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Characterization of DbpA, an *Escherichia coli* DEAD box protein with ATP independent RNA unwinding activity

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ABSTRACT

DbpA is a putative *Escherichia coli* ATP dependent RNA helicase belonging to the family of DEAD box proteins. It hydrolyzes ATP in the presence of 23S ribosomal RNA and 93 bases in the peptidyl transferase center of 23S rRNA are sufficient to trigger 100% of the ATPase activity of DbpA. In the present study we characterized the ATPase and RNA unwinding activities of DbpA in more detail. We report that—in contrast to eIF-4A, the prototype of the DEAD box protein family—the ATPase and the helicase activities of DbpA are not coupled. Moreover, the RNA unwinding activity of DbpA is not specific for 23S rRNA, since DbpA is also able to unwind 16S rRNA hybrids. Furthermore, we determined that the ATPase activity of DbpA is triggered to a significant extent not only by the 93 bases of the 23S rRNA previously reported but also by other regions of the 23S rRNA molecule. Since all these regions of 23S rRNA are either part of the 'functional core' of the 50S ribosomal subunit or involved in the 50S assembly, DbpA may play an important role in the ribosomal assembly process.

INTRODUCTION

Specific RNA–protein interactions often depend on so called 'RNA-chaperones' (1), proteins capable of altering RNA secondary structures. These 'helper-proteins' presumably destabilize or unwind RNA molecules to give them the appropriate structure needed for the correct binding of a specific protein. Members of the DEAD box protein family belong to these 'helper-proteins', and are characterized by the 'DEAD' motif (Asp-Glu-Ala-Asp) as well as by seven other highly conserved amino acid motifs (2). Proteins of this family hydrolyze ATP only in the presence of RNA. New members classified as DEAD box proteins based on sequence homologies alone are generally designated 'putative' ATP dependent RNA helicases, and only a few of those have actually been shown to unwind RNA experimentally. The eukaryotic Initiation Factor 4A (eIF-4A) (3,4) is a true ATP dependent RNA helicase and is generally recognized as the 'prototype' of the DEAD box protein family. eIF-4A plays a key role in unwinding secondary structures of mRNA molecules thus allowing the subsequent binding of the 40S ribosomal subunit to the mRNA which is a prerequisite for the formation of the pre-initiation complex in protein synthesis (4,5). Unlike eIF-4A, however, some DEAD box proteins appear not to be ATP dependent. An example of this is CsdA (also known as deaD; 6), a cold-shock protein in *Escherichia coli* and a member of the DEAD box protein family, which was recently shown to be able to destabilize RNA duplexes in the absence of ATP (7).

To date, five different genes encoding DEAD box proteins have been identified in *E.coli* (8). Among them, DbpA has been designated a 'putative' RNA helicase and has been suggested to play a role in protein biosynthesis and/or ribosome assembly. DbpA hydrolyzes ATP only in the presence of bacterial 23S rRNA (9), and recently it was determined that a region of 93 nucleotides (nt) at the peptidyl transferase center of 23S rRNA is sufficient to stimulate the ATPase activity of the protein (10). The helicase activity of DbpA, however, and its possible effect on protein synthesis or ribosomal assembly has not yet been established. Thus, we characterized DbpA further by determining its ability to destabilize or unwind RNA–RNA and DNA–RNA hybrids. We report here that DbpA indeed possesses an RNA unwinding ability. This RNA unwinding or helix destabilizing activity is not coupled to ATP hydrolysis. By carefully scanning the whole 23S rRNA molecule we found that, in addition to the previously identified 93 bases at the peptidyl transferase center, other regions of the 23S rRNA rich in secondary structure are also able to stimulate the ATPase activity of the protein to significant levels. Since all these regions of 23S rRNA are either part of the functional core of the 50S ribosomal subunit or involved in the 50S assembly, we propose a function of DbpA in the assembly process of this subunit.

MATERIALS AND METHODS

Materials

Enzymes were obtained from Boehringer Mannheim except RNAse H, which was a generous gift from Dr R. Brimacombe. Radiochemicals were obtained from Amersham. Oligonucleotides were synthesized from TIB Molbiol (Berlin).

Cloning, overexpression and purification of DbpA

The gene coding for DbpA (GenBank accession No. X52647) was amplified from *E.coli* DNA by PCR, and after confirming the

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sequence, subsequently cloned into a pET11a expression vector (Novagen). BL21(DE3)LysS cells (11) were transformed with the plasmid and grown at 37°C until they reached an $OD_{560\ nm}$ of 0.4. Overexpression of the protein in a 2 l culture was induced by the addition of IPTG to a final concentration of 1 mM, and a temperature shift from 37 to 30°C in order to avoid formation of insoluble inclusion bodies. After induction the culture was grown for 5 h after which cells were pelleted by centrifugation (3000 *g*, 10 min), resuspended in 20 mM HEPES pH 8.0, 10 mM $MgCl_2$, 300 mM NaCl and disrupted in a French press. Cell debris was removed by centrifugation for 30 min at 10 000 r.p.m. in a Ti45 Beckmann rotor. The supernatant was cleared of ribosomes by another centrifugation step (Ti45 Beckmann rotor, 35 000 r.p.m., 16 h). The clear supernatant was dialyzed against 20 mM HEPES pH 8.0, 125 mM NH_4Cl and applied to a 1.5 × 10 cm DEAE–Sepharose column (Pharmacia). The flow-through was collected, precipitated by the addition of 60% $(NH_4)_2SO_4$ and redissolved in 5 ml 20 mM HEPES pH 8.0, 50 mM NH_4Cl. After dialysis against the same buffer the protein was further purified on an 1.8 × 100 cm ACA44 gel filtration column (IBF Biotechniques). Fractions containing DbpA were collected and dialyzed against 20 mM HEPES pH 8.0, 1.5 M $(NH_4)_2SO_4$. The resulting protein solution was finally applied to a FPLC Phenyl-Superose HR 5/5 (1 ml) column (Pharmacia) and eluted using a linear 1.5 to 0 M $(NH_4)_2SO_4$ gradient with a flow rate of 0.5 ml/min. All purification steps were analyzed on SDS–PAGE (12). Purified DbpA was dialyzed against 20 mM HEPES pH 8.0, 50 mM NH_4Cl and the concentration was determined according to Bradford (13). The protein solution was frozen in liquid nitrogen and stored at –80°C.

