A Nuclear Cap-binding Complex Binds Balbiani Ring Pre-mRNA Cotranscriptionally and Accompanies the Ribonucleoprotein Particle during Nuclear Export

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Abstract. In vertebrates, a nuclear cap-binding complex (CBC) formed by two cap-binding proteins, CBP20 and CBP80, is involved in several steps of RNA metabolism, including pre-mRNA splicing and nuclear export of some RNA polymerase II–transcribed U snRNAs. The CBC is highly conserved, and antibodies against human CBP20 cross-react with the CBP20 counterpart in the dipteran *Chironomus tentans.* Using immunoelectron microscopy, the in situ association of CBP20 with a specific pre-mRNP particle, the Balbiani ring particle, has been analyzed at different stages of pre-mRNA synthesis, maturation, and nucleo-cytoplasmic transport. We demonstrate that CBP20 binds to the nascent pre-mRNA shortly after transcription initiation, stays in the RNP particles after splicing has been completed, and remains attached to the 5' domain during translocation of the RNP through the nuclear pore complex (NPC). The rapid association of CBP20 with nascent RNA transcripts in situ is consistent with the role of CBC in splicing, and the retention of CBC on the RNP during translocation through the NPC supports its proposed involvement in RNA export.

I N the nucleus of eukaryotic cells, messenger RNA precursors (pre-mRNAs)[1] undergo a series of maturation reactions before they are exported to the cytoplasm where the mature messenger RNAs (mRNAs) can direct protein synthesis. One of these maturation events is the addition of a monomethylated guanosine residue to the first encoded nucleotide of the RNA via a 5'-5' triphosphate linkage (Shatkin, 1976). This reaction occurs on all transcripts synthesized by RNA polymerase II shortly after the start of transcription (Salditt-Georgieff et al., 1980; Rasmussen and Lis, 1993). The resulting cap structure is implicated in several aspects of pre-mRNA and mRNA metabolism. The cap structure increases RNA stability, and is required for efficient translation initiation, pre-mRNA splicing, and nuclear export of capped RNAs (Furuichi et al., 1977; Shimotohno et al., 1977; Konarska et al., 1984; Shatkin, 1985; Edery and Sonenberg, 1985; Hamm and Mattaj, 1990; Izaurralde et al., 1992). Some of these effects are mediated by proteins. In the nucleus, a cap-binding complex (CBC) consisting of two proteins, CBP80 and CBP20, has been described (Ohno et al., 1990; Izaurralde et al., 1994, 1995; Kataoka et al., 1994). Studies in HeLa cell extracts and in *Xenopus* oocytes have demonstrated that CBC mediates efficient splicing of pre-mRNA, probably by affecting early stages in spliceosome assembly (Izaurralde et al., 1994). In addition, a direct involvement of CBC in export of U snRNAs has recently been demonstrated (Izaurralde et al., 1995). In the case of mRNA, it has been shown that the presence of a 5' cap structure on the mRNA enhances its rate of export from the nucleus (Hamm and Mattaj, 1990; Jarmolowski et al., 1994), although the effect of the cap on mRNA export is less important than in the case of the U snRNAs. The nuclear CBC is a likely candidate for the mediator of this effect. However, a direct role for CBC in mRNA export has not been demonstrated.

CBP20 and CBP80 were initially characterized in human cells, but homologues of both proteins have been identified in other organisms, including amphibia and insects. CBP20 is a highly conserved protein: the amino acid sequences of human and *Xenopus* CBP20 show 84% identity (Izaurralde et al., 1995), while human and *Drosophila* CBP20 are 75% identical, and *Drosophila* CBP20 is efficiently recognized by anti–human CBP20 antibodies (Lewis, J., and I.W. Mattaj, unpublished data). Thanks to this high degree of conservation, we have been able to use polyclonal antibodies raised against the human CBP20 for in situ studies on insect polytene nuclei. In particular we have used the Balbiani ring system of *Chironomus tentans*

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1. *Abbreviations used in this paper:* BR, Balbiani ring; CBC, cap-binding complex; CBP, cap-binding protein; NPC, nuclear pore complex; pre-mRNA, messenger RNA precursor; RNP, ribonucleoprotein.

(for reviews see Mehlin and Daneholt, 1993; Wieslander, 1994) to analyze in situ the association of CBC with pre-mRNA and mRNA during splicing and export.

The Balbiani rings (BRs) of the dipteran *Chironomus tentans* (*C. tentans*) are puffs of exceptional size in the polytene chromosomes of the larval salivary gland cells. Two large BRs, BR1 and BR2, can be easily identified on the polytene chromosomes. The pre-mRNA synthesized in BR1 and BR2 is 35–40-kb long (75S RNA) and is packaged into large pre-mRNP complexes, the so-called BR RNP particles. The BR particles appear as abundant pre-mRNP complexes in the nucleoplasm of the salivary gland cells (Daneholt, 1982). Due to their structure and extraordinary dimensions, the BR RNP particles can be unambiguously identified in the electron microscope (EM), and their assembly, transport, and disassembly can be directly studied in situ (Skoglund et al., 1983; Mehlin et al., 1992, 1995). Nascent BR pre-mRNA molecules are rapidly packaged into growing RNP fibers that can be observed along the BR transcription units. As transcription progresses, the growing RNP fibers gradually increase in length, and their distal portion becomes folded into a dense globular structure (Skoglund et al., 1983). When transcription is completed, the BR particles are released from the chromosome and can be seen in the nucleoplasm as RNP granules of ∼50 nm (Skoglund et al., 1896). The BR RNP granules are transported to the nuclear envelope, where they unfold and become elongated during translocation through the nuclear pores (Mehlin et al., 1992).

The BR pre-mRNA contains three introns close to the 5' end (introns 1, 2, and 3) and one more intron (intron 4) located 30 kb downstream, close to the 3' end of the pre-mRNA (Wieslander and Paulsson, 1992). Recent studies on the BR1 gene have shown that splicing may occur either cotranscriptionally or after transcription is completed, depending upon the position of the introns in the pre-mRNA (Baurén and Wieslander, 1994). Intron 3 is removed cotranscriptionally from most of the BR pre-mRNA molecules, and in situ studies have shown that spliceosome assembly/disassembly occurs rapidly on the nascent pre-mRNP fiber (Kiseleva et al., 1994). In addition, it has also been shown that most of the BR RNP particles located in the nucleoplasm are fully spliced (Baurén and Wieslander, 1994).

The association of defined RNA-binding proteins with BR RNP particles during successive stages of synthesis, maturation, and export can be directly visualized using immuno-EM methods (Kiseleva et al., 1994; Visa et al., 1996). In this study, we analyze when the nuclear CBC is added to the pre-mRNP complex. It is well established that the formation of the 5' cap occurs cotranscriptionally (Salditt-Georgieff et al., 1980), but the time point at which CBPs bind to the 5' cap has not previously been addressed. The persistence of the association of CBC in fully spliced mRNP complexes in the nucleoplasm and during nucleo-cytoplasmic translocation is also examined.

Materials and Methods

Culturing Conditions

C. tentans was cultured as described by Lezzi et al. (1981) and salivary glands were isolated from fourth instar larvae. *C. tentans* tissue culture cells were grown in suspension at 24°C as previously described (Wyss, 1982). HeLa cells were grown on glass coverslips under standard conditions. For actinomycin D treatments, the cells were incubated in fresh medium containing actinomycin D at a concentration of 5 μg/ml for 70 min. Control cells were incubated in fresh medium without the drug.

