A Novel Ded1-like RNA Helicase Interacts with the Y-box Protein ctYB-1 in Nuclear mRNP Particles and in Polysomes*

Received for publication, January 10, 2006, and in revised form, March 17, 2006. Published, JBC Papers in Press, March 22, 2006, DOI 10.1074/jbc.M600262200

Dmitri Nashchekin 1, Jian Zhao 2, Neus Visa 3, and Bertil Daneholt 4

From the Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, SE-171 77 Stockholm, Sweden

We have characterized a novel mRNA-binding protein, designated hrp84, in the dipteran Chironomus tentans and identified it as a DEAD-box RNA helicase. The protein contains the typical helicase core domain, a glycine-rich C-terminal part and a putative nuclear export signal in the N terminus. The protein belongs to the Ded1 subgroup of DEAD-box helicases, which is highly conserved from yeast (Ded1p) to mammals (DDX3). In tissue culture cells, hrp84 is present both in the nucleus and cytoplasm and, as shown by in vivo UV cross-linking, is bound to mRNA in both compartments. Immunoprecipitation experiments revealed that hrp84 is associated with the C. tentans homologue (ctYB-1) of the vertebrate Y-box protein YB-1 both in the nucleus and cytoplasm, and the two proteins also appear together in polysomes. The interaction is likely to be direct as shown by in vitro binding of purified components. We conclude that the mRNA-bound hrp84:ctYB-1 complex is formed in the nucleus and is translocated with mRNA into the cytoplasm and further into polysomes. As both Ded1 and YB-1 are known to regulate the initiation of translation, we propose that the RNA helicase-Y-box protein complex affects the efficiency of mRNA translation, presumably by modulating the conformation of the mRNP template.

RNA helicases of the DEAD-box family and related families (collectively known as DEXH/H proteins) are involved in many different processes concerning RNA metabolism such as RNA splicing, ribosome biogenesis, RNA transport, translation, and RNA turnover, and they are found in all eukaryotes and most prokaryotes (1–3). RNA helicases are enzymes that unwind double-stranded RNA (dsRNA) molecules in an energy-dependent fashion by hydrolysis of NTP, preferentially ATP. The DEAD-box proteins have a highly conserved helicase core domain, which consists of nine motifs (4). These motifs are involved in ATP binding and hydrolysis, RNA binding, and helicase activity. The core element is usually flanked by divergent N- and C-terminal sequences that are responsible for substrate specificity and/or direct the protein to a specific subcellular location (1, 2, 5). Today, over 500 different DEAD-box protein sequences are present in protein databases (3).

Despite extensive studies, the precise mechanism of action of the DEAD-box proteins is still unclear. In contrast to DNA helicases and DEXH proteins, which are capable of unwinding long stretches of dsDNA/RNA, many DEAD-box proteins are rather poor helicases and seem to disrupt only short RNA duplexes (4, 6). Hence, they are often called RNA unwindases to distinguish them from traditional helicases. Besides their ability to melt regions of dsRNA, DEXH/H proteins can displace protein from RNA or rearrange protein-protein or RNA-protein interactions within an RNA protein complex; these effects are referred to as “RNPase” activity (6–9). Interestingly, this can happen independently of the ability of RNA helicases to unwind dsRNA (10).

In vivo most DEAD-box proteins bind with high specificity to RNA-protein complexes (11), but in vitro they show little RNA sequence specificity (4). Moreover, structural and biochemical studies of the related DEXH-box proteins have revealed that RNA helicases bind to the ribose phosphate backbone of RNA rather than to specific nucleotide bases (12, 13). Therefore, it seems likely that RNA helicases recognize not only the RNA but rather the RNA-protein complex as such. In addition, protein cofactors can interact with RNA helicases to promote target recognition and helicase activity (2). RNA helicases often function within very large RNA-protein assemblies, such as ribosomes and spliceosomes, and can interact with protein factors that recruit the helicases to the appropriate assembly and/or modulate the activity (2, 3). Clearly, to fully understand the activity and function of DEAD-box helicases, specific interacting proteins have to be identified and characterized.

Translation provides striking examples of cooperation between RNA helicases and single-stranded RNA-binding proteins (for discussion, see Ref. 14). One example is the RNA helicase CsdA involved in the cold shock response in Escherichia coli (15). Here the helicase unwinds secondary structures in mRNA, whereas the single-stranded-binding protein CspA keeps the RNA in a single-stranded conformation, which results in efficient translation of the mRNA (16). Another example is the interplay in mammalian cells between the eukaryotic translation initiation factors elf4A and elf4B. elf4A is an RNA helicase that unwinds secondary structures in the 5′-untranslated region (5′-UTR) of the mRNA, which enables the small ribosomal subunit to bind to the cap region of the mRNA and scan along the RNA to reach the start codon AUG (17). However, elf4A on its own is a weak helicase and needs a RNA-binding cofactor, elf4B, to work efficiently (18, 19). elf4B binds to RNA and promotes recruitment of the elf4A-containing complex. It is possible that the elf4A:elf4B complex both unwinds the 5′-UTR and maintains the RNA in a single-stranded conformation to allow binding and scanning of the small ribosomal subunit.

