Studies on Three *E. coli* DEAD-Box Helicases Point to an Unwinding Mechanism Different from that of Model DNA Helicases†

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ABSTRACT: DEAD-box proteins participate in various aspects of RNA metabolism in all organisms. These RNA-dependent ATPases are usually regarded as double-stranded RNA unwinding enzymes, though in vitro this activity has only been demonstrated for a subset of them. Given their high biological specificity, their equivocal unwinding activity may reflect the noncognate character of the substrates used in vitro. Here, we pinpoint other reasons for this elusiveness. We have compared the ATPase and helicase activities of three *E. coli* DEAD-box proteins, CsdA, RhlE and SrmB. Whereas the ATPase activity of all proteins is stimulated (albeit to various degree) by long RNAs, only RhlE is stimulated by short oligoribonucleotides. Consistently, all three proteins can unwind RNA duplexes with long single-stranded extensions, but only RhlE is effective when extensions are short or absent. Another critical constraint concerns the length of the duplex region: in the case of RhlE, the ratio (duplex unwound)/(ATP hydrolyzed) drops 1000-fold upon going from 11 to 14 base pairs, indicating a low processivity. Remarkably, allowing for these constraints, all three proteins can unwind substrates with either 5′ or 3′ extensions (or no extension in the case of RhlE). This behavior, which contrasts with that of well studied SF1 DNA helicases, is discussed in the light of available structural and biochemical data.

DEAD-box proteins, which are characterized by eight to nine conserved amino acid motifs, including the D-E-A-D motif that gave them their name and the newly discovered Q motif, constitute a large family represented in all living organisms from viruses to mammals (*1*−*4*). Together with their close relatives, the DEAH and DExH-box proteins, they participate in many aspects of RNA metabolism (*5*). They belong to the large SF2 helicase superfamily, which itself is related to the SF1 helicase superfamily (*6*). The three-dimensional structures of several SF1 and SF2 proteins have been determined. Despite a low overall sequence homology, all of them are characterized by a structurally conserved core consisting of two homologous domains, the "helicase domains" (*2*, *7*, *8*). In DExH/D proteins (including DEAD-box proteins), this core, which harbors the conserved motifs, is usually flanked by specific amino- and carboxy-terminal extensions that vary widely in length and sequence (*1*).

Members of helicase superfamilies possess either a DNA- or an RNA-dependent nucleotide triphosphatase (NTPase) activity. In the former case, this activity is generally coupled to a robust, sequence-independent unwinding activity. Two possible mechanisms, based mainly on studies with SF1 DNA helicases, have been proposed to account for this coupling. In the active rolling model (*9*), the helicase is at least dimeric, and the monomers adopt two conformations that differ in their affinity for single-stranded or double-stranded nucleic acids. They exchange their conformational states when ATP is hydrolyzed, thus producing a "hand-over-hand" movement that unwinds double-stranded nucleic acid. In the inchworm model, which has been substantiated by structural and enzymatic studies on the *Bacillus stearothermophilus* PcrA helicase (*10*), the helicase, which can be monomeric at all times, uses ATP hydrolysis for translocating unidirectionally along single-stranded nucleic acid. Whenever a double-stranded region is encountered, unwinding occurs; further evidence suggests that the helicase does not simply wait for duplex breathing to move ahead; it also uses ATP hydrolysis for actively destabilizing the duplex (*11*).

From the similarity of their catalytic core, it might be expected that all SF1 and SF2 proteins share a same enzymatic activity. In particular, the DEAD-box proteins, none of which are known to multimerise, would be expected to unwind dsRNA by translocating on ssRNA as PcrA does on ssDNA. Yet, the very question of whether these proteins possess unwinding activity remains somewhat open, since only a subset of them has been shown to unwind synthetic RNA duplexes in vitro (*12*). Even in this case, unwinding is

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usually restricted to duplexes of low stability, showing that DEAD-box proteins are poorly processive as RNA helicases (*13*−*15*). To explain the elusiveness of this activity, it has been pointed out that DEAD-box proteins usually perform very specific roles in vivo and thus may exhibit a high substrate specificity (*2*). This specificity presumably reflects the fact that these enzymes do not normally work in isolation, but together with specific proteins ((*16*, *17*); see ref *4* for review) or RNAs (*18*) that interact with the amino- and carboxy-terminal extensions. These interactions, in turn, would activate the helicase function and direct it to definite cellular targets. The absence of these partners and/or targets in vitro would then explain the difficulty of detecting the unwinding activity. However, even for those DEAD-box proteins that unambiguously behave as RNA helicases, the relevance of the mechanistic information gathered from SF1 helicases remains unclear. Thus, the prototypic DEAD-box protein, the eucaryotic initiation factor 4A (eIF4A), is able to unwind even blunt-end duplexes (*19*, *20*). This property is incompatible with the notion that unwinding requires prior directional movement on a single-stranded substrate. Rather, it fits the view that eIF4A can interact directly with dsRNA, and that it is this interaction that somehow causes unwinding (*19*). However, it is not known whether the ability of eIF4A to unwind blunt-end RNA is shared by other DEAD-box proteins.

The bacterium *Escherichia coli* harbors a total of five DEAD-box proteins (CsdA, DbpA, RhlB, RhlE, and SrmB), all of which possess a core DEAD-box domain of ca. 400 amino acids with high sequence similarity to eIF4A. In contrast, their carboxy-terminal extensions differ in size (from 50 to 250 amino acids) and in sequence, with usually little or no similarity to any other known proteins except orthologs in other bacteria. Of these five proteins, DbpA has been best characterized enzymatically. It is so far unique among DEAD-box proteins in its very strict RNA specificity in vitro. Indeed, its helicase and ATPase activities are confined to RNA molecules encompassing hairpin 92 of 23S rRNA (*13*, *21*). RhlB participates in a multiprotein complex called the "RNA degradosome", within which it assists the 3′→5′ exonucleolytic degradation of structured mRNA regions (*22*−*24*). The other three proteins have been less studied biochemically. CsdA is a cold-shock protein (*25*) involved in the biogenesis of large ribosomal subunits (*26*); in addition, it plays a role in mRNA degradation (*27*). This protein has been reported to possess an in vitro RNA-duplex destabilizing activity that is not coupled to ATP hydrolysis (*25*). SrmB is involved in the assembly of the 50S ribosomal subunit (*28*). In vitro, it shows RNA-stimulated ATPase activity (*29*), and it has recently been found to possess a weak in vitro unwinding activity (*18*). Little is known on the in vivo role or in vitro properties of RhlE.

Taking the less characterized *E. coli* proteins SrmB, CsdA, and RhlE as examples, we attempt here to rationalize the elusiveness of the unwinding activity of DEAD-box proteins. First, we show that short oligonucleotides are inefficient in stimulating the ATPase activity of SrmB and CsdA (but not RhlE). Consistently then, only duplexes carrying relatively long single-stranded extensions can be unwound by SrmB and CsdA. Overlooking this requirement, which may be common among DEAD-box proteins, would have prevented detection of the unwinding activity. Second, for SrmB and CsdA, unwinding is seen whether the extensions are located 3′ or 5′ with respect to the duplex; as for RhlE, it can unwind even blunt-end duplexes, like eIF4A. However, the unwinding efficiency is low and drops precipitously with duplex length. The rationale behind this low processivity and absence of directionality of the unwinding reaction is discussed in the light of available structural and biochemical data on SF1 and SF2 helicases.

EXPERIMENTAL PROCEDURES

*Cloning, Overexpression, and Purification of RhlE, SrmB, CsdA, and CsdA*Δ. The ORFs encoding the *E.coli* DEAD-box proteins CsdA and SrmB were amplified by PCR from plasmids pUC-deaD and pUC-srmB (*27*), using the following primers:

5′-CATGCCATGGCTGAATTCGAAACC-3′ (CsdA forward primer)

5′-TCCGCTCGAGTTACGCATCACCACCGAA-3′ (CsdA reverse primer)

5′-ACATGGCGCCTCTATGACTGTAACGACTTTTTC-3′ (SrmB forward primer)

5′-TCCGCTCGAGTTACTCTTCTGTCGTTTG-3′ (SrmB reverse primer)

After sequencing, the PCR products were digested with *Xho*I and *Kas*I (for SrmB) or *Xho*I and *Nco*I (for CsdA), and ligated into the same restriction sites of the pPROEX-HT expression vector (Invitrogen).

