Evidence for a Posttranscriptional Role of a TFIIICα-like Protein in *Chironomus tentans*

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We have cloned and sequenced a cDNA that encodes for a nuclear protein of 238 kDa in the dipteran *Chironomus tentans*. This protein, that we call p2D10, is structurally similar to the α subunit of the general transcription factor TFIIIC. Using immunoelectron microscopy we have shown that a fraction of p2D10 is located at sites of transcription, which is consistent with a possible role of this protein in transcription initiation. We have also found that a large fraction of p2D10 is located in the nucleoplasm and in the nuclear pore complexes. Using gel filtration chromatography and coimmunoprecipitation methods, we have identified and characterized two p2D10-containing complexes that differ in molecular mass and composition. The heavy p2D10-containing complex contains at least one other component of the TFIIIC complex, TFIIIC-ε. Based on its molecular mass and composition, the heavy p2D10-containing complex may be the Pol III holoenzyme. The light p2D10-containing complex contains RNA together with at least two proteins that are thought to be involved in mRNA trafficking, RAE1 and hrp65. The observations reported here suggest that this new TFIIIC-α-like protein is involved in posttranscriptional steps of premRNA metabolism in *Chironomus tentans*.

INTRODUCTION

Protein-coding RNAs are synthesized as precursors (prem-RNAs), packed into ribonucleoprotein (premRNP) complexes, and processed inside the cell nucleus. Nuclear mRNA export factors are recruited to the mRNPs, which are then transported to the cytoplasm (reviewed by Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999). Several proteins required for mRNA export have been identified, including TAP/Mex67, RAE1/Gle2, Gle1, Dbp5, REF/Aly/Yra1, and UAP56/HEL/Sub2p. The functions of these proteins are not fully understood, but it is known that they promote transport of mRNA to the cytoplasm, presumably by mediating the interactions between the mRNP export cargo and the nuclear pore complex (NPC; reviewed by Cullen, 2000; Cole, 2000; Conti and Izaurralde, 2001; Linder and Stutz, 2001).

The role that nuclear structure plays in mRNP export is not clear, and it is still controversial whether the mRNPs are free to diffuse from the gene to the NPC or whether they interact with nuclear structures. Studies in several experimental systems (such as those carried out by Politz *et al.*, 1998, 1999; Singh *et al.*, 1999) have shown that mRNPs move randomly throughout the nucleus, and a diffusion-based model has been proposed to explain how mature mRNPs are preferentially exported to the cytoplasm (reviewed by Politz and Pederson, 2000). However, there is also evidence for intranuclear retention of mRNPs. For instance, by analyzing in vivo the export of premRNAs that are defective in splicing and/or 3' end formation, Custodio *et al.* (1999) showed that unprocessed premRNAs accumulate at the sites of transcription and proposed that such a retention mechanism might be crucial to prevent the export of aberrant mRNAs (reviewed by Carmo-Fonseca *et al.*, 1999).

The Balbiani ring (BR) genes of the dipteran *Chironomus tentans* and their transcripts, the BR premRNP particles, have been used as a model system to analyze different aspects of gene expression in eukaryotes (reviewed by Wieslander 1994; Daneholt, 2001). In the salivary gland cells of *C. tentans*, the chromatin is organized into giant polytene chromosomes leaving the interchromatin space, or nucleoplasm, free of chromatin fibers and thus amenable to structural analysis. Moreover, in the *C. tentans* salivary gland cells, the assembly, processing, and transport of the BR premRNP

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particles can be directly visualized using transmission elec-
tron microscopy (EM; Skoglund *et al.*, 1983). In a previous
study, we applied electron tomography to analyze in situ
whether BR mRNPs bind to nucleoplasmic structures
(Miralles *et al.*, 2000). We found that approximately one third
of the nucleoplasmic BR particles were in contact with a
fibrous network. We identified one of the proteins located in
such fibers, an evolutionarily conserved protein called hrp65
in *C. tentans*, NonA in *Drosophila melanogaster*, and PSF and
p54[nrb] in human (Miralles *et al.*, 2000 and references therein).
Our observations showed that mRNPs are not always freely
diffusible in the nucleoplasm. However, the tomography
analysis could not determine whether the interaction of the
mRNPs with nucleoplasmic fibers promotes movement of
the mRNP toward the NPC or restricts the movement of the
mRNP, maybe as part of a mechanism for sorting and re-
taining unprocessed premRNPs. To further characterize the
nucleoplasmic structures identified by Miralles *et al.* (2000),
we sought to identify additional nucleoplasmic proteins us-
ing an immunological method that we present here. The
large dimensions of the salivary gland cells allowed us to
isolate the nucleoplasm by manual microdissection, and we
subsequently raised monoclonal antibodies (mAbs) against
nucleoplasmic proteins. Two of the mAbs obtained in this
study, mAbs 2D10 and 1F2, recognize a nuclear protein that
is predominantly located in the nucleoplasm and in the
nuclear envelope. We have called this protein p2D10. Im-
munoscreening of cDNA expression libraries and sequenc-
ing of positive cDNA clones revealed that p2D10 is struc-
turally similar to a general transcription factor, TFIIIC-α. We
also found that p2D10 is associated in vivo with at least one
other component of the TFIIIC2 complex, TFIIIC-ϵ, which
suggests that p2D10 might be the *C. tentans* ortholog of
TFIIIC-α. Moreover, a significant fraction of p2D10 is asso-
ciated with RAE1 and hrp65, two proteins that are thought
to be involved in mRNP trafficking, which suggests that
p2D10 is involved in posttranscriptional events of the RNA
pol II gene expression pathway.

MATERIALS AND METHODS

Animals and Cell Culture

C. tentans were raised under the conditions described by Lezzi *et al.*
(1981). Fourth instar larvae were used for the experiments. For drug
treatments, larvae were grown in the presence of either 10 μg/ml
actinomycin D (Calbiochem, La Jolla, CA) or 85 μg/ml DRB (Sigma
Chemical, St. Louis, MO) for 120 min before dissection and fixation
of the salivary glands.
    C. tentans cells were cultivated as described by Wyss (1982).

Monoclonal Antibody Production

We prepared nucleoplasm by microdissection of salivary gland cells
of *C. tentans* and used this as the antigen for immunization. Salivary
glands were fixed on ice with ethanol-acetic acid 3:1 for ~30 min,
washed with cold 70% ethanol, transferred to ethanol-glycerol 1:1,
and mounted in a dissection chamber with paraffin oil. Nucleoplas-
mic material was pooled and stored at −20°C until use. The immu-
nization was carried out following standard procedures described
by Harlow and Lane (1988). BALB/c mice were injected intraperi-
toneally with the nucleoplasm from ~100 cells in Freund's complete
adjuvant. Two intraperitoneal booster injections were administered
in Freund's incomplete adjuvant at intervals of 2 weeks. One week
after the second booster, the serum was tested by Western blotting

for the presence of antibodies against *C. tentans* nuclear proteins.
Three weeks after this test, the immunized mouse was given a final
intravenous injection of nucleoplasmic material in PBS. Two days
later, the spleen was extracted and macerated, and the spleen cells
were fused to mouse myeloma Sp20 cells following standard pro-
cedures. Hybridoma selection was done in 96-well plates using a
hypoxanthine-, aminopterin-, and thymidine-selective medium as
described by Harlow and Lane (1988). The resulting hybridomas
were screened by Western blot against nuclear extracts. Hybrid-
omas that were positive in this screening were subsequently ana-
lyzed by immunocytology on salivary gland sections. Selected hy-
bridomas were cloned by limiting dilution at least three times. All
mAbs obtained were IgM. MAbs 2D10 and 1F2 were used in the
present study.

