A p50-like Y-box protein with a putative translational role becomes associated with pre-mRNA concomitant with transcription

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Summary

In vertebrates free messenger ribonucleoprotein (RNP) particles and polysomes contain an abundant Y-box protein called p50 (YB-1), which regulates translation, presumably by affecting the packaging of the RNA. Here, we have identified a p50-like protein in the dipteran *Chironomus tentans* and studied its relation with the biogenesis of mRNA in larval salivary glands. The salivary gland cells contain polytene chromosomes with the transcriptionally active regions blown up as puffs. A few giant puffs, called Balbiani rings (BRs), generate a transcription product, a large RNP particle, which can be visualised (with the electron microscope) during its assembly on the gene and during its transport to and through the nuclear pores. The p50-like protein studied, designated Ct-p40/50 (or p40/50 for short), was shown to contain a central cold-shock domain, an alanine- and proline-rich N-terminal domain, and a C-terminal domain with alternating acidic and basic regions, an organisation that is characteristic of p50 (YB-1). The p40/50 protein appears in two isoforms, p40 and p50, which contain 264 and 317 amino acids, respectively. The two isoforms share the first 258 amino acids and thus differ in amino-acid sequence only in the region close to the C-terminus. When a polyclonal antibody was raised against p40/50, western blot analysis and immunocytology showed that p40/50 is not only abundant in the cytoplasm but is also present in the nucleus. Immunolabelling of isolated polytene chromosomes showed that p40/50 appears in transcriptionally active regions, including the BRs. Using immunoelectron microscopy we revealed that p40/50 is added along the nascent transcripts and is also present in the released BR RNP particles in the nucleoplasm. Finally, by UV crosslinking in vivo we showed that p40/50 is bound to both nuclear and cytoplasmic poly(A) RNA. We conclude that p40/50 is being added cotranscriptionally along the growing BR pre-mRNA, is released with the processed mRNA into the nucleoplasm and probably remains associated with the mRNA both during nucleocytoplasmic transport and protein synthesis. Given that the p40/p50 protein, presumably with a role in translation, is loaded onto the primary transcript concomitant with transcription, an early programming of the cytoplasmic fate of mRNA is indicated.

Key words: Y-box protein, YB-1, Pre-mRNP, hnRNP, RNP assembly

Introduction

Post-transcriptional processes like capping, splicing and polyadenylation are closely coupled to the transcription process (Bentley, 2002; Maniatis and Reed, 2002). Even the nucleocytoplasmic transport of mRNA is initiated cotranscriptionally in relation to the action of the RNA polymerase (Lei et al., 2002; Strässer et al., 2002). Therefore, current research on the control of post-transcriptional events is focused on the RNA polymerase complex itself and on the primary transcription products formed.

The nascent pre-mRNA molecule is immediately associated with proteins to form a ribonucleoprotein complex, which is usually called pre-mRNP or hnRNP (heterogeneous nuclear RNP) (for reviews, see Dreyfuss et al., 1993; Krecic and Swanson, 1999). The proteins of the complex, designated hnRNP proteins, comprise about 30 major proteins and a large number of minor proteins. The hnRNP complex forms a 5-10 nm RNP fibril, often referred to as a perichromatin fibril, which can be further packed into spherical particles, the perichromatin granules (Puvion-Dutilleul, 1983; Fakan, 1994). The hnRNP proteins unfold the RNA and keep it extended, thereby facilitating interactions with proteins or protein assemblies. The hnRNP proteins show a general RNA-binding ability and a certain degree of sequence preference. The proteins are not randomly distributed along the RNA, and a given transcript is likely to harbour a specific subset of hnRNP proteins (Matunis et al., 1993; Wurtz et al., 1996). It is known that hnRNP proteins affect splicing and 3′-end processing, retain hnRNA in the nucleus or mediate RNA transport from nucleus to cytoplasm. Furthermore, some of the hnRNP proteins are involved in processes such as translation, mRNA degradation, and transport of mRNA within cytoplasm (for reviews, see Krecic and Swanson, 1999; Nakielny and Dreyfuss, 1999). It is evident, therefore, that the pre-mRNP/mRNP complexes should be looked upon as more-or-less well-organised substrates for both cotranscriptional and

post-transcriptional processes. Although the population of hnRNP proteins as a whole has been extensively studied, there is still little information on the composition and organisation of RNA-binding proteins along specific transcripts and also on how this RNP structure is established and affects cotranscriptional and subsequent events.

The Balbiani ring (BR) genes in the larval salivary glands of the dipteran *Chironomus tentans* form a system that is suitable for analysing the cotranscriptional assembly of a pre-mRNP particle (for a review, see Daneholt, 2001). The salivary gland cells contain four polytene chromosomes, and the transcriptionally active regions appear as chromosome puffs. Two giant puffs, BR1 and BR2, harbour active genes that are 35-40 kb in size and encode large secretory proteins. The assembly of the exceptionally large BR pre-mRNP particles can be visualised using the electron microscope, the completed BR mRNP product can be followed to and through the nuclear pore, and the loading of the BR transcript into polysomes just outside the nuclear pore can be seen. By immunoelectron microscopy the behaviour of several RNA-binding proteins has been studied during the assembly and transport of this specific pre-mRNP particle (Daneholt, 2001). One protein, the hnRNP A1-like protein, hrp36, shows a remarkable flow pattern: it is added to the nascent BR transcript and accompanies the mRNA not only into the cytoplasm but also into polysomes (Visa et al., 1996a). It is not known whether hrp36 affects protein synthesis but it seems reasonable that the loading of hrp36 concomitant with transcription influences not only intranuclear events but also subsequent cytoplasmic events.

To further explore the possibility that cotranscriptional loading of pre-mRNA does influence the nuclear and cytoplasmic fate of the transcript, we studied the behaviour of the *C. tentans* equivalent of an abundant cytoplasmic protein known to regulate translation in somatic cells of vertebrates, the mRNP protein p50 (YB-1) (for a review, see Evdokimova and Ovchinnikov, 1999). Along with the poly(A) binding protein 1 (PABP1), it constitutes the most abundant protein in mRNP particles (Jain et al., 1979), and it is also abundant in polysomes (Minich et al., 1993). The p50 protein binds strongly to mRNA with little or no sequence preference (Minich et al., 1993; Evdokimova et al., 1995). Furthermore, like the hnRNP proteins, it melts secondary structures in the RNA, keeping the mRNA available for interactions (Evdokimova et al., 1995). At high concentrations it blocks translation (Minich et al., 1993; Davydova et al., 1997), whereas it stimulates translation at lower concentrations (Minich and Ovchinnikov, 1992), presumably by modulating the structure of the mRNP complex. The p50 protein exerts its effect at the level of initiation (Evdokimova et al., 1998) by promoting the binding of 40S ribosomal subunits at the initiation codon of mRNA (Pisarev et al., 2002). The p50 protein can also act as a potent cap-dependent mRNA stabiliser (Evdokimova et al., 2001). A p50-like protein has also been found in hnRNPs in the cell nucleus (Sommerville and Ladomery, 1996a), and YB-1 can affect pre-mRNA processing (Chansky et al., 2001). Finally, p50-like proteins are also present in germ cells (Tafuri et al., 1993; Kwon et al., 1993). We conclude that the available information on p50 (YB-1) suggests that this protein should be a good candidate for studying whether a general RNA-binding protein, functioning in the cytoplasm, is already loaded onto pre-mRNA concomitant with transcription.