Antibodies

Chironomus CBP20 was detected using affinity-purified rabbit antibodies raised against recombinant human CBP20 (Izaurralde et al., 1995). Preimmune rabbit serum was used in parallel as negative control. Monoclonal antibody 9H10, kindly provided by G. Dreyfuss, was used to detect hnRNP A1. Nuclear pore complex (NPC) proteins were labeled with monoclonal antibody mAb414 (BAbCO, Richmond, CA). The secondary antibodies for immunofluorescence were anti–rabbit or anti–mouse immunoglobulins conjugated to either TRITC or FITC (Boehringer Mannheim, Mannheim, Germany). In immunoelectron microscopy experiments, the labeling was visualized using anti–rabbit IgG conjugated to 6-nm-colloidal gold particles (Jackson ImmunoResearch Labs., West Grove, PA). Alkaline phosphatase–conjugated antibodies against rabbit immunoglobulins (DAKO A/S, Glostrup, Denmark) were used for Western blot analysis and for immunostaining of polytene chromosomes.

Protein Electrophoresis and Western Blot Analysis

C. tentans tissue culture cells were washed in PBS, resuspended in TNM (10 mM triethanolamine-HCl buffer, pH 7.0, 100 mM NaCl, 1 mM MgCl$_2$) containing 0.2% NP-40 and 0.1 M PMSF, homogenized in a glass tissue grinder with a tight-fitting pestle, and centrifuged at 2,000 *g* for 5 min at 0°C. The supernatant was the cytoplasmic extract used for Western blot analysis. The pellet (nuclei) was washed with TNM, sonicated, and centrifuged at 7,000 *g* for 10 min at 4°C. The supernatant was called RNP extract I. The pellet was resuspended in TNM, digested with 30 ng/ml RNase A for 10 min at room temperature, and centrifuged as before. The supernatant of this last centrifugation together with RNP extract I constituted the nuclear protein extract used for Western blot analysis. Electrophoresis of proteins in SDS-PAGE was carried out using the minigel system (BioRad Labs, Richmond, CA). Proteins were electrophoresed in discontinuous gels (12% acrylamide in the separating gel) and blotted to transfer membranes (Immobilon PVDF, Millipore, Bedford, MA) using a Trans-Blot semidry electrophoretic system (BioRad Labs). Membranes were blocked in PBS containing 10% dry milk powder and incubated with anti-CBP20 antibody (diluted 1:1,500 in PBS containing 1% dry milk powder and 0.05% Tween-20). Labeling was visualized with alkaline phosphatase–conjugated anti–rabbit immunoglobulins using the NBT/BCIP system (Promega, Madison, WI) according to the manufacturer's instructions.

Immunostaining of Polytene Chromosome Squashes

Squash preparations were made as described by Ericsson et al. (1990) with some modifications. The salivary glands were fixed with 4% paraformaldehyde in PBS for 15–20 min and subsequently squashed in 45% acetic acid. The slides were frozen in liquid nitrogen and the coverslips removed. The preparations were then washed extensively with PBS, blocked with 2% BSA in PBS, and incubated with affinity-purified anti-CBP20 antibody diluted 1:500. The secondary antibody was alkaline phosphatase–conjugated (DAKO A/S, Glostrup, Denmark), and the enzymatic reaction was developed using the NBT/BCIP system (Promega). The reaction was stopped by washing the slides in distilled water. The chromosomes were examined and photographed in a Standard microscope (Carl Zeiss, Oberkochen, Germany). For RNase treatments, chromosomes were prepared in the same manner but fixation was only for 5 min. After the glands were squashed and the coverslips removed, half of the slides were incubated in PBS (positive control) and the other half in PBS containing 100 μg/ml of preboiled RNase A (Boehringer Mannheim) for 30 min at room temperature. The slides were then washed in PBS, blocked, and immunostained as above.

Indirect Immunofluorescence

Salivary glands were fixed for 1 h at room temperature in a solution of 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2). Fixed glands were rinsed in the same buffer, infiltrated in 2.3 M sucrose and frozen by immersion in liquid nitrogen. Semithin sections (0.5-μm-thick) were obtained in a Reichert Ultracut S/FC S ultramicrotome system (Reichert,

Wien, Austria), picked up on a drop of 2.3 M sucrose and mounted on glass slides. The sections were incubated in the following solutions: PBS 3 × 5 min, PBSG (PBS containing 0.1 M glycine) for 5 min, 2% BSA in PBS for 20 min, primary antibody (either anti-CBP20 or preimmune serum) diluted 1:500 in PBS supplemented with 0.2% BSA for 1 h, PBS 3 × 5 min, TRITC-conjugated secondary antibody for 1 h, and PBS 3 × 5 min.

HeLa cells grown on coverslips were fixed with 3.7% paraformaldehyde in CKS buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes pH 6.8, 3 mM MgCl₂, 1 mM EGTA) for 10 min, permeabilized with 0.5% Triton X-100 in CSK, and washed 3 × 5 min in PBS. Double labeling was performed according to standard methods. To test the specificity of the immunolabeling, the primary antibody was substituted by preimmune serum in the negative controls.

Samples were examined and photographed in a Zeiss Axioskop fluorescence microscope. Confocal microscopy was performed as previously described (Ferreira et al., 1994) using the EMBL compact confocal microscope (Stelzer et al., 1991).

Immunoelectron Microscopy

Immuno-EM was performed according to the procedure described by Tokuyasu (1980) with minor modifications. The specimens were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 20–25 min. Ultrathin cryosections were picked up on drops of 2.3 M sucrose and deposited on nickel grids coated with formvar and carbon. The sections were incubated at room temperature in the following solutions: PBSG for at least 30 min, 10% newborn-calf serum in PBS for 20 min, first antibody (either anti-CBP20 purified antibody or preimmune serum diluted 1:100 or 1:500) for 1 h, PBSG (4 × 2 min), gold-conjugated secondary antibody (diluted 1:30 in PBSG supplemented with 5% calf serum) for 1 h, and PBSG (4 × 2 min). After staining with 2% aqueous uranyl acetate for 5–7 min, the grids were rinsed with distilled water and embedded in 4% poly vinyl alcohol (9–10 kD, Aldrich-Chemie, Steinheim, Germany) containing 0.4% uranyl acetate. The specimens were examined and photographed in a JEOL 100 CX microscope at 80 kV.

Results

Localization and Transcription-independent Nuclear Accumulation of CBP20 in HeLa Cells