Cloning and Sequencing of Full-Length p2D10

MAb 2D10 was used to screen a random-primed λgt11 cDNA
library from salivary gland cells of *C. tentans* following standard
procedures (Sambrook *et al.*, 1989). Approximately 7.5 × 10^5 clones
were screened, and one antibody-specific clone was isolated,
λ-2D10–1. The insert of λ-2D10–1 was amplified by PCR with λgt11
forward and reverse primers, PCR-labeled with digoxigenin (Roche,
Mannheim, Germany) and used as a probe to screen an oligo(dT)-
primed λZAP cDNA library from tissue culture cells of *C. tentans* as
previously described (Visa *et al.*, 1996a). Approximately 7.3 × 10^5
clones were screened and three positives were isolated: λ-2D10-A,
λ-2D10-B, and λ-2D10-D. The inserts of all three clones were PCR-
amplified with SK and T7 primers and sequenced at least three
times using walking primers. Sequence analysis was performed
with the University of Wisconsin Genetics Computer Group Se-
quence Analysis Program and EGCC extensions to the Wisconsin
Package Sequence Analysis Program. The sequence of p2D10
(AJ313507) was aligned with the sequences of TFIIICα proteins from
Drosophila (Q9VJY7), rat (Q63505), and human (Q12789) using the
PileUp program. The sequence was divided into stretches of amino
acid of length ten, and the percentages of identical residues in at
least 3 out of 4 species were calculated manually.

Subcloning and Expression of Recombinant p2D10

To produce 6xHis-tagged recombinant protein, the cDNA that en-
codes p2D10 (residues 960-2043) was subcloned into the *Nde*I-*Xho*I
sites of pET-21b (Novagen, Madison, WI). The resulting plasmid
was introduced into *Escherichia coli* (DE3). After induction with
isopropyl-β-D-thiogalactopyranoside (IPTG), the recombinant pro-
tein was purified by NTA-agarose affinity chromatography (QIA-
GEN, Hilden, Germany) and analyzed by SDS-PAGE and by West-
ern blotting.

Antibodies

Pep-I and Pep-II antibodies were obtained by immunizing rabbits
with two peptides VNLDKDRTATIDGDP(C) and LNDNGEIPG-
DRKGAG(C) conjugated to keyhole limpet hemocyanin. These pep-
tides corresponded to amino acids 1714–1728 and 990-1005 of
p2D10. Antisera were purified on protein G in a HiTrap affinity
column (Pharmacia Biotechnology, Uppsala, Sweden) and by pep-
tide-affinity chromatography on Sulfolink columns (Pierce, Rock-
ford, IL). MAb Y1D2 against the PEP protein of *D. melanogaster*
(Amero *et al.*, 1991) was kindly provided by S. Amero; this mAb
cross-reacts with the PEP homolog in *C. tentans*, referred to as
hrp120 (N. Visa and B. Daneholt, unpublished results). MAbs 4E9
against hrp65 (Miralles *et al.*, 2000), 2E4 against hrp45 (Wurtz *et al.*,
1996), 4F9 against hrp36 (Wurtz *et al.*, 1996), and 1D3 against hrp23
(Sun *et al.*, 1998) were kindly provided by B. Daneholt. MAb Y12
against the Sm epitope of snRNP core proteins was a gift from J.
Steitz. Rabbit polyclonal antisera against RAE1 and CBP20 were
kindly provided by E. Izaurralde (Sabri and Visa, 2000; Izaurralde *et*

al., 1995). A rabbit serum against TFIIIC-ε was a gift from R.G. Roeder (Hsieh *et al.*, 1999). The antibody against His-tag was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IgM immunoglobulins used for immunoprecipation and anti-IgM antibodies conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HRP) used for Western blot analysis were purchased from Southern Biotechnology Association (Birmingham, AL). Other secondary antibodies conjugated with AP or HRP for Western blotting were purchased from Dako (Glostrup, Denmark). Secondary antibodies conjugated to 6 nm colloidal gold used for immuno-LM and immuno-EM were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Preparation of Cytoplasmic and Nuclear Extracts from C. tentans Tissue Culture Cells

C. tentans tissue culture cells were homogenized in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.2) containing 0.2% Nonident P40 (NP-40) and 100 $\mu g/ml$ PMSF using a glass homogenizer (tight pestle). The homogenate was centrifuged at $2000 \times g$ for 10 min at 4°C. The supernatant was the cytoplasmic fraction. The pellet containing the nuclei was resuspended in PBS containing 100 $\mu g/ml$ PMSF, sonicated three times for 4–5 s each time, and centrifuged as above. The resulting supernatant was the soluble nuclear extract, and it was used as input for the immunoprecipitations, or it was fractionated by gel filtration. For electrophoretic analysis, proteins were precipitated by the addition of 6 volumes of cold acetone and stored at −20°C. For screening of hybridoma supernatants by Western blotting, the nuclear pellet was prepared as described above, resuspended in PBS, sonicated six times for 10 s, and precipitated with acetone. When indicated, the following protease inhibitors were added to all buffers: aprotinin (1 $\mu g/ml$), leupeptin (1 $\mu g/ml$), and pepstatin A (1 $\mu g/ml$). For partial digestion of soluble nuclear extracts with trypsin, the nuclear pellet was resuspended in 10 mM Tris-HCl, pH 7.5, 5 mM KCl, in the absence of protease inhibitors, before sonication and centrifugation. The supernatant was supplemented with $CaCl_2$ to give a final concentration of 10 mM and subsequently incubated with different concentrations (0–50 $\mu g/ml$) of trypsin (Sigma Chemical) for 10 min at room temperature.

SDS-PAGE and Western Blot Analysis

Proteins were separated by SDS-PAGE and stained with Coomassie blue following standard procedures. For Western blotting, proteins were transferred to polyvinylidenefluoride membranes (PVDF; Millipore, Bedford, MA) in Tris-glycine buffer supplemented with 0.02% SDS and 4 M urea using a semidry electophoretic transfer cell (Bio-Rad, Cambridge, MA). Membranes were blocked with 10% nonfat dry milk in PBS and probed with antibodies. Both primary and secondary antibodies were diluted in PBS containing 1% milk and 0.05% Tween 20. The secondary antibodies were conjugated to AP or HRP. Detection of AP conjugates was performed using an NBT/BCIP system. HRP conjugates were detected by chemiluminescence (Amersham Pharmacia Biotechnology, Amersham, UK).

Immunoprecipitation

Immunoprecipitations with mAbs were carried out in two steps. First, the mAb was added to either crude nuclear extract or selected fractions from gel filtration chromatography to give a final concentration of ~10 $\mu g/ml$ in the presence of 0.1% NP-40. The specimen was then incubated for 90 min at 4°C with gentle rotation. Anti-mouse IgM immunoglobulins covalently coupled to protein G-agarose (Zymed Laboratories, South San Francisco, CA) were added, and the rotation was continued for an additional 90 min at 4°C. The agarose beads were washed twice with PBS containing 0.1% NP-40 and once with PBS. In some cases, the beads were washed with PBS supplemented with increasing concentrations of NaCl or Na-deoxy-

cholate (DOC) for 20 min at 4°C and then washed as above. The proteins were eluted with 0.5% SDS at room temperature. The eluted proteins were precipitated with acetone and subsequently analyzed by SDS-PAGE and Western blotting. When peptide-specific antibodies were used for immunoprecipation, the affinity-purified antibodies were covalently cross-linked to protein A-agarose and incubated with protein extracts as indicated above.

In-Gel Digestion and Mass Spectrometry Analysis

The Coomassie-stained bands were excised from the SDS-PAGE gel and subjected to in-gel digestion as described (Hellman, 2000). The peptides were extracted and analyzed by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, using a Bruker Autoflex instrument (Bremen, Germany), equipped with delayed extraction and reflector. The instrument was optimized for peptide analysis and alfa-cyano-4-hydroxy cinnamic acid was used as matrix. Spectra were internally calibrated using autolytic fragments from trypsin. The peptide mass fingerprinting analysis was done using ProFound over the internet and GPMAW software locally.

Gel Filtration

Nuclear extracts were prepared in PBS containing 0.2% NP-40, 100 $\mu g/ml$ PMSF, 1 $\mu g/ml$ aprotinin, 1 $\mu g/ml$ leupeptins, and 1 $\mu g/ml$ pepstatin A. Extract (200 μl), prepared from ~100 ml *C. tentans* cell culture, was fractionated on a Superose HR6 column (Amersham Pharmacia Biotechnology) previously equilibrated with the same buffer. The sample was fractionated at a flow rate of 0.2 ml/min, and fractions of 0.6 ml were collected. The proteins in the fractions were precipitated with acetone and analyzed by Coomassie staining or by Western blotting. For RNase treatment, the nuclear extract was incubated with 25 $\mu g/ml$ RNase A (Roche) at room temperature for 20 min before fractionation.

