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# Dbp5p, a cytosolic RNA helicase, is required for poly(A)$^+$ RNA export

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**The *DBP5* gene encodes a putative RNA helicase of unknown function in the yeast *Saccharomyces cerevisiae*. It is shown here that Dbp5p is an ATP-dependent RNA helicase required for polyadenylated [poly(A)$^+$] RNA export. Surprisingly, Dbp5p is present predominantly, if not exclusively, in the cytoplasm, and is highly enriched around the nuclear envelope. This observation raises the possibility that Dbp5p may play a role in unloading or remodeling messenger RNA particles (mRNPs) upon arrival in the cytoplasm and in coupling mRNP export and translation. The functions of Dbp5p are likely to be conserved, since its potential homologues can be found in a variety of eukaryotic cells.**
*Keywords*: ATPase/DEAD-box protein/mRNA export/ RNA helicase

## Introduction

One of the defining features of eukaryotic cells is the nucleus, which separates the activities of replication and transcription from that of translation. Active and selective nucleocytoplasmic transport is thus required to import and export proteins and RNAs to their functional sites (reviewed in Izaurralde and Mattaj, 1995; Görlich and Mattaj, 1996; Nigg, 1997; Ullman *et al*., 1997). These cargoes must traverse the nuclear envelope via the nuclear pore complexes (NPCs), which are the only known sites for exchange of macromolecules between the nucleus and the cytoplasm (reviewed in Rout and Wente, 1994). Much of our understanding of the nucleocytoplasmic transport came from studies of protein import. Nuclear import is initiated by binding of the nuclear localization signals (NLSs), present on the nucleus-bound proteins, to an NLS receptor, which is a heterodimeric complex composed of importin α and importin β. The tripartite unit then docks to the NPC and is subsequently translocated through the pore by an energy-dependent mechanism (reviewed in Görlich and Mattaj, 1996), which requires the small guanosine triphosphatase (GTPase) Ran (Melchior *et al*., 1993; Koepp and Silver, 1996) and a small homodimeric protein called p10 or NTF2 (Nehrbass and Blobel, 1996). GTP hydrolysis by Ran is thought to promote this translocation process. Importin α then dissociates from importin β and the cargo upon arrival in the RanGTP-rich interior of the nucleus (Görlich and Mattaj, 1996). Recent data suggest that there are multiple independent protein import pathways, each employing distinct members of the β-importin family for import. For example, import of hnRNP A1 protein is mediated by transportin 1 (Pollard *et al*., 1996), and import of some of the ribosomal proteins is facilitated by Kap123p and Pse1p (Rout *et al*., 1997).

In comparison with our knowledge of protein import, less is known about how RNAs exit from the nucleus into the cytoplasm. Micro-injection experiments using the *Xenopus* oocytes have established that RNA export is an energy-dependent and carrier-mediated process (Jarmolowski *et al*., 1994). This approach also provided evidence that export of different classes of RNAs, such as transfer RNAs (tRNAs), 5S ribosomal RNA (rRNA), U small nuclear RNAs (snRNAs) and messenger RNAs (mRNAs), can be competitively inhibited by their cognate RNAs but not affected by other classes of RNAs (Jarmolowski *et al*., 1994). Therefore, it appears that the rate-limiting steps for exit of different RNAs are distinct. Biochemical studies of U snRNA export have revealed that it is mediated, at least in part, by a nuclear cap-binding protein complex (CBP), which is composed of two proteins, CBP80 and CBP20 (Izaurralde *et al*., 1995). In contrast, the 5′ cap appears to be less important in mRNA export (Jarmolowski *et al*., 1994). Furthermore, the export of the partially spliced and unspliced human immunodeficiency virus 1 (HIV-1) mRNAs is mediated by the binding of the viral Rev protein to an RNA element called the Rev response element (RRE) (Daly *et al*., 1989; Zapp and Green, 1989). It was subsequently shown that Rev contains a leucine-rich nuclear export signal (NES), which could also be found in some other exported proteins, such as TFIIIA and protein kinase inhibitor (PKI) (reviewed in Gerace, 1995).

Since nuclear mRNAs are expected to associate with proteins in the form of heterogeneous nuclear ribonucleoprotein particles (hnRNPs) or messenger RNPs (mRNPs), it has been speculated that mRNA export could be mediated by RNA-binding proteins harboring NESs. This proposition has been supported by the finding that hnRNP A1 protein contains a transferrable M9 domain that can confer both rapid protein import and export (Michael *et al*., 1995a) and that hnRNP A1, as well as hnRNP D, E, I and K, shuttle between the nucleus and the cytoplasm (Michael *et al*., 1995b). In addition, a yeast protein Mtr13p/Npl3p/Nop3p, with considerable sequence and structural similarity to mammalian hnRNP proteins, shuttles between the nucleus and the cytoplasm, while its inactivation by mutations causes nuclear accumulation of poly(A)$^+$ RNAs (Singleton *et al*., 1995; Lee *et al*., 1996).

Most recently, a nuclear export receptor, exportin 1 (Crm1p), was identified on the basis of its interactions with both leucine-rich NES and RanGTPase, its shuttling behavior (Fornerod *et al*., 1997; Stade *et al*., 1997) and that *crm1* mutants block poly(A)$^+$ RNA export at the restrictive temperature (Stade *et al*., 1997).

Systematic screening of yeast conditional mutants for poly(A)$^+$ RNA accumulation in the nucleus at the restrictive temperatures has led to the identification of a number of *mtr* (mRNA transport) (Kadowaki *et al*., 1994a) and *rat* (ribonucleic acid trafficking) (Amberg *et al*., 1992) mutants. Some of the corresponding proteins appear to function in mRNA export. For example, several *RAT* genes encode components of NPCs, the nucleoporins (Gorsch *et al*., 1995). A subset of nucleoporins has already been implicated in mRNA export by direct studies of NPCs in both yeast and mammalian cells (reviewed in Doye and Hurt, 1995). In addition, the isolation of the *MTR* genes such as *MTR13*/*NPL3*/*NOP3* (Singleton *et al*., 1995; Lee *et al*., 1996) and *MTR1*/*PRP20 (*Kadowaki *et al*., 1992), which encodes a guanine nucleotide exchange factor, further strengthened the idea that hnRNP-like proteins and the RanGTPase system are likely to take part in mRNA export in yeast. Several other genes identified in these screens encode proteins whose functions are not immediately obvious for mRNA export. For example, Rat1p has a 5′→3′ exoribonuclease activity and may possess DNA strand separation activity (Amberg *et al*., 1992); Mtr2p is a nuclear protein with limited homology to a protein implicated in plasmid DNA transfer in *Escherichia coli* (Kadowaki *et al*., 1994b); and Mtr7p/Acc1p is acetyl coenzyme A (CoA) carboxylase, an enzyme functioning in the *de novo* fatty acid biosynthesis pathway (Schneiter *et al*., 1996).

Since mRNAs are expected to adopt a plethora of RNA structures packaged in the RNPs, their transit through NPCs may require major reorganization of RNA and/or RNP structures, a process predicted to be energy-dependent. Thus, it seems likely that RNA helicases, enzymes that can promote separation of strands in RNA duplexes or removal of secondary structures in single-stranded RNA, may also play a major role in mRNA export. In this regard, a new class of proteins, the DEAD-Box proteins (DBPs) may fulfill this role in mRNA export.

Members of the DBP family are ubiquitous. They share considerable sequence similarities, in particular the Asp–Glu–Ala–Asp (DEAD) motif and its variants (reviewed in Fuller-Pace, 1994). Several of these DBPs, such as the mammalian and yeast eIF4A (Rozen *et al*., 1990; Blum *et al*., 1992), human p68 (Hirling *et al*., 1989), *Xenopus* An3 (Gururajan *et al*., 1994) and *Drosophila* vasa (Liang *et al*., 1994), have been shown to exhibit RNA unwinding activities *in vitro*. Thus, it is generally believed that all DBPs are RNA helicases, whose functions are to modulate specific RNA secondary structures in their respective biochemical pathways, which include translation initiation, nuclear pre-mRNA splicing, and ribosomal biogenesis (reviewed in Fuller-Pace, 1994). There are other families of proteins that are closely related to the DBP family; these are sometimes called the DEAH and the DEXH families and, together with the DBP family, they form the helicase superfamily II (reviewed in Gorbalenya and Koonin, 1993). Two of the DEXH proteins have recently been implicated in mRNA export. The nuclear Mtr4p was identified in the *mtr* mutant screen (Liang *et al*., 1996), while the human nuclear RNA helicase A, a shuttle protein, appears to accompany the simian viral RNAs containing the constitutive transport element (CTE) to the cytoplasm (Tang *et al*., 1997).

In this work, we show that an ATP-dependent RNA helicase, Dbp5p, is essential for export of poly(A)$^+$ RNA from the yeast nucleus. The remarkable cytoplasmic enrichment of Dbp5p suggests that it is likely to play a role in the mRNA export at steps that occur in the cytoplasm.