The localization of CBP80 in HeLa cells has previously been reported (Izaurralde et al., 1994). CBP80 is found essentially uniformly distributed throughout the nucleoplasm and excluded from nucleoli, with a low amount of the protein being found in the cytoplasm. The distribution of CBP20 is similar (Fig. 1, a and b). Like hnRNPA1 (Fig. 1, c and d), CBP20 staining is observed in a punctuate pattern throughout the nucleoplasm, but not in nucleoli. While most of the nucleoplasm is stained, the apparent concentration of CBP20 varies somewhat in different nucleoplasmic regions. There is no detectable increase in CBP20 concentration at the nuclear periphery, i.e., in the region of the nuclear envelope. A small amount of CBP20
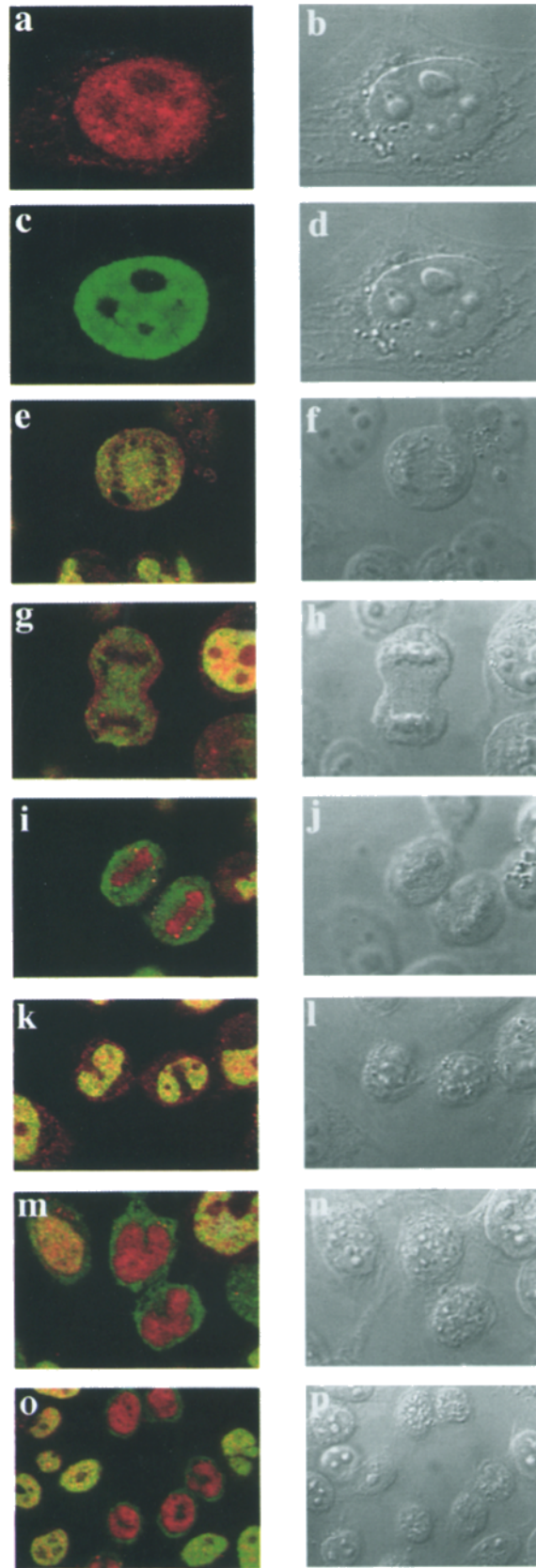

Figure 1. Localization and transcription-independent nuclear accumulation of CBP20 in HeLa cells. HeLa cells were grown in the absence (*a–l*) or presence (*m–p*) of actinomyin D, and double labeled with anti-CBP20 (*red*) and anti-hnRNP A1 (*green*) antibodies. The intracellular distribution of both proteins during the cell cycle was analyzed by confocal microscopy. The localization of CBP20 and hnRNP A1 in nontreated cells during interphase is shown in *a–b* and *c–d*, respectively. During anaphase (*e–f*) and very early telophase (*g–h*) both proteins are cytoplasmic. In late telophase (*i–j*), CBP20 is accumulated in the newly formed nuclei (*red*) while hnRNP A1 (*green*) is still in the cytoplasm. At the beginning of G1, both proteins are nuclear in nontreated cells (*k–l*),

but in the presence of actinomycin D (*m–n* and *o–p*) CBP20 is nuclear while hnRNP A1 remains in the cytoplasm. In all cases, the immunofluorescence images (*left*) are accompanied by the corresponding phase contrast photographs (*right*).

staining is also observed in the cytoplasm (Fig. 1 *a*). During mitosis, both CBP20 and hnRNPA1 staining is seen throughout the cytoplasm (Fig. 1, *e–h*, *e* and *f* showing a cell in anaphase, and, *g* and *h*, a cell in early telophase). CBP20 reaccumulates in the nucleus before hnRNPA1, and at a certain stage in late telophase, cells can be observed where the daughter nuclei stain strongly with anti-CBP20 antibodies but where hnRNPA1 staining is confined to the cytoplasm (Fig. 1, *i* and *j*). The nuclei of early G1 cells stain for both antigens (Fig. 1, *k* and *l*).

It has been reported that some proteins which shuttle between the nucleus and cytoplasm accumulate in the nucleus only when transcription occurs (Piñol-Roma and Dreyfuss, 1991). To determine whether CBP20 behaved in this way, we compared the reaccumulation of CBP20 and hnRNPA1 into daughter cell nuclei in the presence of the transcription inhibitor actinomycin D. Unlike untreated cells (Fig. 1, *k* and *l*), cells which had been incubated in the presence of actinomycin D did not show reaccumulation of hnRNPA1 (Fig. 1, *m–p*). However, CBP20 accumulation in the nuclei of these cells was unaffected. CBP20 nuclear accumulation is therefore not transcription dependent.

Antibodies against Human CBP20 Recognize a Similar Protein in C. tentans

We wished to study the nuclear distribution of CBP20 in more detail, and in particular to examine its association with nascent RNPs and mRNPs undergoing transport to the cytoplasm. The *Chironomus* salivary gland cell nuclei provide an excellent opportunity for such studies. To determine whether the antibodies against the human CBPs could be used for in situ studies in *Chironomus*, we first probed Western blots of *C. tentans* extracts prepared from nuclear and cytoplasmic fractions with anti-CBP20 and anti-CBP80 antibodies. The antiserum against human CBP80 was negative (data not shown). In contrast, two different antisera raised against human CBP20 were positive. Each reacted with a major band of the expected mobility (~18 kD) in *C. tentans* extracts. This 18-kD protein, presumably *C. tentans* CBP20, was the only polypeptide recognized by the affinity-purified anti-CBP20 antibodies in *C. tentans* nuclear extracts (lane *N* in Fig. 2 and data not shown). The cytoplasmic extracts did not contain detectable amounts of the 18-kD protein, but we sometimes observed a band of variable intensity at ~40 kD. This band was labeled weakly and in some extracts was undetectable (lanes *C* in Fig. 2).

Next the intracellular distribution of the 18-kD protein in *C. tentans* was analyzed. For this purpose, indirect immunofluorescence was performed on sections of *C. tentans* salivary glands using anti-CBP20 antibodies and a TRITC-conjugated secondary antibody. Some examples of the results obtained are shown in Fig. 3, *a* and *b*. In agreement with the Western blot analysis reported above, the staining was predominantly nuclear. Inside the nucleus, both the nucleoplasm and the chromosomes were labeled. The staining was quite diffuse but nonuniform, and a banding pattern was often seen on the chromosomes. Significant staining was also observed at the periphery of the nucleus. The nucleoli were not labeled. The nuclear staining seemed to be highly specific as judged from the absence of
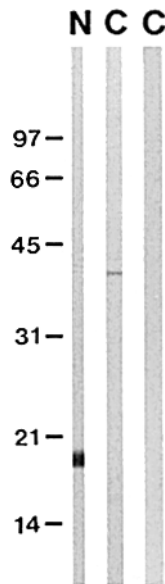

Figure 2. Specificity of anti-CBP20 antibodies in Western blots of *C. tentans* proteins. Proteins of *C. tentans* nuclear (*N*) and cytoplasmic (*C*) extracts were separated by SDS-PAGE and blotted to transfer membranes. The membranes were cut into strips, and the strips incubated with affinity-purified rabbit antibodies against human CBP20. An alkaline phosphatase–conjugated anti–rabbit IgG was used to detect the anti-CBP20 antibody. A major band of ~18 kD was seen in nuclear extracts (*N*) but was undetectable in cytoplasmic extracts (*C*). The two lanes *C* correspond to two cytoplasmic extracts prepared independently. The mobility of molecular mass standards is indicated on the left (kD).

labeling in sections incubated with preimmune serum (Fig. 3 *c*). The cytoplasm of the salivary gland cells was often stained above background levels, but we could not conclude from the present data whether this staining reflected the presence of CBP20 in the cytoplasm, or if it was due to cross-reactivity of the antibodies with the 40-kD polypeptide detected in the Western blots (Fig. 2). Both anti-CBP20 antisera gave similar staining patterns, and the second antiserum also faintly recognized cytoplasmic protein on a Western blot (data not shown). In spite of the obvious differences between HeLa cells and *C. tentans* salivary gland cells, the intranuclear distribution observed in the two cell types was quite similar at a gross level, the only major apparent differences being the ability to detect the polytene chromosome bands and the intense immunostaining at the periphery of the nucleus of the salivary gland cells (see Discussion). As reported above, CBP20 was found in the nucleoplasm of HeLa cells in a relatively uniform distribution (Fig. 1 *a*), and there was no obvious coimmunostaining with an antibody that stained NPC proteins (data not shown).

