Electron Tomography Reveals Posttranscriptional Binding of Pre-mRNPs to Specific Fibers in the Nucleoplasm

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Abstract. Using electron tomography, we have analyzed whether the Balbiani ring (BR) pre-mRNP particles in transit from the gene to the nuclear pore complex (NPC) are bound to any structure that could impair free diffusion through the nucleoplasm. We show that one-third of the BR particles are in contact with thin connecting fibers (CFs), which in some cases merge into large fibrogranular clusters. The CFs have a specific protein composition different from that of BR particles, as shown by immuno-EM. Moreover, we have identified hrp65 as one of the protein components of the CFs. The sequencing of hrp65 cDNA reveals similarities with hnRNP proteins and splicing factors. However, hrp65 is likely to have a different function because it does not bind to nascent pre-mRNA and is not part of the pre-mRNP itself. Taken together, our observations indicate that pre-mRNPs are not always freely diffusible in the nucleoplasm but interact with fibers of specific structure and composition, which implies that some of the posttranscriptional events that the pre-mRNPs undergo before reaching the NPC occur in a bound state.

Key words: pre-mRNA • Balbiani ring particle • hrp65 • mRNA export • nuclear substructure

Introduction

Gene expression in eukaryotes involves both nuclear and cytoplasmic events. Messenger RNA precursors (pre-mRNAs) are cotranscriptionally associated with hnRNP proteins (reviewed by Krecic and Swanson, 1999) and assembled into ribonucleoprotein complexes (pre-mRNPs) that are processed in the nucleus and exported to the cytoplasm where they direct protein synthesis. Transport of pre-mRNPs from the nucleus to the cytoplasm is an active, signal-mediated process that can be subdivided into two steps: (a) intranuclear transport from the sites of transcription to the nuclear envelope, and (b) translocation through the nuclear pore complexes (NPCs). In recent years, many of the signals, adapters, and receptors involved in RNA export have been identified (reviewed by M. Atta and E. Ngmeier, 1998). In the case of pre-mRNA, it has been shown that certain hnRNP proteins, such as hnRNP A1, carry nuclear export signals that confer bidirectional transport across the nuclear envelope (Michael et al., 1995; Izaurralde et al., 1997). However, the mechanistic aspects of pre-mRNA export remain to be elucidated. In the most simple model, the newly synthesized pre-mRNPs would be released from the chromosome upon transcription termination and diffuse through the interchromatin space (also referred to as interchromosomal channel network or nucleoplasm) to the NPCs. Meanwhile, the nuclear export signals in the protein moiety of the pre-mRNP would be recognized by soluble export receptors that would bridge the interaction between the pre-mRNP and the NPC, thus triggering translocation to the cytoplasm (for recent reviews see M. Atta and E. Ngmeier, 1998; Stutz and Rosbash, 1998). However, it is unclear whether the transported pre-mRNPs are freely diffusible through the nucleoplasm or physically attached to nuclear structures. Both diffusion and physical attachment have found experimental support. Studies of RNA transport based on the analysis of transgenes in Drosophila indicated that the intranuclear movement of pre-mRNA occurs isotropically through the interchromosomal channels at rates consistent with diffusion, and suggested that incompletely spliced pre-mRNAs are discriminated at the nuclear surface (Zachar et al., 1993). Recent kinetic studies in two independent systems...
have also shown that at least a large fraction of pre-mRNA move randomly inside the nucleus at rates that are compatible with free diffusion (Politz et al., 1998; Singh et al., 1999; reviewed by D aneholt, 1999). On the other hand, rhodamine-labeled pre-mR NA s microinjected into the nu-
cleus of living cells became localized in discrete nuclear sites rich in splicing factors, and it was shown that the lo-
elocalization was dependent on intron sequences (Wang et al., 1991). The hypothesis that RNA binds to nondiffusible elements was also supported by observations of pre-
RNA tracks extending towards the nuclear envelope and asked whether the pre-
mRNP s show any morphological sign of binding interac-
tions. For this purpose we have used the salivary glands of the dipteran Chironomus tentans (C. tentans). In the nuclei of the salivary gland cells, the chromatin is confined to well-defined polytene chromosomes and thus the nucleo-
plasm is totally free of chromatin fibers. Moreover, in these cells the assembly, transport and disassembly of a specific pre-mR N P, the Balbiani ring (B R) particle, can be directly visualized using transmission electron microscopy (Skoglund et al., 1983). Therefore, the possible binding of pre-mRNP s to nuclear structures can be directly investigated in situ.

The BR genes encode secretory proteins and are ac-

atively expressed in the salivary gland cells in a tissue-spe-
cific manner (reviewed by Wieslander, 1994). The BR pre-
mR NA is exceptionally long (35–40 kb) and is co-

transcriptionally packed into a large pre-mRNP particle. The structure of the BR particle has been previously ana-
yzed by electron tomography (ET) both in situ (Skoglund et al., 1986) and after isolation in sucrose gradients (Lönnroth et al., 1992). The BR particle can be described as a 7-nm RNP fiber folded into a bent ribbon in which four structural domains can be identified (Lönnroth et al., 1992). Domain 1 is the first one to be synthesized and con-
tains the 5’ end of the pre-mRNA, whereas domain 4 contains the 3’ end. Due to their distinct structure and excep-
tional dimensions (50 nm in diameter), the BR particles can be identified in situ by morphological criteria using EM.