The ORF encoding RhlE was excised from pET11-RhlE ((*30*); a gift from Dr. A. J. Carpousis) using restriction enzymes *Nde*I and *Bam*HI and ligated into pET-15b (Novagen) using the same sites.

To overcome CsdA solubility problems (see results), a truncated form of this protein was designed. To this end, the protein was subjected to limited proteolysis with proteases of different specificities (trypsin, chymotrypsin, V8 protease, papain, and bromelain), and the fragments obtained were analyzed on 14% SDS-polyacrylamide gels. A consensus fragment of apparent molecular weight ca. 50 000 Daltons, relatively resistant to further proteolysis, was observed. The corresponding tryptic fragment was characterized by MALDI-TOF mass spectrometry and shown to correspond to CsdA residues 1 to 446 or 448. A truncated version of the *csdA* gene, *csdA*Δ, was then constructed from the pPROEX-HT-CsdA plasmid by PCR, using oligonucleotide 5′-TAGC-CGCTCGAGTTACGCATCTGGCGGTACGAT-3′ and the CsdA forward primer. This amplification changes the *csdA* codon 444 into a stop. The amplified fragment was digested with *Nco*I and *Xho*I and ligated into pPROEX-HT as above. The final construct was verified by sequencing.

For protein overexpression, the *E. coli* strains BL21(DE3) or BL21star (Invitrogen) were transformed with the appropriate plasmids. Cells were grown to an OD_{600} of 0.5−1.0 in LB supplemented with 0.01% ampicillin at 37 °C; they were then induced at 28 °C or 37 °C for 2−4 h with 0.5−1 mM isopropyl-1-thio-β-D-galactopyranoside (the exact conditions were optimized for each protein). Cells were harvested, frozen at −20 °C, thawed, resuspended on ice in "binding buffer" (0.5 M NaCl, 20 mM Sodium/Potassium Phosphate buffer pH 7.4) supplemented with 10 mM imidazole, 1 μg/mL pepstatin (Roche), and tablets of Complete EDTA-free protease inhibitor cocktail (Roche).

The cells were sonicated on ice and the extract was centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was loaded on a HiTrap Chelating HP column (Amersham Pharmacia Biotech) equilibrated at 4 °C with binding buffer + 10 mM imidazole, and the column was extensively washed with binding buffer + 50 mM imidazole until the output OD_{280} leveled off. The his_6-tagged protein was then eluted with binding buffer + 120 mM (for RhlE), 200 mM (for SrmB) or 400 mM (for CsdA and CsdAΔ) imidazole. The eluate was diluted with binding buffer to decrease the imidazole concentration below 50 mM and then supplemented with 0.1 mM EDTA and 5 mM β-mercaptoethanol. His-tags were removed by digestion with the appropriate protease: thrombin (Novagen) for RhlE and a purified his-tagged version of the TEV protease (gifts of Dr. Song Tan (Pennsylvania State University) and Dr. Jennifer Doudna (Yale University)) for CsdA, CsdAΔ, and SrmB. Thrombin was used at 2 U (Novagen definition)/mg of his_6−RhlE during 16 h at 20 °C and TEV protease at a 3%(w:w) protease/protein ratio during 16 h at 20 °C. In the case of thrombin, the protease was removed by incubation with Antithrombin III-agarose beads (Sigma) followed by filtration. DEAD-box proteins were then further purified on a second HiTrap Chelating HP column to remove undigested tagged-protein, TEV protease, released his-tags and contaminants not removed by the first chromatography. The purified proteins were eluted in binding buffer + 50 mM imidazole, dialyzed extensively versus "storage Buffer" (see below), concentrated with Centricon YM-30 (Millipore), flash-frozen in liquid nitrogen by aliquots and stored at −80 °C. The storage buffer was 75 mM KCl, 10 mM Hepes pH 7.5, 0.1 mM EDTA, 1 mM DTT (for RhlE), or 100 mM KCl, 20 mM Hepes pH 7.5, 10%(v:v) glycerol, 0.1 mM EDTA, 1 mM DTT (for SrmB and CsdAΔ). These three proteins could be concentrated to >4 mg/mL in these buffers; on the other hand, CsdA could be not be concentrated beyond 1 mg/mL in a 1M NaCl, 20 mM Hepes pH 7.5, 0.1 mM EDTA, 1 mM DTT buffer. Concentrations of the proteins were calculated from their OD_{280}, using extinction coefficients estimated from their amino acid composition by ProtParam (available at the Expasy Web site). The homogeneity of protein samples was checked by 12% SDS-polyacrylamide gel electrophoresis.

RNA Substrates. *E.coli* tRNA and rRNA, poly dA, polyA, polyC, and polyU were purchased from Sigma, and oligoribonucleotides A_9, A_{16}, and A_{20} from Eurogentec. They were resuspended in 10 mM Tris/HCl pH 8, 1 mM EDTA, and their concentration was estimated from OD_{260}, using extinction coefficients provided by the manufacturers. The oligoribonucleotides used in helicase assays (and in the corresponding ATPase assays) were from Dharmacon Research; they were deprotected following manufacturer's instructions, resuspended in H_2O, and quantified as above. Their sequences, adapted from Rogers et al. (*14*), are arbitrary designed to avoid secondary structures. The sequences are the following:

RNA oligo +1: 5′-GCUUUACGGUG-3′ (11mer)

RNA oligo −1: 5′-CACCGUAAAGC-3′ (11mer complementary to RNA oligo+1)

RNA oligo +2: 5′-GCUUUACGGUGCUA-3′ (14mer)

RNA oligo −2: 5′-UAGCACCGUAAAGC-3′ (14mer complementary to RNA oligo+2)

RNA oligo −3a: 5′-*AACAAAACAAAA*UAGCACCGUAAAGC-3′

(26mer complementary to RNA oligo+1 and +2 with a 5′-single-stranded extension)

RNA oligo −3b: 5′-UAGCACCGUAAAGC*AAAACAAAACAA*-3′

(26mer complementary to RNA oligo+1 and +2 with a 3′-single-stranded extension)

RNA oligo −4: 5′-UAGCACCGUAAAGC*AAAACAAAACAAAACAAAACAA*-3′

(36mer; complementary to RNA oligo+1 and +2 with a 3′-single-stranded extension)

RNA oligo −5: 5′-*AACAAAACAAAACAAAACAAAACAAAACAAAA*UAGCACCGUAAAGC-3′

(46mer; complementary to RNA oligo+1 and +2 with a 5′-single-stranded extension)

The regions of oligos −3 to −5 that lack complementarity to +2 are italicized; they correspond to the same reiterated motif (CA_4).

For unwinding and filter binding assays, RNA oligo+1 and RNA oligo+2 were 5′-^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. Duplex RNA substrates were formed by annealing equimolar amounts of complementary strands in 100 mM KCl, 10 mM Hepes pH 7.5, heating the mixture at 95 °C for 1−2 min and then slowly cooling it to 15 °C; the final concentrations of the oligonucleotides in the annealing mix were 3 μM or 100 μM, depending on the intended use (i.e., high concentrations were needed for ATPase assays but not for the other assays). When preparing substrates for the helicase assays, the concentration of the "cold" strand of the duplex was kept in excess over the ^{32}P-labeled one (6 μM vs 3 μM), to ensure essentially complete incorporation of the label into the duplex (experimentally, we found that the proportion of radioactivity present in the duplexes reached 80−90%). Duplexes were kept at 4 °C and used within a few days of their preparation.