In summary, the results reported above indicated that the antibodies raised against human CBP20 recognize a nuclear protein of ~18-kD in *C. tentans*. The apparent molecular mass of this protein was as expected for CBP20, and its intracellular distribution in *C. tentans* was very similar to that of CBP20 in HeLa cells (see also below). Taken together and considering the strong conservation of CBP20 (see Introduction), these observations strongly suggest that the 18-kD protein recognized by the antibodies in *C. tentans* is the homologue of human CBP20.

CBP20 Is a Chromosomal Protein

Proteins that bind to nascent transcripts can be detected on insect polytene chromosomes using immunochemical methods (e.g., Risau et al., 1983). As a first approach to find out whether CBC binds to nascent RNA, we stained squash preparations of *C. tentans* polytene chromosomes with affinity-purified anti-CBP20 antibodies. An alkaline
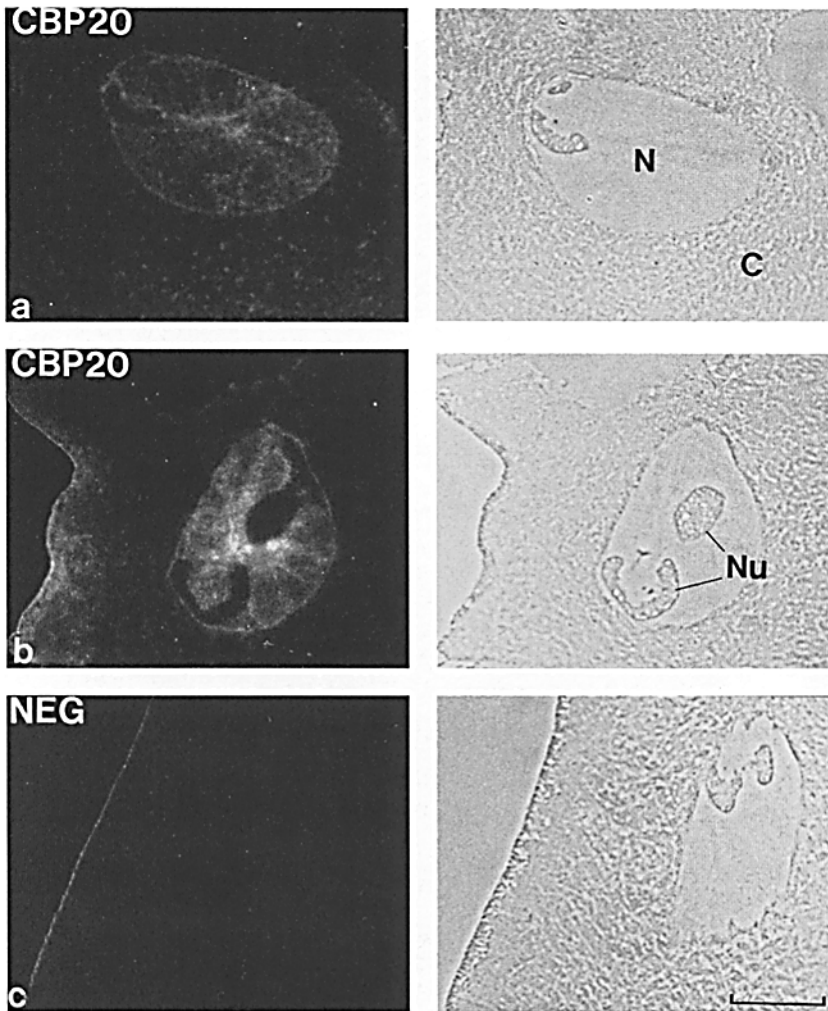
Figure 3. Localization of CBP20 in *C. tentans* salivary gland cells by indirect immunofluorescence. Semithin cryosections of salivary gland cells were stained with anti-CBP20 (*a* and *b*) or preimmune (*c*) antibodies. The antibody-binding sites were visualized using a TRITC-conjugated secondary antibody. For each section, fluorescence (*left*) and phase contrast (*right*) photographs are presented. Note that the labeling is mainly nuclear. The nucleoplasm and the chromosomes are stained, while the nucleoli are negative. Faint labeling is observed in the cytoplasm with both anti-CBP20 and preimmune antibodies. *N*, nucleus; *C*, cytoplasm; *Nu*, nucleolus. The bar represents 20 μm.

phosphatase–conjugated secondary antibody was used to visualize the immunoreactive sites. A discrete banding pattern was observed in chromosomes incubated with anti-CBP20 antibodies (Fig. 4, *a* and *b*) whereas no significant immunoreactivity was seen with preimmune serum (Figs. 4, *c* and *d*). The BR puffs were strongly stained (see arrows in Fig. 4 *a*) and many other loci were also positive. This result was not surprising considering that all RNA polymerase II transcripts bear the 5' cap structure recognized by CBC, and previous immunocytochemical studies showed that antibodies against RNA polymerase II also give a high number of positive bands in *Chironomus* polytene chromosomes (e.g., Sass, 1982). The same pattern of staining was obtained with two affinity-purified antibodies derived from different anti-CBP20 sera (data not shown). To establish that CBP20 binds to nascent transcripts and not to other chromosomal components, control samples were digested with RNase A before the immunostaining. The labeling intensity was heavily reduced in the RNase-treated controls (data not shown), which indicated that at least a major fraction of chromosomal CBP20 is bound to RNA. This type of banded staining is characteristic of many pre-mRNA–binding proteins (e.g., Amero et al., 1992; Matunis et al., 1992; Kiseleva et al., 1994), and we concluded that the CBP20 binds to the nascent transcripts before they are released from the chromosomes.

Binding of CBP20 to the 5' Domain of Nascent BR Pre-mRNP Particles

For a more detailed analysis of the binding of CBP20 to nascent RNA, we studied the presence of CBP20 on the BR transcripts by immunoelectron microscopy. The active BR genes can be visualized in situ by electron microscopy as transcription units with well defined polarity (Skoglund et al., 1983). As illustrated in Fig. 5 *a*, the nascent BR transcripts are packed continuously into RNP fibers of increasing length in the proximal portion of the transcription units. In the middle and distal regions, the 5' domain of the growing RNP particles becomes packed into a dense globular structure. All along the BR genes, the morphology of the nascent BR RNP particles is indicative of their position in the transcription unit.