The BR pre-mRNA has all the features of a typical pre-
RNA and undergoes normal processing. The 5’ end is capped and the nuclear cap binding complex can be de-
tected in situ on the nascent pre-mRNP early after tran-
scription initiation (Visa et al., 1996a). The BR pre-
mRNA contains three short introns located close to the 5’ end that are spliced mostly cotranscriptionally (Baurén and Wieslander, 1994). A fourth intron near the 3’ end is removed at the gene locus, but after 3’ end cleavage, in ~80% of the BR pre-mRNA molecules (Baurén et al., 1998). Kinetic experiments in vivo have shown that BR RNA is efficiently exported to the cytoplasm and is highly

stable, with an average half-life of ~20 h, as expected for a gene that is actively expressed (Edström et al., 1978, and references therein).

In this study, we have investigated whether the BR par-
ticles are bound to any structure that could impair free dif-
fusion through the nucleoplasm. Our results reveal that 31% of the nucleoplasmic BR particles are in contact with thin fibers that in some cases merge into larger nucleoplasmic structures. We have also identified C t-hrp65 (design-
ated hrp65 for simplicity) as one of the protein compo-
nents of these thin fibers, which provides a starting point for determining the biological significance of the bind-
ing interactions reported here (available from GenBank/ EMB L /DDB J under accession number A J 2 4 3 0 1 3 ).

Materials and Methods

Animal and Cell Culture

C. tentans and C. tentans tissue culture cells were cultivated as described previously (Lezzi et al., 1981; Wyss, 1982).

Antibodies

The Ma b 187 is an IgM generated by immunization of Balb/c mice with ssDNA-binding proteins from C. tentans (Wurtz et al., 1996). The Ma b 4E 9 is an IgG1 specific for C t-hrp65 obtained after immunization of mice with pre-mRNP s from C. tentans as described by Sun et al. (1998). 2D-
Western blot assays showed that the antigen recognized by Ma b 4E 9 was the C t-hrp65 protein previously identified by Wurtz et al. (1996). The Ma b 10 3 against hrp23 and 4 F 9 against hrp36 have been characterized elsewhere (Wurtz et al., 1996; Sun et al., 1998). Serum 282-296 is a polycl-
onal antiserum against hrp65 raised in rabbits by immunization with a K L H-conjugated synthetic peptide corresponding to amino acids 282-296 of hrp65 (RK S N D Y Y K A R Q NGPR). A nit-GST monoclonal antibody is from Sigma. Rabbit anti–mouse immunoglobulins used for immuno-
precipitation were purchased from D A K O. Secondary antibodies conjugated with alkaline phosphatase or horseradish peroxidase were also from D A K O. For immuno-E M, secondary antibodies coupled to 6-nm colloidal gold were purchased from A mersham and J ackson Immunoresearch Laboratories.

Electron Tomography

Salivary glands from C. tentans fourth instar larvae were fixed with 2.5% glutaraldehyde in 0.1-M sodium cacodylate-HCl buffer (pH 7.2) containing 0.05-M sucrose. The fixed glands were washed in 0.1-M sodium cacodylate-HCl buffer (pH 7.2), cryoprotected with 2.3-M sucrose in the same buffer, frozen by immersion in liquid nitrogen and stored in liquid nitrogen until further processing. Carbon-coated copper grids (75 x 300 mesh) were glow discharged, incubated with 10-nm colloidal gold solu-
tion (A uraProbe EM GA M IgG G 10; A mersham) diluted 1/3, rinsed with distilled water, and air dried. Cryosectioning was performed at approxi-
ately ~105°C. The sections were picked up with a drop of 2.3-M sucrose in sodium cacodylate buffer (pH 7.2) and mounted on the grids. The grids were stained with 2% aqueous uranyl acetate for 5 min, infiltrated in a so-
lution of 4% polyvinyl alcohol (9–10 kD; A idrich) containing 0.3% uranyl acetate for at least 3-4 min, and picked up with a loop. The excess of liq-
uid was blotted onto a filter paper and the grids were air dried. In control experiments, 20-nm colloidal gold particles were applied on the dry PVA-
embedded sections. The grids were rinsed briefly and allowed to air dry as above.

The specimens were imaged in a Philips CEM 200 FEG transmission electron microscope equipped with a cooled 1,024 x 1,024 pixel CCD chip with a pixel size of 24 μm. A utomatic low-dose tilt series consisting of 61 images (from ~60 to ~60 every 2°) at 24 610 x magnification were re-
corded using the TVIPS computer interface (TVIPS G mbH). The total dose applied to the specimen was ~100 e/Å 2. D efinition of alignment par-
eters, including determination of x, y, and z-coordinates of the used gold markers, was done with an iterative least squares procedure (giving
an alignment error, or gold marker coordinates prediction error, of ~0.6 pixels) and 3D reconstructions were performed according to the filtered back-projection principle at 10 nm resolution as previously described (Skoglund et al., 1986, 1998). The reconstructions were submitted to 10 cycles of refinement against the tilt series data using the Constrained Maximum Entropy Tomography procedure (COMET) (Skoglund et al., 1996). In brief, the COMET refinement generated the most featureless 3D density which projections still fit the observed projections within their variances. The refined reconstructions (~3 nm resolution) were low-pass filtered to 4 nm. The surface rendered images shown in see Fig. 2 d were generated using XT V (Skoglund et al., 1998).