It has also been proposed that RNA helicases cooperate with Y-box proteins to suppress translation during oogenesis in amphibians (14) and in Drosophila (20). Y-box proteins are abundant RNA-binding proteins, which act as molecular chaperones and have been implicated in
transcriptional and translational control (21, 22). In amphibians, the silent mRNAs are stored in mRNP particles (23), which contain the Y-box protein FRGY2 (24, 25) and an oocyte–specific DEAD-box RNA helicase, Xp54 (26). FRGY2 is known to suppress translation (27, 28), and Xp54 to modify translation (29). During oogenesis in *Drosophila*, silent mRNPs are transported from nurse cells to the oocyte and further to specific locations within the oocyte. The mRNP particles in transit contain the Y-box protein Yps (Ypsilon Schachtel) (30), which is similar to the mammalian YB-1, and an RNA helicase, Me31B (20), which is homologous to Xp54 in *Xenopus*. It has been shown that the helicase is required to keep the mRNA repressed (20), and the Y-box protein affects the localization and translation of mRNA (31). Although the information is still limited in both *Xenopus* and *Drosophila*, it is close at hand to suggest that the RNA helicase promotes the binding of the Y-box protein to the mRNA and in this way blocks translation. There is, however, no evidence for a direct interaction between the two proteins, and further work is needed to elucidate the proposed molecular interplay between the two proteins.

Y-box proteins are present in mRNP particles not only in germ cells but also in somatic cells (32). As they are involved in translational control in, e.g. rabbit reticulocytes (33), it is an interesting possibility that the Y-box proteins function in cooperation with an RNA helicase in somatic cells just as proposed to be the case for germ cells.

For several years we have studied the structure, assembly, and transport of messenger RNP particles in the salivary glands of the dipteran *Chironomus tentans* (34). The main advantage with this experimental system is that the gene expression process can be visualized in the electron microscope and followed from the generation of messenger RNP (mRNP) on the gene, via the transport of the mRNP to and through the nuclear pores, to the incorporation of the mRNP into the protein-synthesizing polysomes in cytoplasm. The behavior of several RNA-binding proteins has been characterized in this system (see review, see Ref. 34). Recently, a *C. tentans* homologue (Ct-p40/50) of the mammalian Y-box protein YB-1 was studied, and it was shown that this Y-box protein is added to the mRNA co-transcriptionally and accompanies mRNA from the genes in the nucleus all the way into polysomes in the cytoplasm (35).

In the present study, we have identified a novel RNA helicase in *C. tentans*, which belongs to the Ded1 subfamily of DEAD-box helicases, known to be involved in translation initiation (see "Discussion"). The novel helicase has a molecular mass of 84 kDa and is designated Ct-hrp84, or hrp84 for short. We have further investigated whether hrp84 is associated with the YB-1 homologue in *C. tentans* (here called ctYB-1). The hrp84 protein is bound to mRNA in the nucleus and cytoplasm of epithelial cells grown in culture. It is associated with ctYB-1 in both compartments, and the two proteins also appear together in polysomes. *In vitro* experiments with purified proteins suggested that the interaction between the two proteins is direct. Thus, the mRNA-bound complex between hrp84 and ctYB-1 is formed in the nucleus and seem to accompany the mRNA to cytoplasm and presumably exerts its function in polysomes during the translation of the mRNA. We discuss the putative cooperation between RNA helicases and YB-1 in somatic cells and compare this to the silencing action of similar components in oocytes.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture Cells**—*C. tentans* tissue culture cells were cultivated at 24 °C as previously described (36).

**Generation of Monoclonal Antibody 4E11**—A mouse monoclonal antibody (mAb) 4E11 was obtained as previously described by Sun et al. (37). Briefly, hnRNP complexes were immunoprecipitated from a *C. tentans* nuclear RNP extract by mAb 4F9 against hnRNP protein hrp36 and used as antigen for mouse immunization. Positive clones were identified by screening hybridoma supernatants for binding to proteins in the nuclear RNP extract in Western blot analysis. The mAb 4E11, which recognized an 84-kDa protein, was chosen for further analysis.

**Cell Fractionation, Immunoprecipitation, and Western Blot Analysis**—To obtain nuclear and cytoplasmic extracts, tissue culture cells were washed twice in cold PBS, resuspended in cold PBS containing 0.2% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride (PMSF), homogenized, and centrifuged at 2000 × g for 10 min at 4 °C. The resulting supernatant constituted the cytoplasmic extract. The pellet, which contained the nuclei, was washed once in PBS with PMSF, resuspended in PBS with PMSF, sonicated, and centrifuged at 14,000 g for 20 min at 4 °C. The supernatant was retained as the nuclear extract. For RNase treatment of extracts, RNase A (Roche Applied Science) was added at 1 mg/ml and incubated for 20 min at room temperature.

For immunoprecipitation, nuclear, and cytoplasmic extracts were incubated with mAb 4E11 for 3h at 4 °C and then with rat anti-mouse IgM-Sepharose (Zymed Laboratories Inc.) for 1 h at 4 °C. The beads were washed with PBS containing 0.1% Nonidet P-40 and 0.3% sodium deoxycholate. The proteins were eluted with sample buffer (10% glycerol, 2% SDS, 10 mM dithiothreitol, 0.02% bromphenol blue, 0.05 mM Tris-HCl, pH 6.8).

For Western blot analysis, the samples were boiled in the sample buffer and separated by SDS-PAGE in 10% polyacrylamide gel. After electrophoresis, the proteins were blotted onto transfer membranes (immobilon polyvinylidene difluoride, Millipore) in a Trans-Blot semi-dry electrophoretic transfer apparatus (Bio-Rad). The membranes were blocked for 1 h in PBS containing 10% dry milk powder and incubated for 1 h with the first antibody diluted in PBS containing 1% nonfat dry milk and 0.05% Tween 20. The antibodies were used in the following dilutions: mAb 4E11 (anti-hrp84) and 2E4 (anti hrp45) 1:10, polyclonal anti-hrp84 and monoclonal anti-tubulin (Sigma) 1:1,000, and polyclonal anti-cYB-1 1:10,000. The membranes were washed in PBS containing 0.05% Tween 20 (PBST) three times for 5 min and incubated with the proper peroxidase-conjugated secondary antibody (DAKO) for another hour. After washing, the position of the antibodies was detected by the ECL detection system (Amersham Biosciences).