The presence of CBP20 in growing BR RNP particles was analyzed by immuno-EM on cryosections of whole-mount salivary glands using gold-conjugated secondary antibodies. In spite of the mild fixation conditions required to preserve the immunoreactivity, the morphological preservation was sufficient to enable the identification of growing BR particles in the proximal, middle, and distal portions of the BR genes. All portions of the transcription loops, from proximal to distal, were labeled to some extent. In particular, abundant immunogold markers were
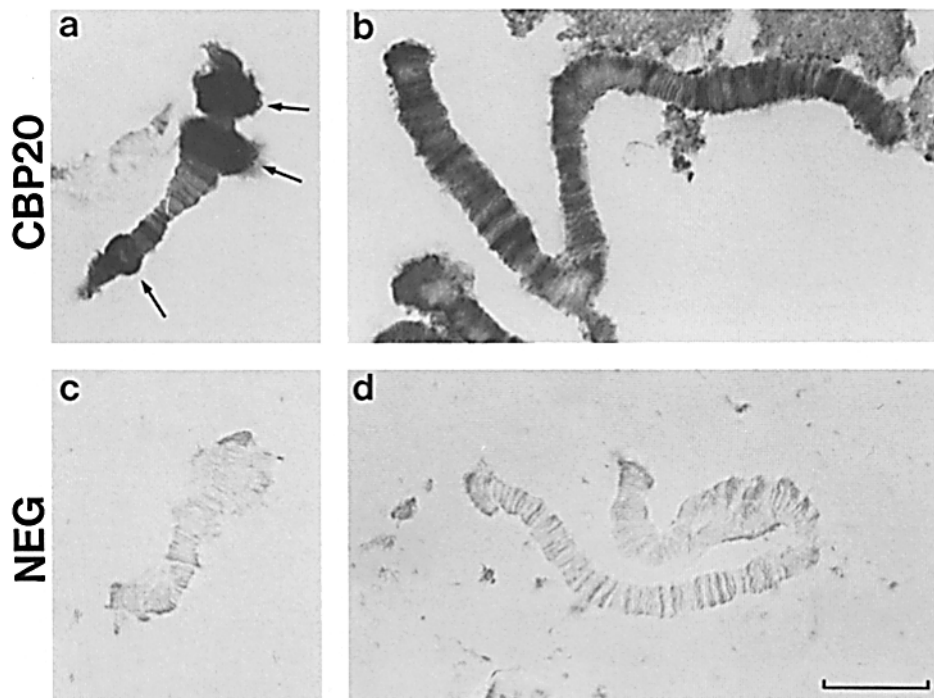
Figure 4. Immunolocalization of CBP20 in *C. tentans* polytene chromosomes. Polytene chromosome squashes were stained with anti-CBP20 antibodies (*a* and *b*) or with preimmune serum (*c* and *d*). The antibody-binding sites were visualized using an alkaline phosphatase–conjugated secondary antibody. Intense anti-CBP20 staining was observed in multiple loci along the chromosomes (*a* and *b*) but not in the negative controls (*c* and *d*). Chromosome IV (*a* and *c*) and chromosome I (*b* and *d*) are shown as examples. The arrows in *a* indicate the location of BR puffs in chromosome IV. The bar represents 20 μm.

seen in BR fibers located in the proximal region of the BR genes (Fig. 5, *b* and *c*). Comparison of labeled fibers with reconstructions of entire BR transcription loops (Masich, S., and B. Daneholt, manuscript in preparation) showed that the dimensions and structure of the shortest growing particles with positive immunolabeling correspond to the RNP particles positioned in the proximal 5–10% of the active gene downstream from the promoter. As the time required for the transcription of a BR gene is ∼20 min (Egyházi, 1975), we conclude that the CBP20 is added to the growing RNP in less than 2 min after the initiation of transcription.

In the middle and distal portions of the BR transcription units, the terminal domain of the growing BR particle becomes packed into a dense globular structure (Fig. 5 *a*). It has previously been shown that the formation of this globular structure is accompanied by major changes in the folding of the basic RNP fiber, but the 5′ end of the pre-mRNA always resides in the 5′ (most distal) domain of the growing BR RNP particle (Skoglund et al., 1983; Mehlin et al., 1992). In the immuno-EM experiments, we observed that the globular portions of the BR particles, but not their stalks, were significantly labeled (Fig. 5, *d–g*). The position of the gold markers was thus consistent with CBP20 being associated with the 5′ domain of the growing BR RNP particles.

The introns located close to the 5′ end of the BR pre-mRNA are spliced cotranscriptionally in most of the BR transcripts, and it has been estimated that the half-life of intron 3 is ∼2.5 min of transcription time (Baurén and Wieslander, 1994). Assuming similar values for introns 1 and 2, all introns located close to the 5′ end of the BR pre-mRNA have been removed from growing BR particles located in the distal part of the BR transcription units. According to our immuno-EM data, these spliced particles still contain CBP20, which suggests that CBC remains on the RNP particles after splicing of introns close to the 5′ end of the pre-mRNA.

BR RNP Complexes during Export

One of the advantages of the immuno-EM technique on sections of whole-mount salivary glands was that we could study in situ not only chromosomal RNPs but also BR RNP particles in the nucleoplasm and at the NPCs. As shown in Fig. 6, *a–c*, nucleoplasmic BR particles were significantly labeled by anti-CBP20 antibodies. These globular particles are known to be mature BR RNPs released from the chromosome and probably in transit from the BR genes to the NPCs. Nucleoplasmic BR particles close to the nuclear envelope were also labeled (Fig. 6 *d*). Apart from BR particles, other nucleoplasmic structures were also recognized by the antibodies. In particular, gold markers were often associated with thin fibrils that most probably represent transcripts from non-BR genes expressed in the salivary glands.

We next addressed whether CBP20 was still present in BR particles in transit to the cytoplasm through the nuclear pores. It is well documented that the globular BR particles become elongated upon interaction with the NPC, and that they are translocated to the cytoplasm as thick ribbons with the 5′ end of the transcript in the lead (Mehlin et al., 1992, 1995). In sections labeled with anti-CBP20 antibodies, we could often see gold markers decorating the leading end of translocating BR particles (Fig. 6, *e–g*). This observation strongly suggests that CBP20 is still attached to the 5′ end of the transcript during nucleo-cytoplasmic translocation.