Immuno-EM on Cryosections of Salivary Glands

Immuno-EM was performed as described by Visa et al. (1996). Dilutions of primary antibodies were 1–10 μg/ml for mAbs, and 1:300 for rabbit pre-immune and immune sera. Secondary antibodies were diluted according to manufacturer’s recommendations. The specimens were examined and photographed in a Philips CM 120 electron microscope at 80 kV under standard high-dose conditions.

Immunostaining of Chromosome Squashes

Salivary glands were fixed in ethanol/acetic acid 3:1 for 5 min and squashed in a drop of 45% acetic acid. The slides were frozen on dry ice, the coverslips pried off, and the preparations were further fixed with 3.7% formaldehyde in PBS for 15 min. The slides were then washed in PBS, dehydrated and stored in absolute ethanol at ~20°C. Before immunostaining, the slides were rehydrated, washed with PBSG (PBS containing 0.1 M glycine) for 10 min, and blocked with a solution of 5% nonfat powder milk in PBSG. Incubations with primary antibodies (0.1–1 μg/ml for mAbs, or 1:1,000 for sera) and alkaline-phosphatase secondary antibody were for 1 h and 40 min, respectively. The labeling was visualized using NBT/BCIP.

Preparation of Protein Extracts

Protein extracts for Western blot analyses were prepared from nuclear and cytoplasmic fractions of cell lines. In brief, the cells were washed in PBS, collected in a glass tissue grinder, homogenized in PBS containing 0.2% NP-40 and 0.1 mM PMSF, and centrifuged at 2,000 g for 10 min at 4°C. The supernatant was the cytoplasmic extract used for Western blotting. The pellet containing the nuclei was resuspended in PBS and sonicated for 60 s.

Immunoprecipitation of Pre-mRNP Complexes

Pre-mRNA complexes were immunoprecipitated from nuclear extracts of C. tentans tissue culture cells as described by Sun et al. (1998) using either mAb 1 D 3 (against hrp23) or mAb 4 E 9 (against hrp65). Control experiments were carried out replacing by PBS either the primary antibody (to detect non-specific binding to the protein A-Sepharose) or the nuclear extract (to identify the immunoglobulin chains in the eluted fraction).

Ion-Exchange Chromatography

C. tentans tissue culture cells were resuspended in PBS containing 0.2% NP-40 and 0.5 mM PM SF, homogenized in a glass tissue grinder and centrifuged at 2,000 g for 10 min at 4°C. The pellet was washed with PBS containing 0.5 mM PM SF, resuspended in buffer I (Tris-HCl pH 7.5, 25 mM NaCl, 0.1% NP-40, 1 mM EDTA, and 0.5 mM PM SF), and sonicated in ice. The extract was finally centrifuged for 30 min at 7,000 g and the supernatant applied to a 2-ml Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) column equilibrated with buffer I. The column was washed with 3 column-volumes of buffer I and the sample was eluted with a linear NaCl gradient (from 25 to 600 mM). Fractions of 1 ml were collected. The proteins in the fractions were precipitated with trichloroacetic acid, washed with acetone, and analyzed by SDS-PAGE GE and Western blotting.

Protein Electrophoresis and Western Blotting

SDS-PAGE GE was performed according to standard procedures using 10% polyacrylamide gels in the Mini-Protein II system (Bio-Rad). 2D-gel electrophoresis was carried out in NEPHGE GE gels using ampholines in the pH 3-10 range. The second dimension was a conventional SDS-PAGE in 10% polyacrylamide gels. The polyacrylamide gels were stained either with Coomassie or silver. For Western blot, the proteins were transferred onto PVDF membranes (Millipore) in Tris-glycine buffer supplemented with 0.02% SDS and 4-M urea using a semi-dry cell (Bio-Rad). The membranes were blocked with 10% dry milk in PBS before incubation with primary antibody. All antibodies were diluted in PBS containing 1% dry milk and 0.05% Tween-20. The secondary antibodies were conjugated to alkaline phosphatase and developed with NBT/BCIP according to standard procedures. In some cases, the detection was based on ECL chemiluminescence (A mersham Pharmacia Biotech) using secondary antibodies coupled to horseradish peroxidase.

Protein Microsequencing

The Coomassie stained band containing p65 was excised from the polyacrylamide gel and proteolytic internal peptides were isolated by in-gel digestion as described (H elman, 1992). In brief, the gel piece was washed and dried, and the protease was forced into the gel matrix during reswelling. A after incubation, peptides were extracted and isolated by reversed phase micro-bore liquid chromatography. Selected peptides were subjected to automated Edman degradation in a PE-A BI Purific 494A amino acid sequencer, following the manufacturer’s instructions.

Molecular Cloning of hrp65

Poly(A)+ RNA was purified from C. tentans tissue culture cells with Oligotex mRNA kit (Qiagen) and reverse-transcribed using an oligo-dT primer. The cDNA was used as a template in a PCR reaction with degenerate oligonucleotides deduced from peptides NFAFLK and TGE - GIVEFAR. This resulted in a product of 260 bp, which was directly cloned into pCR II (Invitrogen) and sequenced. The cloned insert was labeled by PCR with digoxigenin-dUTP (Boehringer Mannheim) and used as a probe to screen a λ-ZAP cDNA library from the salivary glands of C. tentans as previously described (Visa et al., 1996b). Four clones were isolated, purified and their inserts were directly amplified by PCR using λ-ZAP PK and T7 primers. The PCR products were purified and directly sequenced using walking primers and A BI PRISM dye terminator kit (Perkin Elmer). The longest clone, λ-hrp65-1, was sequenced at least three times. Sequence analysis was performed with the University of Wisconsin Genetics computer group Sequence analysis Programs and EGGX extensions to the Wisconsin Package Sequence analysis Programs.