Immunostaining of Salivary Gland Cryosections (Light Microscopy)

Immunostaining of semithin cryosections was performed as previously described (Visa *et al.*, 1996a). Salivary glands from fourth instar larvae were fixed for 60 min at room temperature in 0.1 M cacodylate buffer at pH 7.2 containing 4% paraformaldehyde, cryoprotected with 2.3 M sucrose, and frozen by immersion in liquid nitrogen. Sections of 0.5-μm thickness were cut on a cryoultramicrotome (Ultracut S/FC S; Leica, Heerbrugg, Switzerland) and mounted on glass slides. Before immunolabeling, the sections were incubated in PBS containing 0.1 M glycine and 2% BSA. The first antibody was hybridoma culture medium diluted 1:5 in PBS containing 0.5% BSA. The secondary antibody was an anti-mouse IgM conjugated to 6 nm gold particles (Jackson Immuno Research Laboratories) diluted 1:50 in PBS containing 0.5% BSA. The immuno-gold labeling was silver-enhanced with IntenSE (Amersham Life Science, Arlington Heights, IL). In negative controls, the primary antibody was replaced by either PBS or an unrelated IgM.

Immuno-EM

Immuno-EM was carried out essentially according to the method described by Tokuyasu (1980) with some modifications (Visa *et al.*, 1996a). The specimens were prepared as described above for light microscopy, except that fixation was carried out for 20–25 min in 4% paraformaldehyde and 0.1% glutaraldehyde. The cryosections, 70–80 nm thick, were picked up on drops of 2.3 M sucrose and mounted onto nickel grids coated with formvar and carbon. The grids were floated onto drops of PBS containing 0.1 M glycine and 10% fetal calf serum before incubation with the antibody solutions. Hybridoma cell culture media from 2D10 and 1F2 were diluted 1:2 and 1:10, respectively, in PBS containing 5% fetal calf serum. Two
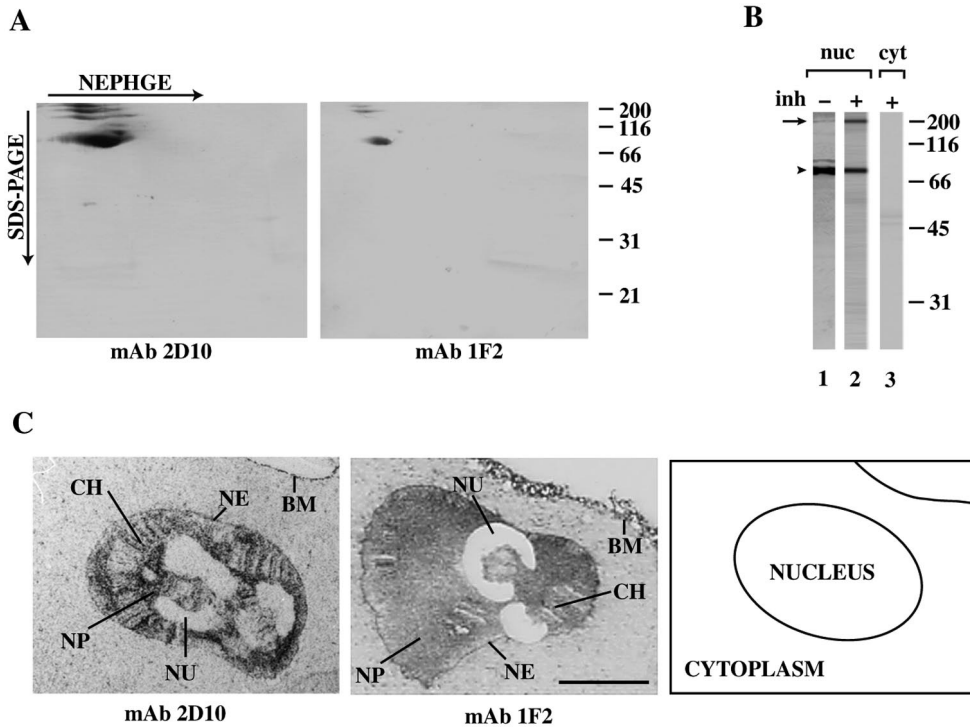
A

mAb 2D10   mAb 1F2

B

C

mAb 2D10   mAb 1F2

Figure 1. MAbs 2D10 and 1F2 recognize a nucleoplasmic protein of *C. tentans*. (A) Nuclear proteins were separated by non-equilibrium pH gradient gel electrophoresis (NEPHGE) using ampholines in the pH 3–10 range. The second dimension was conventional SDS-PAGE. Proteins were analyzed by Western blot with mAbs 2D10 and 1F2. (B) Western blot of proteins in nuclear (nuc) and cytoplasmic (cyt) extracts of *C. tentans* tissue culture cells probed with mAb 2D10. Protein extraction was performed without (lane1) or with (lanes 2 and 3) a cocktail of broad-range protease inhibitors. In nuclear extracts, an intense band at 75–80 kDa (arrowhead) and a weak band at ~220 kDa (arrow) were observed (lanes 1 and 2). In A and B, the mobilities of molecular mass standards in SDS-PAGE are shown on the right. (C) Semithin cryosections of salivary gland cells stained with mAbs 2D10 and 1F2. The antibody-binding sites were detected with a gold-conjugated secondary antibody and visualized after silver enhancement. The results were analyzed and photographed under bright field microscopy. A schematic interpretation of the micrographs is provided on the right. BM, basal membrane; CH, chromosomes; NE, nuclear envelope; NP, nucleoplasm; NU, nucleolus. Bar, 20 μm.

types of negative controls were carried out by replacing the primary antibody either by PBS or by an unrelated IgM. The secondary antibody against mouse IgM, conjugated to 6 nm gold particles (Jackson ImmunoResearch Laboratories), was diluted 1:50 in PBS containing 5% fetal calf serum. After immunolabeling, the sections were stained with 2% aqueous uranyl acetate for 3–5 min and embedded in polyvinyl alcohol (9–10 kDa; Aldrich Chemical, Milwaukee, WI). The specimens were examined and photographed in a Zeiss CEM 902 electron microscope (Thornwood, NY) at 80 KV.

RESULTS

MAbs 2D10 and 1F2 Recognize a Nucleoplasmic Protein in C. tentans

We have used an immunological method to identify new nucleoplasmic proteins in *C. tentans*. Nucleoplasm from fixed salivary glands was manually microdissected, and the isolated nucleoplasmic material was used to immunize mice and to raise mAbs following standard procedures (see MATERIALS AND METHODS). The resulting hybridomas were screened by Western blotting to demonstrate antibody reaction to nuclear proteins prepared from *C. tentans* tissue culture cells. Clones that scored positive in the Western blotting were further screened by immunocytology on sections of salivary gland cells.

Two antibodies, mAbs 2D10 and 1F2, showed very similar patterns in the immunochemical assays. When nuclear extracts of *C. tentans* were analyzed by two-dimensional Western blotting, both mAbs recognized a prominent spot of molecular mass 75–80 kDa and several weaker spots up to ~220 kDa (Figure 1A). The high-molecular-mass spots became stronger when a cocktail of broad-range protease inhibitors was used during extract preparation, as shown for mAb 2D10 in Figure 1B (compare lanes 1 and 2). In cytoplasmic extracts, mAbs 2D10 and 1F2 did not recognize any proteins (lane 3 in Figure 1B and our unpublished results). When nuclear extracts were partially digested with trypsin, mAbs 2D10 and 1F2 showed different patterns of recognition of the degradation products (our unpublished results), which suggests that the epitopes that are recognized by the two mAbs lie on different parts of the protein.

To study the cellular localization of the protein that was recognized by mAbs 2D10 and 1F2, semithin cryosections of salivary gland cells were stained with hybridoma supernatants. The large dimensions of the salivary gland cells of *C. tentans* and their highly organized polytene nucleus allow three distinct nuclear compartments to be identified in the preparations: the polytene chromosomes, the nucleolus, and the nucleoplasm or interchromosome space. MAbs 2D10 and 1F2 labeled extensively the cell nucleus (Figure 1C). The nucleoplasm was the most intensely stained area, and the nuclear envelope was also labeled. The chromosomes displayed a weak, banded staining. A weak labeling was also observed in the cytoplasm. In the negative control, no labeling could be detected in either the nucleus or the cytoplasm of the salivary gland cells (not shown).