ATPase Assays. ATPase activity was measured at 25 °C using a coupled pyruvate kinase−lactate dehydrogenase assay (*31*). In this assay, ATP hydrolysis is continuously coupled to the oxidation of NADH, which is monitored by changes of absorbance at 340 nm. Spectrophotometric measurements were performed either on a Uvikon942 spectrophotometer (Kontron Instruments) or on a Dynatech MR5000 microplate reader (Dynex Technologies). ATP hydrolysis rates were directly derived from linear fits of OD_{340} vs time, assuming an extinction coefficient of 6300 M^{-1} cm^{-1} for NADH. Incubation time was 5−30 min, depending on reaction rate. The concentrations used in the assay were as follows: nucleic acids as indicated, DEAD-box protein 0.05−0.5 μM (this concentration was kept below that of nucleic acids), pyruvate kinase, and lactate dehydrogenase 10 and 20 units/mL respectively (units as defined by the manufacturer), NADH 250 μM, phosphoenolpyruvate 400 μM, ATP/Mg (a stoichiometric mix of $MgCl_2$ and nucleotide) 1.25 mM. Buffer was 75 mM KCl, 10 mM Hepes pH 7.5. Blanks without DEAD-box protein were subtracted

[1] Abbreviations: ds, double-stranded; ss, single-stranded; ORF, open reading frame; TEV, tobacco etch virus; AMP−PNP, 5′-adenylyl-imidodiphosphate; bp, base pairs; nt, nucleotides.

from the measurements. Data from two or three independent experiments were analyzed using KaleidaGraph (Abelbeck Software).

Helicase Assays. Unless otherwise stated, helicase assays were performed at 25 °C under the following conditions: concentrations of DEAD-box protein as indicated in Figure legends, ^{32}P-labeled-duplex RNA 10 nM, unlabeled single-stranded RNA "trap" 200 nM, ATP/Mg (or AMP−PNP/Mg) 2 mM, RNasin (Promega) 2 units/μL. Buffer was 100 mM KCl, 20 mM Hepes pH 7.5, 10%(v:v) glycerol. The RNA trap is the unlabeled counterpart of the labeled duplex strand. This trap, which is present in large molar excess over the duplex, proved absolutely necessary to prevent the corresponding ^{32}P-labeled strand from reannealing during the course of the assay. The DEAD-box protein was added last to start the reaction. After the indicated time, aliquots (4 μL) were removed from the assay, mixed on ice with 1 μL of a stop solution containing 50% glycerol, 20 mM EDTA, 2% SDS and bromophenol blue, and analyzed on 18% native polyacrylamide gels (19:1 acrylamide/bis) in 1xTBE buffer at 4°C. In some cases, "shifted" RNA bands, presumably corresponding to strong DEAD-box protein/RNA interaction that withstand the protein-denaturing conditions of the stop solution, were observed. To eliminate this problem, these samples were mixed with a stop solution supplemented with 2 mg/mL proteinase K and incubated for 5 min at 25 °C before electrophoresis. Gels were visualized and quantified with an FLA-3000 phosphorimager (Fuji); the fraction of ^{32}P-labeled-RNA present in duplex form after time t was taken as R_t(double-stranded)/(R_t(single-stranded) + R_t(double-stranded)), where $R_t(x)$ represents the amount of radioactivity (corrected from background) present in the corresponding spot. Unwinding kinetics was analyzed with KaleidaGraph from two independent experiments.

Filter Binding Assays. Reactions mixtures (100 μL) contained 1 nM of ^{32}P-labeled-RNA, 0 or 1.5 mM AMP−PNP/Mg or ADP/Mg, and variable concentrations of RhlE. Buffer was 75 mM KCl, 10 mM Hepes pH 7.5. The mixture was incubated for 30 min at 25 °C before filtering on a house-made dot-blot apparatus as in (*32*). After quantifications with the FLA-3000 phosphorimager, binding curves were analyzed with KaleidaGraph.

Estimation of Errors. All assays were performed at least in duplicate. In general, the values obtained differed by less than 15% except for very low values (i.e., close to background level) where the relative errors were occasionally higher.

RESULTS

Cloning, Overexpression, and Purification of E.coli DEAD-Box Proteins. The *csdA*, *rhlE*, and *srmB* genes were subcloned into the pET-15b or pPROEX-HT expression vectors, which allow the inducible overexpression of the corresponding proteins with an N-terminal tag. This tag consists of an $(his)_6$ peptide followed by a cleavage site for thrombin (pET-15b) or TEV protease (pPROEX-HT). The overexpressed proteins were purified to homogeneity (Figure 1A) by nickel affinity chromatography, followed by proteolytic removal of the tag and further purification of the cleaved product (see Experimental Procedures). The structure of the different proteins, showing the DEAD-box core and the N- and C-terminal extensions, is schematized in Figure 1B.

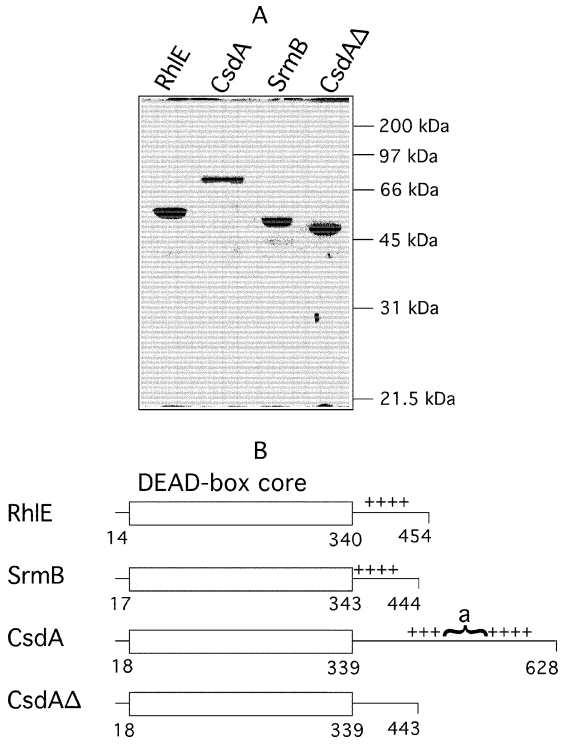

FIGURE 1: The DEAD-box proteins used in this study. (A) PAGE analysis. Proteins (ca. 750 ng) were separated on a 12% SDS-polyacrylamide gel and stained with Commassie blue R-250. Indicated on the right are positions of size markers. (B) The catalytic cores of the four proteins (open rectangles) were defined by the software Pfam (*45*). Nonconserved regions (mostly C-terminal extensions) are drawn to scale as thin lines. Figures below each protein indicate residue numbering. Symbols, +++, represent highly basic regions (i.e., stretches of at least 30 residues with a theoretical pI above 10). The CsdA region marked "a" displays some sequence similarity to the C-terminal extension of the *E.coli* DEAD-box protein DbpA (*18*).

CsdA was found to be poorly soluble under the low salt conditions used in ATPase and helicase assays. Whereas dilute protein solutions can be used for ATPase measurements, helicase assays require higher concentrations. To obviate this difficulty, these assays were performed with a truncated version of the protein (CsdAΔ) lacking most of the C-terminal extension. The C-terminus of CsdAΔ was chosen so that it matches that of the protease-resistant core resulting from limited digestion of the full-length protein (Figure 1B; see Experimental Procedures). CsdAΔ was overexpressed from a shortened version of the *csdA* gene and purified as for other proteins. It was far more soluble than the intact protein at low or moderate NaCl concentration ($\leq$0.15 M). Importantly, the intact and truncated proteins behaved very similarly in ATPase assays (see below).

CsdA, CsdAΔ, RhlE, and SrmB are RNA-Stimulated ATPases that Differ in Their Specificities for RNA. Next, the ATPase activity of the four proteins was compared in the presence or absence of natural RNAs, or of synthetic RNA or DNA homopolymers. In all cases, activity was barely detectable with the isolated proteins, but it was strongly stimulated by a variety of RNA substrates (Table 1; as described in Table legend, the RNA concentrations used correspond in most cases to near-maximal ATPase stimulation). Strikingly, whereas for RhlE the activity varied only

Table 1: Stimulation of the ATPase Activities of RhlE, SrmB, CsdA, and CsdAΔ by Nucleic Acids[a]

nucleic acid stimulator	V_{ATPase} (RhlE)	V_{ATPase} (SrmB)	V_{ATPase} (CsdA)	V_{ATPase} (CsdAΔ)
none	0.6	< 0.5	< 0.5	< 0.5
E.coli tRNA	80	1.2	30	35
E.coli rRNA	90	8	36	40
poly C	105	15	nd	52
poly U	170	1.2	90	70
poly A	130	24	60	64
poly dA	1.8	< 0.5	1.8	0.6
rA_9	45	< 0.5	< 0.5	nd[c]
rA_{16}	110	1.2	1.8	1.0
rA_{20}	120	3.6	1.8	6
RNA oligo-2 (14mer)[b]	110	< 0.5	nd	7
RNA oligo-3a (26mer)[b]	135	1.0	nd	24
RNA oligo-4 (36mer)[b]	130	11	nd	60
RNA oligo-5 (46mer)[b]	130	8	nd	54

[a] ATPase activities are expressed in moles of ATP hydrolyzed per minute per mole of protein. Nucleic acid concentrations are 100 μg/mL for large molecules and 10−15 μM for oligonucleotides; in most cases they allow near-maximal stimulation of ATPase activity. By analogy, the same concentrations were used when little or no stimulation was observed. Results are averaged from two or three independent experiments. [b] For the sequences of the RNA oligonucleotides, see section Experimental Procedures. [c] nd = not determined.