## Results

### *DBP5 encodes an essential putative RNA helicase*

The *DBP5* gene (DDBJ/EMBL/GenBank accession No. U28135) was originally identified in a search for *Saccharomyces cerevisiae* DEAD-box protein genes (Chang *et al*., 1990). The wild-type *DBP5* gene was cloned by colony hybridization with a yeast genomic library using a DNA probe (CA5/6) (Chang *et al*., 1990) corresponding to an internal region of *DBP5*. The 3.9 kb DNA fragment sequenced contained a single 1449 bp open reading frame. The gene product of *DBP5*, Dbp5p, consisted of all of the typical DEAD-box-protein motifs (Figure 1A) and was predicted to be neutral (net overall charge, 0; pI = 7.5), with a molecular mass of 53.8 kDa.

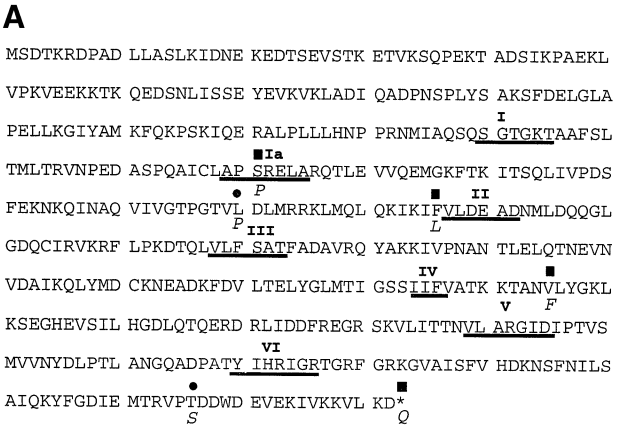
To test whether *DBP5* encodes an essential gene product, we constructed a *dbp5* null allele *in vitro* by replacing an internal 1.2 kb *DBP5* sequence with a *HIS3* selectable marker. This disruption allele was used to transform a wild-type homozygous *his3*$^-$ diploid strain. The resulting His$^+$ transformants were screened for the correct replacement of one of the two genomic copies of *DBP5* by Southern analysis (data not shown). The verified heterozygous diploids were then subjected to sporulation and tetrad dissection. Only two viable His$^-$ haploid spores were recovered in each of the 12 tetrads dissected (data not shown), indicating that *DBP5* is an essential gene.

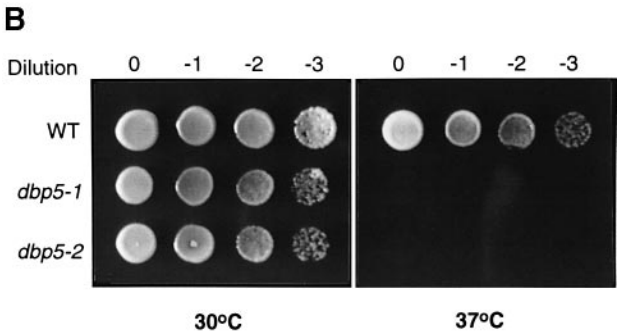
### *Isolation of dbp5 conditional mutants*

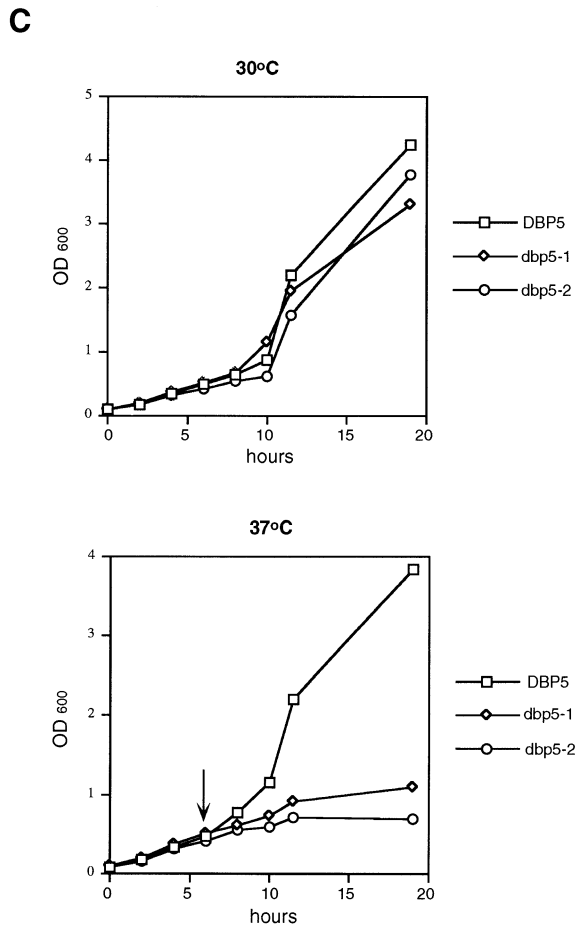
To investigate the function of Dbp5p, we sought to isolate *dbp5* conditional mutants by polymerase chain reaction (PCR) mutagenesis and a plasmid-shuffling method (see Materials and methods). Two *dbp5* temperature-sensitive (Ts$^-$) mutants that failed to grow at 37°C (Figure 1B) were isolated from a screen of ~3000 transformants. In liquid medium, growth of these two mutants can be completely arrested within 2 h of a temperature shift to 37°C (Figure 1C). In both cases, the Ts$^-$ phenotypes were linked to *dbp5* as shown by recloning the *dbp5* inserts for another round of plasmid shuffling. Sequencing analysis revealed that both alleles, *dbp5-1* and *dbp5-2*, contained multiple point mutations (Figure 1A). No attempt was made to assess the contribution of individual mutations to the overall growth phenotypes.

### *Slow reduction of protein synthesis in dbp5 mutants*

*dbp5-1* and *dbp5-2* mutants were screened for biochemical defects associated with other known DEAD-box protein mutants. No splicing defects were detected by Northern




analysis in either mutant after shifting cultures to 37°C for 3 h, in contrast with the aberrant rise of *CRY1* pre-mRNA levels in control strains *prp2* and *prp11*, which harbor splicing mutations (data not shown). We then examined the protein synthesis *in vivo* by measuring [$^{35}$S]methionine incorporation in either *dbp5* mutant at 37°C. Cells were preincubated at the non-permissive temperature for either 15 or 30 min, prior to adding [$^{35}$S]methionine for further incubation at the non-permissive temperature. Protein synthesis in both *dbp5* mutants was found to remain active (Figure 2), but gradually reduced over a period of 1 h (data not shown), in sharp contrast with the rapid cessation of protein synthesis in a *ded1-199* mutant strain defective in translation (Chuang *et al*., 1997). To confirm that Dbp5p is not required for translation, we used an anti-Dbp5p polyclonal antibody (see below) to deplete >95% of Dbp5p in translation extracts prepared from a wild-type strain. A capped luciferase transcript with poly(A) tail was then added to the immunodepleted extract for *in vitro* translation. Translation activity remained unchanged upon Dbp5p-depletion, in contrast with the almost complete loss of translation activity in an extract depleted of Ded1p (data not shown; Chuang *et al*., 1997). Thus, Dbp5p is unlikely to play a direct role in translation, as reported for two yeast DEAD-box proteins, Ded1p (Chuang *et al*., 1997) and Tif1p (Schmid and Linder, 1991).

The slow reduction of protein synthesis suggested that cytoplasmic mRNAs in *dbp5* mutants could be progressively depleted at 37°C. This possibility was investigated by measuring the mRNA turnover rates using an *rpb1-1* temperature-sensitive mutation (Nonet *et al*., 1987) to inhibit the RNA polymerase II (pol II) transcription. At 37°C, the half-lives of the *ACT1*, *CRY1*, *CUP1*, *CYH2* and *PGK1* transcripts in the *rpb1-1·dbp5-1* and *rpb1-1·dbp5-2* double mutants were found to be identical to those measured in the *rpb1-1* mutant (data not shown). Furthermore, stability of the *PGK1* transcript was not changed in either *dbp5* mutant strain at 37°C when transcription was repressed by thiolutin. Thus, it seemed unlikely that Dbp5p is involved in mRNA decay, as noted previously for RhlB (Py *et al*., 1996), a DEAD-box protein in *E.coli*.

### *Rapid nuclear accumulation of poly(A)$^+$ RNA in dbp5 mutants*

Defects in poly(A)$^+$ RNA export could also yield a progressive loss of cytoplasmic mRNAs, which would in turn result in a gradual reduction of protein synthesis.

**Fig. 1.** (**A**) Predicted amino-acid sequence of Dbp5p. Conserved sequence motifs (denoted by roman numerals) found in the helicase superfamilies (Gorbalenya and Koonin, 1993) are underlined. Mutations in the *dbp5-1* (●) and the *dbp5-2* (■) alleles were determined by DNA sequencing and are shown as italicized letters below the Dbp5p sequence. (**B**) Growth phenotypes of *dbp5-1* and *dbp5-2* mutants. Cells were grown to saturation in liquid YPD medium at 30°C, serially diluted (0: no dilution; –1, –2 and –3: 10-, $10^2$- and $10^3$-fold dilution, respectively) and spotted onto YPD plates. A set of two plates were separately incubated at 30 and 37°C. Note that both *dbp5-1* and *dbp5-2* mutant strains grow slightly more slowly than the wild-type strain (WT) even at 30°C. (**C**) Growth curves of wild-type *DBP5*, *dbp5-1*, and *dbp5-2* strains in YPD liquid medium at 30 or 37°C. Cell growth was followed by measuring the optical density at 600 nm ($OD_{600}$). Arrow indicates the time point when cultures were shifted to 37°C.