In summary, the immunocytology and Western blotting results indicated that the mAbs 2D10 and 1F2 recognized a nuclear protein with molecular mass of ~220 kDa in *C. tentans* that we called p2D10. Both mAbs also recognized bands of molecular mass 75–80 kDa, which are proteolytic fragments of p2D10 (see below).

Cloning and Sequencing of a cDNA Encoding p2D10, a TFIIIC-α-like Protein

To characterize p2D10 at the molecular level, mAb 2D10 was used to screen cDNA expression libraries of *C. tentans*. Three different clones were isolated and subsequently sequenced (designated λ-2D10-A, λ-2D10-B, and λ-2D10-D). The clone λ-2D10-D, which had a length of 6300 base pairs, turned out to harbor a 5′ UTR of length 73 nt, an ORF encoding 2043 amino acids, and a 3′ UTR of length 98 nt. The sequences of the other two clones, λ-2D10-A and λ-2D10-B, were included in λ-2D10-D.

To confirm that the ORF encoded by λ-2D10-D was indeed the protein recognized by mAbs 2D10 and 1F2 in nuclear extracts, two polyclonal antibodies were generated against synthetic peptides that encompassed amino acid residues 1714–1728 and 990-1005 of the λ-2D10-D ORF. These two antibodies were designated pep-I and pep-II, respectively. In Western blot assays against nuclear proteins of *C. tentans*, pep-I and pep-II recognized the same bands that had been observed with mAbs 2D10 and 1F2, which confirmed the specificity of the positive clone. Pep-I and pep-II recognized the bands of molecular mass 75–80 kDa (arrowhead in Figure 2A) as well as high-molecular-mass bands up to 220 kDa. The predicted size of the protein encoded by λ-2D10-D was 238 kDa, similar to the apparent mobility of 220 kDa observed in SDS-PAGE.

The fact that all four antibodies against p2D10, including pep-I and pep-II, recognized not only the 220 kDa band but also the 75–80 kDa bands strongly suggested that the 75–80 kDa bands corresponded to proteolytic fragments of p2D10. To confirm this point, the 220 and 75–80 kDa band were analyzed by peptide mass fingerprinting on MALDI-TOF-MS. For this purpose, the sample was immunoprecipitated from a nuclear extract using the pep-II antibody, resolved by SDS-PAGE, and the 75–80 and 220 kDa bands were cut out of the gel and in situ digested with trypsin before mass spectrometry analysis. A total of 21 and 28 peptides were recovered from the 220 and 75–80 kDa bands, respectively, and 12 peptide masses were found in common between the two samples, which suggests the existence of common peptides in the two bands. However, peptide mass analyses of large proteins are usually limited by a high frequency of random matches. For this reason, and in order to further confirm the significance of our mass comparisons, the MH^+ values from the 75–80 kDa band were matched against the amino acid sequences predicted from the p2D10 cDNA and against two other unrelated proteins of similar size, Tpr (267 kDa, Q99968) and myosin (222 kDa, P13535). Disregarding partial digestion products and considering MH^+ values below 1500, eight sequence matches were found in p2D10 whereas only two and three possible matches were found in myosin and Tpr, respectively. This result shows that in spite of the high background levels, the frequency of observed matches between the 75–80 kDa band and p2D10 is two- to threefold higher than expected from random hits. Further-
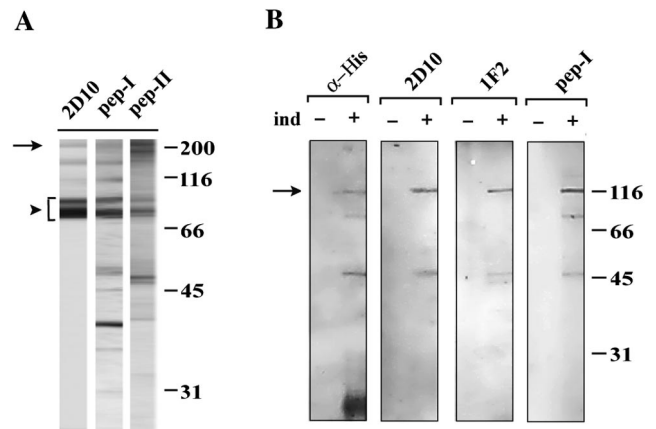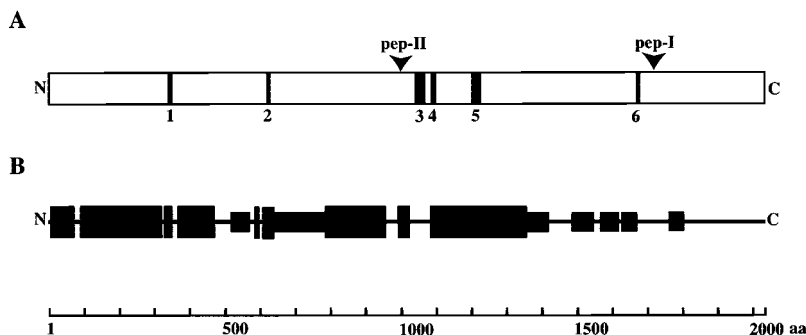

Figure 2. The protein encoded by λ-2D10-D is recognized by mAbs 2D10 and 1F2. (A) Nuclear extracts of *C. tentans* cells were analyzed by Western blot with either mAb 2D10 or two independent peptide-specific antibodies (pep-I and pep-II) raised against sequences encoded in λ-2D10-D. Pep-I, pep-II, and 2D10 recognized bands of molecular mass 75–80 kDa (arrowhead) and also high-molecular-mass bands up to ~220 kDa (arrow). Additional bands, partially due to degradation, were also revealed by pep-I and pep-II. (B) The C-terminal half of λ-2D10-D ORF was expressed in *E. coli* with a His tag at the C terminus and purified on NTA-agarose beads. The purified recombinant protein (arrow) was recognized by antibodies against His, 2D10, 1F2, and pep-I. Extracts prepared before (−ind) or after (+ind) induction of expression were analyzed in parallel. The mobilities of molecular standards expressed in kDa are shown on the right.

more, the most intensive peptide mass peaks in the spectra are those that are common between the 220 and 75–80 kDa bands, supporting the conclusion that these peptides are identical. Furthermore, those peptide mass peaks do not belong to any common contaminating protein such as various keratins. Taken together, the present mass spectrometry analysis and the antibody results reported above support the conclusion that the 75–80 kDa bands are proteolytic fragments of p2D10.

The C-terminal half of the ORF encoded by λ-2D10-D, from residue 960 to residue 2043 (128 kDa), was expressed in *E. coli* with a His tag at the C terminus. The resulting recombinant protein was purified and analyzed by Western blotting. As expected, mAbs 2D10 and 1F2 recognized the recombinant, His-tagged protein (Figure 2B).

The predicted pI of the protein encoded by λ-2D10-D was 8.7. This protein had several putative phosphorylation and glycosylation sites. It also contained several unusual charged stretches (Figure 3A). Two acidic stretches consisting of 8 acidic residues each were found starting from amino acid positions 338 and 1671, respectively (Figure 3A, motifs 1 and 6). Two stretches of basic amino acids were found at amino acid positions 1037–1067 (motif 3) and 1082–1098 (motif 4), containing 14 and 7 lysines, respectively. The ORF of λ-2D10-D also contained a stretch of hydrophobic amino acids (7 serines or leucines in a stretch of length 9 amino acids) starting from residue 625 (motif 2). Except for a short coiled-coil between positions 1203 and 1231 (motif 5), no obvious structural motifs were identified in the λ-2D10-D ORF.

A

B

Figure 3. Primary structure of p2D10 (A) The positions of sequence motifs (motifs 1–6) found in p2D10 are shown schematically. The data were obtained with an integrated search in Prosit, Pfam, and Prints at http://www.expasy.ch and Psort II at http://psort.nibb.ac.jp. Motif 1: Asp/Glu-rich region encompassing residues 338–351. Motif 2: Leu/Ser-rich region at 625–633. Motif 3: Lys-rich region at 1037–1067. Motif 4: Lys-rich region at 1082–1098. Motif 5: coiled-coil corresponding to residues 1203–1231 Motif 6: Asp/Glu-rich region at 1671–1682. The arrowheads show the positions of peptides used to raise the specific antibodies, pep-I and pep-II. (B) Structural conservation of TFIIIC-α. The sequence of p2D10 (AJ313507) was aligned with the sequences of TFIIIC-α proteins from *Drosophila* (Q9VJY7), rat (Q63505), and human (Q12789) with the PileUp program of the GCG package, and the percentage of identity was analyzed as described in MATERIALS AND METHODS. Large and small boxes are correspond to regions with >28% and 14–28% identity, respectively.