In the present study, we identified a *C. tentans* Y-box protein by cDNA cloning. The protein, designated Ct-p40/50 (or p40/50 for short), shows high sequence homology to p50 (YB-1) and has a similar domain organisation. A specific polyclonal antibody was raised against p40/50, and the intracellular distribution of the protein was revealed by immunocytology, immunoelectron microscopy and UV crosslinking. It could be concluded that p40/50 is added to the BR transcript cotranscriptionally, is loaded along the entire RNA molecule and probably remains bound to the mRNA from gene to polysomes. The importance of the cotranscriptional loading process in early programming of the cytoplasmic fate of the mRNA is discussed in this paper.

Materials and Methods

Experimental material

Chironomus tentans larvae were raised in the laboratory. Salivary glands were isolated from rapidly growing fourth instar larvae. A *C. tentans* epithelial cell line was grown in suspension at 24°C as previously described (Wyss, 1982). For initial screening of a *C. tentans* expression library, we used a polyclonal p50 antibody that was previously raised and characterised by one of us (LO) (Evdokimova et al., 1998).

cDNA cloning

A *C. tentans* salivary gland expression λgt11cDNA library was screened according to Alzhanova-Ericsson et al. (Alzhanova-Ericsson et al., 1996) using a p50 polyclonal antibody. A positive clone with a short p50-like sequence was identified and used as template for PCR amplification to generate a DNA probe. This probe was used to screen a *C. tentans* salivary gland oligo dT-primed λZAP (Stratagene, La Jolla, CA) cDNA library to obtain full-length cDNA clones. Two positive clones were selected to determine the sequence of the two isoforms of p40/50.

DNA sequencing and sequence analysis

The cDNA inserts of positive clones were sequenced and analysed as previously described (Sun et al., 1998).

Bacterial expression and purification of p40

The p40 cDNA sequence from the λZAP cDNA library was amplified by PCR (Boehringer Mannheim Roche Diagnostics Scandinavia, Bromma, Sweden). The oligonucleotides were designed to introduce a *Nco*I restriction site at the 5′ end and a *Hin*dIII site at the 3′ end of the PCR product. The p40 fragment was inserted into the expression vector pET21d, which was transformed into Nova Blue cells (Novagen, Darmstadt, Germany). These cells were grown at 37°C in SOC (LB-Broth, 10 mM $MgSO_4$, 20 mM glucose and 10 mM $MgCl_2$) medium for 60 minutes after heat shock at 42°C for 40 seconds, and grown over night at 37°C on LB-Broth agar plates with ampicillin. The positive clones were sequenced, and the correct unmutated clones were transformed as above into BL21(DE3)pLysS cells (Novagen), using LB-Broth agar plates with ampicillin and chloramphenicol. Expression of the fusion protein was induced by the addition of 1 mM IPTG (Boehringer Mannheim), and the bacteria were grown for another 2.5 hours before chilling and pelleting. The bacteria were then washed, pelleted and stored at –70°C.

The p40 protein was purified under denaturing conditions. The frozen bacteria were thawed and incubated in 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8.0) for 2 hours at room temperature. After pelleting the cell debris, the p40 protein was purified from the supernatant using the Ni-NTA system (Qiagen, GmbH, Germany). The

supernatant was incubated with the Ni-NTA resin for 90 minutes and washed in 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 5.9); the protein was eluted in the same buffer adjusted to pH 4.5. The eluted fraction was dialysed against PBS over night and concentrated using Centriprep MWCO 10 (Amicon, Millipore, Bedford, MA). The p40 protein was used to raise a polyclonal antibody in rabbits.

Affinity purification of antibody

The p40 protein solution was coupled to NHS-activated Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol. After incubation with antibody serum, the Sepharose beads were washed with 0.5 M NaCl, 10 mM NaH_2PO_4 (pH 6.8) in PBS to remove unbound proteins. The antibody was eluted with 100 mM glycine-HCl (pH 3.0), which was neutralised immediately with one-tenth volume of 1 M NaH_2PO_4 (pH 8.0). The eluted fractions were dialysed against PBS and stored in PBS with 0.02% sodium azide.

SDS-PAGE and western blot analysis

Isolated salivary glands were transferred to sample buffer (25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromophenol blue, 600 mM Tris-HCl, pH 6.8) and frozen in liquid nitrogen. The frozen sample was homogenised with a mini-homogeniser and boiled.

Extracts were prepared from whole or fractionated tissue culture cells. To obtain a total cell extract, tissue culture cells were pelleted, washed in PBS and resuspended in sample buffer, then vortexed and boiled. To obtain nuclear and cytoplasmic extracts, the cells were washed twice in cold PBS, resuspended in cold TNM buffer (10 mM triethanolamine-HCl, pH 7.0, 100 mM NaCl and 1 mM $MgCl_2$) containing 0.2% NP40 and Complete protease inhibitor cocktail (Roche Diagnostics Scandinavia, Bromma, Sweden), homogenised in a glass tissue grinder provided with a tight-fitting pestle and centrifuged at 2000 ***g*** for 10 minutes at 4°C. The resulting supernatant constituted the cytoplasmic extract and the proteins were precipitated in acetone. The pellet, which contained the nuclei, was washed once in TNM buffer with the protease inhibitor cocktail and further resuspended in PBS with the protease inhibitor cocktail, sonicated, RNase A treated (final concentration 30 ng/ml) and centrifuged at 14,000 ***g*** at 4°C. The supernatant, designated the nuclear extract, was precipitated in acetone. The extracts were dissolved in sample buffer before electrophoresis.

The samples were analysed by electrophoresis in a 10% or 12% polyacrylamide gel containing 0.1% SDS. Western blot analysis was performed as described previously by Sun et al. (Sun et al., 1998). Both the anti-p40/50 serum and pre-immune serum were used in a 1:10,000 dilution.

Immunocytology of salivary glands

Immunostaining on semi-thin cryosections of salivary glands was performed as previously described (Visa et al., 1996a). The salivary glands were fixed in 4% formaldehyde, cryoprotected with 2.3 M sucrose, frozen and sectioned with an Ultracut S/FC4S (Reichert, Leica, Austria). The sections were blocked in 2% BSA in PBS-glycine. The first antibody was either the anti-p40/50 serum or the pre-immune serum (negative control) diluted 1:150, and an antibody against rabbit immunoglobulin G conjugated to 6 nm gold particles (Amersham) diluted 1:50 was used as secondary antibody. The immunogold labelling was silver-enhanced with IntenSETMM (Amersham).

Immunocytology of isolated polytene chromosomes

The isolation of polytene chromosomes from salivary glands, the RNase treatment of the chromosomes and the immunocytological analysis have been previously described (Sun et al., 1998). The affinity-purified anti-p40/50 antibody was used diluted 1:50.

Immunoelectron microscopy of salivary glands

Immunoelectron microscopy was essentially carried out according to the cryomethod described by Visa et al. (Visa et al., 1996a). The specimens were prepared as described above for light microscopy, with the addition of 0.1% glutaraldehyde in the fixation buffer. Ultrathin cryosections were treated with 10% new-born calf serum before incubation with the antibody solutions. The affinity-purified anti-p40/50 antibody and the pre-immune serum were used at a 1:500 dilution. After immunolabelling, the sections were stained with 2% aqueous uranyl acetate and embedded in 4% polyvinyl alcohol (9-10 kDa, Aldrich, WN) provided with 0.4% aqueous uranyl acetate. The specimens were examined and photographed in a Philips CM 120 microscope at 80 kV.