**cDNA Cloning**—A randomly primed Agt11 cDNA library from salivary glands of *C. tentans* was screened with mAb 4E11 (1:100 dilution) using an anti-mouse immunoglobulin conjugated with alkaline phosphatase (Southern Biotechnology Associates, Inc.) and the NBT/BCIP detection system. One of the antibody-specific clones was purified, and the 1.8-kb insert was sequenced and used as template for PCR amplification with Agt11 forward and reverse primers (Promega Biotech). The amplified DNA fragment was purified by MicroSpin™ S-400 HR columns (Amersham Biosciences) and labeled by the DIG DNA Labeling and Detection kit (Roche Applied Sciences). The labeled DNA probe was used to screen an oligo dT-primed *ZAP* cDNA library from the salivary glands of *C. tentans* following the manufacturer’s instructions. Tens of positive clones were detected, and several of them were purified. The inserted cDNAs were PCR-amplified by T3 and T7 primers and purified as described above to determine the size of inserted cDNAs.

**DNA Sequencing and Sequence Analysis**—The amplified DNA fragments were used for sequencing with walking oligonucleotide primers. The DNA Sequencing kit (Dye Terminator Cycle Sequencing) (Applied Biosystems) was applied for DNA sequencing reaction, and the sequencing gel was run on a 373A Automated DNA Sequencer (Applied Biosystems). The DNA sequences were analyzed with the University of Wisconsin Genetics Computer Group (GGC) Sequence Analysis Pro-
In vitro synthesis programs and EGCG extensions to the Wisconsin Package Sequence Analysis programs.

Expression, Purification, and 35S Labeling of Recombinant Proteins—PCR was used to introduce an NdeI site at the 5'-end and a BamHI site at the 3'-end of the hrp84 coding sequence. The amplified cDNA was subcloned into the NdeI and BamHI sites of the pET15b vector (Novagen, Inc.) in-frame with coding sequence for a His tag in the N terminus. ctYb-1 (p40) cDNA was cloned into the NcoI-EcoRI sites of pET30a vector (Novagen) in-frame with coding sequence for an S-tag in the N terminus. The S-tag corresponds to the S-peptide in the N-terminus. The S-tag was used in the construction of recombinant hrp84 for affinity purification.

For generation of 35S-labeled proteins, the in vitro transcription/translation-coupled reticulocyte lysate system from Promega was used. The translated proteins were affinity-purified by Talon metal affinity resin (BD Biosciences) according to the manufacturer’s instructions.

ATPase Assay—Purified hrp84 (0.3 μg) was added to a 5-μl reaction mixture containing PBS, 5 mM MgCl2, 0.4 μg RNA, 0.1 mM cold ATP, and 0.1 μl of [α-32P]ATP. After incubation for 1 h at 37 °C, 1 μl of stop solution (1 mg/ml protease K, 1% SDS, 0.1 mM EDTA) was added, and the reaction mixture was incubated for an additional 20 min at 37 °C. Samples of 2 μl were spotted onto a PEI cellulose plate and developed in 0.6 M potassium phosphate (pH 3.4).

Immunocytology of Tissue Culture Cells—C. tentans tissue culture cells were centrifuged (Cytospin, Shandon Astra Moor, Runcorn, UK) onto glass slides. The cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After washing in PBS, the cells were permeabilized in 0.2% Triton X-100 in PBS for 10 min and washed again in PBS. The cells were blocked with 1% milk in PBS for 10 min and incubated with the first antibody for 1–2 h and rhodamine-conjugated secondary antibody for another hour. Confocal images were taken from 1-μm optical sections with LSM 510 (Zeiss).

Protein-RNA UV Cross-linking—The cross-linking was performed as earlier described (35). Tissue culture cells from 500 ml of medium were centrifuged and suspended in 5 ml of PBS. The cell suspension was irradiated under ultraviolet (UV) light (Stratalinker, Stratagene) to induce covalent protein-RNA cross-links in vivo. The UV light dose was ~0.3 J/cm2. Nuclear and cytoplasmic extracts were prepared, heated at 65 °C in the presence of 0.5% SDS and 1% mercaptoethanol and incubated with oligo(dt) cellulose (Amersham Biosciences) in the presence of 500 mM LiCl. After washing with buffer A (10 mM Tris-HCl, pH 7.5, 500 mM LiCl, 1 mM EDTA, and 0.5% SDS), the bound material of each sample was eluted with buffer A devoid of LiCl. The eluted fractions were incubated with RNase A (50 μg/ml) at 37 °C for 60 min and analyzed by SDS-PAGE and Western blotting.

Polyribosome Analysis—Tissue culture cells in 3 ml of medium were exposed to 50 μg/ml cycloheximide for 10 min, washed twice with cold PBS, resuspended in 150 μl of TKM buffer (10 mM triethanolamine-HCl at pH 7.0, 100 mM KCl, 5 mM MgCl2) and homogenized in a glass tissue grinder. Both PBS and TKM contained 50 μg/ml cycloheximide. The homogenate was centrifuged at 2000 × g for 10 min at 2 °C. The supernatant was loaded onto a gradient of 15% to 40% (w/v) sucrose in TKM. The gradient was incubated at 37 °C for 15 minutes with concomitant measurement of the absorbance at 254 nm. The proteins were precipitated with trichloroacetic acid and submitted to SDS-PAGE and Western blot analysis as described above.