2-fold with the nature of the RNA used, for SrmB, far larger variations were observed. Thus, SrmB was efficiently stimulated by polyA and polyC but not polyU; among heteropolymeric RNA, *E.coli* rRNA was much more efficient than tRNA (Table 1). However, even in the presence of the most efficient RNA stimulators, the ATPase activity of SrmB remained much lower than that of RhlE (Table 1). In all respects, the CsdA (and CsdAΔ) protein behaved intermediately between SrmB and RhlE. In no case could poly(dA) substitute for poly(A) in ATPase activation, consistent with previous observations that DNA cannot stimulate the ATPase activity of eIF4A (*33*).

Interestingly, whereas for all proteins poly(A) stood among the most efficient stimulators, oligoadenylates behaved differently in this respect (Table 1). Even an oligoadenylate as short as $(A)_9$ stimulated the ATPase activity of RhlE efficiently (i.e., to 35% of the level observed with poly(A)), and longer oligoadenylates were nearly as efficient as poly(A) itself. In contrast, oligoadenylates were poor stimulators of SrmB, CsdA, or CsdAΔ: stimulation by $(A)_9$ or $(A)_{16}$, if any, was marginal, and stimulation by $(A)_{20}$ was only a few % of that of poly(A) (Table 1). Because poly(A) and oligoadenylates are not expected to differ in structure, we conclude that RNA must exceed a certain length to stimulate the ATPase activity of these DEAD-box proteins; moreover, this critical length is longer for SrmB and CsdA (or CsdAΔ) than it is for RhlE. This effect was also apparent with oligonucleotides of heterologous sequence. To document this point, we used 14−46-nt oligonucleotides corresponding to one of the strands of the duplexes used in the unwinding assays (see below and Experimental Procedures). For CsdAΔ and SrmB, the activities observed with these oligonucleotides were not strictly in line with those observed with oligoadenylates, further confirming the effect of the RNA sequence on the stimulation of these proteins. However, the major, protein-dependent effect of the oligonucleotide length was still apparent in this series. Thus, whereas the 14-mer was efficient in stimulating the ATPase activity of RhlE only, the 26-mer was efficient for CsdAΔ as well; on the other hand, stimulation of SrmB required the 36- or 46-mers (Table 1).

Unwinding Activity. The three proteins RhlE, CsdAΔ, and SrmB were then assayed for their unwinding activity toward short RNA duplexes (11 or 14 base pairs), carrying or lacking 5′ or 3′ single-stranded extensions. The sequence of the 14 bp duplex was taken from (*14*); the 11 bp duplex, which was derived from the 14 bp duplex by removing 3 external base pairs, is significantly less stable ($\Delta G = -18$ kcal/mol versus −24 kcal/mol (*14*)). As for the extensions, they consist of partial or complete repetitions of the CA_4 motifs; their length is 12, 22, or 32 nucleotides in the case of the 14-mer duplex (see Experimental Procedures). In all cases, 3′ or 5′ extensions were added to the same strand of the duplex (conventionally the lower one in Figures 2 and 3), leaving the complementary (or upper) strand invariable. The latter was first 5′ labeled with ^{32}P, and the two constituent oligonucleotides were annealed as described in Experimental Procedures. Unwinding activities were assayed in the presence or absence of either ATP or of AMP−PNP (a nonhydrolysable ATP analog), using a classical electrophoretic method (*34*) (Figure 2). In a preliminary set of experiments, AMP−PNP was shown to be a competitive inhibitor of RNA-stimulated ATPase activity for all three proteins. Helicase assays were routinely performed in the presence of an excess of the unlabeled upper strand ("cold trap") to prevent the labeled duplex from reassociating after unwinding. As for the concentrations of proteins used, only RhlE could be assayed in conditions where it saturated its substrates (Figure 2A); CsdAΔ and SrmB, which have a lower apparent affinity for RNA (see Figure 3), were used at close to maximal achievable concentration.

RhlE was able to unwind all the 14-mer duplexes used, whatever the length and position (3′ or 5′) of the single-stranded extension present (Figure 2B). In particular, like eIF4A, it was able to dissociate efficiently a blunt-end duplex, indicating that helicase activity does not require a ssRNA handle. In no case was unwinding observed in the absence of ATP or in the presence of AMP−PNP, showing that it requires ATP hydrolysis. In contrast, the unwinding activity of proteins SrmB and CsdAΔ was far more elusive. CsdAΔ was only able to dissociate the 14-mer duplex provided it carried extensions of 12 nt or more (Figure 2B and data not shown); even in this case, compared with RhlE, much higher CsdAΔ concentrations were required to reach the same unwinding activity (cf. Figure 2 legend). Interestingly, although CsdAΔ was unable to unwind the blunt end duplex, it was nearly as efficient in unwinding substrates carrying either 5′ or 3′ extensions (Figure 2B), suggesting that also in this case unwinding does nor require a ss RNA handle of defined polarity. With SrmB, no unwinding was observed with any of the 14 bp duplexes, whatever the nature or length of the extensions (not illustrated). To test the possibility that SrmB is able to unwind weaker duplexes, the assays were repeated with 11 bp duplexes. Even very high concentrations of the SrmB protein were unable to dissociate the blunt-end 11 bp duplex at a rate above background. Similarly, duplexes with the shortest (15 nt) single-stranded extensions were not detectably unwound (Figure 2C and results not shown). In contrast, a weak but unmistakable unwinding activity was observed with 11 bp