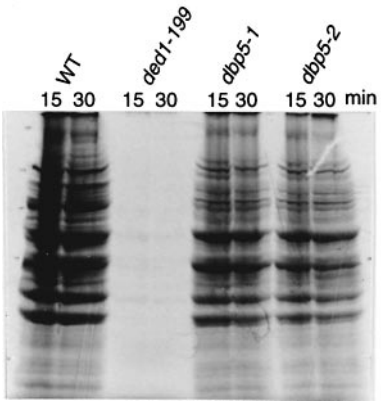



**Fig. 2.** Protein synthesis in *dbp5-1* and *dbp5-2* mutants. Eight units of cells at $OD_{600}$ were resuspended in 1 ml of fresh YPD and incubated at either 15°C (*ded1-199*) or 37°C (WT, *dbp5-1* and *dbp5-2*) for 15 or 30 min prior to the addition of 20 μCi of [$^{35}$S]methionine. After 1 h incubation at the non-permissive temperatures, cells were collected and broken by vortexing with glass beads. Equal amounts of proteins in the supernatant were normalized by Bradford assay and analyzed by electrophoresis on a 10% SDS–polyacrylamide gel and autoradiography.

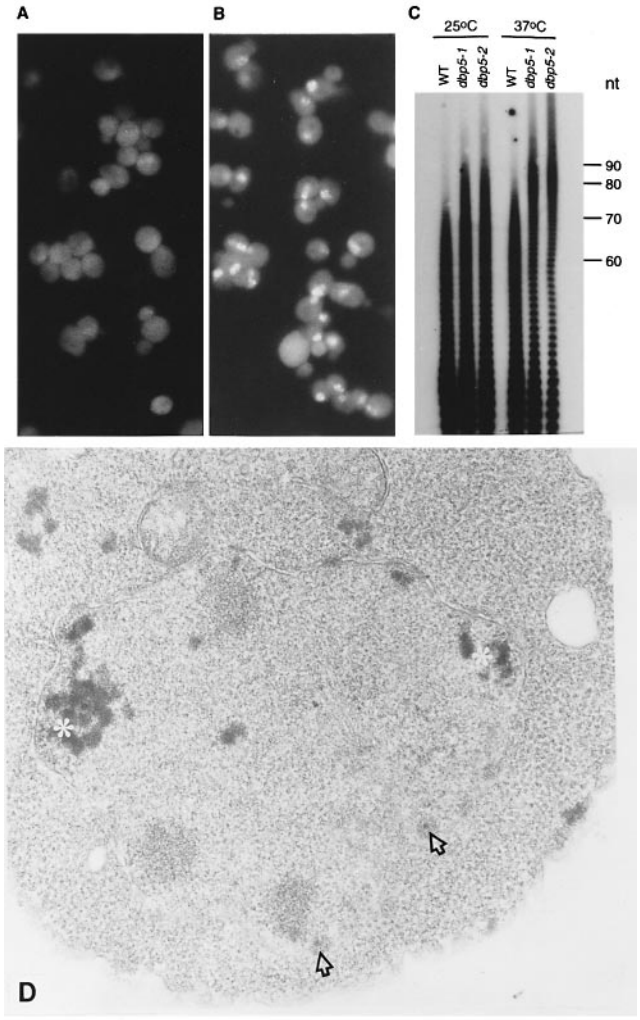



**Fig. 3.** (A and B) Poly(A)$^+$ RNA distributions in *dbp5-1* cells at 23°C (**A**) or at 37°C (**B**). *In situ* hydridization with a biotinylated oligo(dT) probe was carried out 10 min after shifting to 37°C. Probe localization was detected with fluorescein isothiocyanate-conjugated avidin as described by Kadowaki *et al.* (1992). (**C**) Distribution of poly(A) tail lengths in wild-type (WT) strain and *dbp5-1* and *dbp5-2* mutant strains. Poly(A)$^+$ RNAs purified from cells grown to mid-log phase at 25°C and from cells that have been shifted to 37°C for 30 min were 3′-end labeled with [$^{32}$P]Cp and digested with RNase A. The remaining intact mRNA poly(A) tails were separated on a 12% polyacrylamide–7 M urea gel and visualized by autoradiography. Poly(A) tail lengths are indicated in nucleotides (nt). (**D**) Electron microscopy analysis of nuclear structures upon inactivating Dbp5p's function. Thin section of *dbp5-1* after 30 min at 37°C. Note the conspicuous accumulation of amorphous material within the nucleoplasm (stars) as well as the appearance of what appear to be NPCs including a central plug (arrows). Other aspects of ultrastructure appear unchanged.

This possibility was investigated by fluorescent *in situ* hybridization (FISH) using biotinylated oligo(dT) as a probe (Kadowaki *et al*., 1992). *dbp5-1* and *dbp5-2* were incubated at 37°C for 10 min to 3 h and their poly(A)$^+$ RNA distributions were analyzed. At the permissive temperature, fluorescent signals corresponding to the poly(A)$^+$ RNAs distributed faintly over the entire cells (Figure 3A). However, as early as 10 min after the temperature shift, nuclear fluorescent signals can be detected in >80% of cells as distinct spots (Figure 3B), which eventually merged into a single larger staining area and remained stable for >3 h. The observed fluorescence was due to hybridization of the oligo(dT) probe to poly(A)$^+$ RNA, since it could be eliminated by pretreating the fixed cells with a cocktail of RNase, by preincubating cells at 25°C with thiolutin for 1 h, and by repressing pol II transcription in a *rpb1-1·dbp5-1* double mutant at 37°C for 1 h (data not shown). Two observations indicated that the fluorescent signal was a result of nuclear accumulation of poly(A)$^+$ RNA, rather than rapid turnover of the cytoplasmic poly(A)$^+$ RNA. First, the signal persisted for >2 h after the addition of thiolutin, suggesting that the poly(A)$^+$ RNA was unable to exit the nucleus (data not shown). Secondly, the poly(A) tail of poly(A)$^+$ RNA in *dbp5* mutants was longer than that in the wild-type cells (Figure 3C), suggesting a lack of poly(A) shortening thought to occur in the cytoplasm (Sachs, 1993).

Since alterations of nuclear structure have been observed for a number of mRNA export mutants, we studied *dbp5-1* by electron microscopy at 23°C and after 30 min at 37°C. Most aspects of cell ultrastructure appeared normal, suggesting that the observed poly(A)$^+$ RNA accumulation did not result from major structural defects of the nucleus. There was, however, a conspicuous accumulation of amorphous material in the nucleoplasm of the *dbp5-1* mutant cells cultured at 37°C (Figure 3D), similar to what has been observed in some mRNA export mutants such as *mex67-5* (Segref *et al*., 1997). This material did not have an obvious spatial relation to the nuclear envelope; similar amorphous material was occasionally seen in the cytoplasm as well. In addition, some NPCs had what appeared to be an exaggerated central plug.

### *Dbp5p is localized in the cytoplasm and is enriched around the nuclear envelope*

To study the cellular localization of Dbp5p, we raised mono-specific polyclonal antibodies against Dbp5p using recombinant Dbp5p purified from *E.coli* (see Materials and methods). We also constructed yeast strains in which

the wild-type *DBP5* gene was replaced with either a *DBP5*–Protein A (*DBP5–PA*) gene fusion driven by *DBP5*'s own promoter, or a gene fusion producing the hemagglutinin-tagged Dbp5p (*DBP5-HA*) driven by the yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. Both fusion proteins were functional *in vivo*, because the growth rates of their host strains were nearly indistinguishable from that of the wild-type strain. Western analysis using anti-Dbp5p antibody, anti-HA polyclonal antibody and normal immunoglobulin G (IgG) detected a single band corresponding to the wild-type Dbp5p, Dbp5p-HA and Dbp5p–PA in yeast extracts prepared from the corresponding strains (Figure 4A).

Indirect immunofluorescence microscopy (IFM) using anti-Dbp5p antibody with the wild-type cells revealed that Dbp5p was localized predominantly in the cytoplasm and with distinct concentration near the nucleus (Figure 4B, left panels). This localization pattern became even more evident when normal IgG was used to detect Dbp5p–PA (Figure 4B, center panels) and anti-HA monoclonal antibody was used to detect the overproduced Dbp5p-HA (Figure 4B, right panels). Control experiments using preimmune serum, anti-HA antibody and normal IgG to stain the wild-type strain yielded no signal (data not shown). Confocal images of the cells sectioned through the equatorial plane (Figure 4C) consistently demonstrated that the nucleoplasmic region was largely free from staining and that the nuclear rim staining was continuous with a non-uniform cytoplasmic staining. To further confirm the cytoplasmic localization of Dbp5p, we prepared cytosolic and nuclear fractions. Dbp5p and Ded1p (Chuang *et al*., 1997), a cytoplasmic translation factor, were found exclusively in the cytosolic fraction, whereas Nop1p (Schimmang *et al*., 1989), a nucleolar protein, was detected only in the nuclear fraction (Figure 4D).

Inhibition of pol II transcription causes the relocation of the shuttling proteins such as hnRNP A1, A2 and E to the cytoplasm (Michael *et al*, 1995b), and at the non-permissive temperature, the mutant forms of Prp20p are mis-localized to the cytoplasm (Amberg *et al*., 1993). We therefore tested the possibility that Dbp5p may be relocalized to the nucleus or other subcellular locations under similar conditions. The cytoplasmic localization of Dbp5p remained unchanged when RNA transcription was shut down by *rpb1-1* mutation or by preincubating cells with thiolutin for 1 h at the permissive temperature prior to temperature shift (data not shown). In addition, the mutant forms of Dbp5p in *dbp5-1* and *dbp5-2* strains persisted in the cytoplasm after incubating the cells at 37°C for 1 h. Taken together, these data suggest that Dbp5p functions predominantly at sites which are close to, but not tightly associated with, the nuclear envelope.