Protein-Protein Interaction Assay—1 μg of S-tagged ctYB-1 immobilized on 10 μl of S-protein-agarose beads was used for each binding reaction. After pre-equilibration in a binding and washing (BW) buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton), the ctYB-1 beads were incubated with 35S-labeled proteins for 1 h at 4 °C under continuous mixing. The beads were washed five times with 200 μl of BW buffer.

FIGURE 1. Characterization of monoclonal and polyclonal hrp84 antibodies. A, specificity of the monoclonal hrp84 antibody 4E11. Nuclear extract from tissue culture cells of C. tentans was fractionated by SDS-PAGE, blotted, and stained with Coomassie Blue (lane 1) or immunostained with the monoclonal hrp84 antibody (lane 2). B, expression of hrp84 in bacteria, purification of hrp84 and a polyclonal hrp84 antibody. cDNA-encoded hrp84 was expressed in E. coli. The bacterial proteins were fractionated by SDS-PAGE and stained with Coomassie Blue before (lane 1) or after isopropyl-1-thio-β-D-galactopyranoside induction (lane 2). The recombinant hrp84 was analyzed by SDS-PAGE and Western blot analysis using the monoclonal hrp84 antibody (lane 3); the C. tentans nuclear extract was run in parallel (lane 4). A polyclonal antibody was raised against recombinant hrp84 (lane 5) and tested for specificity on nuclear extracts with SDS-PAGE and Western blot analysis (lane 6). The Coomassie Blue-stained nuclear sample is shown (lane 6), and the monoclonal hrp84 antibody was analyzed in parallel (lane 7). C, zymo-blot analysis using affinity-purified, polyclonal hrp84 antibody. Cellular extracts prepared from various organisms were analyzed by SDS-PAGE and immunoblotting using the monoclonal hrp84 antibody. Lane 1, C. tentans salivary gland cells; lane 2, yeast (Saccharomyces cerevisiae) cells; lane 3, Drosophila tissue culture cells; lane 4, Xenopus oocytes; lane 5, mouse liver cells; and lane 6, HeLa cells. Molecular size (kDa) markers are indicated to the left.
Bound proteins were eluted with sample buffer, fractionated by SDS-PAGE, and analyzed by autoradiography.

RESULTS

The Monoclonal Antibody 4E11 Recognizes an 84-kDa Protein—To identify and characterize proteins in nuclear mRNP particles (hnRNP proteins), we raised a series of monoclonal antibodies against hnRNP proteins immunoprecipitated from a C. tentans nuclear RNP extract from tissue culture cells (37). One of the monoclonal antibodies, designated mAb 4E11, specifically recognized an 84-kDa protein in the RNP extract from tissue culture cells as shown by Western blot analysis (Fig. 1A). In the present study, this antibody was used to identify and characterize the corresponding protein by cDNA cloning and to reveal interacting proteins using tissue culture cells.

According to the C. tentans nomenclature established for hnRNP proteins, the 84-kDa protein recognized by mAb 4E11 was designated Ct-hrp84, or hrp84 for short.

Cloning and Sequencing of cDNA Clones Encoding hrp84—Using the mAb 4E11, we screened a randomly primed /H9261 gt11 cDNA expression library made from salivary glands of C. tentans. An antibody-specific clone containing a 1.8-kb insert was sequenced. To get the full-length cDNA, the insert from this clone was amplified by PCR and used to screen an oligo(dT)-primed /H9261 ZAP cDNA library made from salivary glands. Nine positive clones were found with inserts ranging from 2.1 to 2.8 kb.

Sequence analysis of the longest cDNA insert (2.8 kb) showed that the insert contained an open reading frame of 2331 bp with an initiation codon ATG at nucleotides 250–252 and a stop codon TAA at nucleotides 2578–2580, as well as a 3′-non-translated region of 285 nucleotides (accession number DQ 139795). The open reading frame encodes a polypeptide of 776 amino acids with a predicted molecular mass of 86.136 kDa (Fig. 2A).

To demonstrate that the isolated cDNA encodes the hrp84 protein, the recombinant protein was expressed in E. coli. A protein corresponding to hrp84 appeared in Coomassie-stained gels (Fig. 1B, lane 2 versus lane 1). It was shown by Western blot analysis that the expressed protein was recognized by 4E11 and migrated in the gel as hrp84 extracted from C. tentans cells (Fig. 1B, lane 5 versus lane 4). Finally, we purified the recombinant protein (lane 3) and raised a polyclonal antibody. The affinity-purified antibody bound specifically to hrp84 when a nuclear extract from tissue culture cells was studied by Western blot analysis (Fig. 1B, lane 8; cf blot with mAb4E11 in lane 7). Thus, the protein encoded in the cloned cDNA represents full-length hrp84.

hrp84 Is a New Member of the Ded1 Subfamily of DEAD-box RNA Helicases—Data base searches revealed that the hrp84 protein is likely to be a DEAD-box RNA helicase as it contains the nine highly conserved motifs (I, Ia, Ib, II, III, IV, V, VI, and Q), which characterize the members of this family (4) (Fig. 2A).

FIGURE 2. A, predicted amino acid sequence of hrp84. The nine conserved motifs of the DEAD-box RNA helicase family are underlined, and the corresponding designations indicated (Q, I-V). The putative nuclear export signal at the N terminus is boxed. B, schematic representation of the molecular structure of the RNA helicase hrp84.