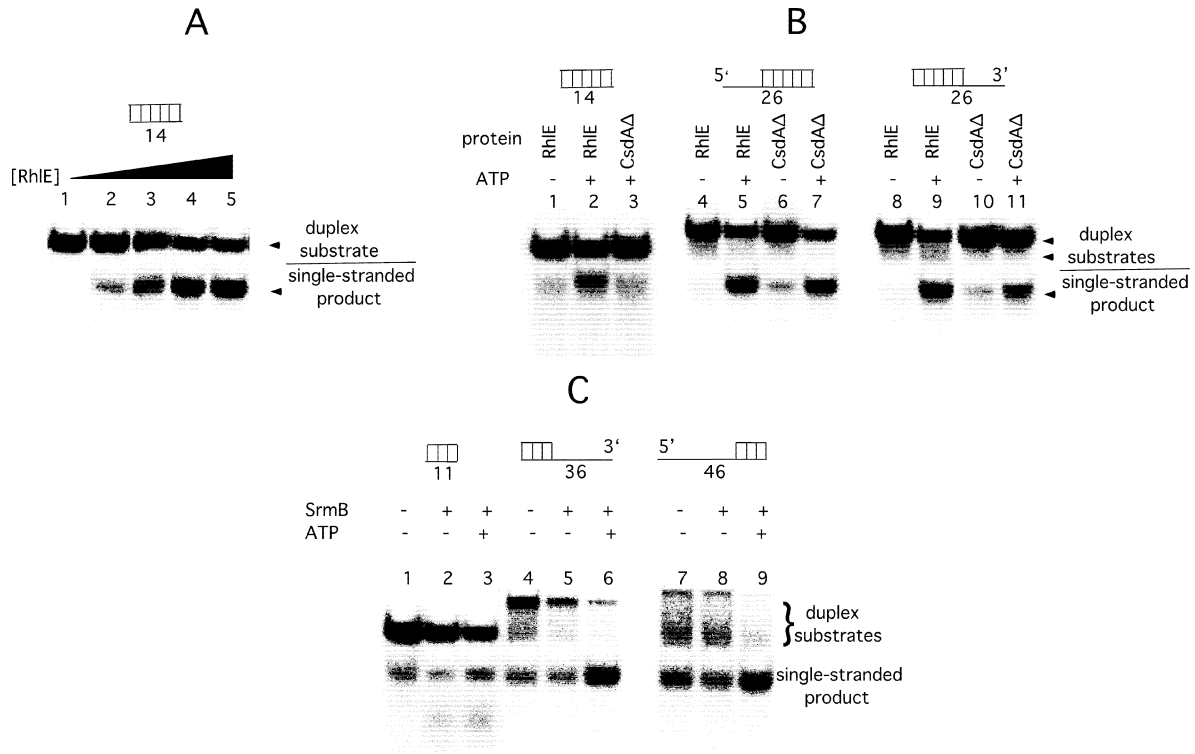

FIGURE 2: RNA unwinding activities of RhlE, CsdAΔ, and SrmB. The duplexes used are schematized above the corresponding lanes; figures underneath refer to the length of the longest strand. (A) Unwinding activity of RhlE toward the 14 base-pairs blunt-end RNA duplex. Incubation was for 60 min at 25 °C in the presence of ATP; RhlE concentration was 0 μM (lane 1), 0.1 μM (lane 2), 0.5 μM (lane 3), 1 μM (lane 4) and 5 μM (lane 5). (B) Unwinding activities of RhlE and CsdAΔ toward a 14 base-pairs RNA duplex. The duplex is either blunt-end (lanes 1−3), or flanked with 12-nucleotide-long single-stranded extensions located either 5′ (lanes 4−7) or 3′ (lanes 8−11) to the duplex. Incubation was for 60 min at 25 °C with 0.7 μM RhlE or 6 μM CsdAΔ. In control reactions (-ATP), AMP−PNP was used instead of ATP. (C) Unwinding activities of SrmB with a 11 base-pairs RNA duplex. Duplex is either blunt-end (lanes 1−3), or flanked with 25-nucleotide-long 3′-single-stranded extensions (lanes 4−6) or with 35-nucleotide-long 5′-single-stranded extensions (lanes 7−9). Incubation was for 10 min at 25 °C in the presence of 2 μM SrmB.

duplexes carrying longer extensions. Again, this activity was observed whether these extensions are located 5′ or 3′ to the duplex (Figure 2C).

As seen above, the ATPase activity of the three DEAD-box proteins respond very differently to individual RNA substrates (Table 1). Therefore, their unequal ability to unwind individual duplexes may reflect a differential stimulation of their ATPase activity. To test this possibility, the ATPase activity of the three proteins was measured in the presence of the same duplexes used in helicase assays (Figure 3). Interestingly, whereas for individual proteins the degree of ATPase stimulation eventually varied widely with the duplex used, it generally remained within a factor of 2 of the stimulation observed with the longest strand from the same duplex (compare Table 1 and Figure 3). This result suggests that the length of the RNA is more important than its ss- or ds-character for ATPase stimulation. In particular, all duplexes used, including the blunt-end 14 bp duplex, were able to stimulate the ATPase activity of RhlE to levels similar to the natural or synthetic RNAs assayed in Table 1 (Figure 3A). As for CsdAΔ, the 14-mer blunt-end duplex was not able to stimulate its ATPase activity, but duplexes carrying extensions of 12 nt or more were, whether these extensions were located 3′ or 5′ to the duplex (Figure 3B). Thus, the unwinding and ATPase activities have identical substrate requirement in this case. It is noteworthy that much higher duplex concentrations were required to stimulate the ATPase activity of CsdAΔ compared with RhlE, suggesting a lower binding affinity. As concerns SrmB, not only was the 11 bp blunt end duplex unable to switch on the ATPase activity, but appending 15 nt extensions was not sufficient either: longer extensions (25 or 35 nt) were required (Figure 3C). Again, these requirements match those observed in the unwinding reaction. Thus, many duplexes that resist unwinding presumably do so because they cannot activate the ATPase activity of the corresponding protein. However, efficient ATPase stimulation was not a guarantee that unwinding will take place. Thus, like their 11 bp counterparts, the 14 bp duplexes carrying long extensions (22 or 32 nt) were able to stimulate the ATPase activity of SrmB (not illustrated). Yet, in contrast to the 11 bp duplexes, they were resistant to unwinding (see above). This suggests that a duplex must fulfill two requirements for being a helicase substrate: it must be able to stimulate the ATPase activity, and it should also not be too stable. This latter point was explored further in the case of RhlE.

RhlE is Comparatively Inefficient in Unwinding Longer Duplexes. To further assess the effect of duplex stability on the helicase reaction, we compared the rate of unwinding of the blunt-end 11 and 14 bp duplexes by RhlE, which exhibits robust activity. To this end, the dissociation of the labeled duplexes (cf. Figure 2) was quantified as a function of time, and the data were used to evaluate the unwinding rates after subtracting the slow spontaneous dissociation observed in the absence of ATP (Figure 4A). Under the conditions used (see Figure 4 legend), the initial rate of unwinding was 70-fold higher for the 11 bp than for the 14 bp duplex. This major impact of duplex length upon unwinding reaction was

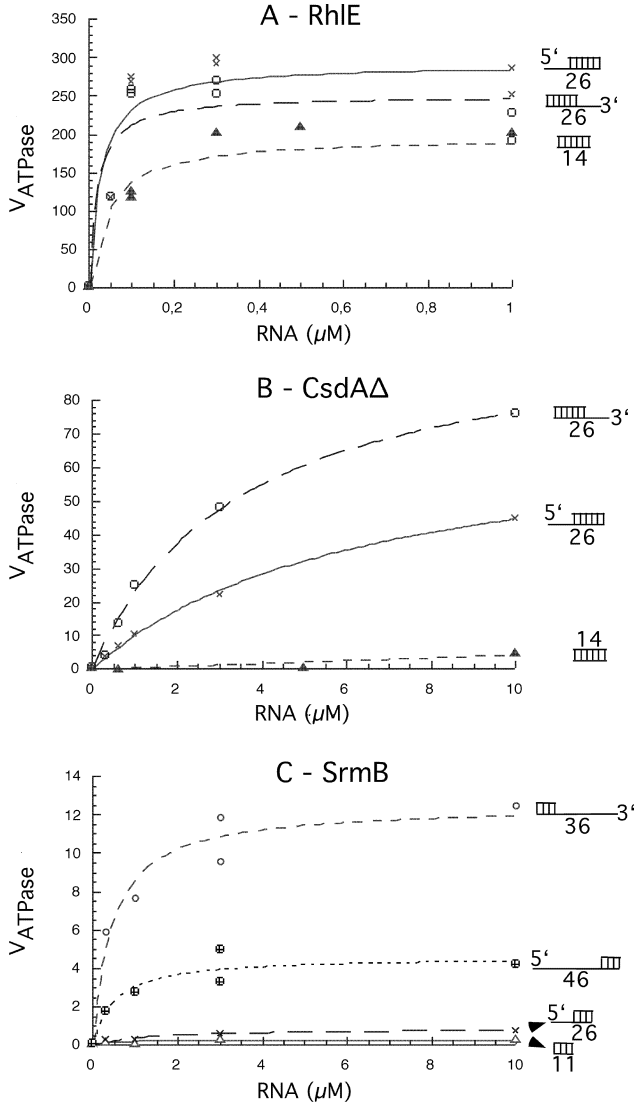

FIGURE 3: Stimulation of ATPase activity by duplexes used in helicase assays. The velocities (V_{ATPase}) of the ATPase reactions (in moles of ATP hydrolyzed per minute and per mole of protein) are shown as a function of the concentration of the duplexes used in the helicase assays. A, B, and C correspond to RhlE, CsdAΔ, and SrmB, respectively. The duplexes used are represented schematically as in Figure 2. The curves represent best fits to equations: $V_{ATPase} = V_{max}/(1+K_{app}/[RNA])$. Note that the abscissa scale in A differs from that of B and C, underlining the higher apparent affinities of RhlE for the duplexes, as compared to CsdAΔ or SrmB.