As shown by Western blot analysis (Fig. 2), the anti-CBP20 antibodies can cross-react with a cytoplasmic protein of ∼40 kD. Although this cross-reactivity was found to be rather weak, we considered the possibility that the
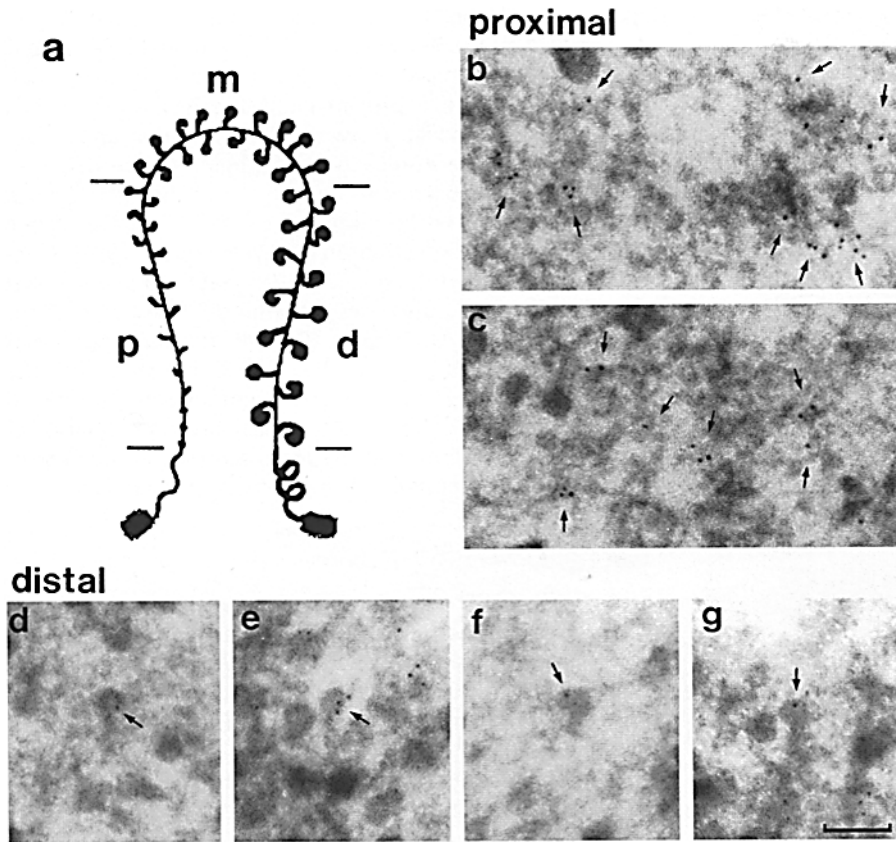
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Figure 5. Binding of CBP20 to nascent BR RNP particles. A schematic representation of the active BR gene is shown in *a*. Note the distinct morphology of the nascent BR RNP particles in the proximal (*p*), middle (*m*), and distal (*d*) portions of the BR transcription unit. The binding of CBP20 to nascent BR particles was analyzed by immuno-EM. Thin cryosections of salivary glands were incubated with anti-CBP20 antibodies, and the binding sites were visualized using a gold-conjugated secondary antibody. Significant immunolabeling was seen in nascent BR particles in all the segments of the active BR genes. Two examples of labeling in the proximal segment are shown in *b* and *c*. Four examples of labeling in distal BR RNP particles are given in *d–g*. Some of the gold markers have been indicated by arrows. The bar represents 100 nm.

labeling observed in the cytoplasmic end of the translocating particles could be due to the 40-kD protein instead of being specific for CBP20. However, a detailed analysis of early translocation stages showed immunogold labeling in particles whose 5' domain has not yet reached the cytoplasm (e.g., Fig. 6 *e*). Since it seems unlikely that a cytoplasmic protein could bind the RNP particle before it traverses the pore, the labeling can most likely be attributed to CBP20. The same results were obtained with affinity-purified antibodies from two independent anti-CBP20 sera. Sections incubated in parallel with the preimmune sera did not show any significant labeling (data not shown). From these results we conclude that CBC, or at least CBP20, remains attached to the 5' cap of the BR transcripts during nucleo-cytoplasmic transport.

Discussion

We have analyzed the association of the CBC with a specific pre-mRNA particle, the BR RNP particle, at different stages of pre-mRNA synthesis and processing. This approach has been possible thanks to the recognition by antibodies raised against the human CBP20 of the corresponding protein in *C. tentans*. A protein of 18 kD was recognized in *C. tentans* nuclear extracts by two independent antibody preparations derived from two different sera against human CBP20. The cross-reativity of the 18-kD protein with anti-CBP20 antibodies, its mobility in SDS-PAGE, its intracellular distribution in salivary gland cells, and its location at the 5' domain of the pre-mRNP particles support the conclusion that the 18-kD protein is the *C.*

tentans homologue to human CBP20. CBP20 is highly conserved in evolution. Although we do not know the sequence of *Chironomus* CBP20, human and *Drosophila melanogaster* CBP20s are 75% identical (Lewis, J., and I.W. Mattaj, unpublished data).

Antibodies raised against human CBP80 did not cross-react with any protein in *C. tentans* extracts, and therefore we could only detect one of the components of the nuclear CBC. However, studies in HeLa cells have previously demonstrated the interdependence between CBP20 and CBP80. The two proteins can be copurified to apparent homogeneity through several different chromatographic columns, and are coimmunoprecipitated by anti-CBP80 antibodies. Furthermore, neither CBP20 nor CBP80 can bind detectably to capped RNA in the absence of each other (Izaurralde et al., 1994, 1995). Therefore, the localization described for CBP20 in the present study is likely to reflect the localization of the whole CBC.

CBC and Pre-mRNP Splicing

In vitro studies using HeLa nuclear extracts (Izaurralde et al., 1994), as well as microinjection experiments in *Xenopus* oocytes (Izaurralde et al., 1995), have shown that the nuclear CBC is involved in splicing of in vitro synthesized pre-mRNA molecules. The effect of the cap structure, and of CBC, is limited to the 5' proximal intron of the pre-mRNA (Ohno et al., 1987; Inoue et al., 1989; Lewis, J., E. Izaurralde, A. Jarmolowski, C. McGuigan, and I.W. Mattaj, manuscript in preparation). It is known that the introns located close to the 5' end of the BR pre-mRNA are
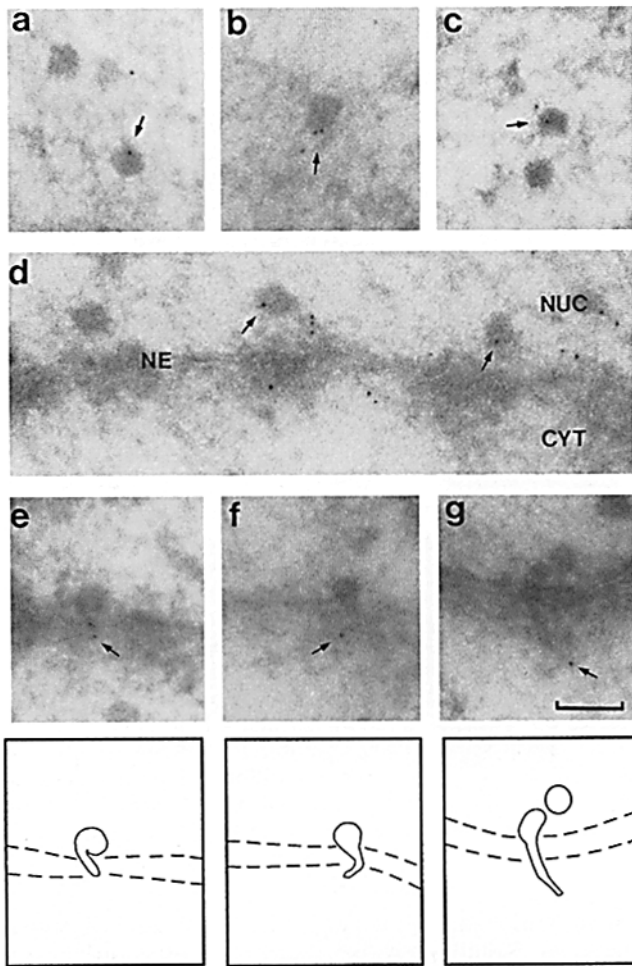
Figure 6. Immuno-EM analysis of CBP20 in BR RNP particles during nucleo-cytoplasmic transport. Thin cryosections of salivary glands were labeled with anti-CBP20 antibody followed by a gold-conjugated secondary antibody. Examples of BR RNP particles in the nucleoplasm of the salivary gland cells are given in *a–c.* The BR RNP particles shown in *d* are located close to the nuclear envelope (*NE*). Three examples of BR RNP particles in transit through the nuclear pores are presented in *e–g,* and schematic interpretations of these translocating particles are given under the micrographs. The arrows show the position of gold markers in BR RNP particles. Note the labeling in the leading domain of the translocating particles shown in *e–g.* The photographs shown in *d–g* are oriented with nucleus (*NUC*) in the top part of the image and cytoplasm (*CYT*) in the bottom. The bar represents 100 nm.

excised cotranscriptionally (Baurén and Wieslander, 1994; Kiseleva et al., 1994). According to these observations, binding of the CBC to the 5′ cap of the pre-mRNA is expected to occur cotranscriptionally. The formation of the 5′ cap structure is an obvious prerequisite for binding and, in fact, the capping reaction is a very rapid event (Salditt-Georgieff et al., 1980; Rasmussen and Lis, 1993). Our observation that CBP20 is a chromosomal protein in *C. tentans* demonstrates that CBP20, and most likely CBC, binds to nascent RNA molecules before transcription is completed.