A

1. MNAYANENGTCGEQQVAGLDINERKSSKTYIPPLHRGEGIESDSLSSNSNDSNQGENLRLDNY 60
2. NRSQGGRDFNWRNRRNNRNRRNNRNQNRQNDYPPFRQQAGGSDFNSGYNNNNNSYNNYN 120
3. NRRYQNGESGERGGSNNWQGGRGSSRTFNSQRGDSRFGPPTQQPQQQQQLDDLQQDSQ 180
4. DEPVNTWQEPQQQODRFFRSNNNNNGGFGKWNQRPAEIDYITIDLPRLDREVSEQELFT 240
5. ANTGINFSKYEDIPVEATQQVXPEHTISFDILTEIIITNMKARYDKPTPQUKAIPI 300
6. ILSQGDLMSCAQGTSQKTAFLIPILNRMLEQASMNPAASNYQRRQKQPYPIGLVLAPTR 360
7. ELATQIYEEAAKFSYRSRMPAVALYGNNNTSEQOMRELDRGCHLVAPGLDDLINRKG 420
8. GLENLRFLVLDDEADRLDMDGFEPQRHIINERDMFPTQQRQLTMFSATFKNQLASEDF 480
9. LSNYIFLAVGRGVTSETNITQTILWVNEENNKRYSYLLDLRSLRREGSDPGYPSDSLTLIFVE 540
10. TKKGADALEEEFLYQNKHFPTISIHGDRSQREDEALKCFRSGDPCIPILVATAVAAARGDLIP 600
11. VKHVINYLPSDVBEYYHIHTRGTGRMGNLGIATSFNEKNRINVSIDLVELLEITNQELPS 660
12. FLEEMANDRYGGPFRRSNSSGGRGNYGGTTAGSRDYRQPQNSQYGNNSGGGRRSGDS 720
13. GSGNSGNNRGYGGGGRGNNRSQGNYGNNSDGYGNNNGYNNHASSGPDWED 776

B

NES Asn-Glu-rich DEAD-box core domain Gly-rich 1 L1 1 21 46 196 284 827 675 776

Ded1-like RNA Helicase Interacts with ctYb-1
and human DBX and DBY (57–58% identity), and yeast Ded1p and Dpb1 (55% identity).

A multiple sequence alignment of hrp84 with the members of the Ded1 group is shown in a supplementary figure. The helicase core domains are almost identical but also the N- and C-terminal regions show common structural features (Fig. 2B). The C-terminal regions are glycine-rich and contain many arginine and serine residues, which form a variable number of RS repeats. In the N terminus, this group of RNA helicases contains a conserved leucine-rich amino acid sequence (LXXX(V/F/L)XXXL) that constitutes a putative nuclear export signal (NES). Thus, based on the structural similarities of hrp84 to the Ded1-like group of DEAD-box proteins, we suggest that hrp84 is a new member of this subfamily.

The polyclonal hrp84 antibody seems to be able to specifically identify the Ded1-like group of proteins as indicated by Western blot analysis of extracts from human, mouse, Xenopus, Drosophila, and yeast (Fig. 1C). This result highlights the striking conservation of this family of proteins from yeast to human.

The hrp84 protein also displays some specific features, in particular an asparagine-rich region (36%, 37/102 amino acids; from amino acid position 46–148) including two RGG repeats, and a glutamine-rich and asparagine-rich region (36%, 37/102 amino acids; from amino acid position 46–148) including two RGG repeats, and a glutamine-rich and asparagine-rich region (36%, 37/102 amino acids; from amino acid position 46–148). This result highlights the striking conservation of this family of proteins from yeast to human.

hrp84 Is an RNA-dependent ATPase—DEAD-box RNA helicases are supposed to be RNA-dependent ATPases (4). To investigate whether hrp84 also has such an enzymatic activity we analyzed the ATPase activity of recombinant hrp84 in vitro in the presence of different polynucleotides (Fig. 3). Poly(A), poly(C), poly(G), poly(U), tRNA, and mRNA were able to stimulate ATPase activity although with somewhat different efficiency. Thus, hrp84 exhibits the predicted RNA-dependent ATPase activity.

hrp84 Is Associated with mRNA Both in Nucleus and Cytoplasm—We first studied the intracellular distribution of hrp84 in C. tentans tissue culture cells using immunocytochemistry. As shown in Fig. 4A, the cytoplasm was heavily decorated with the hrp84 antibody, while the nucleus stained relatively weakly. The two controls showed the predicted result: the hrp45 antibody labeled only the cell nucleus, whereas the preimmune antibody did not result in any immunolabeling (Fig. 4A). We conclude that hrp84 is present in the cytoplasm and presumably also in the nucleus.

Subsequently, we examined nuclear and cytoplasmic extracts from tissue culture cells with SDS-PAGE followed by Western blot analysis (Fig. 4B, lanes 1 and 2). The hrp84 protein seemed abundant in the cytoplasmic fraction, but it was also recorded in the nuclear fraction. To test for purity of the extracts, we used an hrp45 antibody to probe for nuclear material in the cytoplasmic sample and a tubulin antibody for cytoplasmic contamination of the nuclear sample. As expected for non-contaminated fractions, hrp45 was confined to the nuclear fraction (lane 3 versus lane 4), and tubulin to the cytoplasmic fraction (lane 5 versus lane 6). Thus, the Western blot analysis showed that hrp84 is predominantly distributed in the cytoplasm but a minor fraction also resides in the cell nucleus, which is in agreement with the cytological experiment.