Immunoelectron microscopy of isolated polytene chromosomes

The polytene chromosomes were prepared as described above, and the immunoreaction was carried out the same way, except that the anti-p40/50 serum and the pre-immune serum were used at a 1:500 dilution. The specimens were post-fixed, dehydrated, sectioned and stained as previously described (Björkroth et al., 1988).

UV crosslinking of RNA–protein complexes

UV irradiation was performed as previously described (Pinol-Roma et al., 1989) and specified by Visa et al. (Visa et al., 1996a).

Results

Identification of a p50-like Y-box protein in *C. tentans*

A *C. tentans* salivary gland cDNA expression library was screened using a polyclonal antibody against the p50 protein in rabbit reticulocytes (Evdokimova et al., 1998). Positive cDNA clones were isolated and sequenced, and a p50-like protein sequence was identified (accession numbers: AF530577 and AF530578). The corresponding protein was designated Ct-p40/50, or p40/50 for short. The name alludes to our finding that there are two isoforms of the protein –p40 and p50 – which are named on the basis of their electrophoretic mobilities (see below). The amino acid sequences of the two isoforms are presented in Fig. 1A. The shorter isoform, p40, consists of 264 amino acids (28 kDa) and the longer one, p50, has 317 amino acids (34 kDa). The two isoforms share the first 258 amino acids but differ in their most C-terminal ends. The specific C-terminal ends of p40 and p50 have six amino acids and 59 amino acids, respectively (Fig. 1A).

The p40/50 protein shows high sequence homology to the rabbit reticulocyte p50 protein (and human YB-1) and other proteins of the Y-box protein family (Wolffe et al., 1992; Wolffe, 1994; Sommerville and Ladomery, 1996a; Matsumoto and Wolffe, 1998; Graumann and Marahiel, 1998; Sommerville, 1999; Evdokimova and Ovchinnikov, 1999). The p50 isoform of p40/50, which is about the same length as the rabbit p50 protein (317 and 324 amino acids, respectively), has an amino acid sequence that is 47% identical to that of the rabbit p50 protein. Furthermore, p40/50 displays the same three-domain organisation as rabbit p50 and other Y-box

proteins: an N-terminal domain, a central cold-shock domain (CSD) and a C-terminal domain (Fig. 1B).

The N-terminal domain is rich in alanine and proline, as is the same domain in rabbit p50 (Fig. 1A). It has been proposed that this domain could be responsible for the ability of rabbit p50 to bind to actin filaments in the cytoplasm (Ruzanov et al., 1999).

The CSD of p40/50 comprises 74 amino acids (55-128; boxed region in Fig. 1A) and is 81% identical to the CSD in rabbit p50, suggesting that this domain in particular is highly conserved during evolution. Two RNA binding motifs, RNP1 and RNP2, are present in the p40/50 protein like in other CSDs (Matsumoto and Wolffe, 1998). The CSD is known to be responsible for the nucleic acid binding activity of p50 and other Y-box proteins (Wolffe et al., 1992; Graumann and Marahiel, 1998).

The C-terminal domain of p40/50 is rich in glycine and arginine and contains many RG repeats (Fig. 1A), which is a characteristic feature of invertebrate Y-box binding proteins, e.g. the *Drosophila yps* protein (Thieringer et al., 1997), the *Schistosoma* SMYB1 protein (Franco et al., 1997) and the planarian DjY1 protein (Salvetti et al., 1998). However, its similarity to the C-terminal domain of the mammalian p50 protein is also striking – both have alternating acidic and basic regions and several clusters of arginine (Fig. 1A). The alternating charge distribution is usually not observed in invertebrate Y-box binding proteins (e.g. Thieringer et al., 1997). Thus, the C-terminal domain of Ct-p40/50 shares features with both vertebrate and invertebrate Y-box binding proteins.

The two p40/50 isoforms differ in their length of the C-terminal tail but both show the same high content of glycine and arginine throughout the entire length of the tail (Fig. 1A). It has been proposed that the C-terminal domain can contribute to RNA binding through an interaction between its positive charge (which is due to the presence of arginine clusters), and the negatively charged RNA phosphate groups (Bouvet et al., 1995; Ladomery and Sommerville, 1994; Murray, 1994). A high glycine content with several RGG repeats is also likely to promote binding to RNA – in particular, in combination with other types of RNA-binding domains (Burd and Dreyfuss, 1994). Furthermore, the C-terminal domain is thought to be responsible for protein–protein interactions due to the alternating modules of acidic and basic amino acids (Wolffe et al., 1992; Swamynathan et al., 1998). Finally, the C-terminal domain also seems to mediate self-multimerisation by protein–protein interactions (Wolffe et al., 1992; Evdokimova and Ovchinnikov, 1999).

Generation of a specific anti-p40/50 antibody

The rabbit p50 antibody recognised not only the *C. tentans* p40/50 protein but also a few additional proteins in western blot analysis (data not shown). Therefore, we had to raise a more specific antibody for studying the intracellular distribution of p40/50 in *C. tentans*. The λZAP p40 cDNA sequence was amplified by PCR and inserted into a pET21 expression vector. The p40 isoform was expressed, purified and used to raise a polyclonal antibody. The specificity of the antibody was tested by western blot analysis of salivary gland and tissue culture cell extracts using the anti-p40/50 serum (Fig. 2A). In the salivary gland extract (lane 2), two bands were recorded and the corresponding proteins were designated p40 and p50 according to their apparent molecular masses in kDa. The more rapidly migrating protein probably corresponds to the p40 isoform of p40/50, given that it migrated in the gel as the recombinant p40 isoform (lane 1). The more slowly migrating protein was identified as the p50 isoform by a p50-specific antibody (D.N., T.S., Elizaveta Kovrigina, L.O. and B.D., unpublished). The apparent molecular masses (40 and 50 kDa) are evidently considerably higher than the values predicted for the two isoforms from the amino acid sequences (28 and 34 kDa, respectively). Such an aberrant migration is probably due to a