Expression of hrp65 in Bacteria

The λ-hrp65-1 clone was used as a template in a PCR reaction to amplify the complete hrp65 open reading frame, using Pfu polymerase (Stratagene) and oligonucleotides designed to introduce a BamHI and a SalI restriction site at the 5’ and 3’ ends of the amplified product, respectively. Upon restriction enzyme digestion, the amplified product was cloned into pGEX-4-T1 plasmid (A mersham Pharmacia Biotech) and sequenced to verify correct coding sequence. pGEX-4-T1 and pGEX-4-T1-hrp65-1 were transformed into BL21 bacterial strain and expression of GST and GST-hrp65 proteins was induced with 1 mM IPTG for 2 h. Purification of recombinant proteins was carried out with glutathione-Sepharose beads (A mersham Pharmacia Biotech), as described by the manufacturer.

Results

In the salivary gland cells of C. tentans, the expression of the BR genes can be monitored using electron microscopy and the assembly and transport of BR particles can be directly analyzed in situ (Fig. 1 a). EM observations suggested that BR particles in transit from the gene to the NPC could be associated with thin fibers that extended into the surrounding nucleoplasm (Fig. 1 b). Moreover, a monoclonal antibody (mAb 1 B 7) was found that labeled the nucleoplasmic fibers associated with BR pre-mRNPs (Fig. 1 c). Such fibers were best preserved in cryo-sections of glands fixed in isotonic conditions (for details see Materials and Methods), although they were also visible in thin sections of plastic-embedded specimens prepared according to classical EM procedures (e.g., Fig. 6 in Skoglund et al., 1983). Single EM images cannot resolve overlapping struc-
The morphology of a typical salivary gland cell in a thin cryosection is shown in Fig. 2 b. The images for tomographic reconstructions were recorded from nucleoplasmic regions randomly selected at low magnification. We used 11 independent data sets, and all putative BR particles in each set were selected and reconstructed. Fig. 2 c shows the central tilt (0°) from a data set and illustrates the area selected for the reconstruction of one BR particle. After reconstruction and COMET refinement, the resolution achieved was in the 3-nm range. To facilitate the interpretation of the results in the scope of this study, we low-pass filtered the reconstructed densities to 4 nm. From 106 reconstructions, 52 could be unambiguously identified as BR particles by structural criteria and were further analyzed; the other 54 were discarded. The 52 reconstructed BR particles were classified in three groups (Table I). The first group contained particles that were apparently not in contact with any other structure (Fig. 2 d, I). The second class contained BR particles that were associated with large complexes (Fig. 2 d, II). This group of complex particles was rather heterogeneous: all the reconstructions in this group could be identified as BR particles but the attached structures were of different types. It was impossible to further interpret these complex BR particles due to the little knowledge available about the structure of macromolecular complexes involved in pre-mRNA processing. The third group included BR particles associated with thin fibers (Fig. 2 d, III, IV, and V). Most of the BR particles in this third class presented only one fiber. Only 2 out of 16 BR particles were found with two fibers (not shown). All the fibers had uneven thickness in the 5–7-nm range and were slightly convoluted. In 7 out of 16 cases, the fibers...
Figure 2. Electron tomographic analysis of nucleoplasmic BR particles. (a) Control experiment to detect possible shrinkage of the specimen during data collection (see text for details). The graph shows the z-values (in pixels) of 16 gold markers in the first (tilts 1-31) and second (tilt 31-61) halves of a tilt series. The 16 gold markers are represented along the x-axis of the graph. Note that a decrease in z-value can be observed for some gold markers, whereas others show increased z-values in tilts 31-61. Therefore, no consistent shrinkage was detected during the data collection. (b) Section through a salivary gland cell as seen in the EM at low magnification. The cytoplasm (Cy) and the nucleus can be easily recognized. The data sets for tomographic reconstructions were recorded from nucleoplasm areas (Np) avoiding nucleolus (Nu) and chromosomes (Ch). (c) Part of the central tilt of one of the data sets used for ET. The cube represents the volume selected for the reconstruction of one putative BR particle. The BR particle appears as a dense granule in the center of the cube. The arrows point at some of the gold markers used in the alignment of the 61 images that constitute one full data set. (d) Analysis of reconstructed BR particles. The numbers on the images (1–4) refer to the domains of the BR particles. The BR particle in II is associated with a large complex. BR particle in III, IV, and V are in contact with fibers that extend into the nucleoplasm. The fiber in V is continuous with a fibrogranular cluster (FGC) located in the upper left corner of the image. Bars: (b) 5 μm; (c) the side of the cube is 150 pix; 1 pix = 0.975 nm; (d) 20 nm.
Tomographic Reconstructions from 11 Independent Data Sets

Table I. Classification of Nucleoplasmic BR RNPs Based on Tomographic Reconstructions from 11 Independent Data Sets

| Data set | Number of reconstructed BR particles | Number of BR particles in each class |
|----------|--------------------------------------|-------------------------------------|
|          | Free       | Complex | With fiber |          |
| 1        | 8          | 2       | 4          | 2        |
| 2        | 2          | 1       | 0          | 1        |
| 3        | 7          | 2       | 2          | 3        |
| 4        | 6          | 1       | 4          | 1        |
| 5        | 2          | 0       | 0          | 2        |
| 6        | 7          | 2       | 3          | 2        |
| 7        | 3          | 1       | 2          | 0        |
| 8        | 2          | 0       | 2          | 0        |
| 9        | 7          | 1       | 2          | 4        |
| 10       | 5          | 2       | 3          | 0        |
| 11       | 3          | 1       | 1          | 1        |

Total: 52 13 23 16
% in each class: 25 44 31

The criteria used for classification are described in the text.