To determine whether hrp84 is bound to poly(A) RNA (mRNA) both in the nucleus and cytoplasm, C. tentans tissue culture cells were exposed to UV-light, which cross-links protein–RNA complexes in vivo (47). Nuclear and cytoplasmic fractions were prepared and heated in the presence of SDS and mercaptoethanol to break non-covalent associations. The cross-linked RNA-protein complexes were collected onto oligo(dT) cellulose, and after washing the RNAs were eluted. The samples were further digested with RNase A and examined with SDS-PAGE followed by Western blotting using the monoclonal hrp84 antibody. As shown in Fig. 4C (lanes 1 and 2), hrp84 was cross-linked to both nuclear and cytoplasmic poly(A) RNA. The hrp84 protein did not co-purify with the mRNA when the ultraviolet was omitted (Fig. 4C, lanes 3 and 4). Thus, both the nuclear and cytoplasmic hrp84 is bound to mRNA.

To decide whether hrp84 is present in polysomes, cytoplasmic extracts were prepared and the polysomes resolved by sedimentation in sucrose gradients. The position of polysomes, monosomes, and ribosomal subunits were determined by A260 measurements (Fig. 5A) and by agarose gel electrophoreses of RNA in the collected fractions (data not shown). The hrp84 protein was analyzed by immunoblotting of the various fractions. Fig. 5B shows that hrp84 was present in the polysome region although most of the protein was recorded in the lighter fractions (fractions 12–15). As suggested by cross-linking experiments (data not shown), the relatively large amount of hrp84 in the top fractions is probably at least to a considerable extent due to release of loosely bound hrp84 from the polysomes during the extraction procedure (cf. loosely bound hrp36 in Ref. 48). The appearance of hrp84 in the polysome-containing fractions was sensitive to EDTA treatment (Fig. 5B), which causes the dissociation of ribosomes from mRNA (Fig. 5A). We conclude that hrp84 is associated with polysomes. In conclusion, hrp84 is bound to mRNA both in the nucleus and cytoplasm. At least some hrp84 remains linked to polysomes during translation.

hrp84 Interacts Directly with the Y-box Protein ctYb-1—The observed behavior of hrp84 is similar to that earlier reported for another C. tentans RNA-binding protein, the Y-box protein ctYb-1. This protein is also bound to mRNAs sequences both in the nucleus and cytoplasm and is associated with the endoplasmic reticulum (35) We now also show that ctYb-1 cosediments with polysomes in an EDTA-sensitive manner, suggesting that ctYb-1 is present in polysomes (Fig. 5C).

To determine whether hrp84 and ctYb-1 are closely associated both in the nucleus and cytoplasm, we immunoprecipitated hrp84 from the nuclear and cytoplasmic extracts and tested for co-precipitation of ctYb-1 by immunoblotting. As shown in Fig. 6A, ctYb-1 was coupled to hrp84 in both the nuclear and the cytoplasmic samples (lanes 3 and 5). Addition of RNase A to the extracts before immunoprecipitation did not prevent ctYb-1 from co-precipitating with hrp84 (Fig. 6A, lanes 4 and 6, respectively). Immunoprecipitation by a rat antimouse antibody did not bring down ctYb-1 (lane 7). These results suggested that hrp84 and ctYb-1 are linked to each other in a complex in the nucleus as well as in the cytoplasm.
To test whether hrp84 can directly interact with ctYB-1, we studied the in vitro binding of purified, radioactively labeled hrp84 to ctYB-1 immobilized on agarose beads. His-tagged hrp84 was synthesized and 35S-labeled in a cell-free rabbit reticulocyte lysate, and the protein was subsequently purified. In addition, the abundant C. tentans hnRNP protein hrp36 (48), used as control in the binding experiment, was expressed and purified in the same way. ctYB-1-coupled agarose beads were incubated with purified 35S-labeled hrp84 and hrp36, and washed, and the bound proteins were released, fractionated by SDS-PAGE and detected by autoradiography. hrp84 was detected predominantly in the bound fraction (Fig. 6B, lane 2), whereas hrp36 was essentially confined to the unbound fraction (lane 3). None of the proteins were able to bind to the beads lacking ctYB-1 (lane 4). We conclude that hrp84 and ctYB-1 interact directly with each other.

To decide whether hrp84 interacts with ctYB-1 in polysomes, we prepared a cytoplasmic extract, separated the RNP complexes by sucrose gradient centrifugation and determined whether hrp84 and ctYB-1 co-precipitated along the gradient. The gradient fractions were pooled into three portions: polysomes (I), monosomes, ribosomal subunits, and free mRNPs (II), and the remaining top fractions (III) (Fig. 7A). The three portions were treated with RNase and immunoprecipitated with the monoclonal hrp84 antibody. The proteins were analyzed with SDS-PAGE and immunoblotting. As shown in Fig. 7B, ctYB-1 co-immunoprecipitated with hrp84 in the polysome (I) and the monosome/ribosomal subunit/mRNP fraction (II) but also to some extent with hrp84 in the supernatant (III). Thus, our results suggest that hrp84 is bound to ctYB-1 in polysomes. In addition, the hrp84/ctYB-1 complex appears in the 30–100 S complexes, presumably in free mRNP particles.

DISCUSSION

hrp84, a DEAD-box Protein—In the present study we have identified a putative DEAD-box RNA helicase in C. tentans, designated hrp84.
The hrp84 protein exhibits the nine conserved motifs that characterize the helicase core of DEAD-box proteins. This helicase core has been extensively studied and shown to be responsible for binding of ATP and RNA and for ATP hydrolysis and RNA helicase activity (4). In the present study, we have shown that hrp84 binds to RNA in vivo and that it can hydrolyze ATP in vitro in an RNA-dependent manner.