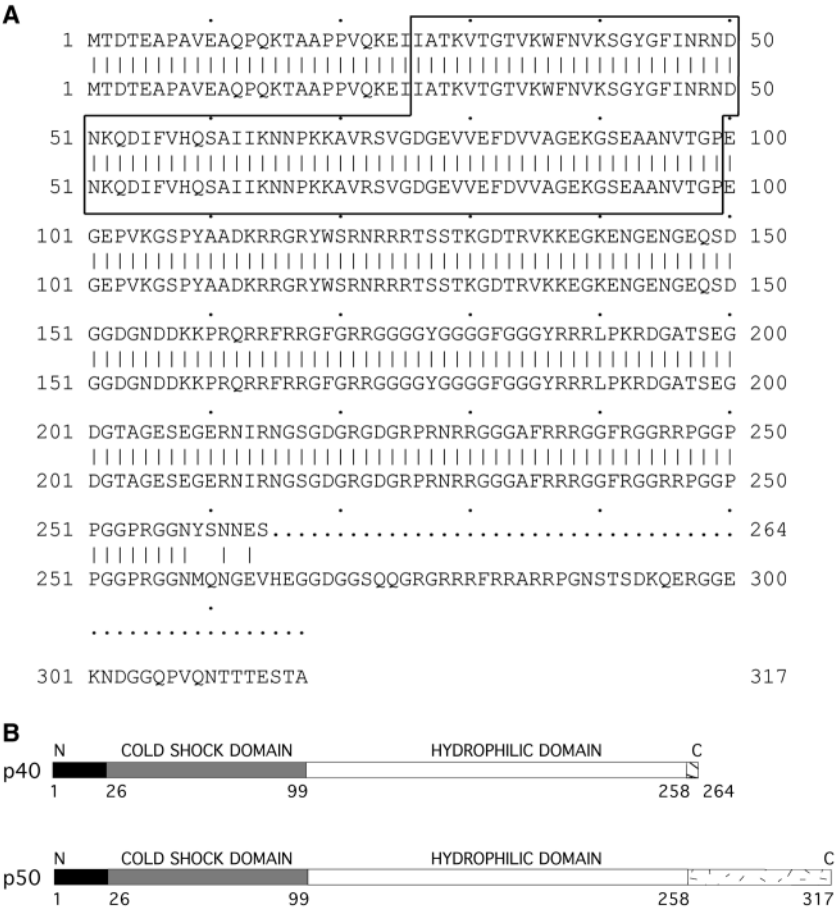

Fig. 1. Predicted amino acid sequences of the two isoforms of the *C. tentans* p40/50 protein. (A) The upper sequence corresponds to the p40 isoform and the lower sequence corresponds to the p50 isoform. The sequences are identical for the first 258 amino acids. The predicted cold-shock domain is boxed. (B) Schematic presentation of the domain organisation of the two isoforms of the p40/50 protein.

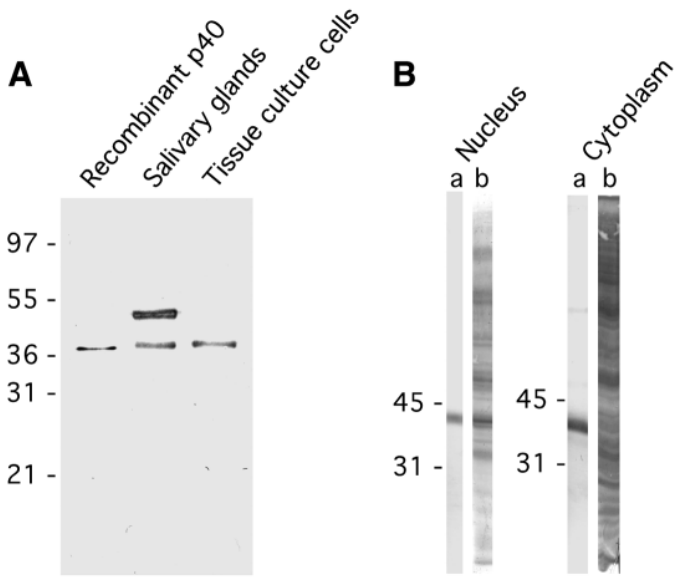

Fig. 2. Western blot analysis of the p40/50 protein. (A) Comparison of p40/50 in *C. tentans* salivary glands and tissue culture cells (total extracts). Recombinant p40 served as control. An anti-p40/50 serum was used as primary antibody and an alkaline phosphatase-conjugated antibody as secondary antibody. (B) Demonstration of p40/50 in nuclear and cytoplasmic extracts from *C. tentans* tissue culture cells. The whole nuclear extract and 1/10 of the cytoplasmic extract were loaded onto SDS-polyacrylamide gels and blotted. The blots were either immunostained using an affinity-purified p40/50 antibody followed by an alkaline phosphatase-conjugated antibody (a), or stained with Coomassie blue (b). The positions of molecular mass standards are shown to the left in kilodaltons.

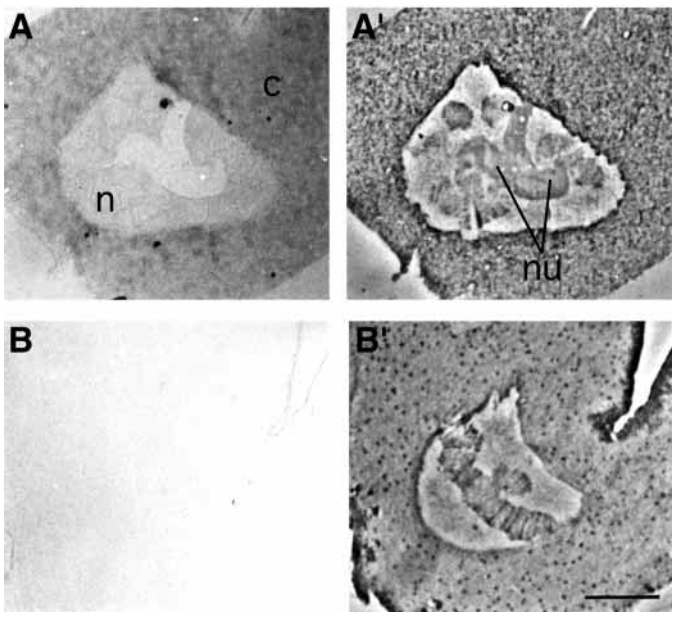

Fig. 3. Immunocytological localisation of p40/50 in *C. tentans* salivary gland cells. Semi-thin cryosections of salivary glands were prepared and immunostained with an affinity-purified antibody to p40/50 (A,A′) or a pre-immune serum (B,B′) as primary antibody and a gold-conjugated antibody as secondary antibody. The immunogold labelling was silver-enhanced and photographed both in bright-field (A,B) and phase-contrast (A′,B′) microscopy. n, nucleus; c, cytoplasm; nu, nucleolus. Bar, 20 μm.

high content of charged amino acids and has been observed for p50 and other Y-box proteins (e.g. Murray et al., 1992). Apart from the two p40/50 bands, the anti-p40/50 antibody recognised no other proteins. In the tissue culture cell extract, only the p40 isoform was observed (lane 3). We conclude that the newly raised polyclonal p40/50 antibody is specific to the p40/50 protein.

To reduce the background in subsequent immunocytological and immunoelectron microscopy experiments, the serum antibody was affinity-purified on p40-Sepharose. The purified antibody showed the same specific immunolabelling pattern as the anti-p40/50 serum in western blots (Fig. 2B and data not shown).

p40/50 is present in the nucleus and abundant in the cytoplasm

To elucidate the cellular distribution of p40/50, we first studied nuclear and cytoplasmic extracts from *C. tentans* tissue culture cells by western blotting (Fig. 2B). The protein is predominantly located in the cytoplasm (note that only 1/10 of the cytoplasmic extract was loaded onto the gel in Fig. 2B), but it is also present in the cell nucleus.