A search of nonredundant databases was performed with BLAST and FASTA. The best hits were the general transcription factors TFIIIC-α from human (hTFIIIC-α) and rat (rTFIIIC-α) with 28% identity. A similar level of homology was also found for the product of the CG7099 gene of *D. melanogaster* (Q9VJY7). The product Q9VJY7 of *D. melanogaster* has not been characterized, but it appears to be the *Drosophila* ortholog of TFIIIC-α, as indicated by database searches that compared the hTFIIIC-α against the *D. melanogaster* genome database. No other related proteins were found in the databases.

Comparing the primary structure of the TFIIIC-α proteins of human, rat, and *Drosophila* showed that the most conserved sequences were located within the N-terminal two-thirds of the protein, whereas the C-terminal third was much less conserved. A higher degree of conservation at the N-terminus was also found when p2D10 was compared with known TFIIIC-α proteins (Figure 3B).

We wanted to determine whether p2D10 was associated in vivo with components of the TFIIIC complex. Human TFIIIC-α is the largest subunit of the TFIIIC2 factor, which is one of the components of the Pol III transcription machinery (reviewed by Geiduschek and Kassavetis, 2001; Huang and Maraia, 2001). One of the TFIIIC2 subunits, TFIIIC-ε, is conserved (Hsieh *et al.*, 1999), and this allowed us to use antibodies against hTFIIIC-ε for Western blot analyses in *C. tentans*. A nuclear extract was prepared from *C. tentans* tissue culture cells, and mAb 2D10 was used to immunoprecipitate p2D10. As controls, either the antibody or the nuclear extract were excluded from the immunoprecipitation. The immunoprecipitated proteins were resolved by SDS-PAGE and either stained with Coomassie or analyzed by Western blotting. Coomassie-staining revealed that at least 10 polypeptides were coimmunoprecipitated with p2D10 (Figure 4A). A polyclonal antibody against hTFIIIC-ε reacted with a major band of approximate molecular mass 50 kDa when the immunoprecipitated material was analyzed by Western blotting (Figure 4B). The molecular mass of the hTFIIIC-ε is ~63 kDa (Hsieh *et al.*, 1999), whereas the protein that is believed to be the TFIIIC-ε homolog in *Caenorhabditis elegans* (GenBank accession no. Z35603) is 50 kDa (Hsieh *et al.*, 1999). Therefore, the apparent molecular mass of the protein recognized by antihTFIIIC-ε in *C. tentans* (50 kDa) agrees with the size of TFIIIC-ε in invertebrates, and we

suggest that this protein is the counterpart in *C. tentans* of TFIIIC-ε (ctTFIIIC-ε).

Coprecipitation of other TFIIIC2 factors could not be tested because of lack of evolutionary conservation.

In summary, the p2D10 protein is structurally similar to TFIIIC-α and is associated in vivo with at least one other component of the TFIIIC2 complex. Although it remains to be proven whether p2D10 is active as transcription factor, our present results suggest that p2D10 is the *C. tentans* ortholog of TFIIIC-α (ctTFIIIC-α).

Association of p2D10 with Nucleoplasmic Proteins

The location of p2D10 predominantly in the nucleoplasm of the salivary gland cells and the significant labeling observed at the nuclear envelope (Figure 1C) led us to analyze whether p2D10 is associated with other nuclear proteins previously characterized in *C. tentans*. We carried out immunoprecipitation experiments with mAb 2D10 as described above, and the immunoprecipitated proteins were eluted, separated by SDS-PAGE and probed with a panel of antibodies against nuclear proteins (Figure 4C). All of the available antibodies against p2D10 (mAb 2D10, mAb 1F2, pep-I, and pep-II) were positive against the immunoprecipitated material, as expected (our unpublished results). The following antibodies against other nuclear proteins were tested: mAb Y1D2 against hrp120/Pep (Amero *et al.*, 1991), mAb 4E9 against hrp65 (Miralles *et al.*, 2000), mAb 2E4 against hrp45 (Alzhanova-Ericsson *et al.*, 1996), mAb 4F9 against hrp36 (Visa *et al.*, 1996a), mAb 1D3 against hrp23 (Sun *et al.*, 1998), anti-hRAE1 polyclonal antibody recognizing *C. tentans* RAE1 (Sabri and Visa, 2000), mAb Y12 against the Sm epitope of snRNP core proteins (Pettersson *et al.*, 1984), and a polyclonal antibody against the cap-binding protein CBP20 (Visa *et al.*, 1996b). The hrp45 and hrp36 proteins were highly abundant in the extracts that were used as input for the immunoprecipitations, but they were not detected in the eluate (Figure 4C, lanes 3 and 4, respectively). The Sm-core proteins and the CBP20 were not coimmunoprecipitated either (lanes 7 and 8, respectively). Instead, four other proteins, hrp120, hrp65, hrp23 and RAE1, coimmunopurified with p2D10 in a very reproducible manner (Figure 4C, lanes 1, 2, 5, 6).

Figure 4. Identification of proteins coimmunoprecipitated with p2D10. (A) Immunoprecipitation on nuclear extract from tissue culture cells of *C. tentans* with mAb 2D10. The eluted proteins were separated by SDS-PAGE and stained with Coomassie blue. Bands corresponding to identified proteins are indicated on the right. HC and LC correspond to the heavy and light chains, respectively, of mAb 2D10. The p26 protein is a *C. tentans* protein similar to *Drosophila* Q9VIX5 (Kiesler, Sabri and Visa, unpublished observations). (B) The presence of TFIIIC-ε was analyzed by immunoblotting on either nuclear extract (input) or immunoprecipitated (IP) proteins using antihTFIIIC-ε antibody. A main band of 50 kDa (arrow) corresponding to TFIIIC-ε was observed in both the input and the immunoprecipitated material. (C) Proteins immunoprecipitated by mAb 2D10 were analyzed by Western blot using antibodies against different proteins, as indicated above the lanes. (D) Proteins immunoprecipitated with pep-II were analyzed by Western blot as in C. The additional bands observed with the anti-RAE1 antibody (lane 7) are due to cross-reactivity of the secondary antibody with pep-II, as shown by the control with only secondary antibody shown in lane 8. (E) Immunoprecipitations were performed as in A, and the bound proteins were washed with either PBS or PBS containing increasing concentrations of NaCl or DOC for 20 min before elution. The eluted proteins were subsequently analyzed by Western blot with antibodies against hrp120, hrp65, hrp23, and RAE1.
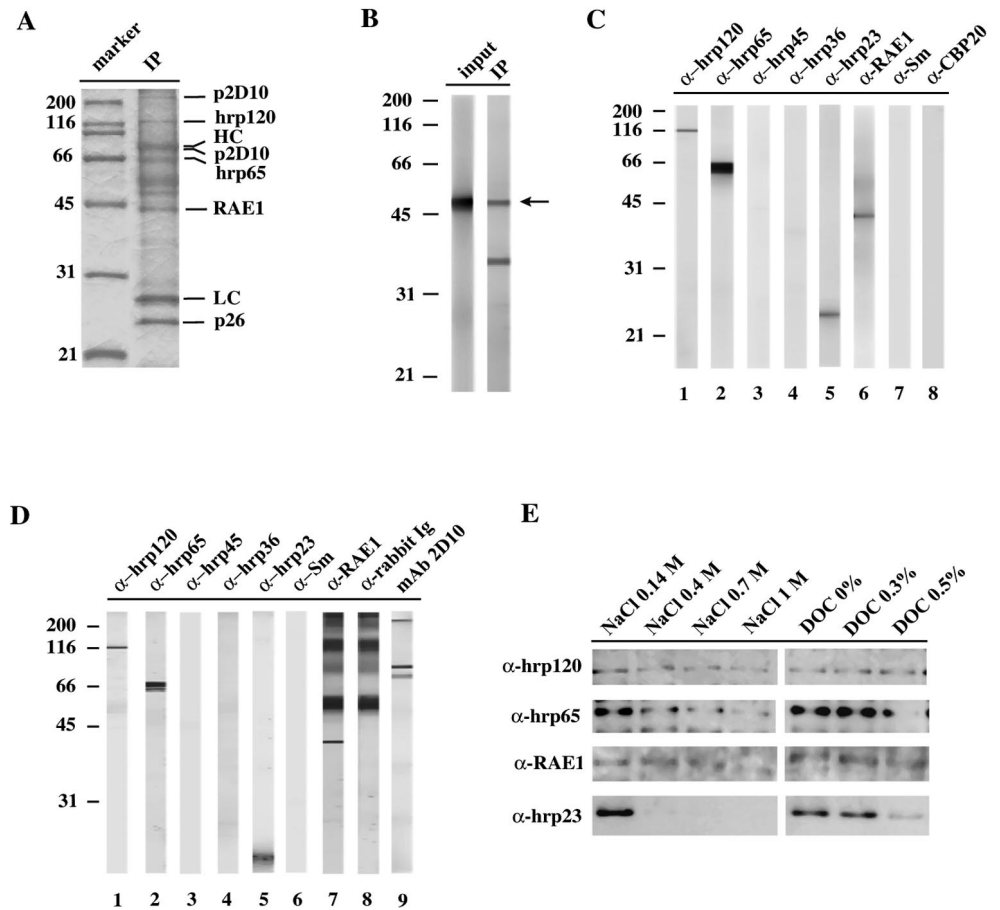
The coimmunoprecipitated experiments were also carried out with mAb 1F2 and pep-I (Figure 4D). Identical results were obtained in all cases irrespective of the antibody used for immunoprecipitation, which supports the specificity of the coimmunoprecipitation results.