The localization of Dbp5p in the cytoplasm raises the question of whether it may also play a role in protein import. We therefore examined the localization pattern of a reporter protein consisting of an NLS from Mtr4p fused to the N-terminus of *E.coli* β-galactosidase (Liang *et al*., 1996). The newly synthesized reporter protein was found to localize exclusively in the nucleus of *dbp5-2* mutant after shifting cultures to 37°C for 90 min (Figure 5). In addition, we observed that there was no cytoplasmic accumulation of Nop1p in *dbp5* mutants after 3 h at 37°C (data not shown). Although these data appear to suggest that some proteins continue to enter the nucleus upon Dbp5p's inactivation, the rapid cessation of mRNA export in *dbp5* cells at 37°C may have made it difficult for us to detect the reporter protein accumulated at a low level in the cytoplasm (see also Discussion).

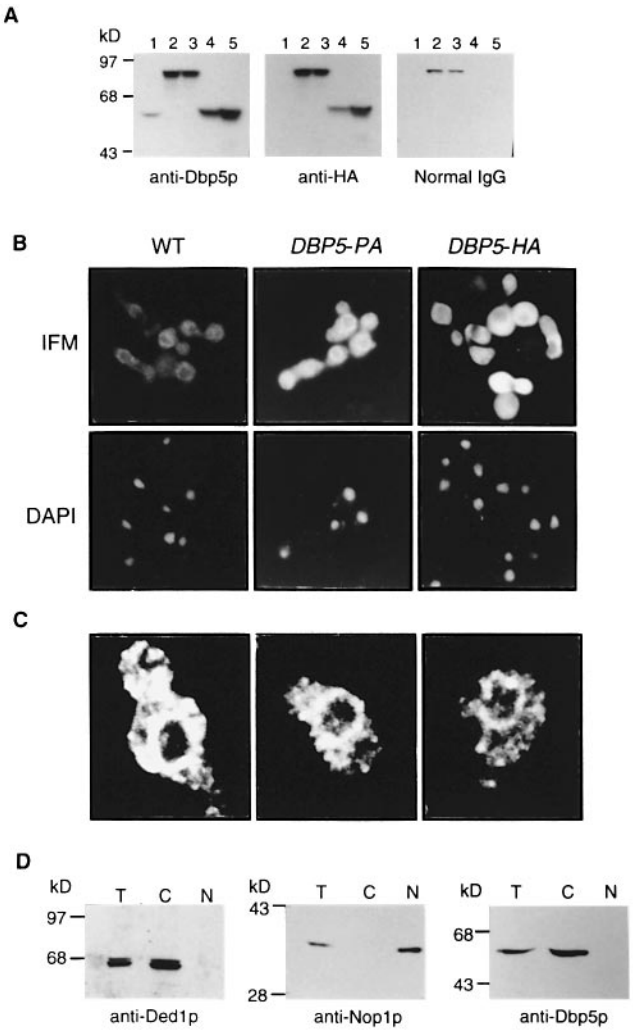



**Fig. 4.** Cytosolic localization of Dbp5p. (**A**) Immunoblot analysis of crude extracts from the wild-type strain (lane 1), the *DBP5–PA* strain (lanes 2 and 3) or from the *DBP5-HA* strain (lanes 4 and 5). Immunoblots were developed with anti-Dbp5p polyclonal antiserum, anti-HA polyclonal antibody or normal rabbit serum (normal IgG) at 1:5000 dilution, followed by the addition of Protein G–horseradish peroxidase conjugate (Bio-Rad) at 1:5000 dilution and chemiluminescent detection (ECL system, Amersham). The *DBP5-HA* gene fusion was driven by the yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter ($P_{GPD}$) and was carried on either centromeric (lane 4) or 2 μm (lane 5) plasmids. Mol. wt standards are indicated on the left. (**B**) IFM of the wild-type (WT), *DBP5-PA* or *DBP5-HA* expressing cells. Cells were stained with either the anti-Dbp5p antiserum (WT), purified normal rabbit IgG (*DBP5-PA*) or anti-HA antibody (*DBP5-HA*) at 1:200 dilution and then with Cy3-conjugated goat antibody to rabbit IgG (WT and *DBP5-PA*) or with Texas Red-conjugated goat antibody to mouse IgG (*DBP5-HA*) at 1:50 dilution. Staining of the same cells by 4′, 6-diamino-2-phenylindole (DAPI) is also shown. (**C**) Detection of Dbp5p by confocal IFM in wild-type cells. Single cells sectioned through the equatorial plane are shown. (**D**) Subcellular fractionation of Dbp5p in a wild-type diploid strain. Total cellular extract (T) was separated into cytosolic (C) and nuclear (N) fractions and analyzed by immunoblotting. Antisera against Ded1p, a cytosolic translation factor, and Nop1p, a nucleolar protein, were used as controls to monitor the effectiveness of the fractionation procedure.

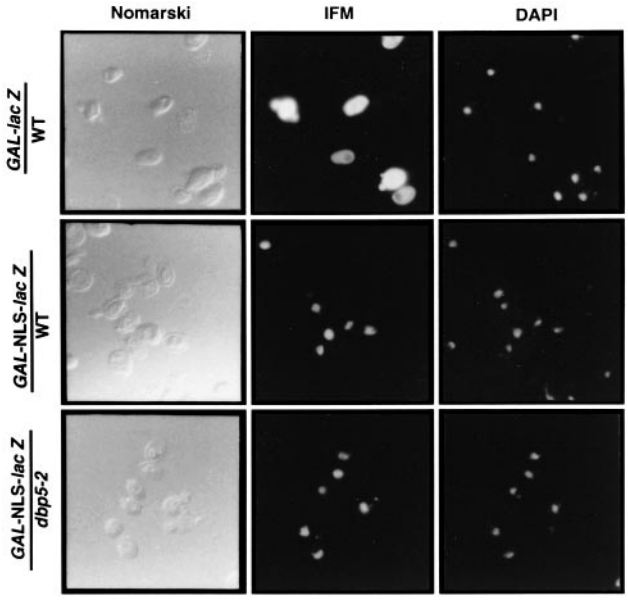



**Fig. 5.** Import of reporter proteins in wild-type and *dbp5-2* cells. Two reporter proteins, one consisting of an NLS derived from Mtr4p fused to β-galactosidase (*GAL*–NLS-*lac*Z) and the other lacks of the NLS (*GAL–lac*Z), were used in these experiments. Both reporter genes were driven by the yeast *GAL10* promoter (*GAL*). Wild-type (WT) and *dbp5-2* strains were grown initially in medium containing raffinose to an $OD_{600}$ of 0.7. The harvested cells were resuspended in medium containing galactose and incubated at 25°C for 90 min to overproduce the reporter proteins. To inactivate the function of Dbp5p, cells were then incubated at 37°C for additional 90 min prior to processing for immunolocalization experiments with anti-β-galactosidase antibody. Normarski, cells viewed by Normarski optics; IFM, cells viewed using a Texas Red filter; DAPI, cells viewed using a DAPI filter.