To further study the intracellular distribution of p40/p50, salivary gland cryosections were immunolabelled with the p40/50 antibody and a gold-conjugated secondary antibody, silver-enhanced, and studied under the light microscope (Fig. 3A). The cytoplasm (c) was heavily stained, confirming that the p40/50 protein is abundant in the cytoplasm. A weaker and nonuniform immunolabelling was detected in the nucleus (n). This staining seemed to correspond to the positions of the chromosomes and the nucleoplasm, whereas the nucleoli (nu) were unstained. The pre-immune serum was used as negative control and did not show any labelling (Fig. 3B). Thus, the immunocytology study of the salivary gland cells confirmed the cell fractionation result that p40/50 is abundant in the cytoplasm and more sparsely in the nucleus. However, the distribution in the nucleus is uneven; p40/50 is located on the polytene chromosomes and in the nucleoplasm but seems to be absent from the nucleoli.

p40/50 is present in Balbiani rings and other chromosomal puffs and is associated with RNA

As our study was crucially dependent on the appearance of p40/50 in the Balbiani rings (BRs), we continued to investigate the localisation of p40/50 on the isolated giant salivary gland chromosomes using immunocytology. Fig. 4 shows the immunolabelling of two of the four chromosomes: chromosome IV with the giant puffs BR1, BR2 and BR3 (Fig. 4A), and chromosome I (B). The BRs and a large number of smaller puffs along the chromosomes were stained (Fig. 4A,B), but not the nucleoli on chromosomes II and III (data not shown). The pre-immune serum was used as negative control (Fig. 4C,D). To determine whether p40/50 was associated with RNA in the BRs and the other puffs, isolated chromosomes were also treated with RNase (Fig. 4E,F). The immunosignal was essentially abolished by this treatment, showing that the

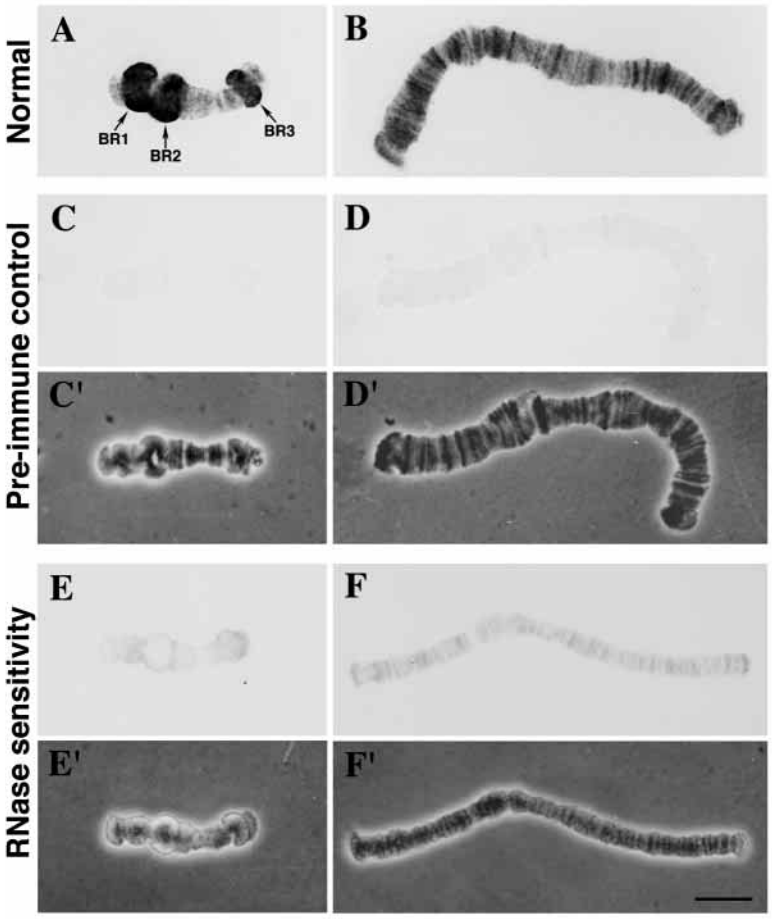

Fig. 4. Immunolocalisation of the p40/p50 protein on isolated chromosomes from *C. tentans* salivary gland cells. Polytene chromosomes were isolated and incubated with an affinity-purified p40/p50 antibody (A,B) or pre-immune serum (C,C′ and D,D′), and subsequently incubated with a gold-conjugated secondary antibody. The immunogold labelling was silver-enhanced, and the specimens were photographed both in bright-field (A-F) and phase-contrast (C′-F′) microscopy. Some chromosomes were RNase A treated before immunolabelling (E,E′ and F,F′). A,A′,C,C′,E,E′, chromosome IV; B,B′,D,D′F,F′, chromosome I. The three Balbiani rings (BR1-3) have been indicated in (A). Bar, 10 μm.

presence of p40/50 in the puffs was dependent on RNA. Thus, p40/50 is likely to be associated with RNA at a large number of transcription sites, including the Balbiani rings.

p40/50 is associated with BR mRNA during assembly and transport

To further study the relation of p40/50 to the specific transcription product generated in the BRs, the BR RNP particles, we performed immunoelectron microscopy on cryosections from salivary glands. Gold particles (arrows), indicating the position of the p40/50 proteins, were detected in the BRs (Fig. 5A), the nucleoplasm (Fig. 5B) and abundantly in the cytoplasm (Fig. 5C). The immunosignal in the BRs, nucleoplasm and cytoplasm was determined relative to that in a pre-immune serum control, and it amounted to 10×, 5× and 75×, respectively, that of the control. The signal in the nucleoli was close to background. Thus, the overall distribution of the immunolabelling examined at the ultrastructural level confirmed that p40/50 was present in chromosome puffs, in the nucleoplasm and abundantly in the cytoplasm, but absent from the nucleoli.

The ultrastructural analysis was then focused on the BR RNP particles. As shown schematically in Fig. 5, lower part, the BR RNP products can be seen in statu nascendi in the BRs and after release from the genes as granules, 50 nm in diameter, in the nucleoplasm. The particles unfold when passing through the nuclear pore, and the exiting mRNP fibre associates immediately with ribosomes to form polysomes, which anchor to the endoplasmic reticulum (ER) (for a review, see Daneholt, 2001). Both growing RNP particles (Fig. 5A) and released particles (Fig. 5B) are immunolabelled (barred arrows in Fig. 5A,B, respectively). The label in the cytoplasm is to a large extent, but not exclusively, localised at the tubular ER, but as the unfolded mRNP fibril cannot be identified in the cytoplasm, it is not possible to show directly that the immunolabel is due to p40/50 bound to mRNP in polysomes. We conclude that p40/50 becomes bound to BR pre-mRNA co-transcriptionally and remains bound to the released BR RNP particles in the nucleoplasm.

p40/50 is added along the BR transcript during transcription

To further investigate the behaviour of p40/p50 during the assembly of the BR particle on the gene, polytene chromosomes were isolated and studied by immunoelectron microscopy. A pre-embedding immunolabelling procedure was used, giving essentially no background labelling (50× lower than the specific immunosignal).

An overview of a BR region is shown in Fig. 6. Proximal (p), middle (m) and distal (d) portions of the BR genes have been indicated in the figure and should be compared with the schematic drawing presented in Fig. 6B. In the proximal region the RNP filament is folded into an RNP fibre, whereas in the middle and distal regions the 5′ end of the filament is being further packed into a globular structure, which is growing in size along the gene.

