleva et al., 1997; Sun et al., 1998). The antibodies against T7-tag and His-tag were from Novagen and Santa-Cruz Biotechnologies, respectively. Rabbit anti-mouse immunoglobulins used for immunoprecipitation and secondary antibodies conjugated with alkaline phosphatase used for western blot analysis were purchased from Dako. Secondary antibodies conjugated to 6 nm colloidal gold used for immuno-LM and immuno-EM were purchased from Jackson ImmunoResearch Laboratories.

Cloning and sequencing of full-length Ct-RAE1 cDNA

Semi-nested PCR amplification was performed in two steps using Taq DNA polymerase (Roche). For the first reaction, genomic DNA from *C. tentans* tissue culture cells was used as a template with the oligonucleotides F1 (5′TTCGAGGTC GCCTCGCCGCCGGANGA3′) and R1 (5′AGTTTTGTTCTG GCRTCYTTRTCCCA3′). The product of this first PCR was used as a template in the second reaction with primers F1 and R2 (5′CACCACGGCCATCGG ATACTCNACRTC3′). F1 and R2 primers were based on a *Drosophila* EST sequence with accession number AA141856, nt 8–33 and 443–470, respectively. R1 was based on nt 1085–1110 of the human RAE1 cDNA sequence (accession number U84720). The final PCR product, 650 bp long, was labeled with digoxigenin-dUTP (Roche) and used as a probe to screen a lambda-Zap cDNA library from tissue culture cells of *C. tentans* as previously described (Visa et al., 1996a). Two positive clones were obtained, λ-RAE1-1 and λ-RAE1-2. The λ-RAE1-1 clone was sequenced using walking primers with the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech). The ORF of λ-RAE1-1 was sequenced at least three times. Sequence analysis was performed with the University of Wisconsin Genetics Computer Group Sequence Analysis Program and EGCG extensions to the Wisconsin Package Sequence Analysis Programs.

Subcloning and expression of Ct-RAE1

The ORF of λ-RAE1-1 was cloned into PET21a (Novagen) for expression of recombinant Ct-RAE1 in *E. coli*. A cDNA fragment encoding amino acids 1–342 was amplified by PCR with Pfu DNA polymerase (Stratagene) using λ-RAE1-1 as a DNA template and primers that introduced a 5′ *Bam*HI site and a 3′ *Hin*dIII site. Upon restriction enzyme digestion, the PCR product was inserted into the *Bam*HI-*Hin*dIII digested vector using standard methods. To avoid the C-terminal His-tag of PET21a, the reading frame was shifted by linearizing the plasmid with *Hin*dIII, filling in the 5′ overhangs with Pfu DNA polymerase (Stratagene), and religating the blunt ends. The resulting recombinant protein contained only a T7-tag in the N-terminus. Expression in *E. coli* Bal21 was induced by addition of phage λ-CE6 (5–10 particles/cell), followed by incubation at 37 °C for 2 h.

Preparation of cytoplasmic and nuclear extracts from *C. tentans* tissue culture cells

Cytoplasmic and nuclear protein extracts were prepared as described before (Miralles et al., 2000). Briefly, the *C. tentans* tissue culture cells were homogenized in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.2) containing 0.2% NP-40 and 0.1 mM phenylmethylsulfonyl (PMSF) using a glass homogenizer (tight pestle) and centrifuged at 2,000 × *g* for 10 min at 4 °C. The supernatant was the cytoplasmic fraction. The pellet containing nuclei was resuspended in PBS containing 0.1 mM PMSF and sonicated for 60 s. The proteins in each fraction were precipitated by addition of 6 vol of cold acetone and stored at −20 °C.

SDS-PAGE and western blot analysis

Proteins were separated by SDS-PAGE in polyacrylamide gels and stained with Coomassie blue according to standard procedures. For western blot, proteins were transferred to polyvinylidene fluoride membranes (PVDF; Millipore) in tris-glycine buffer supplemented with 0.02% SDS and 4 M urea using a semidry electrophoretic transfer cell (Bio-Rad). Membranes were blocked with 10% nonfat dry milk in PBS and probed with antibodies. Both primary and secondary antibodies were diluted in PBS containing 1% milk and 0.05% Tween 20. The secondary antibodies were conjugated to alkaline phosphatase. For detection, NBT/BCIP was used according to standard procedures.