RNA-binding assay

The binding of DbpA to RNA was determined by the retention of ^{32}P-labeled RNA on nitrocellulose filters. Reactions contained 20 pmol protein and 50 pmol RNA unless otherwise stated. Reactions were carried out in binding buffer (20 mM HEPES pH 8.0, 5 mM Mg-acetate, 50 mM NH_4Cl, 1 mM DTT) in a final volume of 100 µl. When 200 mM ammonium chloride was present in the assay, the binding of rRNA was reduced to 20% compared to the binding in the absence of ammonium. Therefore, 50 mM ammonium chloride was chosen arbitrarily for these assays. The ammonium dependence of the binding remained consistently the same regardless of the kind of rRNA (23S, 16S or 5S rRNA) used in the assays. Under the conditions used, RNA is not retained on nitrocellulose filters unless it is bound to protein. The reaction mix was incubated at 37°C for 15 min, then stopped by the addition of 2 ml ice-cold wash buffer (same as binding buffer with the addition of 0.1 mM EDTA) and immediately applied to nitrocellulose filters. The reaction tubes were rinsed twice with 2 ml ice-cold wash buffer. Filters were dried under an infrared lamp and radioactivity was determined by liquid scintillation counting.

Cross-linking of DbpA to ^{32}P-labeled ribosomal RNA and [α-^{32}P]ATP

The cross-linking experiments were performed based on Pause *et al*. (14). DbpA was cross-linked to either 16S or 23S ^{32}P-labeled rRNA, or [α-^{32}P]ATP. The amount of DbpA used for all these experiments was 20 pmol. For the cross-link of DbpA to ATP, 100 pmol [α-^{32}P]ATP (1 µCi) were incubated with DbpA for 5 min at 37°C in Tris–HCl pH 7.5, 50 mM KCl, 10% glycerol, with or without the addition of unlabelled tRNA or 23S rRNA. For the cross-link of DbpA to rRNA, 50 pmol (1 µCi) of 16S or 23S ^{32}P-labeled rRNA were incubated with DbpA for 5 min at 37°C in 20 mM HEPES–KOH pH 7.5, 10 mM NH_4Cl, 5 mM Mg-acetate and 1 mM DTT, with or without the addition of unlabelled tRNA and/or ATP. The reaction mixtures were then placed on ice and UV-irradiated for different times (Fig. 1), using four 65W lamps (Sylvania G8T5, 330nm) placed 2 cm away from the samples. After irradiation, the reaction mixtures were incubated with 10 µg RNAse A for 10 min at 37°C. Cross-linked products were analyzed by autoradiography after separation by 10% PAGE.

Preparation of ribosomal subunits and *in vivo* ^{32}P-labeled ribosomal RNA

Ribosomal subunits were prepared according to Rheinberger *et al*. (15). Ribosomal RNA was prepared by phenol extraction following a standard protocol (16).

Conditions for the preparation of ^{32}P-labeled ribosomes were as described by Stiege *et al*. (17). Cultures of *E.coli* MRE600 (40 ml) were inoculated with 2 ml of an overnight preculture and 5 mCi ortho-[^{32}P]-phosphate was added. Cells were grown until the amount of incorporated radioactivity reached 80% and then harvested by centrifugation. Bacteria were lysed by sonication and cell extracts were layered onto 10–40% linear sucrose gradients in 10 mM Tris–HCl pH 7.8, 0.3 mM Mg-acetate, 150 mM KCl and then centrifuged at 4°C in a Beckmann SW27 rotor for 16 h at 20 000 r.p.m. After gradient fractionation, radioactivity of the fractions was determined and fractions containing 50S and 30S ribosomal subunits, respectively, were pooled and precipitated with 2 vol ethanol. ^{32}P-labeled rRNA was obtained from the radioactive labeled subunits by phenol extraction (16).

RNAse H digestion of ribosomal RNA

Defined RNA fragments were obtained by digestion with ribonuclease H (18). Pairs of DNA oligonucleotides (10–15mers) containing complementary sequences to 23S rRNA were mixed in equimolar amounts with the target RNA in 15 mM Tris–HCl pH 7.8, 50 mM NH_4Cl, 1 mM $MgCl_2$, 0.1 mM DTT and heated to 55°C for 10 min. A predetermined amount of RNAse H was added and samples were incubated for a further 30 min at 55°C. The reaction was stopped by the addition of EDTA and SDS to a final concentration of 1 mM and 0.1%, respectively. RNA fragments were separated on 5% denaturing polyacrylamide gels and cut out under UV light. Extraction from the gel was achieved by stirring gel pieces overnight at 4°C in 10 mM Tris–HCl pH 7.8, 2 mM EDTA, 0.1 M Na-acetate, 0.1% SDS and 50% of phenol. RNA fragments were recovered by ethanol precipitation (16).