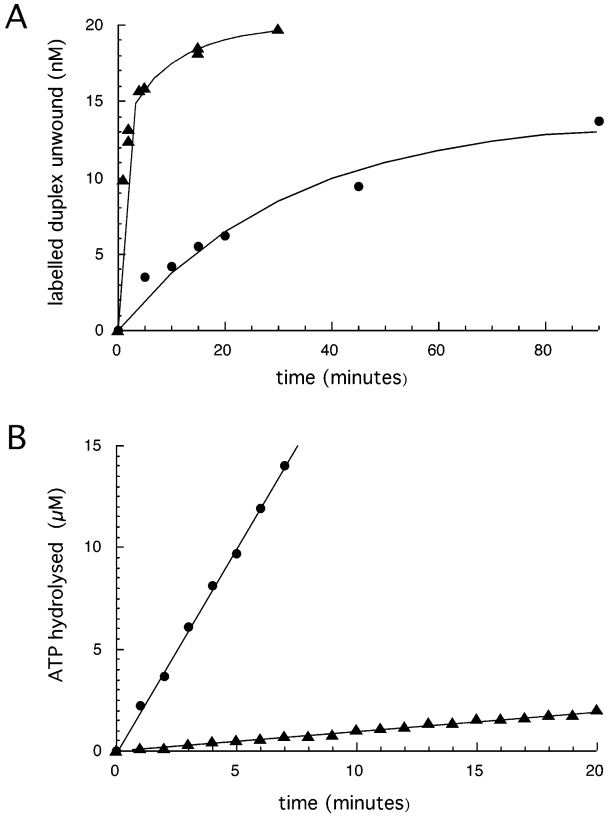

FIGURE 4: Comparison of the unwinding and ATPase activities of RhlE in the presence of blunt-ended duplexes. Triangles and circles correspond to the 11- and 14-base-pairs duplexes, respectively. (A) Unwinding was assayed at 25 °C with 0.7 μM RhlE, 20 nM ^{32}P-labeled duplex, and 190 nM cold RNA trap (see text). The curves represent the best fits to equations: [dsRNA unwound] = $[dsRNA]_0 \cdot (1-\exp(-V_0 \cdot \text{time}/[dsRNA]_0)$. From these curves, the initial velocities of unwinding (V_0) were estimated to 12 nM/min and 0.2 nM/min for the 11- and 14-bp duplexes, respectively. (B) ATPase activity was monitored under the same conditions as unwinding, except that the cold RNA trap was omitted. Since the enzyme is present in large excess over RNA, the ATPase activity arising from the duplex must be the same whether the cold trap is present or not. Because the duplex reanneals rapidly under these conditions (see experimental procedures), its concentration remains constant, hence the constant rate of the ATP consumption over time. This rate (V'_0) is 0.1 μM/min for the 11-bp duplex and 2 μM/min for the 14-bp duplex. The energetical yield of unwinding was calculated from V_0 and V'_0 (see text).

even more striking when it was compared with the ATPase activity in each case. Indeed, with the concentration used in the unwinding assay, the 14 bp duplex was 20-fold more efficient than the 11 bp duplex for ATPase stimulation (Figure 4B). Comparison of the unwinding and ATPase rates then shows that the unwinding of one 11 bp duplex is accompanied, on the average, by the hydrolysis of 10 ATP molecules, whereas for the 14 bp duplex this figure raises to 10^4. As an interpretation for this highly variable yield, we propose that RhlE can only unwind a few base-pairs at the time of each ATP hydrolysis, and that this limited unwinding is readily reversible. Whereas for short duplexes it will frequently lead to complete dissociation, for longer ones the most probable outcome is the reannealing of the few opened base-pairs, hence the very low dissociation yield. On the basis of different evidence, previous work with other DEAD-box proteins have also led to the conclusion that these proteins are very poorly processive as RNA helicases (*13*, *19*).

Unwinding Corresponds to a Bona-Fide ATPase-Driven Helicase Activity. One recurrent concern about helicase assays is that they are usually conducted under conditions of excess enzyme, casting doubt on the fact that helicase activity is really enzymatic. Indeed, duplex dissociation might just reflect the preferential binding of the protein to ssRNA. Besides the fact that unwinding is ATP-dependent, two arguments argue against this view in the case of RhlE. First, when cold trap was omitted from the helicase assay so that the dissociated oligonucleotides are free to reanneal, no unwinding was observed, suggesting that the separated oligonucleotides are not kept apart by protein binding (not illustrated). Second, using a filter-binding assay, we have measured the affinities of RhlE for the blunt-end 14-mer duplex used in helicase assays or for its constituent single-

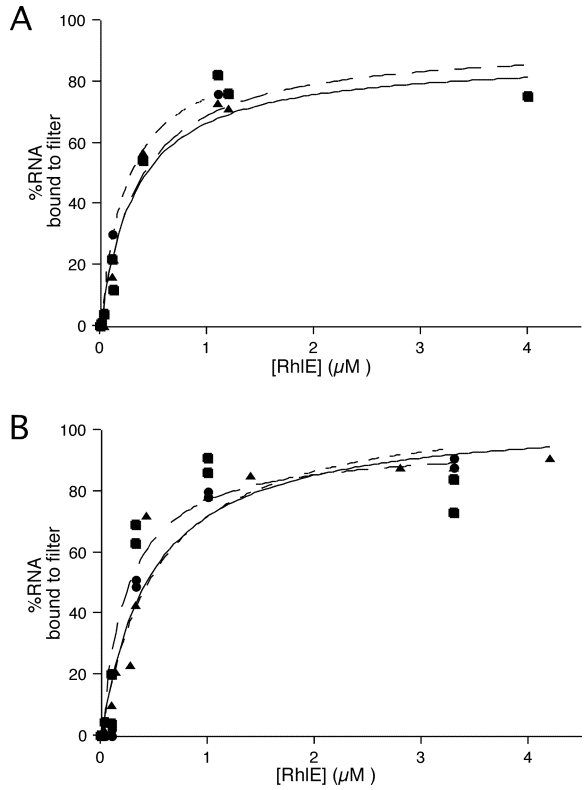

FIGURE 5: Binding of RhlE to the 14-base-pairs blunt-ended RNA duplex (A), and to one of its constitutive strands (RNA oligo+2; see Experimental Procedures) (B), were measured by a filter binding assay. The reaction mixtures contained either 1.5 mM AMP−PNP/Mg (squares), 1.5 mM ADP/Mg (circles), or no nucleotide (triangles). The curves represent best fits to a simple binding equilibria.

strand oligonucleotides, in the presence or absence of either ADP or AMP−PNP. In no case was preferential binding of RhlE to ss RNA observed (Figure 5), further supporting the enzymatic character of the helicase activity.

DISCUSSION

In this article, we have compared the in vitro properties of three DEAD-box proteins from *E. coli*, RhlE, SrmB and CsdA (for convenience most experiments were actually done with a C-terminally truncated version of CsdA, CsdAΔ). All three proteins exhibit ATPase activity when incubated with a variety of RNA species, a property presumably shared by all DEAD-box proteins. Yet, whereas the various RNAs tested were nearly equivalent in stimulating RhlE, with SrmB and CsdAΔ they were not. Indeed, oligoadenylates down to the 9-mer stimulated the ATPase activity of RhlE almost like long poly(A) molecules, whereas for CsdA and SrmB, they were completely inefficient (Table 1). This differential response was not limited to oligoadenylates: it was also observed with two blunt-end 11- or 14 bp RNA duplexes, which stimulated the ATPase activity of RhlE but not CsdA or SrmB. Not unexpectedly then, only RhlE was able to unwind these duplexes in an ATP-dependent reaction. In contrast, these same duplexes, when bearing long enough 5′ or 3′ single strand extensions, could stimulate the ATPase activity of CsdA and SrmB; meanwhile, unwinding was observed. However, with SrmB, unwinding was restricted to the weaker (11 bp) duplex, and even with RhlE, the amount of ATP hydrolysis required for unwinding the 14-mer duplex was several orders of magnitude larger than that for the 11 bp duplex. Altogether, the experiments with oligoadenylates suggest the existence of a minimal length beyond which RNA molecules cannot stimulate ATPase; this minimal length differs markedly among DEAD-box proteins. The experiments with duplexes fit the view that provided they meet the length requirement for ATPase stimulation, duplexes can be unwound by the DEAD-box proteins whether they carry 5′ or 3′ ss extensions, or even no extension at all; however, unwinding is restricted to duplexes of low stability. These two points will be discussed separately.