Identification of hrp65 as a Putative Component of the Nucleoplasmic Fibers

The mAb b 1B7 labeled the nucleoplasmic fibers by immuno-EM (Fig. 1 c) as well as other cell structures in both nucleus and cytoplasm (data not shown). Western blot assays revealed that mAb b 1B7 recognizes three major polypeptides of ~35, 65, and 95 kD in the nuclei of C. tentans cells, as well as a 180-kD polypeptide in the cytoplasm (Fig. 3 a).

Wurtz et al. (1996) had identified in C. tentans a basic protein of 65 kD, designated Ct-hrp65 (or hrp65 for simplicity), that communoprecipitated with hrp36 and hrp45 and was recognized by mAb b 4E9. Immunoprecipitation assays followed by two-dimensional Western blot analysis indicated that the 65-kD protein recognized by mAb b 1B7 was the hrp65 protein reported by Wurtz et al. (1996). As shown in Fig. 3 b, the hrp65 protein isolated from a nuclear extract by immunoprecipitation was recognized by mAb b 1B7. The two-dimensional gel analysis also indicated that hrp65 was not a single polypeptide but a family of immunologically related isoforms with slight variations in size and pI.

The results reported above suggested that hrp65 was a good candidate to be a component of the pre-mRNP-associated fibers. With the aim of confirming this possibility, we performed immuno-EM experiments with the mAb 4E9 specific for hrp65. However, this antibody was found to be sensitive to the glutaraldehyde fixation used for imaging.

Figure 3. The 65-kD protein recognized by mAb b 1B7 is hrp65. (a) Specificity of mAb b 1B7 in Western blot of C. tentans proteins. Proteins of nuclear (N) and cytoplasmic (C) fractions were separated by SDS-PAGE, blotted to transfer membranes and probed with mAb b 1B7. (b) Reactivity of mAb b 1B7 against the hrp65 protein immunoprecipitated by mAb 4E9. Pre-mRNP complexes were immunoprecipitated from C. tentans nuclear extracts with mAb b 4E9 specific for hrp65. The immunoprecipitated proteins were analyzed by 2D Western blot using mAb b 1B7 (top). The bottom panel shows the specificity of mAb b 4E9 in 2D-bLOTS of C. tentans nuclear proteins. Note that both mAbs recognize the same group of spots in the basic region of the gel. Approximate antibody concentrations: 1 μg/ml for 1B7, 0.2 μg/ml for 4E9, and 1 μg/ml for the alkaline phosphatase-conjugated secondary antibody. The mobility of molecular mass standards is indicated in kD.
muno-EM and failed to give any labeling in this type of assays. The localization of hrp65 in pre-mRNP-associated fibers was confirmed later on using an anti-hrp65 antibody raised against a synthetic peptide (see below).

Cloning of hrp65 cDNA

The hrp65 protein was partially purified by ion-exchange chromatography from a nuclear extract made from C. tentans tissue culture cells. Fractions containing hrp65 were identified by Western blot using mAb 1B7 and pooled together. Hrp65 was resolved by SDS-PAGE, excised from the gel and subjected to proteolytic digestion. Six peptides were isolated and sequenced with the following results: MQLEEEKLEAQM, ELF5, IRDEQYYMR, AVIVVDDR, TTGEIGEVAR and NFAFLK. Degenerate oligonucleotides corresponding to these amino acid sequences were used in different combinations to prime C. tentans cDNA in a PCR reaction (see Materials and Methods). A 260-bp PCR product obtained with oligonucleotides corresponding to peptides TTGEIGEVAR and NFAFLK was labeled and used to screen a C. tentans salivary gland cDNA library. Four positive clones were identified. The longest one, λ-hrp65-1, was found to have a 2,200-bp insert that included a 5’UTR (91 nt), an open reading frame (1,605 nt) capable of encoding a protein of 535 amino acids, a 3’UTR (513 nt) and a poly(A) stretch. There were five in-frame stop codons upstream of the first initiation codon, which perfectly matched the consensus translation initiation site for eukaryotic RNAs (Kozak, 1986).

The protein encoded by clone λ-hrp65-1 had a predicted molecular mass of 62 kD and contained several putative phosphorylation and glycosylation sites. The estimated isoelectric point of the protein was 9.64, in agreement with the basic behavior of hrp65 in NEPHGE (Fig. 3b; Wurtz et al., 1996).