To assess the stability of the interactions detected, the immunoprecipitations were followed by highly stringent washes with buffers containing up to 1 M NaCl or 0.5% DOC (Figure 4E). Hrp120, hrp65, and RAE1 were associated with p2D10 in the presence of either NaCl or DOC. In contrast, hrp23 could not be detected in association with p2D10 at high ionic strength ($\geq$0.4 M NaCl).

In summary, our results indicate that p2D10 is in a complex with ctTFIIIC-ε (Figure 4B) and that p2D10 is associated with hrp120, hrp65, hrp23, and RAE1 (Figure 4, C and D). CtTFIIIC-ε is a component of the Pol III transcription machinery, whereas hrp120, hrp65, hrp23, and RAE1 are involved in processing and/or transport of premRNAs. This means that our results suggest that p2D10 functions in both Pol III and Pol II gene expression pathways.

p2D10 Is Associated with hrp65 and RAE1 in a Complex that Contains RNA

The results described above suggest that p2D10 has several functions, and this led us to expect that the p2D10 protein would be found in different complexes of defined composition. To check this hypothesis, nuclear extracts were fractionated in a size-exclusion Superose HR6 chromatography column. Proteins in each fraction were analyzed by Western blotting using mAb 2D10. As shown in Figure 5, p2D10 was detected in a heavy peak with a molecular mass of 1800–2000 kDa (fractions 12–13) and in a second much broader peak extending from ~1100 to <160 kDa (fractions 16–27). The 220-kDa band recognized by mAb 2D10 was visible in the heavy peak but not in the other 2D10-positive fractions, which contained only the 75–80 kDa proteolytic fragments.

To check whether any of the p2D10 complexes contained RNA, a nuclear extract was treated with RNase A, fractionated, and analyzed as above. The result is shown in Figure 5C. The same amount of nuclear extracts was loaded in Figure 5, B and C. The heavy peak remained unchanged
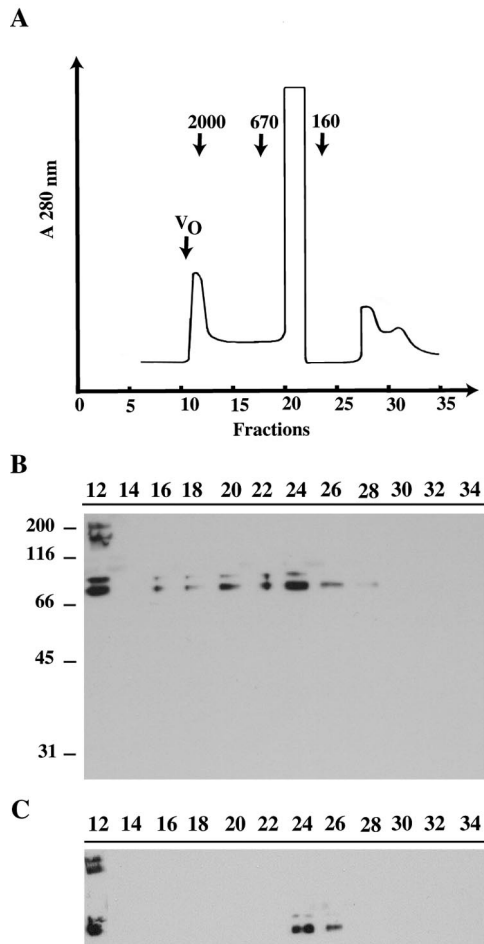
A

B

C

Figure 5. Chromatographic analysis of TFIIIC-α–containing complexes. Nuclear proteins from tissue culture cells of *C. tentans* were fractionated on a gel filtration Superose HR6 column. (A) The chromatogram, showing the fractionation of some molecular mass standards, in kDa, used for calibration. V_O: void volume. (B) Fractions were pooled two by two (for example, lane 12 contains fractions 12 and 13), separated by SDS-PAGE, and analyzed by immunoblotting using mAb 2D10. The mobilities of molecular mass standards in SDS-PAGE are shown on the left. (C) Nuclear extract was preincubated with 25 μg/ml RNase A for 20 min before chromatography and Western blot analysis as in B.

after RNase digestion. The light peak, however, became much narrower and was restricted to fractions 24–27, corresponding to a mass of <160 kDa. We concluded that the light peak was a ribonucleoprotein complex that contained both protein and RNA.

The finding that a TFIIIC-α-like protein was present in two complexes of different sizes and compositions encouraged us to analyze in more detail the association of p2D10 with proteins involved in either transcription or posttranscriptional events in the two different complexes. For this purpose, nuclear extracts were fractionated as described above, and selected fractions were used as the input to immunoprecipitation assays with mAb 2D10. The fractions were pooled in four groups, fractions 12–14, 16–18, 20–22,
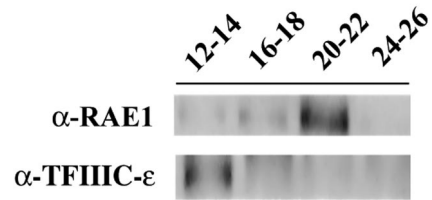

Figure 6. Coimmunoprecipitation of TFIIIC-α–associated proteins from selected gel filtration fractions. After fractionation of the nuclear extract on a Superose HR6 column, selected fractions were pooled in four groups including fractions 12–14, 16–18, 20–22, and 24–26 and used for immunoprecipitation with mAb 2D10. The eluted proteins were analyzed by Western blot using antibodies against RAE1 and TFIIIC-ε.

and 24–26. All immunoprecipitations were performed under identical conditions. The eluted proteins were analyzed by Western blotting with antibodies against the general transcription factor ctTFIIIC-ε and the putative RNA export factor RAE1. As shown in Figure 6, the association of p2D10 with ctTFIIIC-ε was restricted to fractions 12–14, whereas p2D10 was associated with RAE1 in fractions 20–22. The same type of analysis was carried out with antibodies against hrp65, which was also preferentially detected in fractions 20–22 (our unpublished results). On the other hand, hrp120 was associated with the heavy p2D10-containing complex (unpublished observation). The association of hrp23 with the p2D10 complexes could not be determined after gel filtration, probably because of the lower affinity of the interaction.