Stimulation of the ATPase Activity. Why are some DEAD-box proteins unable to use oligoribonucleotides—including the synthetic duplexes used in unwinding assays—for switching on their ATPase activity? It is unlikely that these proteins are unique in that their helicase core cannot bind short oligonucleotides, because available data suggest that the structure of this core is highly conserved and can bind single-stranded nucleic acids as short as 8−10 nt or even less (*2*, *33*, *35*, *36*). Alternatively, these proteins may only be functional as multimers. Some of them (e.g., RhlE) would multimerise on their own, whereas others (e.g., CsdA or SrmB) would require the presence of a long RNA molecule to bridge individual monomers. However, as noted above, there is at present no evidence that DEAD-box proteins can multimerise (yet the *Methanococcus jannaschii* equivalent of eIF4A packs as a dimer in the crystal (*37*)). A more attractive possibility is that, besides the conserved RNA-binding track located within the helicase core, some of these proteins possess at least another RNA binding site in less conserved regions of the core or in the N- and C-terminal extensions (cf. Figure 1B). The binding of RNA to this secondary site(s) would then strengthen RNA binding to the core, provided the RNA template is long enough to bridge the two sites. Supporting this view, the C-terminal extension of the DEAD-box protein DbpA is thought to encompass a binding site for helix 92 of 23S rRNA (*18*). In this case, only RNA molecules carrying extensions fused to helix 92 (either 5′ or 3′) can act as ATPase stimulators or as substrates for the unwinding reaction, presumably because the binding of helix 92 to the C-terminal domain facilitates the interaction of RNA with the helicase core (*13*). Similarly, SrmB and CsdA (or CsdAΔ) might also bind RNA (albeit with little or no specificity) through additional sites besides the RNA-binding track of the helicase core, and this binding would help delivering it to the core. In contrast, for RhlE, binding of RNA to the core would be strong enough for proficient interaction even without the help of extra binding site(s). Further work is needed to evaluate this hypothesis.

Mechanism of Helicase Action. The 3D structure of the helicase domains from SF1 and SF2 proteins is highly conserved, arguing for a common enzymatic mechanism. The "inchworm" mechanism, as proposed for the hepatitis C virus SF2 helicase NS3 (*35*) and further documented by structural studies on the *B. strearothermophilus* SF1 DNA helicase PcrA (*10*), may therefore apply to most of SF1 or SF2 helicases, at least to those that are monomeric. In this mechanism, depending upon which nucleotide—ADP or ATP—is bound, the distance between the two helicase domains increases or decreases; meanwhile, the affinity of the two domains for ssDNA alternates, so that the protein

uses ATP hydrolysis to translocate directionally on ssDNA. Whenever ds regions are encountered, they must be peeled off as a consequence of this translocation; however, PcrA appears to also use ATP hydrolysis for actively destabilizing the duplex (*10*). At first sight, our observations with DEAD-box proteins are at odds with such scenario. Whereas for SrmB or CsdA, the stimulation of ATPase by duplexes carrying ss extensions could reflect directional translocation over these extensions, it is difficult to imagine how this might result in the unwinding of duplexes irrespective of their position − 5′ or 3′ − with respect to the extension. Even more striking is the case of RhlE, for which ATPase stimulation and unwinding are observed even without any ss extensions. Moreover, the ability to unwind duplexes whatever the polarity of ss extensions do not seem uncommon among DEAD-box proteins. Thus, like RhlE, eIF4A has ATPase and unwinding activities on blunt-end duplexes (*19*, *20*), and besides CsdA and SrmB, several other DEAD-box proteins can use duplexes carrying either 5′ or 3′ extensions (e.g., DbpA (*13*), p68 (*38*), or RH70 (*39*)). In contrast, some DEAD-box proteins have been reported to require ss extensions of defined polarity for unwinding (e.g., see refs *40*, *41*) or to be unable to use dsRNA for ATPase stimulation (*32*). Whether these exceptions are real or reflect the particular experimental conditions used is not clear at present.

How to reconcile the fact that dsRNA unwinding by the DEAD-box proteins studied here do not require ss extensions of defined polarity, with the hypothesis that unwinding is driven by a translocation reaction, as observed with PcrA? One possibility is that SrmB, CsdA or RhlE can translocate not only on ssRNA but also on dsRNA, and that translocation on dsRNA somehow causes its unwinding. Translocation on ds nucleic acid can easily be accounted for by the inchworm mechanism, provided the helicase core can accommodate ds nucleic acid. Indeed, it has been documented for one particular SF2 helicase, RecG (*42*, *43*), and it has been suggested to hold for many others as well (*8*). It should be stressed, however, that whereas a protein that translocates specifically on ssRNA has by necessity an unwinding action, translocation on ds-RNA will only cause unwinding if the protein possesses a dedicated device for separating the strands behind it. For instance, residues from the N-terminal extension of RecG appear to strip away the two strands as the motor moves over dsDNA (*42*). The fact that for the three DEAD-box proteins studied here unwinding activity drops so precipitously with duplex length would then suggest that such a strand-separating device, if any, is inefficient. Assuming that each translocation cycle causes only partial unwinding, this unwinding would then be easily reversible. The probability that it results in complete dissociation would decrease exponentially with duplex stability, resulting in a very low unwinding "processivity", as observed.

Alternatively, other interpretations can be proposed, particularly since ds-RNA translocation has not been documented with DEAD-box proteins. Duplexes carrying either 3′ or 5′ extensions (or no extension at all) could still be unwound by ss-RNA translocation if sufficient fraying occurs at duplex extremities for the helicase to bind even in the absence of appropriate extensions. We consider this possibility unlikely because the duplex carrying a ss-extension of appropriate polarity would presumably be unwound much more efficiently than its counterparts with the inappropriate extension, which would rely upon ds-RNA fraying; yet this odd behavior has not been observed here. Another interpretation, which does not require translocation at all, has been put forward by Rogers et al. in the case of eIF4A (*19*). In this simple model, the protein interacts with ds-RNA statically; ATP hydrolysis then leads to local unwinding of the duplex, which can lead either to complete dissociation or to reannealing, depending upon duplex stability. This model, which is clearly compatible with our results, would however imply that PcrA-like enzymes and DEAD-box proteins use very different mechanisms for unwinding. It is noteworthy that a distinctive feature common to this model and the ds-translocation mechanism is the existence of a direct interaction between DEAD-box proteins and ds-RNA during the unwinding process.

CONCLUSIONS

The in vivo relevance of the unwinding activities documented here for SrmB, CsdA, or RhlE remains elusive. Because long stretches of dsRNA are presumably rare in *E. coli*, these activities may well be biologically significant despite their weakness. Alternatively, these proteins may associate in vivo with specific partners that strengthen their helicase activity. This situation would be reminiscent of eIF4A, the helicase activity of which is stimulated by its association with eIF4B and eIF4H (*17*). However, it is also possible that the biological roles of these proteins do not require dsRNA unwinding, but simply their activity as molecular motors. Interestingly, it has been shown that the NS3 helicase can use ATP hydrolysis to disrupt RNA-protein interactions (*44*). Such an activity, which does not necessarily require dsRNA unwinding, may pinpoint an important role of these proteins in the rearrangement of ribonucleoprotein assemblies.

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REFERENCES

1. Linder, P., Lasko, P. F., Ashburner, M., Leroy, P., Nielsen, P. J., Nishi, K., Schnier, J., and Slonimski, P. P. (1989) Birth of the D-E-A-D box, *Nature 337*, 121−122.
2. Tanner, N. K., and Linder, P. (2001) DExD/H box RNA helicases: from generic motors to specific dissociation functions, *Mol. Cell 8*, 251−262.
3. Tanner, N. K., Cordin, O., Banroques, J., Doère, M., and Linder, P. (2003) The Q motif: a newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis, *Mol. Cell 11*, 127−138.
4. Silverman, E., Edwalds-Gilbert, G., and Lin, R. J. (2003) DExD/H-box proteins and their partners: helping RNA helicases unwind, *Gene 312*, 1−16.
5. de la Cruz, J., Kressler, D., and Linder, P. (1999) Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families, *Trends Biochem. Sci. 24*, 192−198.
6. Gorbalenya, A. E., and Koonin, E. V. (1993) Helicases: amino acid sequence comparisons and structure−function relationships, *Curr. Opin. Struct. Biol. 3*, 419−429.
7. Caruthers, J. M., and McKay, D. B. (2002) Helicase structure and mechanism, *Curr. Opin. Struct. Biol. 12*, 123−133.