To obtain specific antibodies against hrp65 that could be used in immuno-EM, antibodies were raised in rabbits against a synthetic peptide encompassing amino acid residues 282–296 from hrp65 (serum 282–296). As shown in Fig. 4a, the serum 282–296 solely recognized a protein of 65 kD that comigrated with the protein labeled by mAb 4E9, whereas no band was observed with the pre-immune serum. To further demonstrate that the protein encoded by the λ-hrp65-1 clone was indeed the hrp65 protein recognized by mAb 4E9, the open reading frame of λ-hrp65-1 was fused in-frame with the GST protein and the hrp65-GST fusion protein was expressed in E. coli. The GST protein alone was expressed in parallel. The recombinant proteins were purified and analyzed by Western blotting with an anti-GST monoclonal antibody, mAb 1B7, mAb 4E9 and serum 282–296, as indicated above the lanes. Note that the hrp65-GST fusion protein is recognized by all the antibodies, whereas the GST alone is only labeled by the anti-GST antibody. The mobility of molecular mass standards is indicated in kD.

The Sequence of hrp65

The amino acid sequence predicted from the cDNA clone λ-hrp65-1 revealed a central region with two classical RNA-binding domains (RBD1 and RBD2) containing the RNP-1 and RNP-2 consensus sequences (Burd and Dreyfuss, 1994), and two auxiliary domains (Fig. 5a). The amino-terminal auxiliary domain was rich in asparagine (21%), glycine (13%), lysine (10%), and proline (10%), and did not reveal any significant sequence similarity to any other known protein. The hrp65 carboxy-terminal domain was rich in glycine (23%), asparagine (20%), glutamine (14%), and arginine (10%). Some of these amino acids were organized in two glycine clusters with five interspersed GGN tripeptides.

Searches in the SWISS-PROT database (Bairoch and Boeckmann, 1993) revealed that hrp65 had a strong sequence similarity to RNA-binding proteins. The highest similarity score was obtained with the Drosophila NoNa protein (Jones and Rubin, 1990; von Besser et al., 1990). The amino acid identity between the two proteins was 36% and the similarity 64%. The human p54nrb and PSF proteins (Dong et al., 1993; Patton et al. 1993) also proved to be very similar to hrp65, with ~35% identical and 48% similar amino acid residues. Most of this extensive sequence homology resided within a central region of 320 amino acids, the so-called DBHS (Drosophila behavior and human splicing) domain (Dong et al., 1993), which is a common feature of NoNa, p54nrb, PSF, and hrp65 (Fig. 5b). The DBHS domain includes the two RBDs plus additional 100 amino acids downstream of unknown function.
Localization of hrp65

The serum 282-296 was used to confirm the location of the hrp65 protein in the nucleoplasmic fibers. We performed immuno-EM experiments using cryo-sections of salivary gland cells from C. tentans. The sections were first incubated with the serum 282-296 and subsequently with a gold-conjugated secondary antibody. As expected, the gold markers were mainly located in the nucleus and the nucleoplasmic fibers were often decorated by gold markers (Fig. 6a), which confirmed that hrp65 was indeed a component of these novel structures. Moreover, the immuno-EM analysis revealed that hrp65 was not present along the whole length of the fiber but exclusively in the segment close to the BR particle.

The possible presence of hrp65 in the BR particle itself, and not only in the fiber, could not be totally ruled out. A fraction of hrp65 could be buried in the pre-mRNP and inaccessible to the 282-296 peptide antibody, or post-translationally modified in a way that could impair antibody recognition. However, we favor the interpretation that hrp65 is not present in the BR particle because labeling with mAb 1B7 was also seen preferentially on the fibers (Fig. 1c).

Comparing the immuno-EM results obtained with mAb 1B7 (Fig. 1c) and serum 282-296 (Fig. 6a), it is clear that...
the former resulted in a stronger signal. This difference might be explained by signal amplification due to the fact that 1B7 is an IgM. On the other hand, taking into account the complex specificity of mAb 1B7 (e.g., Fig. 3 a), we cannot exclude the possibility that mAb 1B7 could recognize additional proteins also located in the nucleoplasmic fibers.

We also investigated whether the hrp65 protein was bound to the BR particles cotranscriptionally. To answer this question, preparations of polytene chromosome were immunostained with anti-hrp65 antibodies (Fig. 7). The results revealed a number of immunoreactive loci but the large puffs BR1 and BR2 on chromosome IV, where the BR genes are transcribed, were clearly negative. Identical results were obtained with mAb 4E9 and serum 282-296. The pre-immune serum was negative. These results demonstrated that hrp65 does not bind to BR pre-mRNPs on the chromosome. We conclude that the interaction between hrp65 and the BR particles occurs posttranscriptionally.

**Discussion**

Using ET we have studied the 3D structure of BR particles in transit from the gene to the NPC. Our data revealed that nucleoplasmic BR particles constitute a very heterogeneous population. There is a fraction of apparently free BR particles (~25%), a population of BR particles associated with large complexes (44%) and a third population bound to fibers (31%) that in some cases merge into an intricate fibrogranular meshwork. A cooström et al. (1978), 80–100% of the BR RNA is exported to the cytoplasm, which implies that all three types of BR particles do eventually leave the nucleus and can therefore be regarded as different intermediates in the processing/export pathway.

The population of BR particles associated with fibers reveals the existence of two novel structures: a thin connecting fiber (CF) that is in contact with the BR particle, and a fibrogranular cluster (FGC) to which the CF becomes occasionally attached. CFs and FGCs are structurally continuous but the observation that hrp65 is present exclusively in CFs supports the interpretation that CFs and FGCs are different structures.