Studies in different systems have indicated that the 5′ cap is primarily required for efficient splicing of 5′ proxi-

mal introns (Ohno et al., 1987; Inoue et al., 1989; Lewis J., E. Izaurralde, A. Jarmolowski, C. McGuigan, and I.W. Mattaj, manuscript in preparation). Unfortunately, the exact kinetics of removal of the 5′ proximal intron in the BR pre-mRNA (intron 1) is unknown. However, the formation and disappearance of spliceosomes in the 5′ region of the BR transcription unit take place within less than 5 min (Kiseleva et al., 1994), and intron 3 is known to be excised within 2.5 min after its transcription (Baurén and Wieslander, 1994). Considering these obervations, it is reasonable to assume that splicing of intron 1 also occurs within the first few minutes of transcription. Analysis of the size and morphology of nascent BR RNPs suggests that CBP20 interacts with the pre-mRNP within 2 min after the start of transcription, which indicates that CBC binds to these transcripts before splicing begins, consistent with the proposed role of the nuclear CBC in cotranscriptional splicing of the 5′ proximal intron.

CBC and Pre-mRNP Export

After transcription is completed, pre-mRNA molecules are released to the nucleoplasm and must move to the nuclear envelope. In the case of BR transcripts, it is known that most of the BR RNP particles located in the nucleoplasm are fully spliced (Baurén and Wieslander, 1994; Kiseleva et al., 1994). The results of our immuno-EM studies have shown that many nucleoplasmic BR particles are labeled by anti-CBP20 antibodies, and from this observation we conclude that the CBC remains attached to the 5′ cap of the spliced BR RNP particles during transport from the chromosome to the nuclear envelope.

The indirect immunofluorescence on *C. tentans* cells showed significant labeling at the nuclear periphery of the salivary gland cells, which raised the intriguing possibility that in these cells a fraction of CBP20 might be stably localized at the nuclear envelope. However, double-labeling experiments performed in HeLa cells clearly showed that CBC was not concentrated at the nuclear envelope (Fig. 1 and unpublished data). Because of this, and after examination of the *C. tentans* nuclei by immuno-EM, we favor the explanation that the peripheral immunofluorescence seen in these nuclei is due to accumulation of BR RNP particles at the nuclear envelope, probably due to rate-limiting events early in the process of translocation of these large, very abundant, RNPs. This interpretation is supported by the high density of BR RNP particles seen close to the nuclear envelope, by the observation that these BR RNP particles are labeled by antibodies against CBP20, and by the fact that similar peripheral staining was seen using antibodies against *Chironomus* hnRNP proteins that associate with BR RNPs (data not shown).

In relation to export of mRNA, the most interesting finding is the presence of CBP20 at the leading end of RNP particles that are being translocated through NPCs. In spite of technical limitations due to cross-reactivity of the anti-CBP20 antibodies used in this study with cytoplasmic proteins, the labeling seen in BR RNP particles at the nuclear side of the nuclear envelope, as well as the presence of CBP20 in early translocating particles whose 5′ domain is still located within the NPC, support the conclusion that CBP20, and therefore probably CBC, is present in the RNP particles during nucleo-cytoplasmic transloca-

tion. The mere presence of CBC in exported particles cannot be taken as a proof of functional significance. However, together with previous reports that documented a stimulation of mRNA export rate by the 5' cap (Hamm and Mattaj, 1990; Jarmolowski et al., 1994), our results support an involvement of the CBC in export of mRNA. In this context, it is noteworthy that the 5' end of BR RNPs is always in the leading end when BR RNAs pass through NPCs (Mehlin et al., 1992). This raises the possibility that the cap structure with its associated proteins could be involved in the early recognition of the RNP particle at the pore complex or in the translocation process itself.

Several reports from different laboratories (Rozen and Sonenberg, 1987; Ohno et al., 1990; Izaurralde et al., 1994), and also our present data, showed that CBP20 and CBP80 are mainly nuclear proteins. A complex of cytoplasmic proteins that bind to the cap (eIF-4F) that are different from the nuclear CBC has been characterized in mammalian and yeast cells (for reviews see Shatkin, 1985; Rhoads, 1988; Sonenberg, 1988). It is still an open question when eIF-4F replaces nuclear CBC. A fraction of eIF-4E, the component of eIF-4F that actually binds to the cap, has recently been found in the cell nucleus (Lang et al., 1994), suggesting that eIF-4E might bind to RNA before it leaves the nucleus. However, our observation is that the nuclear CBC is exported as part of the BR RNP particle, making exchange on the cytoplasmic side of the NPC more probable.

The immuno-EM study indicated that CBC is transported to and through the NPC, although we cannot definitively say whether the complex is released from the distal side of the NPC into the cytoplasm. Taken together, these observations suggest that the nuclear CBC is reimported into the nucleus rapidly after leaving the RNP particle. No experiments have been done so far to determine directly whether CBC exhibits shuttling behavior, but our experiments on actinomycin D–treated cells show that, unlike the prototypical shuttling hnRNP protein hnRNPA1, nuclear localization of CBC does not depend on ongoing transcription. CBP80 carries a functional nuclear localization signal (Izaurralde et al., 1994, 1996; Weis et al., 1995), and the nuclear CBC could therefore be reimported to the nucleus via the canonical NLS-dependent pathway, i.e., in a way that would be independent of transcription.

References

Amero, S.A., G. Raychaudhuri, C.L. Cass, V.W. Van, W.J. Habets, A.R. Krainer, and A.L. Beyer. 1992. Independent deposition of heterogeneous nuclear ribonucleoproteins and small nuclear ribonucleoprotein particles at sites of transcription. *Proc. Natl. Acad. Sci. USA.* 89:8409–8413.

Baurén, G., and L. Wieslander. 1994. Splicing of Balbiani Ring 1 gene pre-mRNA occurs simultaneously with transcription. *Cell.* 76:183–192.

Daneholt, B. 1982. Structural and functional analysis of Balbiani ring genes in the salivary glands of *Chironomus tentans*. In Insect Ultrastructure. Vol I. R. King and H. Akai, editors. Plenum Publishing Corporation, NY. 382–401.

Edery, I., and N. Sonenberg. 1985. Cap-dependent RNA splicing in a HeLa nuclear extract. *Proc. Natl. Acad. Sci. USA.* 82:7590–7594.

Egyházi, E. 1975. Inhibition of Balbiani ring RNA synthesis at the initiation level. *Proc. Natl. Acad. Sci. USA.* 72:947–950.

Ericsson, C., U. Grossbach, B. Björkroth, and B. Daneholt. 1990. Presence of histone H1 on an active Balbiani ring gene. *Cell.* 60:73–83.

Ferreira, J.A., M. Carmo-Fonseca, and A.I. Lamond. 1994. Differential interaction of splicing snRNPs with coiled bodies and interchromatin granules during mitosis and assembly of daughter cell nuclei. *J. Cell Biol.* 126:11–23.