The immunolabel is present in all three segments of the BR gene (Fig. 6A). We also show examples of proximal (Fig. 6C), middle (Fig. 6D,E) and distal (Fig. 6F,G) regions, along with interpretations. We noted that immunolabel is present in all segments and to approximately the same extent. As the label appears already early in the proximal region (Fig. 6C), p40/50 is present at or close to the 5′ end of the transcript. It is, however, not restricted to this region (cf. e.g. the exclusive 5′ end labelling of the cap-binding protein CBP20 in Visa et al. (Visa et al., 1996b). The gold particles seem rather to be randomly bound to the growing BR particles, indicating that p40/50 appears along the entire RNA molecule. As the degree of labelling is not increased along the gene it seems likely that the epitope becomes to a large extent concealed during the packing of the RNP filament into a compact particle. The p40/p50 labelling pattern

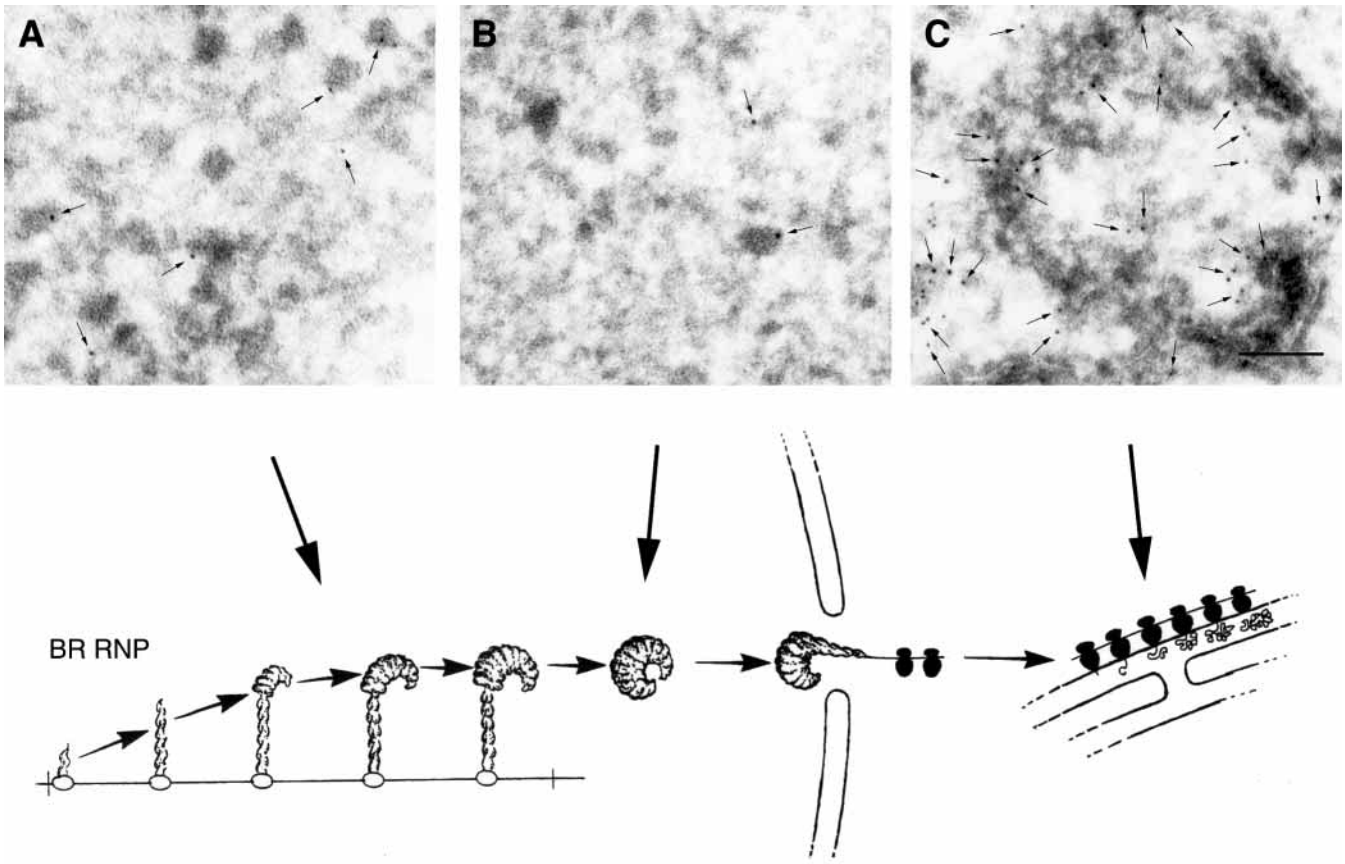

Fig. 5. Immunoelectron microscopic localisation of p40/50 in Balbiani rings (A), nucleoplasm (B) and cytoplasm (C) in salivary gland cells. Ultrathin cryosections of salivary glands were incubated with a p40/50 antibody, a gold-conjugated secondary antibody was added to visualise the labelled sites, and the sections were finally embedded in polyvinyl alcohol. Small arrows indicate the position of gold particles; the barred arrows denote specifically labelled BR particles. A schematic drawing outlining the assembly and transport of BR particles is shown below the electron micrographs. Large arrows relate the electron micrographs to the corresponding stage during mRNA biogenesis. Bar, 100 nm.

would then be similar to that shown earlier for the hnRNP A1-like hrp36 (Visa et al., 1996a), which is known to be distributed along the RNA molecule (Kiseleva et al., 1997). It is not excluded that all the p40/50 protein is added early during transcription and subsequently continuously redistributed along the growing transcript, but we regard this as less probable. Thus, we conclude that p40/p50 is added to the RNA transcript early during transcription and is probably further added to the growing transcript in parallel with the growth of the transcript.

p40 is bound to poly(A) RNA both in nucleus and cytoplasm

To determine whether p40/50 is connected to poly(A) RNA both in the nucleus and the cytoplasm, we carried out UV crosslinking experiments with *C. tentans* tissue culture cells following the procedure devised by Pinol-Roma et al. (Pinol-Roma et al., 1989). The cultured cells were irradiated with UV light and separated into cytoplasmic and nuclear fractions. The samples were treated with 0.5% SDS at 65°C, and the poly(A) RNA with its covalently bound proteins was isolated with oligo-(dT) chromatography. The RNA was degraded with RNase, and the proteins further studied by SDS-PAGE and western blot analysis. The previously studied hnRNP protein hrp36 (Visa et al., 1996a) binds to mRNA and serves as a positive control in this study. Non-UV-irradiated cells were studied in parallel. The results showed that poly(A) RNA bound to the p40/50 protein in both the nuclear and the cytoplasmic fractions after UV treatment (Fig. 7A). Neither hrp36 nor p40/50 copurified with RNA when the UV irradiation was omitted (Fig. 7B). Thus, the p40/50 protein is directly bound to poly(A) RNA both in the nucleus and in the cytoplasm of living cells.

General conclusion

The immunoelectron microscopy results showed that p40/50 is added along the BR transcript concomitant with transcription, and it accompanies the completed and released BR mRNA into the nucleoplasm. The p40/50 protein is abundant in the cytoplasm and, to a considerable extent, located at the ER, where BR mRNA resides in the cytoplasm. As the crosslinking experiment showed that p40/50 is bound to poly(A) RNA both in the nucleus and the cytoplasm, it seems plausible that p40/50 remains associated with the mRNA also during the exit into the cytoplasm and during the protein synthesis at the ER.