CFs can be observed under different preparative conditions (i.e., hydrophobic plastic embedding and hydrophilic cryosections), which argues against the possibility that they could be artifacts. The specificity of the CFs is also supported by the observation that they have a preferential attachment site on the BR particle, and by their specific protein composition. Our present data is based on 3D reconstructions of small nucleoplasmic volumes and therefore we cannot state neither the dimensions of full FGCs nor whether they are interconnected or associated with other nuclear structures. Further research is required to elucidate FGC’s structure, composition and function.

Even in the absence of kinetic data, the structural results presented here provide relevant information about binding interactions that impose restrictions on the type of movement of the BR particles. We can infer the following conclusions. First, the structure of the so-called free BR particles is compatible with the proposal that intranuclear transport of pre-mRNPs is based on diffusion phenomena. Second, at least some of the pre-mRNPs in transit from the gene to the nuclear pore interact with specific nuclear structures, i.e., CFs and FGCs. Third, the BR particles attached to FGCs via CFs cannot diffuse freely at rates estimated for single BR particles. We cannot rule out the possible diffusion of a complex containing BR particles, CFs and FGCs. However, the diffusion properties of such a large complex would differ significantly from those of single pre-mRNPs. Fourth, from the existence of apparently free BR particles not in contact with CFs, and considering that hrp65 is absent from the BR transcription sites, we can also conclude that the interaction between CFs and BR particles is a transient, posttranscriptional event.

In summary, our results are compatible with the proposal that a large fraction of pre-mRNPs are free to diffuse in the nucleoplasm (reviewed by Danelhoft, 1999) but we also provide direct evidence for physical interactions between the pre-mRNP and other nuclear structures. These observations imply that nucleoplasmic pre-mRNPs are not always freely diffusible and that some of the posttranscriptional events that the pre-mRNP undergoes before reaching the NPC occur in a bound state.
The hrp65 Protein

We have identified hrp65 as one of the protein components of the CFs associated with BR particles. The hrp65 protein had been previously identified by Wurtz et al. (1996) in a 2D-electrophoretic analysis of immunoprecipitated hnRNP proteins. We have now cloned and sequenced a cDNA encoding hrp65 and the primary sequence of this new protein is presented here.

The hrp65 protein has two features in common with hrps present in the BR particles, such as hrp23 (Sun et al., 1998), hrp36 (Visa et al., 1996b), and hrp45 (Alzhanova-Ericson et al., 1996). First, all these proteins have a modular primary structure containing one or two RBDs. Second, they can all be immunoprecipitated from nuclear extracts, which suggested that they are part of the same hnRNP complexes (Wurtz et al., 1996). However, our immuno-EM study suggests that hrp65 is located in the fibers that are connected to the BR particle and not in the BR particle itself. Moreover, hrp23, hrp36, and hrp45 bind to the BR pre-mRNA cotranscriptionally, as expected for hnRNP proteins, whereas we failed to detect binding of hrp65 to the chromosomal BR pre-mRNA, in agreement with observations by Singh, O. P., N. Visa, and B. Daneholt (manuscript in preparation). In conclusion, due to its different localization, hrp65 cannot be classified as a typical hnRNP protein.

Another interesting finding is the presence of a DBHS domain in the hrp65 protein and the high similarity to other proteins such as P54nuRNP, PSF, and NoNa/Bj6. The PSF protein is a human splicing factor associated with the polypyrimidine tract-binding protein that can also bind directly to the polypyrimidine tract of mammalian introns (Patton et al., 1993). The human P54nuRNP protein can bind to both mRNA and DNA, and it can act as a transcription activator (Basu et al., 1997). Mutations in the gene that encodes NoNa/Bj6 have pleiotropic effects in the central nervous system of Drosophila (Rendahl et al., 1992, and references therein) and it has been proposed that the NoNa/Bj6 function is related to splicing regulation (Dong et al., 1993). In summary, these proteins appear to play different roles in gene expression, and the mere presence of a DBHS domain in hrp65 does not provide conclusive information about its function. The auxiliary domains are likely to be essential to define the functional properties of each protein, and it is interesting to note that the amino-terminal domain of hrp65 is not similar to any protein in the databases.

Although hrp65 is not present in the BR puffs, we could detect hrp65 in a number of other loci in the polytene chromosomes (Fig. 7). This observation suggests that the formation of CFs on pre-mRNPs from other genes might occur cotranscriptionally. Alternatively, and considering the role of P54nuRNP in transcription (Basu et al., 1997), the chromosomal fraction of hrp65 could be engaged instead in transcription regulation.

CFs Mediate Binding of Pre-mRNPs to Other Nucleoplasmic Structures

CFs associated with nucleoplasmic BR particles can be of variable length, and only some of them make contact with FGCS. The most simple interpretation of these observations is that CFs assemble on the pre-mRNP particle and subsequently interact with pre-existing FGCS in the nucleoplasm. We propose that once the assembly of a CF has been initiated, an elongation process takes place by binding of additional protein molecules to the growing CF. Considering that the longest CFs are 50–100 nm long, and that an average globular protein has a diameter of ~3 nm, the longest CFs could contain ~100 protein molecules. On the other hand, from the immuno-EM experiments we infer that hrp65 cannot be the only component of the CFs because antibodies against hrp65 label exclusively the segment of the CFs close to the BR particles. Thus, additional protein/s must be present in the CFs, at least in the distal segment.