8. Singleton, M. R., and Wigley, D. B. (2002) Modularity and specialization in superfamily 1 and 2 helicases, *J. Bacteriol. 184*, 1819−1826.
9. Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997) Major domain swiveling revealed by the crystal structures of complexes of E. coli Rep helicase bound to single-stranded DNA and ADP, *Cell 90*, 635−647.
10. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) Crystal structures of complexes of PcrA DNA helicase with a DNA substrate indicate an inchworm mechanism, *Cell 97*, 75−84.
11. Soultanas, P., Dillingham, M. S., Wiley, P., Webb, M. R., and Wigley, D. B. (2000) Uncoupling DNA translocation and helicase activity in PcrA: direct evidence for an active mechanism, *EMBO J. 19*, 3799−3810.
12. Rocak, S., and Linder, P. (2004) DEAD-box proteins: The driving forces behind RNA metabolism, *Nat. Rev. Mol. Cell Biol. 5*, 232−241.
13. Diges, C. M., and Uhlenbeck, O. C. (2001) *Escherichia coli* DbpA is an RNA helicase that requires hairpin 92 of 23S rRNA, *EMBO J. 20*, 5503−5512.
14. Rogers, G. W. J., Richter, N. J., and Merrick, W. C. (1999) Biochemical and kinetic characterization of the RNA helicase activity of Eukaryotic Initiation Factor 4A, *J. Biol. Chem. 274*, 12236−12244.
15. Lorsch, J. R., and Herschlag, D. (1998) The DEAD box protein eIF4A. 1. A minimal kinetic and thermodynamic framework reveals coupled binding of RNA and nucleotide, *Biochemistry 37*, 2180−2193.
16. Vanzo, N. F., Li, Y. S., Py, B., Blum, E., Higgins, C. F., Raynal, L. C., Krisch, H. M., and Carpousis, A. J. (1998) Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome, *Genes Dev. 12*, 2770−2781.
17. Rogers, G. W. J., Richter, N. J., Lima, W. F., and Merrick, W. C. (2001) Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F, *J. Biol. Chem. 276*, 30914−30922.
18. Kossen, K., Karginov, F. V., and Uhlenbeck, O. C. (2002) The carboxy-terminal domain of the DExD/H protein YxiN is sufficient to confer specificity for 23S rRNA, *J. Mol. Biol. 324*, 625−636.
19. Rogers, G. W. J., Lima, W. F., and Merrick, W. C. (2001) Further characterization of the helicase activity of eIF4A. Substrate specificity, *J. Biol. Chem. 276*, 12598−12608.
20. Du, M. X., Johnson, R. B., Sun, X. L., Staschke, K. A., Colacino, J., and Wang, Q. M. (2002) Comparative characterization of two DEAD-box RNA helicases in superfamily II: human translation initiation factor 4A and hepatitis C virus nonstructural protein 3 (NS3) helicase, *Biochem. J. 363*, 147−155.
21. Fuller-Pace, F. V., Nicol, S. M., Reid, A. D., and Lane, D. P. (1993) DbpA: a DEAD-box protein specifically activated by 23S rRNA, *EMBO J. 12*, 3619−3626.
22. Khemici, V., and Carpousis, A. J. (2004) The RNA degradosome and poly(A) polymerase of *Escherichia coli* are required in vivo for the degradation of small mRNA decay intermediates containing REP-stabilizers, *Mol. Microbiol. 51*, 777−790.
23. Py, B., Higgins, C. F., Krish, H. M., and Carpousis, A. J. (1996) A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome, *Nature 381*, 169−172.
24. Miczak, A., Kaberdin, V. R., Wei, C. L., and Lin-Chao, S. (1996) Proteins associated with RNase E in a multicomponent ribonucleolytic complex, *Proc. Natl. Acad. Sci. U.S.A. 93*, 3865−3869.
25. Jones, P. G., Mitta, M., Kim, Y., Jiang, W., and Inouye, M. (1996) Cold-shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*, *Proc. Natl. Acad. Sci. U.S.A. 93*, 76−80.
26. Charollais, J., Dreyfus, M., and Iost, I. (2004) CsdA, A cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit, *Nucleic Acids Res.*, in press.
27. Iost, I., and Dreyfus, M. (1994) mRNAs can be stabilized by DEAD-box proteins, *Nature 372*, 193−196.
28. Charollais, J., Pflieger, D., Vinh, J., Dreyfus, M., and Iost, I. (2003) The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in *Escherichia coli*, *Mol. Microbiol. 48*, 1253−1265.
29. Nishi, K., Morel-Deville, F., Hershey, J. W. B., Leighton, T., and Schnier, J. (1988) An eIF-4A-like protein is a suppressor of an *Escherichia coli* mutant defective in 50S ribosomal subunit assembly, *Nature 336*, 496−498.
30. Raynal, L. C., and Carpousis, A. J. (1999) Poly(A) polymerase I of *Escherichia coli*: characterization of the catalytic domain, an RNA binding site, and regions for the interaction with proteins involved in mRNA degradation, *Mol. Microbiol. 32*, 765−775.
31. Bessman, M. J. (1963) Deoxynucleotide monophosphate kinases, *Methods Enzymol. 6*, 166−176.
32. Iost, I., Dreyfus, M., and Linder, P. (1999) Ded1p, a DEAD-box protein required for translation initiation in *Saccharomyces cerevisiae*, is a RNA helicase, *J. Biol. Chem. 274*, 17677−17683.
33. Peck, M. L., and Herschlag, D. (1999) Effects of oligonucleotide length and atomic composition on stimulation of the ATPase activity of translation initiation factor eIF4A, *RNA 5*, 1210−1221.
34. Pause, A., and Sonenberg, N. (1992) Mutational analysis of a DEAD box RNA helicase − the mammalian translation initiation factor eIF-4A, *EMBO J. 11*, 2643−2654.
35. Kim, J. L., Morgenstern, K. A., Griffith, J. P., Dwyer, M. D., Thomson, J. A., Murcko, M. A., Lin, C., and Caron, P. R. (1998) Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding, *Structure 6*, 89−100.
36. Levin, M. K., and Patel, S. S. (2002) Helicase from hepatitis C virus, energetics of DNA binding, *J. Biol. Chem. 277*, 29377−29385.
37. Story, R. M., Li, H., and Abelson, J. N. (2000) Crystal structure of a DEAD box protein of the hyperthermophile *Methanococcus jannaschii*, *Proc. Natl. Acad. Sci. U.S.A. 98*, 1465−1470.
38. Huang, Y., and Liu, Z. R. (2002) The ATPase, RNA unwinding and RNA binding activities of recombinant p68 RNA helicase, *J. Biol. Chem. 277*, 12810−12815.
39. Lee, C. G. (2002) RH70, a bidirectional RNA helicase, co-purifies with U1snRNP, *J. Biol. Chem. 277*, 39679−39683.
40. Flores-Rozas, H., and Hurwitz, J. (1993) Characterization of a new RNA helicase from nuclear extracts of HeLa cells which translocates in the 5′ to 3′ direction, *J. Biol. Chem. 268*, 21372−21383.
41. Yu, E., and Owttrim, G. W. (2000) Characterization of the cold stress-induced cyanobacterial DEAD-box protein CrhC as an RNA helicase, *Nucleic Acids Res. 28*, 3926−3934.
42. Singleton, M. R., Scaife, S., and Wigley, D. B. (2001) Structural analysis of DNA replication fork reversal by RecG, *Cell 107*, 79−89.
43. Mahdi, A. A., Briggs, G. S., Sharples, G. J., Wen, Q., and Lloyd, R. G. (2003) A model for dsDNA translocation revealed by a structural motif common to RecG and Mfd proteins, *EMBO J. 22*, 724−734.
44. Jankowsky, E., Gross, C. H., Shuman, S., and Pyle, A. M. (2001) Active disruption of an RNA-protein interaction by a DExH/D RNA helicase, *Science 291*, 121−124.
45. Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., Eddy, S. R., Griffiths-Jones, S., Howe, K. L., Marshall, M., and Sonnhammer, E. L. (2002) The Pfam Protein Families Database, *Nucleic Acids Res. 30*, 276−280.

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