Preparation of RNA/RNA hybrids

Fragments of 16S and 23S rRNA were generated using the 'RNAse H method' as described above. To allow the formation of RNA hybrids, fragments with complementary sequences were combined in each assay. In order to follow hybrid formation and

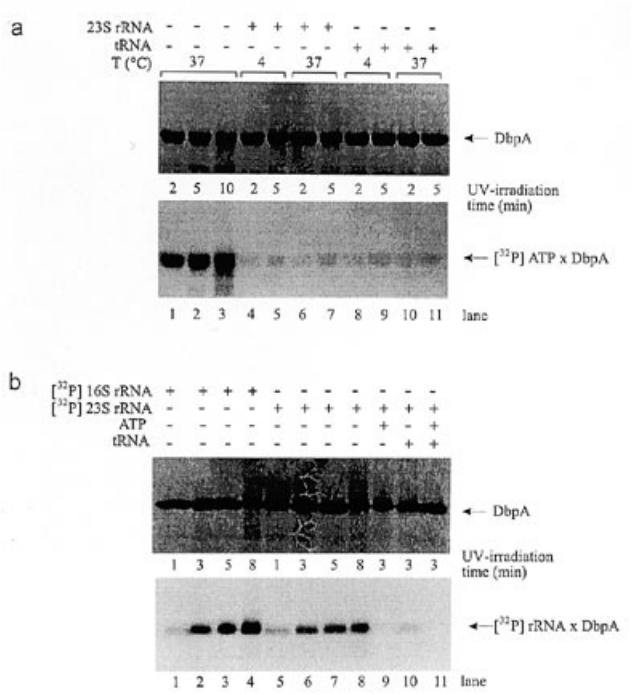

Figure 1. Cross-linking of DbpA to ATP or rRNA. All cross-links were formed with 20 pmol DbpA. After irradiation, the reaction mixtures were incubated with 10 µg RNAse A for 10 min at 37°C. In the top panels of (a) and (b) cross-linked samples were separated by PAGE and stained with Coomassie blue. The bottom panels are duplicate gels of (a) and (b), respectively, subjected to autoradiography. Cross-links to either [α-^{32}P]ATP or 23S [^{32}P]-rRNA were greatly reduced when the cross-linking experiments were performed in the presence of unlabeled 23S rRNA or ATP, respectively. (**a**) Cross-link of DbpA to 100 pmol [α-^{32}P]ATP. Lanes 1, 2 and 3, DbpA was cross-linked to [α-^{32}P]ATP by UV-irradiation for different periods of time; lanes 4–7, 10 pmol 23S rRNA was incubated with [α-^{32}P]ATP and DbpA at 4°C (lanes 4 and 5) or 37°C (lanes 6 and 7) before UV irradiation. Lanes 8–11, 300 pmol tRNA instead of 23S rRNA, was incubated with [α-^{32}P]ATP and DbpA at 4°C (lanes 8 and 9) and 37°C (lanes 10 and 11) prior to the UV irradiation. (**b**) Cross-link of DbpA to 16S or 23S [^{32}P]-rRNA (50 pmol). Lanes 1–4, cross-link to 16S [^{32}P]-rRNA using the indicated UV-irradiation times; lanes 5–8, cross-link to 23S [^{32}P]-rRNA using the indicated UV-irradiation times; lane 9, cross-linking to 23S [^{32}P]-rRNA was performed in the presence of ATP (5 mM); lane 10, cross-linking to 23S [^{32}P]-rRNA was performed in the presence of tRNA (300 pmol); lane 11, cross-linking was performed in the presence of ATP (5 mM) and tRNA (300 pmol).

subsequent unwinding, one of the two fragments was ^{32}P-labeled (see preparation of ^{32}P-labeled ribosomes). For lengths and positions of RNA sequences see Table 2. Hybridization was achieved as follows: 50 pmol of each RNA fragment were mixed in a buffer containing 20 mM Tris–HCl pH 7.6, 150 mM KCl and heated to 65°C for 2 min. After slow cooling to 37°C, samples were divided into three parts, one of which served as a control for successful hybridization and the other two for monitoring unwinding activity. To each of these samples 30 pmol DbpA was added, either in TMK buffer (20 mM Tris–HCl pH 7.6, 2.5 mM $MgCl_2$, 50 mM KCl) or in TMAK buffer (same as TMK with the addition of 1 mM ATP). Samples were incubated at 37°C for 20 min, chilled on ice and separated on 7.5% non-denaturing polyacrylamide gels at 4°C. Gels were dried and subjected to autoradiography.

Preparation of DNA/RNA hybrids

DNA oligonucleotides were selected based on their ability to bind to distinct regions of the 16S or 23S rRNA and were labeled at their 5′-end using [γ-^{32}P]ATP and T4 polynucleotide kinase (16). 20 pmol labeled oligonucleotide was mixed with 35 pmol ^{32}P-labeled 16S or 23S rRNA in 20 mM Tris–HCl pH 7.6, 150 mM KCl, heated to 55°C for 15 min and slowly cooled to 37°C. The sample was divided into three aliquots and the subsequent unwinding reaction was carried out as described above. Samples were separated on 3% composite acrylamide–agarose gels (19) and gels were dried and subjected to autoradiography.