The substrate specificity and the regulation of DEAD-box helicases are likely to be dependent on the C- and N-terminal regions of the helicase (3). These regions are more extended in hrp84 and the other members of the Ded1 subfamily than in DEAD-box proteins in general (38) and should, therefore, be well suited to permit recognition of a complex RNP substrate and to bind protein cofactors. Some general structural features in hrp84 and the other members of the Ded1 subfamily support such a notion. In the C-terminal end there is a glycine-rich region, which has been observed in many RNA-binding proteins and shown to be involved in protein-protein interactions (e.g. in hnRNP A1; (49)). In this region, there are also several arginine-serine repeats, which are characteristic of the RS domains in the C terminus of SR proteins, where they are responsible for interactions with other proteins (50). However, experimental information is still very limited as to specific molecular interactions involving members of the Ded1 subfamily. It has e.g. been shown that DDX3, the mammalian homolog of hrp84, interacts with CRM1 (51) and the HCV core protein (52, 53) through the C-terminal region. We conclude that further studies on hrp84 and the other members of the Ded1 subfamily are needed to permit precise conclusions regarding the functional significance and mode of action of the terminal regions.

The hrp84 protein and other members of the Ded1 subfamily harbor a putative leucine-rich nuclear export signal (NES) in the N-terminal region. The putative NES can indeed function as an NES, as shown for An3 (54), but that other members of the Ded1 subfamily could be transferred from nucleus to cytoplasm by other mechanisms involving interaction with CRM1 (51, 55).

Members of the Ded1 subfamily of the DEAD-box proteins seem to be involved in translation. Most information is available on Ded1, the yeast member of the subfamily. It has been shown that Ded1 is an essential protein and required for translation initiation (56, 57). The human, mouse, and Drosophila homologues can substitute for Ded1 in genetic complementation assays, which suggests that the cellular function of this subfamily of RNA helicases is conserved (39, 56, 58). It is, therefore, indicated that all members of the Ded1 subfamily can work as translation initiation factors. In addition, it was recently shown in Drosophila that the bel recessive lethal phenotype is similar to that produced by mutations in other translation initiation factors such as eIF4A and eIF4E.
Ded1-like RNA Helicase Interacts with ctYb-1

FIGURE 7. Interaction between hrp84 and ctYB-1 in polysomes. A, C. tentans cytoplasmic extract was centrifuged in a sucrose gradient and separated into three fractions: polyribosomes (I), monoribosomes, ribosomal subunits and mRNPs (II), and supernatant (III). B, proteins in each fraction were immunoprecipitated with the monoclonal hrp84 antibody and analyzed by Western blotting using an hrp84 or a ctYB-1 antibody.

(39), which again suggests a role for the Ded1 group of helicases during translation.

The hrp84&ctYB-1 Complex and Its Function during Translation—In the present study we have demonstrated that the helicase hrp84 is bound to mRNA and is associated with the Y-box protein ctYB-1. As suggested by in vitro experiments, the interaction between hrp84 and ctYB-1 is direct, and we propose that there is an mRNA-bound hrp84&ctYB-1 complex within the cell. The complex is present in the nucleus and cytoplasm and appears in polysomes in the cytoplasm. We assume that the complex remains bound to the mRNA in polysomes, but our experiments do not rule out the possibility that it could have shifted position to the ribosomes during protein synthesis.

The finding that the hrp84&ctYB-1 complex ends up in polysomes is in good agreement with the current view that Ded1-like helicases participate in translational control. Furthermore, there is also strong evidence suggesting that the partner in the complex, the Y-box protein, affects the translation process. As mentioned in the Introduction, Y-box proteins are likely to be involved in silencing mRNA during oogenesis (21, 22). Furthermore, in somatic cells Y-box proteins seem to play a role in translation as a global modulator (33). In rabbit reticulocytes, the Y-box protein YB-1 (p50) is present in free mRNPs as well as in polysomes (59), and the amount of the protein affects the intensity of translation: low levels stimulate translation (60–62), whereas high levels result in repression (59, 60, 62, 63). YB-1 binds along mRNA with little or no sequence specificity (33), and it disrupts RNA secondary structure (32, 65). Thus, evidence from both somatic and germ cells suggest that not only the RNA helicase hrp84 but also the Y-box protein ctYB-1 is likely to play a role in translation in C. tentans. It is, therefore, most interesting that the two components appear together in polysomes.

The fact that hrp84 and ctYB-1 form a complex in polysomes suggests that they act as partners to control translation. It is close at hand to suggest that the RNA helicase-Y-box protein complex exerts its function through the ability of the Y-box protein to modulate the overall structure of the mRNP complex, and in this way control translation (62, 66–68). One possibility would be that hrp84 unwinds duplex structures in mRNA, while ctYB-1 keeps the mRNA in a single-stranded conformation (14). The helicase could then act as a modulator of Y-box binding to the template. Another possibility would be that the helicase works as an RNPase, stabilizing or disrupting Y-box protein mRNA complexes (see Introduction). It has been proposed that phosphorylation of either the Y-box protein and/or the helicase could play a critical role in promoting mRNA-protein or protein-protein interactions (for discussion, see Ref. 14). It is then interesting to note that phosphorylation of YB-1 by the serine/threonine protein kinase Act was recently shown to activate translation of silent mRNAs (69). Even if the RNA helicase-Y-box protein complex modulates the overall mRNP structure, the critical functional effects are recorded at the 5'- and/or the 3'-ends of the mRNA because the Y-box protein-mediated control affects initiation of translation and not elongation or termination (61, 63). The binding of translation initiation factors and the small ribosomal subunit is crucial (62), but also the scanning of the small ribosomal subunit from the cap region to the initiation codon could be influenced by the YB-1 protein (61). Notably, it has been revealed that also yeast Ded1 facilitates the scanning process along the 5'-untranslated regions (71). Evidently, further experiments are required to establish the proposed close interplay between the RNA helicase and the Y-box protein during translation.