In summary, p2D10 is found in vivo in at least two different complexes. One of the complexes, referred to as the heavy p2D10 complex, has a mass of about 1800 kDa, is not sensitive to RNase digestion and contains at least one transcription factor, ctTFIIIC-ε. The second p2D10 complex, which is called the light p2D10 complex, contains RNA and at least two other proteins, RAE1 and hrp65, involved in premRNA metabolism.

p2D10 Is Located in the Nucleoplasm and at Nuclear Pores

We also determined the subcellular location of the p2D10 protein in the salivary gland cells of *C. tentans* by immuno-EM. Thin cryo-sections of salivary glands were immunolabeled with either mAb 2D10 or mAb 1F2, with an unrelated IgM being used as a negative control. Labeling was visualized with a gold-conjugated secondary antibody. The same distribution of labeling was obtained with both mAbs. However, labeling with mAb 2D10 was weak in the immuno-EM assays, and for this reason the results shown were obtained with mAb 1F2. The distribution of p2D10 in the different nuclear compartments is illustrated in Figure 7. In the chromosomes, the dense chromatin was not labeled, whereas some interbands were labeled (Figure 7B). The nucleolus, in particular the granular component, was also weakly labeled (Figure 7C). The highest density of labeling was found in the nucleoplasm and at the nuclear envelope, which agrees with the distribution seen in the light microscope, shown in Figure 1C. The nucleoplasmic BR particles of the salivary gland
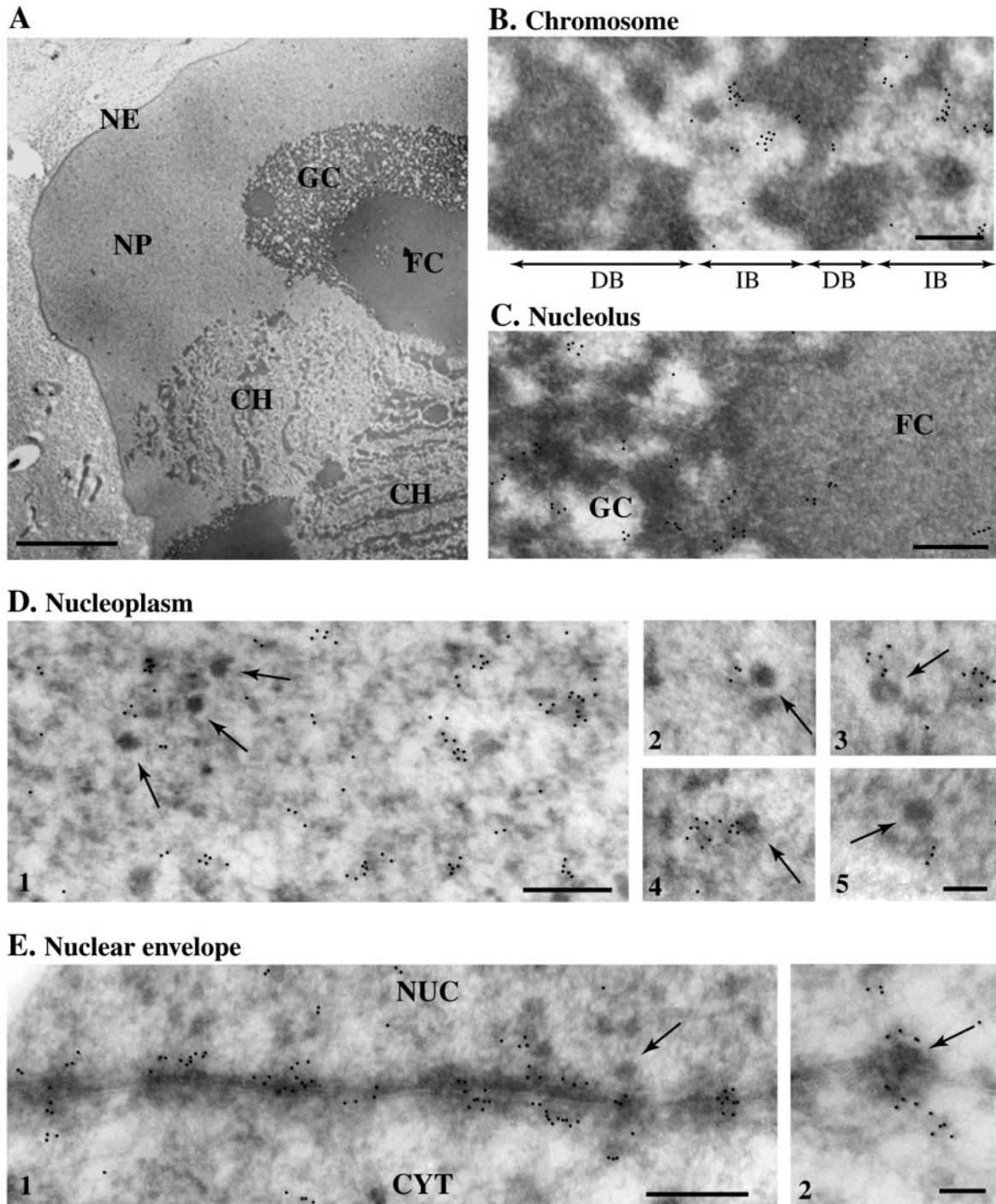
Figure 7. Immuno-EM analysis of p2D10 in salivary gland cells of *C. tentans*. Thin cryosections of salivary glands were stained with mAb 1F2 and with a secondary antibody coupled to colloidal gold. (A) Overview of a salivary gland cell as seen in the EM at low magnification. CH, chromosome; FC, fibrillar component of the nucleolus; GC, granular component of the nucleolus; NE, nuclear envelope; NP, nucleoplasm. Bar, 5 μm. (B) In the chromosomes, labeling was concentrated in the interbands (IB), whereas the dense chromatin bands (DB) were not labeled. Bar, 300 nm. (C) In the nucleolus, labeling was observed mainly in the granular component (GC). Bar, 300 nm. (D) Examples of labeling in the nucleoplasm. The arrows point at BR RNP particles located in the nucleoplasm. Bar, D1: 300 nm; D2–5: 100 nm. (E) Labeling along the nuclear envelope. The arrows point at BR RNP particles located at the NPC. The nuclear (NUC) and cytoplasmic (CYT) compartments are indicated. Bars, 300 and 100 nm in E1 and E2, respectively. A black dot was pasted onto each of the original gold markers to facilitate the visualization of the results.

cells, which are known to contain a premRNA molecule associated with hnRNP proteins (reviewed by Daneholt, 2001), were not labeled (Figure 7D1). The nucleoplasmic labeling was often observed on fibrillar structures located close to the BR particles (Figure 7D, 2–5). These labeled structures correspond to connecting fibers (CFs) and fibrogranular clusters (FGCs) described previously (Miralles *et al.*, 2000). Interestingly, the CFs also contain hrp65 (Miralles *et al.*, 2000).

Most of the nuclear pore complexes (NPCs) were intensely labeled (Figure 7E), with gold particles decorating both the nuclear and the cytoplasmic sides of the nuclear envelope. This labeling was highly specific, and a total absence of gold was observed in the negative controls (our unpublished results). The BR particles in transit through the NPC were not significantly labeled; the NPC itself was labeled, but not the exported mRNP cargo (Figure 7E2).

The location of RAE1 at the NPC depends on ongoing RNA pol II transcription (Pritchard *et al.*, 1999) and is related to the presence of an export cargo at the NPC (Sabri and Visa, 2000). To determine whether the association of p2D10 with the NPC shows the same behavior, fourth instar larvae were treated with transcription inhibitors, actinomycin D or DRB, before dissection of salivary glands and immuno-EM analysis. Neither actinomycin D nor DRB had any effect on the location of p2D10 at the NPC (our unpublished results).

In summary, the in situ studies demonstrate that p2D10 is located in the chromosomes but also present in other nuclear compartments that contain hrp65 and RAE1, such as the nucleoplasm and the nuclear envelope. Moreover, the location of p2D10 at the NPC does not depend on Pol II transcription.