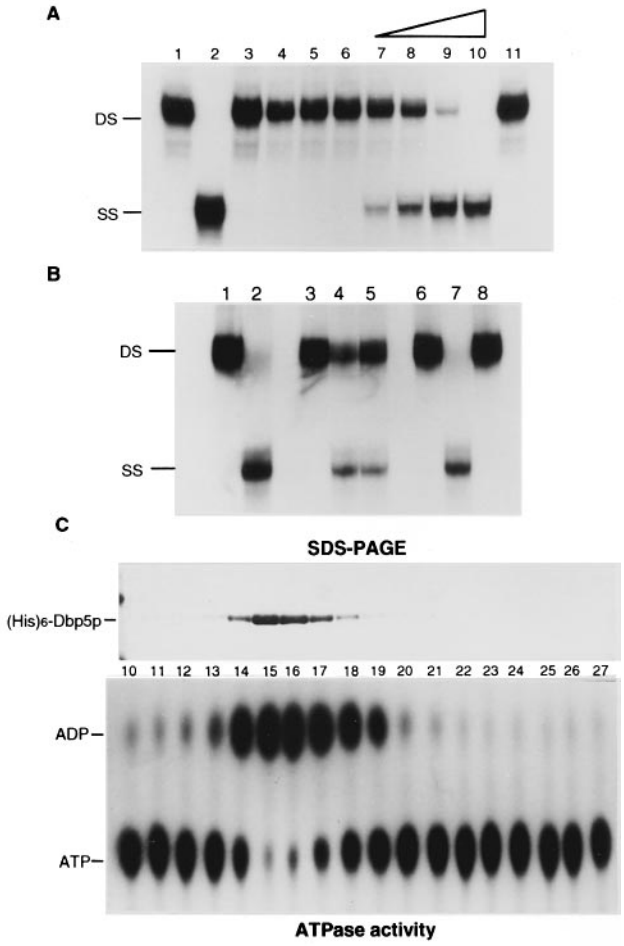



**Fig. 6.** Enzymatic assays of Dbp5p. (**A**) RNA unwinding activity of immunoprecipitated Dbp5p–PA. Increasing amounts of the cytosolic fractions isolated from a *DBP5–PA* diploid strain were incubated with IgG–Sepharose (lanes 7–10) and immunoprecipitates were assayed for RNA unwinding activity at 37°C using an RNA substrate containing both 5′ and 3′ single-stranded overhangs flanking a 10 bp alternating G–C duplex (Rozen *et al.*, 1990). Cytosolic fractions from an isogenic wild-type strain (lane 4) or from the *DBP5–PA* strain but omitting ATP (lane 5) or $Mg^{2+}$ (lane 6) in the unwinding assay were used as controls. Neither IgG–Sepharose (lane 3) nor Protein A–Sepharose (lane 11) alone can unwind the RNA duplex. Double-stranded RNA substrate (DS) (lane 1) can be denatured into the single-stranded form (SS) by boiling (lane 2). (**B**) Inactivation of the RNA unwinding activity of the immunoprecipitated Dbp5-2p–PA. Cytosolic fractions isolated from wild-type *DBP5* (lanes 3 and 6), *DBP5–PA* (lanes 4 and 7) and *dbp5-2–PA* diploid strains (lanes 5 and 8) were assayed for IgG–Sepharose-precipitated RNA unwinding activity. Reactions were carried out either at 30°C without preincubation (lanes 3–5) or at 37°C with preincubation at 37°C for 15 min. Double-stranded RNA substrate (lane 1). Heat-denatured ssRNA (lane 2). (**C**) Intrinsic RNA-dependent ATPase activity of Dbp5p. Recombinant Dbp5p with a six-histidine tag [$(His)_6$-Dbp5p] was purified to homogeneity by ammonium sulfate fractionation, DEAE–Sepharose chromatography, immobilized metal affinity chromatography (ProBond, Invitrogen) and gel filtration on a Superdex 200 column. Fractions 10–27 from the final gel filtration column were analyzed by SDS–PAGE (top) and assayed for ATP hydrolysis in the presence of poly(I) (bottom). ADP was separated from ATP by thin-layer chromatography (TLC) on PEI-cellulose.

### *RNA-dependent ATPase and ATP-dependent RNA unwinding activities of Dbp5p*

Several DEAD-box proteins exhibit RNA unwinding activities *in vitro* (Fuller-Pace, 1994). To evaluate the RNA helicase activity of Dbp5p, we incubated the cytosolic fraction prepared from the *DBP5–PA* strain with IgG–Sepharose and assayed for the RNA unwinding activity directly on the IgG–Sepharose beads. Almost 100% of the RNA substrate could be unwound in a dose-dependent manner when increasing amounts of the cytosolic fraction were used for immunoprecipitation (Figure 6A, lanes 7–9). The observed RNA unwinding activity required both ATP (lane 5) and $Mg^{2+}$ (lane 6). This RNA unwinding activity was probably derived from the Dbp5p moiety of the immunoprecipitated Dbp5p–PA, as IgG–Sepharose beads incubated with extracts prepared from the wild-type strain (lane 4), IgG–Sepharose beads alone (lane 3) or PA–Sepharose beads alone (lane 11) yielded no detectable RNA unwinding activity. In addition, using extracts prepared from a *dbp5-2–PA* temperature-sensitive strain, we showed that the IgG–Sepharose-associated RNA unwinding activity was active at 30°C (Figure 6B, lane 5), but could be completely inactivated by pre-incubating the reaction at 37°C for 15 min (lane 8). Thus, the observed RNA unwinding activity is derived from Dbp5p, rather than from other factors co-precipitated with Dbp5p. When tested under the same conditions, the purified recombinant Dbp5p [$(His)_6$-Dbp5p] (Figure 6C, top) exhibited no RNA unwinding activity, suggesting a scenario analogous to that of the mammalian eIF4A, which requires eIF4B as a cofactor for RNA unwinding (Rozen *et al.*, 1990). The purified $(His)_6$-Dbp5p had an RNA-dependent ATPase activity (Figure 6B, bottom), which could be activated by poly(U), poly(A), poly(I), poly(C), poly(G) and tRNA, but not by poly(dT) or plasmid

DNA (data not shown). Under the same conditions, only negligible amounts of GTP, CTP and UTP hydrolysis could be detected (data not shown).

### *Evolutionary conservation of Dbp5p*

We searched the DDBJ/EMBL/GenBank database for potential Dbp5p homologues and identified three candidates in *Schizosaccharomyces pombe* (accession No. Q09747; 60.3% sequence identity), *Dictyostelium discoideum* (accession No. AF002677; *helC*; 47.2% sequence identity) and mouse (accession No. L25125; 48.4% sequence identity) (Figure 7A). Interestingly, disruption of the *helC* gene has been shown to lead to developmental asynchrony, failure to differentiate and aberrant morphogenesis in *D.discoideum* (L.M.Machesky, personal communication). The likelihood that Dbp5p is evolutionarily conserved was further examined using the anti-Dbp5p polyclonal antibody to survey protein extracts prepared from *Xenopus laevis* oocytes, *Drosophila melanogaster* early embryos, HeLa cells and three other cell lines. Except for the human Jurkat cell line, the antibody reacted strongly with a single protein in each case (Figure 7B), suggesting that the functions of Dbp5p may be conserved among higher eukaryotes.