ATPase activity assay

ATPase activity was determined using 15 pmol DbpA and various amounts of 23S rRNA or 23S rRNA fragments as indicated in the figure legends. The reaction contained 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP and 0.05 µCi [γ-^{32}P]ATP in a final volume of 50 µl. The reaction mixture was incubated at 37°C for 30 min and stopped by the addition of 300 µl 7% activated charcoal powder (Merck) suspended in 50 mM HCl and 5 mM H_3PO_4. Samples were mixed and centrifuged at 10000 r.p.m. for 10 min in a tabletop centrifuge. Aliquots (250 µl) were taken from the supernatant and the radioactivity was measured using a liquid scintillation counter (Packard). The background radioactivity was determined in a similar reaction without protein and subtracted from all other reactions. Obtained values were converted to pmol [γ-$^{32}P_i$] released/min.

RESULTS

Binding of rRNA and ATP to DbpA

DbpA binds to various RNAs, but hydrolyzes ATP only in the presence of 23S rRNA, one of the three rRNAs found in prokaryotic ribosomes (9). However, the extent of binding of DbpA to the remaining two rRNA molecules, the 16S rRNA and the 5S rRNA, has not been reported. Thus, we purified rRNA and DbpA to homogeneity and tested the extent to which it was able to bind to isolated 23S, 16S and 5S rRNAs in a filter binding assay. DbpA bound to radioactive labeled 5S, 16S as well as 23S rRNA molecules (Table 1). Although binding of DbpA to rRNA was dependent on the ammonium salt concentration (data not shown), DbpA showed no binding preference for any of the rRNA species tested under the ionic conditions used. Radioactive labeled rRNAs could be chased by adding unlabeled competitor rRNA.

Table 1. Amount of rRNA bound to DbpA

rRNA type	pmol rRNA/pmol of DbpA
23S rRNA	0.26
16S rRNA	0.20
5S rRNA	0.28

5′-radioactively labeled 23S, 16S and 5S rRNA (50 pmol) was incubated with DbpA (20 pmol) and the extent of binding was determined by filter binding assays (see Materials and Methods). No significant difference could be detected in the binding of DbpA to the different rRNAs.

Table 2. DNA probes and rRNA fragments used in the helix destabilizing experiments

Hybrid type	23S rRNA	16S rRNA
DNA–rRNA	175–188	**760–776**
(DNA-probe hybridized	1415–1430	**996–1012**
to rRNA at position)	**1704–1720**	1356–1376
	2528–2552	**1391–1406**
rRNA–rRNA	1625–1736	1302–1453
(rRNA fragment pairs forming	1966–2054	1453–1542
doubled stranded hybrids)	**2442–2546**	**1416–1445**
	2546–2670	**1454–1494**
	2494–2528	
	2532–2587	

DNA–rRNA hybrids, DNA oligonucleotides complementary to the rRNA positions listed above were hybridized to the respective rRNA.
RNA–RNA hybrids, RNA fragments corresponding to the positions shown were generated by RNAse H digestion of either 23S or 16S rRNA. Pairs of fragments known to hybridize based on the secondary structure model of 16S and 23S rRNA were used to generate the RNA-RNA hybrids.
DNA–rRNA hybrids as well as the pairs of rRNA fragments shown in bold in this Table correspond to the fragments shown in Figure 2. Numbers represent the base number in the 16S or 23S rRNAs.

It has been well established that DbpA hydrolyzes ATP only in the presence of 23S rRNA, but not in the presence of 5S or 16S rRNA (9). To better understand the interaction of DbpA with ATP and rRNA, UV cross-linking experiments were performed by independently cross-linking radioactive ATP, 16S rRNA or 23S rRNA to DbpA. Competition experiments were then carried out where cross-linking to radioactive ATP (Fig. 1a) and rRNA (Fig. 1b) were performed in the presence of increasing amounts of competitor, i.e. unlabeled rRNA (23S rRNA or tRNA) or ATP, respectively. Our results indicated that DbpA can be cross-linked to ATP, 23S and 16S rRNA and that these cross-links are competitive, since addition of unlabeled 23S rRNA or tRNA greatly reduced the cross-link of DbpA to [^{32}P]ATP (Fig. 1a) and addition of unlabeled ATP or tRNA equally reduced the cross-link to [^{32}P]-23S rRNA (Fig. 1b).

Since DbpA showed no exclusive binding specificity for 23S rRNA, and cross-links of rRNA and ATP were competitive, we decided to investigate the helicase activity of DbpA for 23S rRNA, 16S rRNA, as well as other RNA molecules in the presence and absence of ATP.

Hybrid destabilizing activity of DbpA

Although DbpA has been described as a 'putative' RNA helicase due to sequence homology to other members of the DEAD box protein family (9), its helicase activity has never been proven. Thus, we investigated the ability of DbpA to destabilize DNA–rRNA as well as rRNA–rRNA hybrids.

To test the destabilizing activity of DbpA for DNA–rRNA hybrids, we used radioactively labeled 16S and 23S rRNA in conjunction with radioactively labeled DNA oligonucleotide probes selected in base of their ability to hybridize to rRNA. We selected four DNA probes that independently hybridized to 23S rRNA and four others that hybridized to 16S rRNA. The DNA probes used in these experiments and the positions to which they hybridize with rRNA are listed in Table 2. DNA–rRNA hybrids were shown to be stable by PAGE (Fig. 2a, lanes 5 and 8; Fig. 2b, lanes 3, 5 and 7).

When radioactively labeled DNA–rRNA hybrids, DNA probe 'A' + 23S rRNA or DNA probe 'B' + 23S rRNA were incubated with DbpA, the protein was capable of destabilizing both hybrids. This can be seen in Figure 2a by the appearance of the band corresponding to the DNA probes. Surprisingly, addition of ATP was not necessary to trigger the destabilizing activity, but instead seemed to slightly hamper the unwinding reaction as judged from Figure 2a (compare lane 6 with 7 and lane 9 with 10). By performing the corresponding experiments with 16S rRNA instead of 23S rRNA, we found that DbpA could destabilize DNA probes 'C', 'D' and 'E' hybridized to 16S rRNA as well (Fig. 2b). DNA–16S rRNA hybrids showed the same response to ATP as described above (data not shown). Thus, the destabilizing activity of DbpA appears to be independent of the type of ribosomal RNA used, and ATP is not needed for the unwinding activity.