DISCUSSION

We report here the molecular cloning of a cDNA that encodes for p2D10, a 238-kDa nuclear protein that shares many properties with TFIIIC-α, the largest subunit of the TFIIIC2 factor. The assembly of transcription complexes on Pol III promoters requires at least two multimeric factors, TFIIIB and TFIIIC, in addition to Pol III itself (reviewed by Geiduschek and Kassavetis, 2001; Huang and Maraia, 2001). In mammals, TFIIIC can be resolved into two complexes of specific compositions and functions, designated TFIIIC1 and TFIIIC2. The TFIIIC2 complex, which is required for promoter recognition (Dean and Berk, 1988), consists of five subunits of molecular mass of ~220, 110, 102, 90, and 63 kDa (Yoshinaga *et al.*, 1989; Sinn *et al.*, 1995; Wang and Roeder, 1996). We have shown that p2D10 coimmunoprecipitates with at least one other component of TFIIIC2, TFIIIC-ϵ. We have also shown by immuno-EM in the salivary gland cells of *C. tentans* that a fraction of p2D10 is located in the nucleoplasm and at the nuclear envelope. Moreover, our results show that p2D10 is associated with hrp65 and with RAE1, two RNA-binding proteins that are thought to be involved in intranuclear transport and/or retention of premRNA. Our data suggest that this new TFIIIC-α-like protein may be involved in posttranscriptional steps of premRNA metabolism.

p2D10, a TFIIIC-α-like Protein in C. tentans

Three experiments support the conclusion that mAbs 2D10 and 1F2 recognize p2D10, a TFIIICα-like protein in *C. tentans*. First, two independent peptide-specific antibodies raised against sequences that are encoded in the p2D10 cDNA gave the same labeling pattern as mAbs 2D10 and 1F2 in Western blotting. Second, recombinant p2D10 expressed in *E. coli* was recognized by mAbs 2D10 and 1F2. Third, the mAb 2D10 could immunoprecipitate another component of the TFIIIC2 complex, ctTFIIIC-ϵ, from crude nuclear extracts. Although the function of p2D10 in transcription remains to be demonstrated, the observations summarized above suggest that the protein that is recognized by mAbs 2D10 and 1F2 is not only structurally similar but also functionally related to TFIIIC-α.

Four different antibodies against p2D10, two mAbs and two peptide-specific antibodies, have been used in the present study. In Western blots of *C. tentans* proteins, all four antibodies recognized prominent, very intense bands of 75–80 kDa in addition to the full-length p2D10 that migrates to ~220 kDa. Short p2D10 isoforms may exist in *C. tentans*, but a comprehensive screening of cDNA expression libraries failed to reveal any other related proteins. Based on this observation, on the specificity of our antibodies, and on mass spectrometry data, we conclude that the 75–80 kDa proteins detected in our Western blots correspond to proteolytic fragments of p2D10.

Complexes that Contain p2D10

The human Pol III holoenzyme has an apparent molecular mass of ~1500 kDa (Wang *et al.*, 1997). We have identified a complex of similar mass in *C. tentans*. This complex contains p2D10 and ctTFIIIC-ϵ, and we refer to it as the heavy p2D10 complex. The Pol III machinery in insects has not been characterized. However, the mass of the heavy p2D10 complex, and the fact that it contains both p2D10 and ctTFIIIC-ϵ, suggest that the heavy p2D10 complex might be the Pol III holoenzyme of *C. tentans*. Our immuno-EM experiments have shown that a fraction of p2D10 is located in the chromosomes and specifically in the interbands, where actively transcribed genes are known to be located (Sass and Bautz, 1982). This distribution is consistent with p2D10 having a role in transcription.

We have also detected another complex that contains p2D10. This complex has a lower molecular mass than the first and is more abundant in the nuclear extracts as judged from the intensity of labeling in Western blots. We have termed this complex the light p2D10 complex. Two other proteins, RAE1 and hrp65, were found in the light p2D10 complex. Hrp65 belongs to a family of RNA-binding proteins that includes PSF and p54[nrb]/NonO in mammals (Dong *et al.*, 1993; Patton *et al.*, 1993), and Bj6/NonA in *Drosophila* (von Besser *et al.*, *1990*). The function of these proteins is still controversial, but they appear to play several roles in the expression of class II genes. The human PSF was initially identified as a premRNA splicing factor (Patton *et al.*, 1993), and more recent reports have suggested a role for both PSF and p54[nrb]/NonO in transcription regulation (Mathur *et al.*, 2001 and references therein) and nuclear retention of hyper-edited RNA (Zhang and Carmichael, 2001). In *C. tentans*, hrp65 was identified as a component of

the nucleoplasmic fibers that are associated with premRNP particles in transit from the gene to the NPC (Miralles *et al.*, 2000). The RAE1 protein, also called Gle2p or mrnp41, is highly conserved in eukaryotes and is involved in mRNA export (reviewed by Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999; Cullen, 2000). A recent report has suggested that RAE1 plays a role in mitotic regulation (Wang *et al.*, 2001). RAE1 is located at the NPC through direct binding to a nucleoporin, Nup116p/NUP98, and it is thought to bridge the interaction between the mRNA and the NPC (Bailer *et al.*, 1998; Pritchard *et al.*, 1999; Sabri and Visa, 2000).

The light p2D10 complex eluted in a broad peak, with molecular masses ranging from 1100 to <160 kDa. The molecular mass of the complex following RNase digestion was <160 kDa. Thus, we conclude that the light TFIIIC-α complex in native, nondigested extracts is a heterogeneous complex that contains both protein and RNA. The drastic reduction in size caused by RNase digestion suggests that the RNA contained in the light p2D10 complex is relatively long, maybe rRNA or mRNA. We favor the hypothesis that the light p2D10 complex contains mRNA (or premRNA), because the other proteins detected in this complex, RAE1 and hrp65, have been found in association with mRNA (Bharathi *et al.*, 1997; Kraemer and Blobel, 1997; Miralles *et al.*, 2000).

We could not detect the full-length p2D10 protein in the light p2D10 complex. This may be due to proteolytic degradation during our experiments, or it may suggest that a specific cleavage product of p2D10 binds, directly or indirectly, to hrp65 and RAE1 in a ribonucleoprotein complex in vivo. Interestingly, the human TFIIICα protein is specifically cleaved both in vitro and in vivo by the poliovirus 3C protease, which results in stable proteolytic fragments of ~90 and 125 kDa (Shen *et al.*, 1996). It will be interesting to determine whether the 75–80 kDa bands observed in the case of p2D10 also originate from specific cleavage events mediated by viral or cellular proteases.

Subcellular Location and Posttranscriptional Role of p2D10

Our coimmunoprecipitation experiments show that p2D10 and hrp65 are associated, as are p2D10 and RAE1, but we have not demonstrated the existence in vivo of a ternary complex containing all three proteins. The light p2D10 complex is likely to be heterogeneous and to include different subcomplexes. Indeed, the fact that the proteins identified in this complex are found in several subcellular locations suggests that the interactions between these proteins are dynamic and are different in different cell compartments.

The immuno-EM analysis presented in Figure 7 reveals that the nucleoplasmic fraction of p2D10 is widely distributed in connecting fibers (CFs) and fibrogranular clusters (FGCs) that are in contact with the BR particles (Miralles *et al.*, 2000). The hrp65 protein is also found in the CFs, but it is confined to the proximal part of the fibers, close to the BR particles (Miralles *et al.*, 2000). Thus, hrp65 and p2D10 might interact with each other in the CFs, maybe bridging the binding of the BR particles to the FGCs. It is interesting to point out that hrp23, which coimmunoprecipitates with both p2D10 and hrp65, is one of the major RNA-binding proteins in the BR particles (Sun *et al.*, 1998). Thus, it is tempting to speculate that the binding of BR particles to the CFs is maintained by the interaction between hrp23 and hrp65,

and, in turn, the CFs are associated with the large FGCs by interactions between hrp65 and p2D10.

The RAE1 protein is predominantly located at the nuclear envelope, which suggests that the interaction between p2D10 and RAE1 occurs in a later step of the gene expression pathway. We have shown that p2D10 remains associated with the NPC after transcription inhibition, whereas RAE1 is not found at the nuclear envelope if transcription is inhibited (Pritchard *et al.*, 1999). These findings indicate that the binding of p2D10 to the NPC cannot be mediated by RAE1.

To our surprise, a fraction of p2D10 was located in the granular compartment of the nucleolus, as shown by immuno-EM. The hrp23 displays a very similar nucleolar localization (Sun *et al.*, 1998), which suggests that p2D10 localizes to the nucleolus through its association with hrp23.

The spatial relationships of p2D10 with BR particles observed in the immuno-EM experiments suggests that the p2D10 protein functions in the intranuclear transport or retention of mRNP particles, maybe participating in the delivery of the mRNP export cargoes to the nuclear pore. The further characterization of direct protein–protein and protein–RNA interactions in the light p2D10 complex remains as goal for the future and will contribute to our understanding of the role of this complex in mRNA biogenesis.

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