Discussion

Structure and putative role of p40/50

The *C. tentans* p40/50 resembles rabbit p50 (human YB-1)

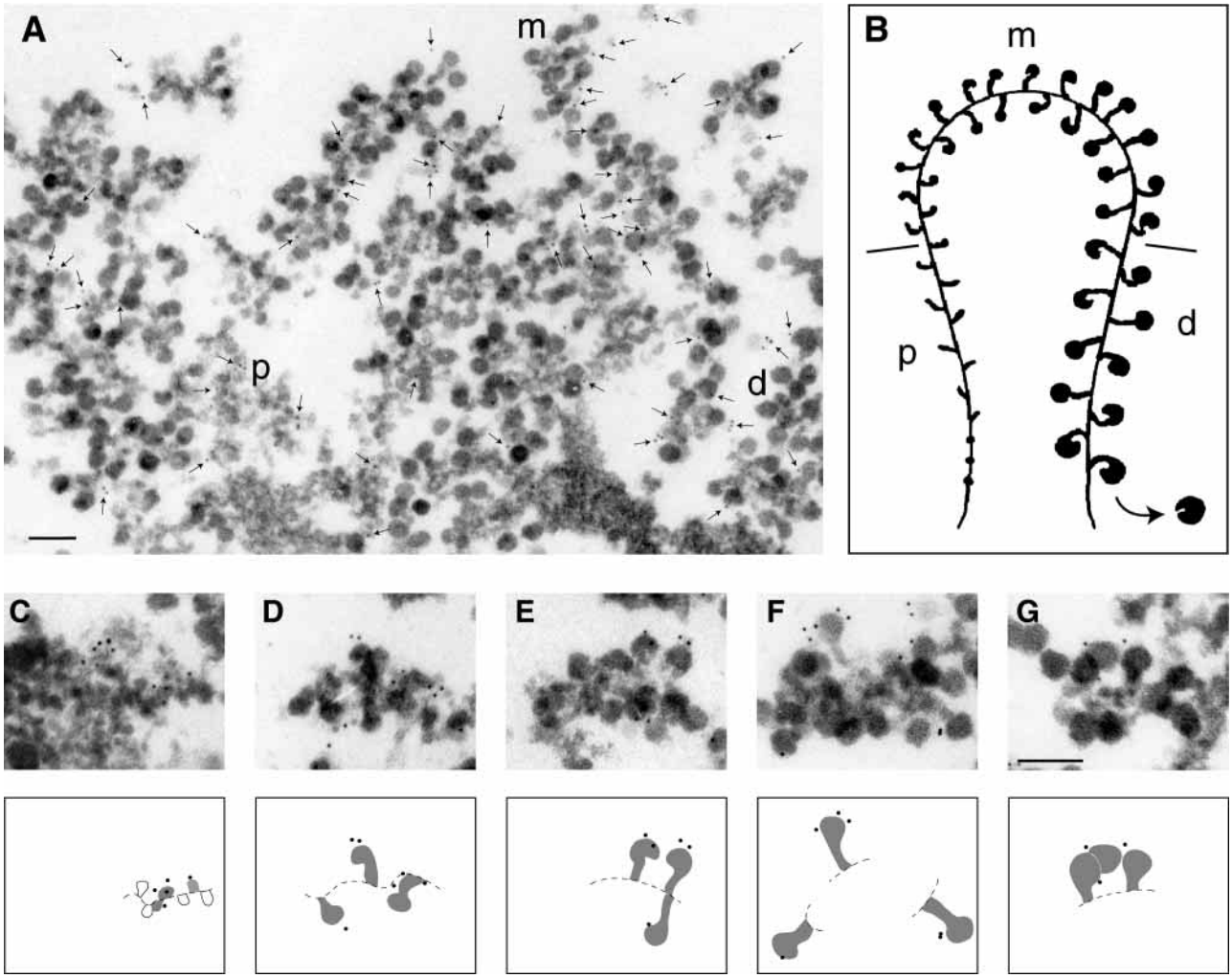

Fig. 6. Immunoelectron microscopic localisation of p40/50 along a transcriptionally active BR gene. Polytene chromosomes were isolated, incubated with a p40/50 antibody and detected with a secondary antibody conjugated to 12 nm colloidal gold markers. (A) Section through a Balbiani ring showing several segments of active genes. Examples of proximal (p), middle (m) and distal (d) segments have been indicated. Arrows mark the position of gold particles. (B) Schematic drawing of an active BR gene. Growing particles in the promoter-proximal (p), middle (m) and promoter-distal (d) portions of the gene are shown, as well as the chromatin axis. (C-G) Immunolabelled proximal (C), middle (D,E) and distal (F,G). segments of BR genes. Schematic drawings are shown below the electron micrographs, with examples of labelled nascent RNPs (dark) and unlabelled RNPs (outlined). The chromatin axis is indicated by a short dashed line. Bars, 100 nm.

(overall amino acid sequence identity 47%) (Wolffe et al., 1992; Evdokimova et al., 1995) and exhibits a similar three-domain organisation with an N-terminal domain rich in alanine and proline, a central highly conserved CSD (74 amino acids; 81% sequence identity), and a C-terminal domain with alternating acidic and basic regions and clusters of arginine. The rabbit p50 (and human YB-1) belongs to the Y-box binding protein family, which comprises many proteins involved in transcriptional control, DNA repair and replication, as well as in mRNA translation, storage and localisation (for reviews, see Wolffe, 1994; Sommerville and Ladomery, 1996a; Evdokimova and Ovchinnikov, 1999). The main common feature of these proteins is the central CSD.

The Y-box binding proteins are capable of binding to one or more types of nucleic acids: single- or double-stranded DNA or RNA (Murray, 1994; Ladomery and Sommerville, 1994). We have strong evidence that Ct-p40/50 is bound to RNA. The p40/50 protein appears to be associated with transcriptionally active regions, the chromosome puffs, and it is released from isolated chromosomes on RNase treatment, suggesting that p40/50 is associated with RNA. In addition, the protein appears in growing RNP particles on the BR genes and remains associated with the released BR RNP particles in the nucleoplasm. Furthermore, the recombinant Ct-p40 and Ct-p50 proteins bind to BR RNA in gel-shift experiments (D.N., T.S., Elizaveta Kovrigina, L.O. and B.D., unpublished). Finally, our crosslinking experiments showed that p40/50 is bound to both nuclear and cytoplasmic poly(A) RNA in vivo. There is no p40/50 in the nucleolus, suggesting that it is not present on RNA polymerase I transcripts. Thus, p40/50 is bound to RNA polymerase II transcripts in *C. tentans*.

The Ct-p40/50 and p50 (YB-1) are not only structurally similar but also show the same cellular distribution, i.e. they

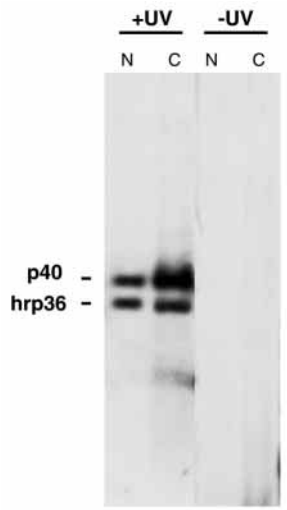

Fig. 7. UV-crosslinking of p40/50 to nuclear and cytoplasmic poly(A) RNA in vivo. Nuclear (N) and cytoplasmic (C) extracts were prepared from both UV-irradiated (+UV) and control (–UV) tissue culture cells. The crosslinked proteins were collected with oligo-(dT) chromatography, fractionated by SDS-PAGE and analysed by western blotting. Antibodies against p40/50 and hrp36 (positive control) were used in the western blot.

are both predominantly cytoplasmic. Furthermore, they are both bound to poly(A) RNA in the cytoplasm and are present in polysomes. It seems likely, therefore, that Ct-p40/50 has a role in translation initiation similar to that of rabbit p50 (see Introduction).