In order to test DbpA's helicase activity for rRNA–rRNA hybrids, we used the 'RNAse H method' to generate a family of defined fragments from 23S as well as 16S rRNA. To avoid contamination with other rRNA regions, the individual fragments were separated by denaturing gel electrophoresis, and pairs of fragments were chosen based on their ability to form stable helices according to the secondary structure models for both rRNAs (20,21). The ability of every pair to form an rRNA–rRNA duplex was confirmed by gel electrophoresis (Fig. 2c, lanes 2 and 5). DbpA was able to unwind these rRNA–rRNA hybrids (Fig. 2c, 16S lanes 3 and 4, and 23S lanes 6 and 7) regardless of the presence or absence of ATP, and independent of whether 16S or 23S rRNA fragments were used. Similar observations were made for all other DNA–rRNA and rRNA–rRNA hybrids listed in Table 2.

To determine whether DbpA was able to destabilize (unwind) not only RNA–RNA or DNA–RNA hybrids containing rRNA but any RNA, we added DbpA to a RNA–RNA hybrid produced by hybridizing oligo(A) (12–15 bases long) to poly(U) molecules (Fig. 2b, lanes 9–12). DbpA was unable to destabilize this RNA–RNA hybrid. Furthermore, DbpA was unable to destabilize a DNA–RNA hybrid generated by hybridizing a DNA probe to the mRNA that codes for the ribosomal protein L1 from *Haloarcula marismortui* (data not shown).

It is important to note that all the hybrid destabilizing experiments were performed with nearly equal amounts of protein and RNA–RNA hybrids. Therefore, in a typical experiment the ratio of DbpA (30 pmol) to the maximum amount of hybrid that could be formed (20 pmol) was never greater than 1.5. As can be judged from the results in Table 1, this excess of DbpA is not enough to saturate the rRNA molecules, thus making it unlikely that the destabilizing effect could be caused solely by the binding of DbpA to the rRNA molecules.

Our experiments showed clearly that DbpA has helix destabilizing activity, and that it cannot discriminate between 16S or 23S rRNA. Furthermore, the helix destabilizing activity is not coupled to the ATPase activity.

23S rRNA regions able to stimulate the ATPase activity of DbpA

For mapping regions of 23S rRNA able to stimulate ATPase activity of DbpA, we used a different approach from that described