Many questions arise in relation to the molecular mechanisms that result in the formation of CFs. We would like to understand what is the signal or feature of the BR particle that triggers the assembly of the CF, whether this feature is RNA or protein, and whether the hrp65 protein is involved in this initial recognition step. The hrp65 protein has two RNA-binding domains and PSF is able to bind to polypyrimidine-rich sequences (Patton et al., 1993). These two facts suggest that hrp65 can bind to the pre-mRNA directly. However, binding of hrp65 to the pre-mRNA would result in the presence of hrp65 at the sites of transcription and in our experiments hrp65 was not detectable at the chromosomal level. Therefore, we favor the hypothesis that hrp65 does not bind to the BR pre-mRNA directly and that there is a previous step, maybe binding of another protein to the BR particle, before hrp65 can be incorporated into the new CF.

The BR pre-mRNA particle is a bent ribbon in which domains 1 and 4, containing the 5' and 3' ends of the pre-mRNA, respectively, are in close proximity (Skoglund et al., 1986). It is remarkable that most of the CFs observed in this study make contact with the BR particle just in the region where domains 1 and 4 come together. This suggests that the assembly of the CF could start by recognition of unique features of the 5' or 3' ends. Alternatively, the CFs might be related to the presence of introns or splicing factors. This possibility would also be compatible with attachment of the CF to domains 1 or 4 because the BR pre-mRNA contains three introns close to the 5' end and one intron close to the 3' end, whereas domains 2 and 3 include exonic sequences exclusively (Wieslander, 1994).

In summary, our results suggest the following series of events leading to the attachment of BR particles to FGCS (Fig. 8). First, the assembly of an CF is initiated on the BR particle. Second, the initial CF is elongated by addition of protein molecules to the growing end. Third, the free end of the CF makes contact with a FGC. Whether the BR particle is subsequently released, or actively transported to the NPC in a nondiffusible state remains to be elucidated.

Structural Complexity of the Nucleoplasm

The results of our ET study provide direct evidence for the high structural complexity of the nucleoplasmic compartment, which appears to contain complex aggregates of interconnected fibers and granules that we refer to as FGCS. Moreover, BR particles interact with these FGCS before being exported to the cytoplasm, which argues in favor of
mRNPs is sorted/processed/transported (4). The BR particle is subsequently released into the nucleoplasm (5a) and can reach the NPC by free diffusion (6). Alternatively, the BR particle might be transported to the NPC in a guided manner (5b).

a role for these structures in pre-mRNA metabolism. It is important to notice that neither the CFs nor the FGCs are true chromatin fibers: in the polynucleotide nuclei used in our study the DNA is located exclusively in the polynucleotide chromosomes, which leaves the nucleoplasm free of chromatin.

The key molecules involved in pre-mRNA synthesis, processing, and export appear to be highly conserved during evolution, and thus CFs and FGCs are not likely to be exclusive of insect polynucleate cells. Some nuclear structures have been described that could be related to CFs and FGCs. One of them is the nuclear matrix (reviewed in Pederson, 1998). Although we have not performed any detailed comparison, the nucleoplastic structures reported in this study do not resemble the filaments of the nuclear matrix. Perhaps the most remarkable difference is that FGCs appear as relatively compact structures that occupy discrete regions of the nucleoplasm, whereas the nuclear matrix preparations reveal a meshwork of fibers that extend throughout the whole interior of the nucleus (e.g., Fey et al., 1986).

The second type of structures that should be considered in this context are the interchromatin granules found in a variety of plant, animal, and fungal cells for review see Puvion and Puvion-Dutilleul, 1996). The interchromatin granule clusters (IGCs) are known to be dynamic structures that serve as storage and/or reassembly sites for splicing factors (Misteli and Spector, 1998). Moreover, IGCs are likely to play additional roles in sorting/transport of pre-mRNAs. Interestingly, several studies have shown that pre-mRNAs accumulate transiently in IGCs during intranuclear transport (e.g., Puvion-Dutilleul et al., 1997; Bridge et al., 1996). These findings suggest that the Chironomus FGCs could be the insect counterpart of the mammalian IGCs.

The NPC-attached filaments containing Tpr protein (Cordes et al., 1997) also share some features with the nucleoplasmic fibers reported here. In Drosophila, the Tpr protein is not only located at the nuclear periphery but it extends deep inside the nucleus and reaches the periphery of the chromosomes (Zimowska et al., 1997).

Moreover, a recent report has shown that two Tpr homologues in yeast, Mlp1p and Mlp2p, are part of intranuclear structures that might be involved in the translocation of macromolecules between the nucleoplasm and the NPC (Strambio-de-Castillia et al., 1999). It will be interesting to investigate whether CFs and FGCs are related to the Tpr filaments.

The most interesting question arising from our conclusions is the functional significance of the CFs in relation to pre-mRNA processing and/or export. The binding interaction that we report must necessarily affect the mobility of the pre-mRNPs (Nakielny and Dreyfuss, 1996), by the presence of introns in the pre-mRNA (Legrain and Rosbash, 1989), or by incomplete 3’ end processing (Custódio et al., 1999). Alternatively, the CFs might be involved in active, guided transport of pre-mRNPs from the nucleoplasm to the NPC in a late step of intranuclear transport (Strambio-de-Castillia et al., 1999, and references therein). The functional characterization of CFs, and in particular the identification of proteins interacting with hrp65, should help to elucidate the molecular composition of CFs and to understand their role in mRNA biogenesis.

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