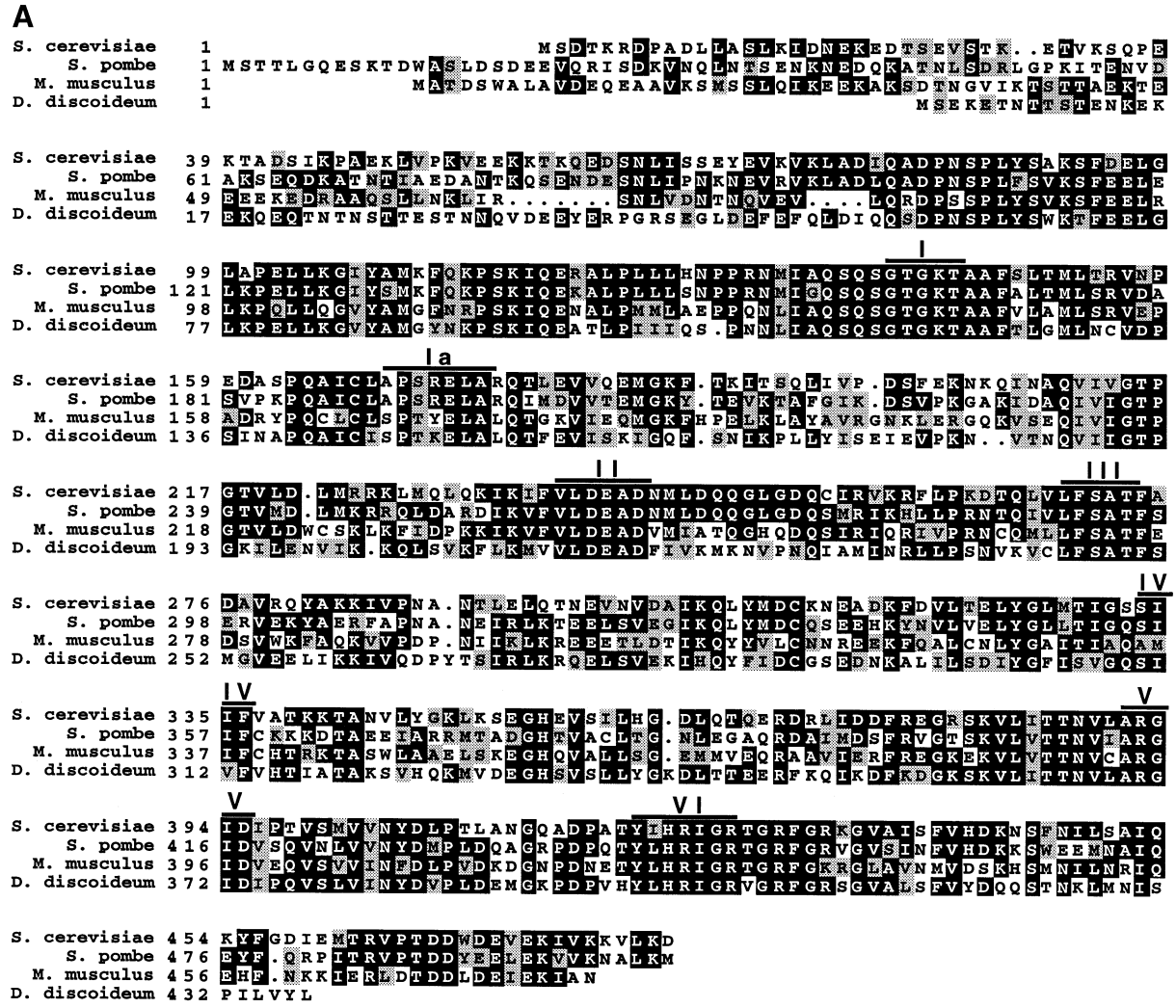
## Discussion

During or immediately after transcription, nascent pre-mRNA transcripts are rapidly packaged into RNPs, which are likely to be the bona fide substrates for export. A number of observations have led to the suggestion that RNA-binding proteins play a key role in mRNA export. For example, it has been reported that the shuttling of some hnRNP proteins can be arrested by inhibition of pol II transcription (Michael *et al*., 1995b), that an M9 domain in hnRNP A1 protein is responsible for its rapid export and import (Michael *et al*., 1995a) and that an hnRNP-like protein was found to escort the Balbiani ring (BR) granules, the giant-size pre-mRNPs found in the salivary glands of *Chironomus tentans*, from the nucleus to the cytoplasm (Visa *et al*., 1996). A general model for mRNA export can therefore be advanced as follows (see also Ullman *et al*., 1997): after binding of specific proteins to mRNA, the export process is initiated by the binding of the 'exportins', or the NES receptors, to NESs present in the protein moiety of the mRNP complex. The exportins in turn mediate the interaction of the mRNP cargo with NPC, through which the mRNP cargo translocates, with the participation of RanGTPase, to the cytoplasm. Upon their arrival in the cytoplasm, the mRNP cargo is then released from exportins and most of the RNA-binding proteins are recycled back to the nucleus for a new round of export. If this itinerary is relatively uniform for most mRNAs, proteins which are capable of altering or normalizing the structure of the mRNAs or mRNPs would be expected to play a role in the export process as well. These may include nuclear proteins which package the mRNA into mRNP and facilitate their interactions with exportins, NPC proteins which influence the RNA–protein interactions (Teixeira *et al*., 1997), and cytosolic proteins which mediate the release or displacement of the mRNP cargo, thereby facilitating the recycling of RNA-binding proteins.

In this work, we show that mutations in a novel DEAD-box protein gene, *DBP5*, cause a dramatic and rapid block of poly(A)$^+$ RNA export. Most strikingly, we show that Dbp5p is not localized to the nucleoplasm, but rather is concentrated in the cytoplasm with significant enrichment surrounding the nuclear envelope. What would be the role of Dbp5p in mRNA export, considering its characteristic RNA unwinding activity *in vitro*? It is possible that Dbp5p is required for 'unpackaging' the mRNP cargo. Upon regulation by appropriate cytosolic factors, Dbp5p may alter the secondary or tertiary structure of exported mRNAs and, therefore, affect their association with specific proteins. Such events may release the mRNP cargo, in a scenario akin to the role of importin β in mediating the release of the exported snRNAs (Görlich *et al*., 1996). Thus, when it cannot function, critical proteins that normally return to the nucleus for recruiting or packaging export cargo can no longer do so, resulting in a rapid accumulation of poly(A)$^+$ RNA in the nucleus. Alternatively, if unpackaging does not occur, mRNA carriers associated with mRNA may still return to the nucleus but may be unable to accept additional mRNA. Consistent with this latter possibility, at least one putative mRNA carrier, Mtr13p/Npl3p appears to continue to cycle between the nucleus and cytoplasm in the *dbp5* mutants (A.M. Tartakoff, unpublished).

Studies of the export of the BR granules in *C.tentans* by electron microscopy suggests that protein synthesis may be initiated immediately upon translocation of the mRNA into the cytoplasm, since ribosomes can be seen attached to the leading end of the RNP fibril (reviewed in Daneholt, 1997). The fact that Dbp5p is concentrated around the nuclear envelope raises an interesting possibility that it may play a role in coupling mRNA export to translation. It seems unlikely that Dbp5p is directly involved in translation, since we have shown that protein synthesis in both *dbp5-1* and *dbp5-2* mutants remains active for some time after inactivating the activity of Dbp5p *in vivo* (Figure 2), and that *in vitro* depletion of Dbp5p does not impact on the programmed cell-free protein synthesis (data not shown). Thus, it is more likely that Dbp5p may assist in remodeling or altering the conformation of the packaged mRNP, which can then be committed to translation. In this way, the function of Dbp5p is similar to the chromatin-remodeling complexes, SWI/SNF (Peterson and Tamkun, 1995) and RSC (Cairns *et al*., 1996), both of which exhibit DNA-dependent ATPase activity, and to three of the yeast DEAH-box proteins, Prp2p (Kim *et al*., 1992), Prp16p (Schwer and Guthrie, 1992) and Prp22p (Company *et al*., 1991), which have been shown to alter the spliceosome conformation during splicing reaction (reviewed in Staley and Guthrie, 1998).

We also noted that a substantial amount of Dbp5p is distributed in a non-uniform manner throughout the cytoplasm. This 'patch-like' distribution is somewhat reminiscent of the nuclear distribution pattern of mRNA splicing factors, such as the SR protein SC35, which is localized in a network of large nuclear objects known as 'speckles'. Speckles are thought to be the storage or assembly sites containing the highest concentration of splicing factors (reviewed in Singer and Green, 1997). Thus, it is tempting to speculate that there may be different populations of Dbp5p existing in the cytoplasm, one of



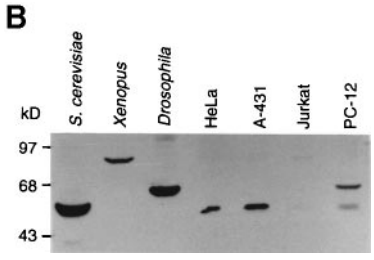




**Fig. 7.** (**A**) Sequence alignment of Dbp5p homologues. Protein sequences of Dbp5p (*S.cerevisiae*) homologues found in *S.pombe*, *Mus musculus* and *D.discoideum* were aligned using the PILEUP program in the GCG package and further adjusted by importing into the BOXSHADE server (http://ulrec3.unil.ch/software/BOX_form.html) for graphic presentation. Conserved sequence motifs (denoted by roman numerals) are indicated. Black boxes, sequence identities; gray boxes, sequence similarities. (**B**) Evolutionary conservation of Dbp5p. Cellular extracts prepared from various organisms were analyzed by immunoblotting. The immunoblot was developed with anti-Dbp5p polyclonal antiserum at 1:5000 dilution and Protein G–horseradish peroxidase conjugate (Bio-Rad) at 1:5000 dilution, followed by chemiluminescent detection of potential Dbp5p (*S.cerevisiae*) homologues. *Xenopus*, stage VI oocytes from *X.laevis*; *Drosophila*, early (1 h) embryos from *D.melanogaster*; A-431, epidermoid carcinoma cell line (human); Jurkat, acute T-cell leukemia cell line (human); PC-12, adrenal pheochromocytoma cell line (rat). Mol. wt standards are indicated on the left.

which is concentrated in the patch-like areas and the other is enriched around the nuclear envelope. The patch-like areas may represent the storage sites for Dbp5p, from which Dbp5p molecules are recruited to the nuclear periphery for participating in mRNA export. In this regard, it is interesting to note that Dbp5p is a remarkably abundant protein constituting almost 0.1% of the total cellular protein in the yeast cell (S.S.-I Tseng and T.-H. Chang, unpublished), as are the highly abundant mammalian splicing factors, such as SR proteins. Alternatively, the patch-like areas may contain a subpopulation of Dbp5p that has accompanied the exported mRNAs to their sites of translation. The yeast Pub1p, an RNA-binding protein in the nucleus and the cytoplasm, was found to localize in a markedly discontinuous pattern in the cytoplasm. Interestingly, Pub1p is thought to be stably bound to a translationally inactive subpopulation of mRNAs within the cytoplasm (Anderson *et al*., 1993).

It has long been proposed that the energy requirement for mRNA export is derived from GTP hydrolysis by Ran

(reviewed in Koepp and Silver, 1996). We find that the purified recombinant Dbp5p exhibits a characteristic RNA-dependent ATPase activity, similar to all characterized DEAD-box proteins. When assayed under the same condition, Dbp5p hydrolyzes only a negligible amount of GTP. Thus, our data suggest that ATP hydrolysis by Dbp5p may be used as an energy source to modulate the RNA or RNP structure during mRNA export. The finding that the immunoprecipitated Dbp5p–PA, but not the purified recombinant Dbp5p, can unwind RNA duplex *in vitro* raises the possibility that other cofactors may be required to regulate or activate Dbp5p's RNA unwinding activity *in vivo*. This is consistent with our observation that Dbp5p is part of a large complex (S.S.-I Tseng and T.-H.Chang, unpublished). It remains to be established whether the RNA unwinding activity we observed is truly required for mRNA export. To this end, we are in the process of isolating *dbp5* mutants which can uncouple the ATPase activity from that of the RNA unwinding activity.

The majority of the proteins found to be involved in mRNA export are localized either in the nucleus or in NPC. There are, however, several proteins that are primarily in the cytosol. These include the GTPase-activating protein (GAP), such as the yeast Rna1p, which maintains cytosolic Ran in the GDP-bound state (Koepp *et al*., 1996); Mtr7p/Acc1p, an acetyl CoA carboxylase required for stable integration of the NPC into the nuclear envelope (Schneiter *et al*., 1996); Mtr10p, a protein distantly related to importin β which is required for the nuclear localization of Mtr13p/Npl3p (Pemberton *et al*., 1997; A.M. Tartakoff, unpublished); and the yeast eIF5A homologue, Hyp2p, which has been implicated in the export of HIV-1 Rev protein (Bevec *et al*., 1996). Our discovery that a cytosolic RNA helicase, Dbp5p, is essential for mRNA export not only emphasizes the importance of various RNA helicases, including the nuclear Mtr4p (Liang *et al*., 1996) and RNA helicase A (Tang *et al*., 1997), in the export process, but also underscores our limited understanding of the cytosolic events occurring subsequent to mRNA export yet preceding translation initiation.