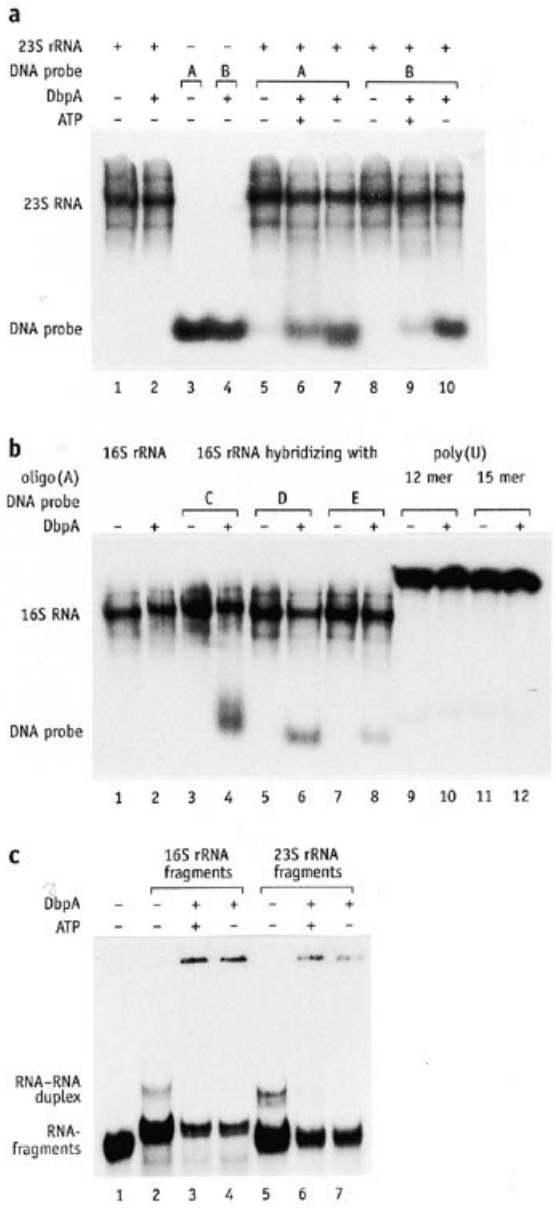

Figure 2. Hybrid destabilizing activity of DbpA. For the experiments in (a) and (b), 20 pmol of the 5′[^{32}P]-labeled DNA probes (Table 2) were hybridized to 35 pmol [^{32}P]-rRNA. For the experiments in (c), hybrids were formed by mixing 50 pmol of each one of the 16S or 23S rRNA fragments able to build rRNA duplexes (see Table 2). **(a)** Ability of DbpA to destabilize DNA–23S rRNA hybrids. [^{32}P]-23S rRNA used for the experiments (lane 1) was incubated with DbpA (lane 2). Lane 3, 20 pmol ^{32}P-DNA-probes 'A'; lane 4, 20 pmol [^{32}P]- DNA-probe '**B',** thus corresponding to the theoretical maximum of helix destabilization. Lane 5, DNA-probe 'A' hybridized to 23S rRNA; lane 6, DNA probe 'A' hybridized to 23S rRNA in the presence of 30 pmol DbpA and ATP; lane 7, DNA probe 'A' hybridized to 23S rRNA in the presence of DbpA but without ATP. Same as lanes 5–7 but with DNA-probe 'B' instead of 'A' (lanes 8–10). DbpA was able to destabilize all the hybrids tested regardless of the presence or the absence of ATP (lanes 6, 7, 9 and 10). **(b**) Ability of DbpA to destabilize DNA–16S rRNA hybrids, as well as oligo(A)-poly(U) hybrids. [^{32}P]-16S rRNA used for the experiments (lane 1) was incubated with DbpA (lane 2). DNA probes 'C', 'D' and 'E' were hybridized to [^{32}P]-16 rRNA in the absence of DbpA (lanes 3, 5 and 7, respectively). Lanes 4, 6 and 8, 30 pmol DbpA was added to the DNA–16S rRNA hybrids in the presence of ATP. Lane 9, 12mer [^{32}P]-oligo(A) was hybridized to poly(U) 3000 nucleotides long; lane 10, DbpA was added to the 12mer oligo(A)-poly(U) hybrid. Lanes 11 and 12, same as lanes 9 and 10 but using 15mer [^{32}P]-oligo(A) hybridized to poly(U) instead of 12mer. DbpA was able to destabilize DNA–16S rRNA hybrids (lanes 4, 6 and 8). However, oligo(A)–poly(U) hybrids were not destabilized in the presence of DbpA (lanes 10 and 12). **(c**) Ability of DbpA to destabilize rRNA–rRNA hybrids. Lane 1, [^{32}P]-23S rRNA fragment 2494–2528 before duplex formation; lane 2, 16S rRNA duplex (Table 2). Lane 3, 16S rRNA-duplex incubated in the presence of DbpA (20 pmol) and ATP; lane 4, 16S rRNA-duplex incubated in the presence of DbpA only. Lane 5, 23S rRNA duplex (from Table 2). Lane 6, 23S rRNA-duplex incubated in the presence of DbpA (20 pmol) and ATP; lane 7, 23S rRNA-duplex incubated in the presence of DbpA without ATP. DbpA was able to unwind 16S (lanes 3 and 4) as well as 23S rRNA hybrids (lanes 6 and 7) independent of ATP. All other 23S or 16S rRNA duplexes from Table 2 were destabilized by DbpA in a similar way.

by Nicol and Fuller-Pace (10). To screen individual regions of 23S rRNA, 26 different fragments were generated by RNAse H digestion of native 23S rRNA. Thus, it was possible to assay the ATPase activity with 'native' instead of transcribed 23S rRNA, avoiding the risk of incorrect folding of 23S rRNA during *in vitro* RNA synthesis by T7 RNA polymerase (22). Moreover, RNA fragments produced from native ribosomal RNA still contain all modified nucleotides which presumably play an important role in the maintenance of the structure and/or function of rRNA (23).

We identified a region of 23S rRNA similar to that reported by Nicol and Fuller-Pace (10), that was able to stimulate 100% of the protein's ATPase activity compared to intact 23S rRNA (Fig. 3, fragment D, nucleotides 2500–2600). In addition, however, we discovered that four other regions of 23S rRNA were able to trigger up to 60% of the ATPase activity (Fig. 3, fragments A, B, C and E). It can be seen in Figure 3 (bottom part) that the regions stimulating ATPase activity are not concentrated in any particular portion of the molecule but are rather scattered all along 23S rRNA. Although these regions display no sequence consensus, their common feature appears to be a high extent of stem–loop structures.

Because of these results, we investigated whether DbpA could play a role in one of the different steps of protein biosynthesis. We detected no influence of DbpA on either the initiation or elongation steps of protein synthesis. Nevertheless, DbpA displaced the equilibrium of 70S ⇔ 50S + 30S in the direction of the free subunits during protein synthesis (data not shown). However, an effect of DbpA in *in vivo* protein translation seems not very likely since the concentration of DbpA in the cell is very low compared to the number of ribosomes (24).

DISCUSSION

Proteins containing the conserved DEAD box motifs are assumed to hydrolyze ATP while unwinding RNA (25). However, some DEAD box proteins need the interaction with other proteins in order to exert their ATPase or helicase activities. For example, eIF-4A needs to interact with eIF-4B in order to display ATPase and helicase activity (reviewed in 26). The *E.coli* protein RhlB also shows no ATPase activity unless it is part of the so-called 'degradosome' (27,28). Here we show that DbpA—in spite of being a DEAD box protein—possesses a helix destabilizing activity which is ATP independent. Thus, DEAD box proteins such as DbpA and CsdA (7) display ATPase independent RNA helix destabilizing activity, and apparently need no interaction with other proteins in order to display ATPase or RNA destabilizing activity. If we consider that five DEAD box proteins have been found in *E.coli* to date (29), and that the two analyzed