We conclude that the *C. tentans* p40/50 protein resembles the rabbit p50 protein and is a typical member of the Y-box binding protein family. Like rabbit p50, the p40/50 protein binds to RNA polymerase II transcripts and is predominantly located in the cytoplasm; presumably, it is involved in the regulation of translation.

p40/50 accompanies mRNA from gene to polysomes

Western blot analysis and immunocytology showed that the p40/50 protein is present both in the nucleus and in the cytoplasm. The immunocytology analysis of isolated polytene chromosomes further argued that the protein is already added to pre-mRNA during transcription, suggesting that p40/50 is coupled to the mRNA sequence during the whole lifespan of the mRNA. Such a hypothesis could be directly tested in the *C. tentans* system by analysis of a specific transcription product, the BR mRNP particles.

By applying immunoelectron microscopy, we found that the p40/50 protein becomes associated with the growing transcripts on the BR genes already in the proximal portion of the gene, and is added all along the growing transcript. In this context, it should be pointed out that the Y-box proteins FRGY2a/b have also been detected on nascent transcripts along lampbrush loops in *Xenopus* oocytes (Sommerville and Ladomery, 1996b). In *C. tentans*, the p40/50 protein could be observed bound to the BR particles when these appear in the nucleoplasm. The BR mRNA is further translocated as an RNP complex through the nuclear pores, and appears in the cytoplasm, where the BR mRNA is immediately engaged in protein synthesis (Mehlin et al., 1992). The giant BR mRNA-containing polysomes are associated with the rough ER and are responsible for the synthesis of the major protein product of the salivary gland cells, the giant-sized secretory proteins (Case and Wieslander, 1992). The unfolded BR mRNP fibril could not be visualised in the cytoplasm, and therefore, it is not possible to relate p40/50 to the BR RNA-containing polysomes directly by using the electron microscope. However, we could observe that p40/50 is located mostly at the rough ER in the salivary gland cells. Furthermore, as the UV crosslinking experiment showed that p40/50 is coupled to poly(A) RNA in both the nucleus and the cytoplasm of tissue culture cells, it seems likely that the ER-associated p40/50 is bound to the BR mRNA involved in the synthesis of secretory proteins. We conclude that the p40/50 protein is probably coupled to the BR RNA all the way from the gene via the nuclear pores to the polysomes located on the ER.

The fact that many – maybe all – RNA polymerase II transcripts are loaded cotranscriptionally with p40/50 suggests that not only BR mRNA is associated with p40/50 in the cytoplasm. We noted that cytoplasmic poly(A) RNA readily forms crosslinks with p40/50 in tissue culture cells, further supporting such a notion. Moreover, p40/50 is present in all the larval tissues studied (salivary glands, stomach, intestine, colon, Malpighian tubules and imaginal disks) (D.N., T.S., Elizaveta Kovrigina, L.O. and B.D., unpublished). Thus, not only BR mRNA, but probably many more mRNAs leave the nucleus associated with p40/50.

p40/50 and cotranscriptional assembly of pre-mRNP

As the immunolabelling of the polytene chromosomes showed that p40/50 is added onto a large number of growing primary transcripts, it is believed to participate in the formation of pre-mRNP (hnRNP) complexes in general. The detailed analysis of the long nascent transcripts on the BR genes further showed that p40/50 is being added along the growing transcripts and is not restricted to the 5′ end region, for example. This is a distribution similar to that previously recorded for several important hnRNP proteins (Daneholt, 2001). In fact, there are several striking similarities between the p40/50 homologue, the reticulocyte p50 protein and the A/B type hnRNP proteins – the predominant RNA-binding proteins in the pre-mRNP complexes (Dreyfuss et al., 1993; Krecic and Swanson, 1999). Although p50 and A/B type hnRNP proteins have different amino acid sequences, they both have at least one RNA-binding domain of the RNP consensus type (CS-RBD, also called RNA recognition motif, RRM) and a C-terminal auxiliary domain with both RNA- and protein-binding ability (for a review, see Dreyfuss et al., 1993). Like hnRNP proteins, p50 melts secondary structures, facilitates annealing of complementary RNA and shows little or no sequence specificity for RNA (see Introduction). It seems likely, therefore, that p40/50, like the A/B type hnRNP proteins, is bound to many different RNA sequences and packages the RNA into an RNP fibril, which will constitute the substrate on which the various molecular machines, such as those of splicing, transport and translation, are likely to operate.

Initially, it was believed that the hnRNP proteins were essentially functioning in a more global manner as general packaging proteins, but more and more information is accumulating to suggest that the hnRNP proteins exert more specific roles apart from packaging the RNA (e.g. Krecic and Swanson, 1999). For example, the hnRNP A1 can influence the outcome of alternative splicing (Mayeda and Krainer, 1992; Mayeda et al., 1994) and probably also mediates mRNA transport (Michael et al., 1995). Another hnRNP protein, hnRNP C, contains a nuclear retention signal, and thus could

act as a regulator of export of RNA (Nakielny and Dreyfuss, 1996), whereas others, including hnRNP A1, K and E, enter cytoplasm and affect protein synthesis, mRNA localisation and mRNA stability (Krecic and Swanson, 1999). In the BR system, the hnRNP A1-like hrp36 enters polysomes and remains there during translation, which suggests that it plays a defined role during translation (Visa et al., 1996a). It has been suggested that general RNA-binding proteins like hnRNPA1 can improve the efficiency of translation by suppressing false initiations of translation along the message (Svitkin et al., 1996). Thus, it seems that the hnRNP proteins are not only packaging proteins –they can also play many, and even multiple, roles in gene expression. Regarding p50 (YB-1), there is evidence that the protein can affect splicing in vertebrate cells (Chansky et al., 2001). However, the most important role of p50 seems to be to regulate translation (see Introduction). As many mRNAs, maybe all, are concerned (Evdokimova et al., 1995; Davydova et al., 1997), p50 exerts a global effect on translation and presumably it acts by governing the organisation of the mRNA in polysomes and free mRNP particles (see Evdokimova and Ovchinnikov, 1999). It is plausible, but remains to be shown, that p40/50 behaves like p50 (YB-1).

The general picture emerging from analysis of hnRNP proteins and which is further strongly supported by the present study of *C. tentans* p40/50 is that the post-transcriptional fate of an mRNA could, to a large extent, be determined when the primary transcription product, the pre-mRNP complex, is assembled (Daneholt, 2001). A similar conclusion has been reached by Wolffe and co-workers in studies of the oocyte-specific Y-box protein FRGY2 in *Xenopus* (for a review, see Matsumoto and Wolffe, 1998). Clearly, the RNP structure regulates many co- and post-transcriptional events in the cell nucleus (Dreyfuss et al., 1993; Krecic and Swanson, 1999). However, as some RNA-binding proteins like p40/50 and the hnRNP A1-like hrp 36, and perhaps shuttling hnRNP proteins in general, accompany mRNA into cytoplasm, it is possible that at least some structural features in pre-mRNP are maintained in cytoplasmic mRNP, governing the functional options for mRNA (see Matsumoto and Wolffe, 1998). Thus, the present study of p40/50 highlights the possibility that the cotranscriptional loading of RNA-binding proteins onto pre-mRNA could represent an early programming of the transcript and influence not only post-transcriptional processes in the nucleus but also the fate of mRNA in the cytoplasm.

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