Furuichi, Y., A. LaFiandra, and A.J. Shatkin. 1977. 5' Terminal structure and mRNA stability. *Nature (Lond.).* 266:235–239.

Hamm, J., and I.W. Mattaj. 1990. Monomethylated cap structures facilitate RNA export from the nucleus. *Cell.* 63:109–118.

Inoue, K., M. Ohno, H. Sakamoto, and Y. Shimura. 1989. Effect of the cap structure on pre-mRNA splicing in Xenopus oocyte nuclei. *Genes & Dev.* 3: 1472–1479.

Izaurralde, E., C. McGuigan, and I.W. Mattaj. 1996. *Cold Spring Harbor Symp. Quant. Biol.* In press.

Izaurralde, E., J. Lewis, C. Gamberi, A. Jarmolowski, C. McGuigan, and I.W. Mattaj. 1995. A cap-binding protein complex mediating U snRNA export. *Nature (Lond.).* 376:709–712.

Izaurralde, E., J. Lewis, C. McGuigan, M. Jankowska, E. Darzynkiewicz, and I.W. Mattaj. 1994. A nuclear cap binding protein complex involved in pre-mRNA splicing. *Cell.* 78:657–668.

Izaurralde, E., J. Stepinski, E. Darzynkiewicz, and I.W. Mattaj. 1992. A cap binding protein that may mediate nuclear export of RNA polymerase II–transcribed RNAs. *J. Cell Biol.* 118:1287–1295.

Jarmolowski, A., W.C. Boelens, E. Izaurralde, and I.W. Mattaj. 1994. Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.* 124:627–635.

Kataoka, N., M. Ohno, K. Kangawa, Y. Tokoro, and Y. Shimura. 1994. Cloning of a complementary DNA encoding an 80 kilodalton nuclear cap binding protein. *Nucleic Acids Res.* 22:3861–3865.

Kiseleva, E., T. Wurtz, N. Visa, and B. Daneholt. 1994. Assembly and disassembly of spliceosomes along a specific pre-messenger RNP fiber. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:6052–6061.

Konarska, M.M., R.A. Padgett, and P.A. Sharp. 1984. Recognition of cap structure in splicing in vitro of mRNA precursors. *Cell.* 38:731–736.

Lang, V., N.I.T. Zanchin, H. Lünsdorf, M. Tuite, and J.E.G. McCarthy. 1994. Initiation factor eIF-4E of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 269: 6117–6123.

Lezzi, M., B. Meyer, and R. Mähr. 1981. Heat shock phenomena in *Chironomus tentans*. I: In vivo effects of heat, overheat, and quenching on salivary chromosome puffing. *Chromosoma.* 83:327–339.

Matunis, M.J., E.L. Matunis, and G. Dreyfuss. 1992. Isolation of hnRNP complexes from *Drosophila melanogaster*. *J. Cell Biol.* 116:245–255.

Mehlin, H., and B. Daneholt. 1993. The Balbiani ring particle: a model for the assembly and export of RNPs from the nucleus? *Trends Cell Biol.* 3:443–447.

Mehlin, H., B. Daneholt, and U. Skoglund. 1992. Translocation of a specific premessenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography. *Cell.* 69:605–613.

Mehlin, H., B. Daneholt, and U. Skoglund. 1995. Structural interaction between the nuclear pore complex and a specific translocating RNP particle. *J. Cell Biol.* 129:1205–1216.

Ohno, M., H. Sakamoto, and Y. Shimura. 1987. Preferential excision of the 5' proximal intron from mRNA precursors with two introns as mediated by the cap structure. *Proc. Natl. Acad. Sci. USA.* 84:5187–5191.

Ohno, M., N. Kataoka, and Y. Shimura. 1990. A nuclear cap binding protein from HeLa cells. *Nucleic Acids Res.* 18:6989–6995.

Piñol-Roma, S., and G. Dreyfuss. 1991. Transcription-dependent and transcription-independent nuclear transport of hnRNP proteins. *Science (Wash. DC).* 253:312–314.

Rasmussen, E.B., and J.T. Lis. 1993. In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes. *Proc. Natl. Acad. Sci. USA.* 90:7923–7927.

Rhoads, R. 1988. Cap recognition and the entry of mRNA into the protein synthesis initiation cycle. *Trends Biochem. Sci.* 13:52–56.

Risau, W., P. Symmons, H. Saumweber, and M. Frasch. 1983. Nonpackaging and packaging proteins of hnRNA in *Drosophila melanogaster*. *Cell.* 33:529–541.

Rozen, F., and N. Sonenberg. 1987. Identification of nuclear cap specific proteins in HeLa cells. *Nucleic Acids Res.* 15:6489–6500.

Salditt-Georgieff, M., M. Harpold, S. Chen-Kiang, and J.J. Darnell. 1980. The addition of 5' cap structures occurs early in hnRNA synthesis and prematurely terminated molecules are capped. *Cell.* 19:69–78.

Sass, H. 1982. RNA polymerase B in polytene chromosomes: immunofluorescent and autoradiographic analysis during stimulated and repressed RNA synthesis. *Cell.* 28:269–278.

Shatkin, A. 1976. Capping of eukaryotic mRNAs. *Cell.* 9:645–653.

Shatkin, A. 1985. mRNA cap binding proteins: essential factors for initiation of translation. *Cell.* 40:223–224.

Shimotohno, K., Y. Kodama, J. Hashimoto, and K.I. Miura. 1977. Importance

of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis. *Proc. Natl. Acad. Sci. USA.* 74:2734–2738.

Skoglund, U., K. Andersson, B. Björkroth, M.M. Lamb, and B. Daneholt. 1983. Visualization of the formation and transport of a specific hnRNP particle. *Cell.* 34:847–855.

Skoglund, U., K. Andersson, B. Strandberg, and B. Daneholt. 1986. Three-dimensional structure of a specific premessenger RNP particle established by electron microscope tomography. *Nature (Lond.).* 319:560–564.

Sonenberg, N. 1988. Cap-binding proteins of eukaryotic messenger RNA: functions in initiation and control of translation. *Prog. Nucleic Acid Res. Mol. Biol.* 35:174–207.

Stelzer, E.H.K., I. Wacker, and J.R. DeMey. 1991. Confocal fluorescence microscopy in modern cell biology. *Semin. Cell Biol.* 2:145–152.

Tokuyasu, K.T. 1980. Immunocytochemistry on ultrathin frozen sections. *Histochem. J.* 12:381–403.

Visa, N., A.T. Alzhanova-Ericsson, X. Sun, E. Kiseleva, B. Björkroth, T. Wurtz, and B. Daneholt. 1996. A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. *Cell.* 84: 253–264.

Weis, K., I.W. Mattaj, and A.I. Lamond. 1995. Identification of hSRP1 α as a functional receptor for nuclear localization sequences. *Science (Wash. DC).* 268:1049–1053.

Wieslander, L. 1994. The Balbiani ring multigene family: coding repetitive sequences and evolution of a tissue specific cell function. *Prog. Nucleic Acid Res. Mol. Biol.* 48:275–313.

Wieslander, L., and G. Paulsson. 1992. Sequence organization of the Balbiani ring 2.1 gene in Chironomus tentans. *Proc. Natl. Acad. Sci. USA.* 89:4578–4582.

Wyss, C. 1982. *Chironomus tentans* epithelial cell lines sensitive to ecdysteroids, juvenile hormone, insulin and heat shock. *Exp. Cell Res.* 139:309–319.