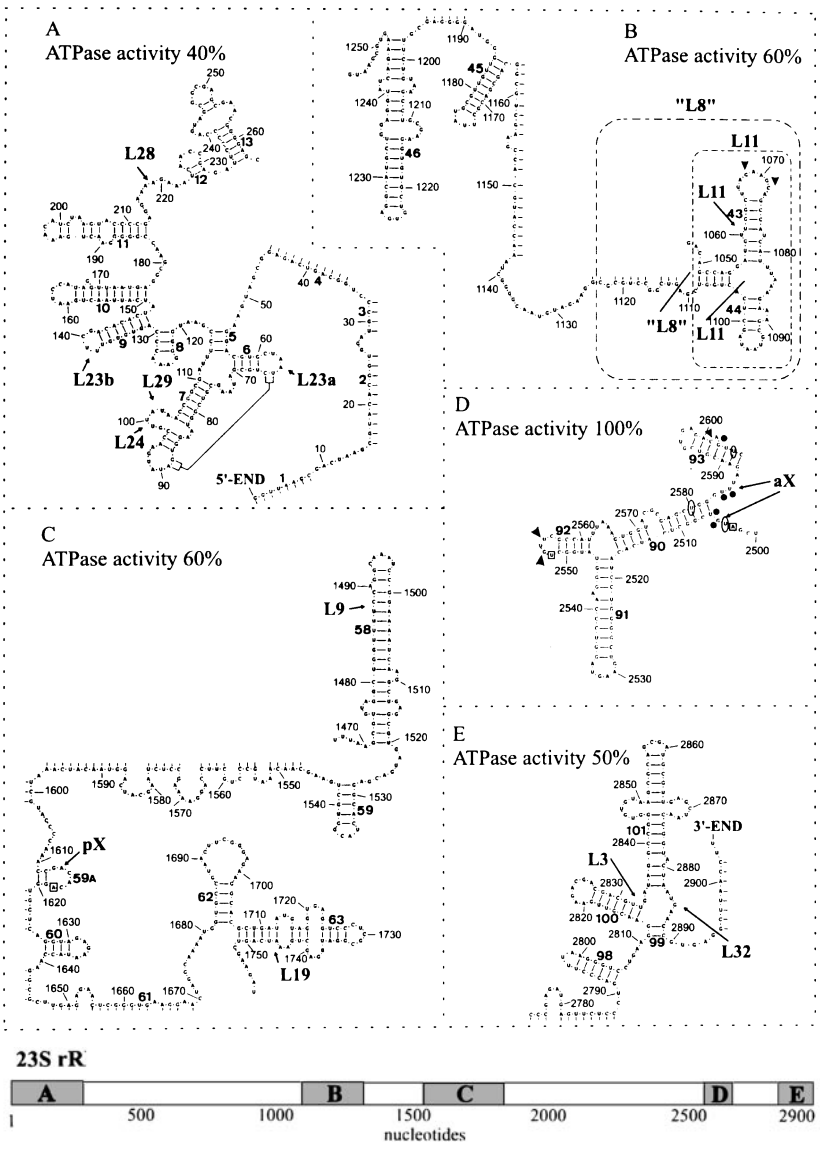

Figure 3. 23S rRNA fragments triggering ATPase activity of DbpA. ATPase activity was measured by incubating DbpA with 23S rRNA fragments: A (nucleotides 1–265), B (nucleotides 1240–1055), C (nucleotides 1465–1760), D (nucleotides 2500–2600) or E (nucleotides 2770–2904). ATPase activity found for fragments A, B, C and E is indicated with respect to the ATPase activity of DbpA obtained for the whole 23S rRNA, which was taken as 100% activity. Fragment D showed 100% activity as previously reported (10). An schematic representation of the 23S rRNA showing the respective location of the fragments, is depicted at the bottom of the figure. The 23S rRNA structure for the fragments is according to Brimacombe *et al.* (18) and the helix numbering (in bold) corresponds to Leffers *et al.* (40). Cross-links between rRNA and ribosomal proteins are indicated by an arrow, and the names of the ribosomal proteins are given as L*n*, where *n* corresponds to the number of the ribosomal protein. tRNA footprint sites are indicated by filled triangles (A-site) and circles (P-site). Modified nucleotides are indicated by a square (methylation) or an ellipse (pseudouridines). Cross-links to the aminoacyl or peptide moieties of the growing amino acid chain are designated as aX and pX, respectively. For a detailed review see Brimacombe (39).

for helicase activity (i.e. DbpA and CsdA) can destabilize RNA helices in the absence of ATP, it is clear that ATPase and helicase activities are not necessarily coupled in all DEAD box proteins.

Koonin and Rudd (30) suggested that some proteins that have evolved from the helicase superfamily I contain only the N-terminal motifs of the DEAD box proteins thought to be responsible for ATP hydrolysis, and can exist separately from proteins containing only the C-terminal motifs needed for the helicase activity. DbpA has an unusual 70 amino acid C-terminal domain consisting of 25% positively charged residues and 29% small (Gly/Ala) residues (24). Therefore, it is possible that the protein's C-terminal region is responsible for RNA binding without inducing ATP hydrolysis, whereas the domain that induces the 23S rRNA dependent ATP hydrolysis is linked to the central DEAD domain (31).

Zinc fingers and cysteine rich metal-binding domains are known to interact with nucleic acids (32). Interestingly, DbpA possesses a putative metal-binding cysteine-rich sequence motif: Cys-Val-Val-Phe-Cys-Asn-Thr-Lys-Lys-Asp-Cys-Gln-Ala-Val-Cys (Cys-X3-Cys-X5-Cys-X3-Cys) between the 'SAT' and 'ARGXD' DEAD-

box conserved motifs. Thus, one could speculate that DbpA has two different RNA binding domains, one of which is specific for 23S rRNA probably due to the putative metal-binding motif, and the other is located at the C-terminal domain of the protein which is responsible for the non-specific RNA binding due to the positive charges of its residues. If this putative metal-binding sequence lies structurally close to the ATPase (or DEAD) motif, the dependence of the 23S rRNA specific binding and the ATPase activity of DbpA can be explained.