## Materials and methods

### *Cloning and sequencing of DBP5*

A YCp50 genomic library was screened by bacterial colony hybridization for *DBP5*-containing clones using a $^{32}$P-labeled DNA probe, *CA5/6* (Chang *et al*., 1990). Potential positives were re-screened twice prior to restriction analysis and Southern analysis using the same *CA5/6* probe. On the basis of the Southern results, a 3.9 kb *Spe*I–*Xba*I fragment thought to encompass the entire *DBP5* gene was subcloned into pBluescript KS (–) vectors (Stratagene) in both orientations to yield pCA5001 and pCA5002. Two sets of nested deletion clones, one from each orientation, were constructed using an ExoIII/Mung Bean Deletion Kit from Stratagene. Single-stranded DNA (ssDNA) templates from these clones were then sequenced by dideoxy chain termination method. A total of 3972 nucleotides was determined.

### *Construction of yeast strains*

*DBP5* carried on a *Bam*HI–*Not*I (both were from polylinker) fragment from pCA5001 was recloned into pBluescript KS(+) vector to yield pCA5013 for constructing the *dbp5::HIS3* allele. An internal 1.2 kb *Eco*RI–*Eco*RV fragment from the *DBP5* coding region was replaced with a 1.7 kb *Eco*RI–*Ecl*136II fragment containing *HIS3* to yield pCA5014. To replace the chromosomal *DBP5* by homologous recombination, a 4.5 kb *Sac*I–*Apa*I fragment containing the *dbp5::HIS3* allele from pCA5014 was transformed into a *his3*$^-$ diploid strain, YPH274 (*MAT***a**/α, *ura3-52*/*ura3-52*, *lys2-801*/*lys2-801*, *ade2-101*/*ade2-101*, *trp1-*Δ*1*/*trp1-*Δ*1*, *his3-*Δ*200*/*his3-*Δ*200*, *leu2-*Δ*1*/*leu2-*Δ*1*; Sikorski and Hieter, 1989). Yeast genomic DNAs isolated from the His$^+$ transformants were used to screen by Southern analysis for transformants in which one of the two *DBP5* genes was replaced by the *dbp5::HIS3* allele. The probe used was a *Pst*I DNA fragment isolated from pCA500. The resulting heterozygous strain, YTC150, bears the following genotype: *MAT***a**/α, *dbp5::HIS3*/*DBP5*, *ura3-52*/*ura3-52*, *lys2-801*/*lys2-801*, *ade2-101*/*ade2-101*, *trp1-*Δ*1*/*trp1-*Δ*1*, *his3-*Δ*200*/*his3-*Δ*200*, *leu2-*Δ*1*/*leu2-*Δ*1*. Transformation of strain YTC150 with pCA5005 (*DBP5*/*CEN*/*URA3*) yielded strain YTC151. Subsequent sporulation of strain YTC151 allowed us to isolate strain YTC152 [*MAT***a**, *ura3-52*, *lys2-801*, *ade2-101*, *trp1-*Δ*1*, *his3-*Δ*200*, *leu2-*Δ*1*, *dbp5::HIS3*, (pCA5005)].

### *Screening conditional mutants*

A 1.6 kb DNA fragment was PCR-amplified using primers DBP5-3 (ccaccttggatccgaatgag; *Bam*HI site, underlined) and DBP5-5 (cttttgtatacgagctcttttactgt; *Sac*I site, underlined) on pCA5001 template. Primer DBP5-3 is complementary to sequence adjacent to the initiation codon of *DBP5*, and primer DBP5-5 is complementary to a region ~200 bp downstream from the stop codon. Mutagenic conditions developed by Cadwell and Joyce (1992) were used for PCR amplification. The PCR product was digested with *Avr*II and *Sac*I. The resulting 1.2 kb *Avr*II–*Sac*I fragment, covering all the conserved motifs, was then used to replace a corresponding fragment in pCA5022 (*DBP5*/*CEN*/*LEU2*). Approximately 6000 bacterial transformants were pooled for preparing a mutant DNA library to transform into yeast strain YTC152 (see above). Selection for pCA5005 (*URA3*), which carries the wild-type *DBP5* gene, was omitted, allowing this plasmid to be lost freely. Transformants were replica-plated onto three 5-FOA plates which were then incubated at 30, 37 and 16°C, respectively (Sikorski and Boeke, 1991). Conditional mutants were picked and colony-purified on 5-FOA plates. Two tight Ts$^-$ mutants, *dbp5-1* and *dbp5-2*, were isolated after screening ~3000 colonies. To confirm the linkages between the Ts$^-$ phenotypes and the *dbp5* alleles, the mutagenized plasmids were recovered and the *dbp5* mutant alleles were recloned and reintroduced into YTC152 for another round of 5-FOA counterselection. Mutations in the *dbp5* mutant alleles were determined by DNA sequencing.

### *Purification of (His)$_6$-Dbp5p and production of anti-Dbp5p antibody*

A 1.4 kb *Bam*HI (introduced by DBP5-3 primer)–*Hin*dIII fragment containing the *DBP5* coding region was cloned into pRSET-C to create pCA5012 for transformation into bacterial strain BL21 (DE3). Cells were grown to 0.5 unit at $OD_{600}$ prior to the addition of IPTG to 0.1 mM for 3 h at 37°C. Cells from 7.5 l culture were harvested, washed once with 50 mM Tris–HCl (pH 7.5) and stored at –75°C. Cells were thawed on ice, resuspended in 30 ml of lysis buffer [40 mM HEPES–NaOH (pH 7.5), 200 mM NaCl, 1 mM EDTA, 10% glycerol, one proteinase inhibitor tablet (Boehringer Mannheim) per 50 ml buffer], and treated with lysozyme (100 μg/ml) on ice for 15 min. Three cycles of sonication/freeze–thaw, each consisting of three 10 s sonication bursts at a medium intensity setting, flash freezing in liquid nitrogen and rapid thawing in a 37°C water bath, were employed to lyse the cells. The crude lysate was cleared by centrifugation at 12 000 r.p.m. in an SS34 rotor (Sorvall) for 10 min. Nucleic acids in the supernatant were removed by addition of polyethylenimine to 0.2% with constant stirring in the cold room for 30 min, followed by centrifugation at 18 000 r.p.m. (SS34) for 30 min at 4°C. Proteins in the cleared lysate were then precipitated by $(NH_4)_2SO_4$ at 60% saturation and collected by centrifugation at 18 000 r.p.m. for 1 h. The protein pellet was resuspended in buffer A [40 mM HEPES–NaOH (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol and proteinase inhibitors], dialyzed against two changes of buffer A in the cold room overnight, and applied onto a 50 ml DEAE–Sepharose (Pharmacia) column. The flow-through fraction was then loaded onto a 30 ml ProBond (Invitrogen) column, washed with 10 bed volumes of buffer A, and the bound $(His)_6$-Dbp5p was eluted by a linear imidazole gradient (10–300 mM). $(His)_6$-Dbp5p that eluted at 150 mM imidazole was collected and dialyzed against buffer A and further purified by gel filtration on a Superdex 200 column using a Pharmacia SMART system. Purified $(His)_6$-Dbp5p was used to raise rabbit polyclonal antibody and for subsequent enzymatic assays.

### *Detection of Dbp5p, Dbp5p-HA and Dbp5p–PA by immunoblots*

A nine amino acid HA epitope was fused to the C-terminus of Dbp5p by PCR using primers DBP5-3 and DBP5-4 [acacaagatctcta-(agcgtaatctggaacatcgtatgggtaatc)-ctttaacactttcttaa; *Bgl*II site, underlined;

HA epitope, in parentheses]. The 3′ sequence of DBP5-4 is complementary to the last 17 nucleotides of the *DBP5* coding sequence. A 1.4 kb DNA fragment amplified from pCA5001 using Expand High Fidelity polymerase mix (Boehringer Mannheim) was digested with *Bam*HI and *Bgl*II and cloned into expression vectors pRS315-pG1 and pRS425-pG1 (Chuang *et al*., 1997; Weaver *et al*., 1997) to yield pCA5018 and pCA5020. To construct the *DBP5–PA* clone, an *Aat*II site was introduced immediately upstream of the stop codon by PCR using primers DBP5-1 (tacttaccaccttagaaagctttgagtgatacaaagag) and DBP5-6 (attatgtactgaattctagacgtcatcctttaacac; *Aat*II site, underlined). The PCR product was digested into a 1.3 kb *Avr*II–*Eco*RI DNA fragment, which was used to replace a corresponding fragment in pCA5013 (see above) to yield pCA5023. A 651 bp *Aat*II DNA fragment (Chuang *et al*., 1997) encoding the Protein A moiety was then inserted into the *Aat*II in pCA5023 to yield pCA5032. Strain YTC152 was transformed with pCA5018, pCA5020 and pCA5032. Transformants were streaked on 5-FOA plates to yield strains YTC159, YTC160 and YTC433, respectively.