A Role for the hrp84&ctYB-1 Complex during Nucleocytoplasmic Transport of mRNPs?—On the basis of the distribution of the hrp84&ctYB-1 complex within the cell, we have concluded that the mRNA-associated hrp84&ctYB-1 complex is formed already in the nucleus and accompanies the mRNA from the cell nucleus all the way into polysomes in the cytoplasm. Such a conclusion would be in good agreement with an earlier study of the nucleocytoplasmic flow of the YB-1 in C. tentans (35). Also in the case of Xenopus oocytes, it is indicated that the Y-box protein FRG2 (27), and the RNA helicase Xp54 (72) get associated already with nascent RNA on the lampbrush chromosomes and presumably remain bound to mRNA during translation of the message from nucleus to cytoplasm. This early association with mRNA brings up the issue whether the RNA helicase-Y-box protein complex plays a role also within the nucleus and/or during nucleocytoplasmic transport.
One possibility would be that the helicase-Y-box protein complex takes part in RNA splicing. In fact, an early yeast genetic study suggested that the RNA helicase Ded1 is involved in splicing (73). In addition, in a proteomic study Ded1 was recorded in yeast spliceosomes (74). Moreover, it has been shown that YB-1 can stimulate alternative splicing in humans by binding to exon enhancers (75). However, more information is needed to establish that Ded1 and/or YB-1 are important factors in mRNA splicing.

A perhaps more attractive possibility would be that the RNA helicase-Y-box protein complex plays a crucial role during nucleocytoplasmic transport by keeping mRNA silent. Such an option is supported by analysis of mRNP transport between nurse cells and the oocyte in Drosophila (20). Normally, mRNA in the mRNPs is repressed during transport and does not become activated until the mRNPs reach their final polar destination in the oocyte. If the RNA helicase Me31B, a homologue to Dh1 in yeast (20), is absent from the mRNP particles, no effect on transport can be noted but remarkably, translation of the mRNA is initiated already in the nurse cells. Thus, a key function of the RNA helicase-Y-box protein complex could be to form a non-translatable mRNP complex during transport. Evidently, the subsequent fate of the mRNAs in the cytoplasm depends on cell type. In germ cells, the mRNA could remain silent for a considerable period as in amphibian oocytes or be activated just after having reached its final location in the cytoplasm as in Drosophila oocytes. In somatic cells, the activation could take place already at the exit from the nucleus (e.g. in the salivary gland cells in C. tentans; (76) or be delayed until the mRNPs reach specific locations within the cytoplasm (e.g. in dendrites of neurons; Ref. 70, see also Ref. 64). If the RNA helicase-Y-box protein complex does play such a repressive role during transport, the nature and spatial organization of the release of the repression would be a fascinating topic to further explore.

Acknowledgments—We thank Lars Wieslander for providing us with cDNA libraries. We are grateful to Lise-Marie Fjelkestam and Oxana Nashchekina for technical assistance.

REFERENCES

1. de la Cruz, J., Kresse, D., and Linder, P. (1999) Trends Biochem. Sci. 24, 192–198
2. Silverman, E., Edwalds-Gilbert, G., and Lin, R. J. (2003) Exp. Cell Res. 284, 431–443
3. Rocak, S., and Linder, P. (2004) Nat. Struct. Biol. 11, 1197–1211
4. Jamieson, D. J., Rahe, B., Pringle, J., and Beggs, J. D. (1991) J. Biol. Chem. 266, 13467–13472

Ded1-like RNA Helicase Interacts with ctYb-1
Ded1-like RNA Helicase Interacts with ctYb-1

Yaminsky, I. V., Vasiliev, V. D., and Ovchinnikov, L. P. (2004) *Nucleic Acids Res.* **32**, 5621–5635

68. Evdokimova, V., Ruzanov, P., Imataka, H., Raught, B., Svitkin, Y., Ovchinnikov, L. P., and Sonenberg, N. (2001) *EMBO J.* **20**, 5491–5502

69. Evdokimova, V., Ruzanov, P., Anglesio, M. S., Sorokin, A. V., Ovchinnikov, L. P., Buckley, J., Triche, T. J., Sonenberg, N., and Sorensen, P. H. (2006) *Mol. Cell. Biol.* **26**, 277–292

70. Gardiol, A., Racca, C., and Triller, A. (2001) *Results Probl Cell Differ.* **34**, 105–128

71. Berthelot, K., Muldoon, M., Rajkowitsch, L., Hughes, J., and McCarthy, J. E. (2004) *Mol. Microbiol.* **51**, 987–1001

72. Smillie, D. A., and Sommerville, J. (2002) *J. Cell Sci.* **115**, 395–407

73. Hayashi, N., Seino, H., Irie, K., Watanabe, M., Clark, K. L., Matsumoto, K., and Nishimoto, T. (1996) *Mol. Gen. Genet.* **253**, 149–156

74. Stevens, S. W., Ryan, D. E., Ge, H. Y., Moore, R. E., Young, M. K., Lee, T. D., and Abelson, J. (2002) *Mol. Cell* **9**, 31–44

75. Stickeler, E., Fraser, S. D., Horig, A., Chen, A. L., Berget, S. M., and Cooper, T. A. (2001) *EMBO J.* **20**, 3821–3830

76. Mehlin, H., Daneholt, B., and Skoglund, U. (1992) *Cell* **69**, 605–613