Moreover, the fact that DbpA can be cross-linked to ATP in the absence of RNA (Fig. 1a) and vice versa (Fig. 1b) indicates that the binding of one of the substrates does not require the binding of the other. In this respect, DbpA is different from eIF-4A where cross-linking to RNA is dependent on ATP hydrolysis and is the driving force for productive binding to RNA (14). It is possible that ATP hydrolysis of DbpA is used to destabilize the interaction between 23S rRNA and the putative metal-binding motif after RNA-unwinding or destabilization. It has been shown for RNA helicase A for example, that the ATP consuming step is the release of the RNA (33). Moreover, since the binding of DbpA to RNA is rather non-specific (9) (Table 1) and DbpA concentration in the cell is very low (24), a rapid turnover of DbpA could be ensured after its binding to 23S rRNA if ATP was the driving force for binding release.

Frequently 'non-ribosomal' proteins have been suggested to play a role in the ribosome assembly process (34,35). These proteins could be a possible cause for the dramatic differences observed between *in vivo* and *in vitro* ribosome assembly time, temperature and ionic conditions (36). For example, the chaperone DnaK has been implicated in ribosome assembly in *E.coli* (37). In addition, two putative RNA helicases (of the DEAD box protein family) have been recognized in *E.coli* as gene-dosage dependent suppressors of temperature sensitive mutants in genes of ribosomal proteins. One of these, SrmB, is a suppressor of a temperature-sensitive mutation in the ribosomal protein L24(38), and the other is encoded by the deaD gene that codes for CsdA, a cold shock ribosome-associated protein capable of suppressing a temperature sensitive mutation linked to the ribosomal protein S2 (6,7). However, to date no DEAD box protein has been identified that has a direct influence on the *E .coli* ribosome assembly process.

Our analysis of the 23S rRNA fragments triggering the ATPase activity of DbpA shows that—aside from the region previously identified by Nicol and Fuller-Pace (10)—additional regions can stimulate this activity. It is interesting to note that these regions are spread across the 23S rRNA molecule and show no apparent consensus sequence. However, all of them are rich in stem–loop structures, and all of these 23S rRNA regions are either part of the 'functional center' of the 50S ribosomal subunit (39) or have been found to be related to ribosomal proteins playing a leading role in the assembly process (36). The functional center of the 50S subunit or 'core structure' is rich in ribosomal RNA, contains nearly all of the 23S rRNA modified nucleotides found so far, and has a complex structural organization (23,39). As an example, the ribosomal protein L23 cross-links to nucleotides A63 and U138 in helices 6 and 9 of 23S rRNA (40,41). These helices are part of fragment A shown in Figure 3. Cross-linking experiments with a photoaffinity-analogue of the antibiotic puromycin identified L23 as a target protein, and therefore, L23 is hypothesized to be localized in the vicinity of the peptidyl transferase center of the 50S ribosomal subunit (42). Interestingly L24, one of the two 50S assembly initiator proteins (43) has also been cross-linked to fragment A, implicating this region as essential during the early assembly steps.

Cross-links to L11 and 'L8' (the pentameric complex formed by L7/L12 and L10) have been found for fragment B (Fig. 3). These proteins are an essential part of the GTPase center of the 50S subunit also known as the 'translocation' domain, the place where the elongation factors have been mapped to (44,45). Footprint sites to tRNA (A-site) have been also found in helix 43 (Fig. 3, fragment B) (46,47).

Fragment C (bases 1585–1603) of Figure 3 which has been identified in our experiments to stimulate the ATPase activity of DbpA to a significant extent, contains parts of helices 51–54 of 23S rRNA where L23 binds to (48). Cross-linking experiments with nascent peptides identified position A1614 in helix 59a, corresponding to the cross-linking site pX in fragment C (Fig. 3), as being in close contact with the growing peptide chain when it leaves the ribosome (49). Fragment C contains a modified base in position A1618. L9 has been cross-linked to helix 58 in fragment C (41), and antibodies against L9 inhibited protein synthesis almost completely (50). L9 has also been cross-linked to L28 (fragment A) and these two proteins (L9 and L28) have been cross-linked to L2 (51,52), an essential component of the peptidyl transferase center (53,54).

Fragment D in Figure 3 belongs to the peptidyl transferase center and contains multiple footprint sites for tRNA (A-site and P-site) (46,47) as well as many modified nucleotides (23,55,56).

Fragment E (Fig. 3) shows a cross-link to ribosomal protein L32, which has been mapped to the vicinity of the peptidyl transferase center (57) as well as to L3, that together with L24 is one of the two ribosomal proteins that leads the assembly of the 50S subunit (43).

In summary, it seems reasonable to assume that all regions of 23S rRNA described above are highly interrelated within the three-dimensional structure of the 50S ribosomal subunit. Therefore, we propose that DbpA may play an important role in the assembly of the 'functional center' of the 50S subunit, possibly by assisting the correct folding of these regions. At early assembly stages, DbpA could interact with the different regions of the 23S rRNA prior to the binding of ribosomal proteins to the 23S rRNA molecule. At the assembly level, the interaction of DbpA with 23S rRNA could destabilize certain regions of the 23S rRNA molecule, thus, lowering activation energy barriers and allowing the binding of the ribosomal proteins under thermodynamically favorable conditions. Based on all these findings as well as on preliminary results on the effect of DbpA in *in vitro* assembly of the 50S subunit (N. Böddeker, C. Glotz. and F. Franceschi, unpublished results), we hypothesize that DbpA could play a major role in ribosome assembly, specifically in the assembly process of the 'active center' of 50S ribosomal subunits.

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