To detect the Dbp5p-HA and Dbp5p–PA by immunoblotting, yeast strains were grown in 100 ml of YPD medium to 1 unit at $OD_{600}$. Cells were harvested and broken with glass beads in lysis buffer (50 mM Tris–Cl pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mM PMSF). 100 μg of total yeast proteins were separated on a sodium dodecyl sulfate–10% polyacrylamide gel (10% SDS–PAGE) and then transferred to a nitrocellulose membrane by electroblotting. Immunoblots were developed with either anti-Dbp5p polyclonal antiserum or normal rabbit serum (normal IgG) at 1:5000 dilution, or anti-HA polyclonal antibody (BAbCO) at 1:5000 dilution, followed by the addition of Protein G–horseradish peroxidase (HRP) conjugate (Bio-Rad) at 1:5000 dilution and chemiluminescent detection (ECL system, Amersham).

### Microscopy

Haploid *dbp5* strains were used in FISH analysis as described (Kadowaki *et al*., 1992). IFM was carried out as described (Chuang *et al*., 1997; Weaver *et al*., 1997) using strains YPH274 (Sikorski and Hieter, 1989) and YTC433 (*DBP5–PA*; see above) for staining with anti-Dbp5p polyclonal antiserum and with purified normal rabbit IgG, respectively. Detection of Dbp5p-HA was done in the haploid background using monoclonal anti-HA antibody (BAbCO). The detection of the wild-type Dbp5p in YPH274 was also carried out using a Bio-Rad confocal microscopy system (MRC-600), in which the laser beam was set to serially section cells in a depth of 1.25 μm. For thin-section electron microscopy, haploid *dbp5-1* mutant cells were fixed by addition of formaldehyde and glutaraldehyde (final concentrations 2 and 0.5%, respectively) to the culture medium, followed by sedimentation, continued aldehyde fixation, spheroplasting and osmication. After embedding in Polybed 812, thin sections were stained with lead citrate and uranyl acetate and examined in a Jeol CX100 electron microscope. Protein import assay was carried out as described (Gorsch *et al*., 1995) using wild-type and *dbp5-2* strains carrying a *lac*Z reporter construct described in Liang *et al*. (1996).

### Subcellular fractionation of Dbp5p

Diploid strain YPH274 was grown in 500 ml of YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C to an $OD_{600}$ of 0.7. Cells were harvested and spheroplasted with zymolyase 100T (Seikagaku America), and regenerated in 500 ml of YPD supplemented with 1 M sorbitol at 30°C for 1 h. The regenerated spheroplasts were broken by 12 strokes of douncing in a glass dounce homogenizer (Kontes) and fractionated exactly as described (Wise, 1991). For immunoblot analysis, equal volumes of the nuclear and cytosolic fractions were separated by 10% SDS–PAGE. Monoclonal anti-Nop1p antiserum was from J.Aris and used at 1:5000 dilution. Polyclonal anti-Ded1p and anti-Dbp5p antisera were also used at 1:5000 dilution.

### RNA unwinding assay

5–20 μl of cytosolic fraction (protein concentration, 3 mg/ml) was incubated with 20 μl (bed volume) of IgG–Sepharose (Pharmacia) at 4°C for 3 h. The mixture was extensively washed eight times with 1 ml of HMSN buffer [25 mM HEPES–NaOH (pH 7.5), 5 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.25 M sucrose, 0.05% NP-40, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM pepstatin] followed by three additional washes with 1× unwinding buffer [17 mM HEPES–NaOH (pH 7.5), 1 mM $MgCl_2$, 2 mM DTT, 1 mM spermidine, 0.3% PEG8000]. Each wash consisted of 10 min rocking on a nutator at 4°C. A 20 μl unwinding reaction was assembled and added to the washed beads and incubated at 37°C for 20 min. A typical unwinding reaction (Rozen *et al*., 1990) contained 1× unwinding buffer, 1 mM ATP, 0.1 mM GTP, 5% glycerol, 150 mM KCl, 40 U RNasin, 10 μg tRNA and 50 fmole $^{32}$P-labeled dsRNA substrate. One μl of stop buffer (10 mg/ml proteinase K, 10% SDS) was then added to 10 μl supernatant to terminate the reaction by incubating at 37°C for 20 min. Reactions were analyzed by 10% native polyacrylamide gel (19:1) electrophoresis. Inactivation of the RNA unwinding activity of Dbp5-2p–PA was achieved by preincubating the reaction without ATP and dsRNA substrate at 37°C for 15 min followed by the addition of ATP and dsRNA and incubation at 37°C for 20 min.

### ATPase assay

A 10 μl reaction containing the purified $(His)_6$-Dbp5p in 40 mM HEPES–NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 0.1 mg/ml poly(I), 1 mg/ml BSA, 10 μM cold ATP and 0.1 μl [α-$^{32}$P]ATP (specific activity = 3000 Ci/mmol) was assembled. After incubation for 1 h at 30°C, reactions were terminated by adding 4 μl of stop buffer (1 mg/ml proteinase K, 1% SDS, 0.1 M EDTA) and incubated for 20 min at 37°C. For TLC analysis, 2 μl of the reaction mixture was spotted on a PEI-cellulose plate and developed by 0.6 M potassium phosphate (pH 3.4).

### Measurement of mRNA turnover

YTC152 was crossed to yRP693 (*MAT*α, *rpb1-1*, *ura3*, *leu2*; obtained from C.Decker) for isolating a haploid segregant (*MAT***a** *dbp5::HIS3*, *rpb1-1*, *ura3*, *leu2*, pCA5005), which was transformed with plasmids harboring the *dbp5-1* and *dbp5-2* alleles. Counterselection of pCA5005 by 5-FOA yielded *rpb1-1·dbp5-1* and *rpb1-1·dbp5-2* double mutants. Cells were grown in YPD to 0.6 unit at $OD_{600}$, shifted to 37°C for 0.5, 1, 2 and 3 h, respectively, prior to extraction of total RNAs for Northern blotting using probes hybridized to the *ACT1*, *CRY1*, *CUP1*, *CYH2* and *PGK1* transcripts. For thiolutin method, 3 μg/ml of thiolutin was added to cell cultures at an $OD_{600}$ of 0.6 unit and incubated for 1 h prior to shifting to 37°C for 15, 30, 45 and 60 min.

### Protein synthesis in dbp5 mutants

Wild-type, *ded1-199*, *dbp5-1* and *dbp5-2* strains were grown to an $OD_{600}$ of 0.5 unit in YPD. Eight units of cells at $OD_{600}$ were harvested, resuspended in 1 ml of fresh YPD and incubated at either 15°C (*ded1-199*) or 37°C (*dbp5-1* and *dbp5-2*) for 15 or 30 min prior to the addition of 20 μCi of L-[$^{35}$S]methionine (>1000 Ci/mmol; NEN). After 1 h incubation at the non-permissive temperatures, cells were collected at room temperature and washed once in 1 ml Buffer I [50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100 and 50 μg/ml cycloheximide] in a microfuge tube. The washed cell pellet was resuspended in 200 μl Buffer I and ~500 μl of glass beads were added. Cells were broken by vortexing for 12 cycles, each consisting of 30 s of vortexing and 1 min of cooling on ice, in the cold room. Cell debris was clarified by two low-speed centrifugations at 3000 r.p.m. for 2 min each in an SS34 rotor. Protein concentration in the supernatant was then determined by Bradford assay. A total of 120 μg of total proteins of each sample were fractionated by 10% SDS–PAGE. After electrophoresis, the gel was fixed in 50% methanol–10% acetic acid for 30 min, soaked in 1 M salicylic acid for 1 h, vacuum dried and exposed directly to the X-ray film. Experiments testing the requirement of Dbp5p for translation *in vitro* were carried out as described (Chuang *et al*., 1997) using anti-Dbp5p polyclonal antibody for immunodepletion followed by examining the translational activities of the depleted extracts by luciferase assay.

### Determination of poly(A) tail length

The preparation of poly(A)$^+$ RNAs from the wild-type, *dbp5-1* and *dbp5-2* cultures shifted to 37°C for 30 min, their labeling by [$^{32}$P]Cp and the analysis of the poly(A) tail length by RNase A digestion, were done as described by Sachs and Davis (1989).

### Detection of potential Dbp5p homologs by immunoblots

*Xenopus* oocyte extract was prepared by mixing oocytes with 1.3 volumes of homogenization buffer [40 mM Tris–HCl (pH. 7.5), 10 mM $MgCl_2$, 7% sucrose, 2 mM DTT, 1% Triton X-100, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin] in a dounce homogenizer followed by 12 strokes of douncing. After centrifuging at 5000 and at 13 000 r.p.m. for 10 and 30 min each in an SS34 rotor, the cytosolic fraction between the lipid layer and the pellet was recovered. HeLa cells were grown to 90% confluence, harvested, washed once in phosphate-buffered saline, resuspended in 500 μl of RIPA [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 10 mM $Na_2HPO_4$, 10 mM NaF, 0.5% deoxycholate, 0.1% SDS, 1% NP-40, 10% glycerol, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin] and kept on ice for 5 min. After centrifugation in a microfuge at 13 000

r.p.m. and 4°C for 10 min, the supernatant was then recovered. Protein extract from *Drosophila* early embryos was a gift from E.Gottlieb. Protein extracts from A-431, Jurkat and PC-12 cells were obtained from M.Coggeshall. For immunoblotting, ~100 µg protein extract was fractionated by 10% SDS–PAGE. The immuno-detection of Dbp5p homologues was performed using anti-Dbp5p polyclonal antibody (1:5000 dilution) and HRP-conjugated anti-rabbit IgG (from donkey; Amersham) (1:5000 dilution).

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