Immunostaining of salivary gland cryosections (LM)

Immunostaining on semithin cryosections was performed as previously described (Visa et al., 1996a). Salivary glands from fourth instar larvae were fixed in 0.1 M cacodylate buffer at pH 7.2 containing 4% paraformaldehyde for 60 min at room temperature, cryoprotected with 2.3 M sucrose, and frozen by immersion in liquid nitrogen. 0.5-μm-thick sections were obtained in a cryoultramicrotome (Ultracut S/FC S, Reichert) and mounted on glass slides. Before immunolabeling, the sections were incubated in PBS containing 0.1 M glycine and 2% BSA. The first antibody, either anti-RAE1 serum or preimmune serum, was diluted 1:500 in PBS. The secondary antibody was an anti-rabbit IgG conjugated to 6 nm gold particles (Jackson ImmunoResearch Laboratories) diluted 1:50 in PBS containing 0.5% BSA. The immunogold labeling was silver enhanced with IntenSE™ (Amersham).

Immuno-EM on cryosection of salivary glands

Immuno-EM was carried out essentially according to the method described by Tokuyasu (1980) with few modifications (Visa et al., 1996a). The specimens were prepared as described above for light microscopy except that fixation was for 20–25 min in 4% paraformaldehyde and 0.1% glutaraldehyde. Thin cryosections, 70–80 nm thick, were picked up on drops of 2.3 M sucrose and deposited onto nickel grids coated with formvar and carbon. The grids were floated on drops of PBS containing 0.1 M glycine and 10% new-born calf serum before incubation with the antibody solutions. Pri-

mary antibodies, either anti-RAE1 or preimmune sera, were diluted 1:500 in PBS. The secondary antibody was an anti-rabbit IgG conjugated to 6 nm gold particles (Jackson ImmunoResearch Laboratories) diluted 1:50 in PBS containing 5% calf serum. After immunolabeling, the sections were stained with 2% aqueous uranyl acetate for 5 min 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 quantitative analyses, randomly selected areas covering different cell compartments were photographed at a magnification of 20,000×. The density of labeling in each cell compartment (nuclear envelope, nucleoplasm, chromosome, and nucleolus) was manually counted for each nucleus and given as number of gold particles per μm^2. In the case of nuclear envelope, the region analyzed was a 0.4-μm-wide band centered at the central plane of the nuclear envelope (0.2 μm towards the nucleus and 0.2 μm towards the cytoplasm); this area was calculated by multiplying the length of nuclear envelope by 0.4 μm. The length of nuclear envelope was measured on the photographs using a digital measuring meter. In the conditions of our study, using two antibodies and 6-nm gold markers, the antigen could be located as far as 25 nm from the center of the gold marker, although in most cases the distance was expected to be much shorter. Thus, for the purpose of our study, we defined labeled BR particles as those that were located within 20 nm of a gold marker, measured from the surface of the BR particle. Labeling of BR particles was expressed as percentage of labeled BR particles in each location. Results summarized in Tables I, II, and III are based on the analysis of data from two independent experiments. Eleven and five different nuclei were analyzed for anti-RAE1 and preimmune serum, respectively.

Immunoprecipitation of pre-mRNP complexes

Pre-mRNP complexes were immunoprecipitated from RNP extracts of *C. tentans* tissue culture cells as described by Sun et al. (1998) using monoclonal antibody 1D3 specific for Ct-hrp 23. In brief, the immunoprecipitation was carried out in two steps. First, mAb 1D3 was added to the RNP extract to a final concentration of ~10 μg/mL in the presence of 0.1% NP-40 and 0.1 mg/mL *E. coli* tRNA, and incubated for 90 min at 4 °C with gentle rotation. Rabbit anti-mouse immunoglobulins covalently coupled to protein G-agarose (Zymed Laboratories, Inc.) were added and the rotation was continued for an additional 90 min at 4 °C. The agarose beads were washed twice with PBS containing 0.1% NP-40 and once with PBS. The proteins were eluted with 0.5% SDS at room temperature. The eluted proteins were precipitated with acetone and subsequently analyzed by SDS-PAGE and western blotting.

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