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# Characterization of the autoantigen La (SS-B) as a dsRNA unwinding enzyme

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## ABSTRACT

**During the analysis of the La (SS-B) autoantigen for catalytic activities an ATP-dependent double-stranded RNA unwinding activity was detected. Both native and recombinant La proteins from different species displayed this activity, which could be inhibited by monospecific anti-La antibodies. La protein was able to melt dsRNA substrates with either two 3′-overhangs or a single 3′- and a 5′-overhang. Double-stranded RNAs with two 5′-overhangs were not unwound, indicating that at least one 3′-overhang is required for unwinding. Sequence elements of the La protein that might be involved in dsRNA unwinding, such as an evolutionarily conserved putative ATP-binding motif and an element that is homologous to the double-stranded RNA binding protein kinase PKR, are discussed.**

## INTRODUCTION

Sera from patients with autoimmune diseases such as systemic lupus erythematosus or primary Sjögren's syndrome frequently develop autoantibodies to RNA- or DNA-binding proteins (1). One of the targets of such autoantibodies is the so-called La (SS-B) protein. The La protein can be found associated with all primary RNA polymerase III transcripts including precursor molecules of ribosomal 5S RNA, the tRNAs, some 7S RNAs, as well as a portion of the U1 and U6 snRNA (2–4). Common to all primary RNA polymerase III transcripts is the 3′-terminal oligo(U)-tail, which is transcribed during the transcription termination step. These oligo(U)-tails were shown to be a binding site for the La protein (5). In most cases the binding of the La protein to the primary RNA polymerase III transcripts is only transient, as their respective oligo(U)-tails are removed after transcription during an unknown processing step. However, some small cytoplasmic La RNAs retain their oligo(U)-tails even after transport to the cytoplasm (6). In addition to the oligo-uridylated RNA polymerase III transcripts an association of the La protein with some non-oligouridylated RNAs has been reported especially for some viral RNAs including the leader RNAs of vesicular stomatitis virus and rabies virus (6,7).

Recently, the La protein was shown to be involved in internal initiation of translation of poliovirus mRNA and to alleviate translational repression by the TAR element in HIV-1 mRNA (reviewed in ref. 8). As a hypothetical mode of action a dsRNA unwinding mechanism has been suggested (9). In a previous report it was described that the La protein is able to melt DNA–RNA hybrids (10). This activity could explain how the La protein might release the primary RNA polymerase III transcripts from their DNA template (10–12). During these experiments an activation of the La protein-associated ATPase activity was not only observed for DNA–RNA hybrids but also for poly(I)·poly(C), an observation which was confirmed by a recent report of Xiao and co-workers (13) who have shown that La is able to unwind dsRNA to single-stranded forms. In this report we extend these observations and show that La is an ATP-dependent helicase that unwinds dsRNA substrates with either two 3′-overhangs or a single 3′- and 5′-overhang.

## MATERIALS AND METHODS

### Materials

Isopropyl β-D-thiogalactopyranoside (IPTG), anti-human IgG (γ-chain specific) conjugated with alkaline phosphatase (A3150; 8 U/ml), anti-mouse IgG developed in sheep [$(Fab')_2$ fragments] adsorbed with human serum proteins (A-0532) were obtained from Sigma (St Louis, MA, USA). The Enhanced Chemiluminescence (ECL)-Western blotting detection reagents were from Amersham-Buchler (Braunschweig, Germany). 4-Nitro-blue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) from Roth (Karlsruhe, Germany). [$^{32}$P]UTP (specific radioactivity 3000 Ci/mmol) from Dupont (Dreieich, Germany). Dinatrium 3-(4-methoxyspiro {1,2-dioxetan-3,2′-(5′-chloro) tricyclo[3.3.1.1.$^{3,7}$] decan}-4-yl) phenyl phosphate (CSPD) was obtained from Tropix (Bedford, MA, USA). pBluescript II SK(–) and KS(–) were obtained from Stratagene (Heidelberg, Germany). pGEM-4Z was from Promega (Heidelberg, Germany). T7-, SP6- and T3 RNA polymerase, pSPT 19 and various restriction enzymes were obtained from Boehringer Mannheim, (Mannheim, Germany).

### Anti-La antibodies and sera

Monospecific anti-La antibodies were isolated by affinity-purification from serum W10 (14) using recombinant human La protein covalently linked to Sepharose 4B. Elution of the bound

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antibodies was performed as described previously (10,15–17). The anti-La mAb La4B6 was raised against recombinant human La protein and recognizes the sequence SKGRRFKGKGKGN in the C-terminal part of the La protein (18).

### Preparation of cell extracts

For the preparation of total cell extracts, monolayer human XPTA cells and mouse 3T3 fibroblasts (ATCC CCL 92) were grown to confluency in a culture flask. The cells were washed and 500 µl hot SDS–PAGE sample solution (0.1 M $Na_2HPO_4$, pH 8.3; 20% [w/v] sucrose, 1% SDS, 1 mM PMSF) was added per 200 ml flask. The lysed cells were heated in an Eppendorf tube for 5 min at 95°C and the resulting protein extract was centrifuged for 15 min at 11 000 *g* and frozen at –80°C until use.

### Preparation of the purified La protein fractions

The La protein was biochemically purified as described (16,19) using heparin- and poly(U)-Sepharose chromatography followed by immunoaffinity chromatography on anti-La Sepharose. The native rat La proteins were isolated from either total rat liver extracts (female Brown Norway) or from extracts obtained by solubilisation of isolated rat liver nuclei or nuclear envelopes with Triton X-100 (20). The nuclei and nuclear envelopes were prepared by the following procedure (all steps at 4°C, unless stated otherwise). Rat liver was minced with a pair of scissors and homogenized in 4 vol 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$, 250 mM sucrose, 1 mM PMSF. The homogenate was filtrated through four layers of cheese cloth. After centrifugation at 800 *g* for 10 min, the pellet was washed with the same buffer and resuspended in 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$, 2.1 M sucrose, 1 mM PMSF. After centrifugation for 60 min at 70 000 *g*, the pelleted nuclei were resuspended and applied to a 5 ml sucrose cushion. Nuclei were obtained by centrifugation for 30 min at 70 000 *g*. The pelleted nuclei were resuspended in 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$, 250 mM sucrose, 1 mM PMSF at a concentration of ~$5 \times 10^8$ nuclei/ml. For preparation of nuclear envelopes the nuclei were incubated with 250 µg/ml DNase I for 60 min at 30°C, after which the nuclei were collected again by centrifugation (800 *g*, 10 min). Nuclei were resuspended in 10 mM Tris–HCl, pH 7.4, 0.2 mM $MgSO_4$, 1 mM PMSF and subsequently 4 vol 2 M NaCl in 10 mM Tris–HCl, pH 7.4, 0.2 mM $MgSO_4$, 1 mM PMSF were added. 2-Mercaptoethanol was added to a final concentration of 1% and after an incubation for 15 min the nuclear envelopes were collected by centrifugation (1600 *g*, 30 min). Then the NaCl extraction step was repeated without the addition of 2-mercaptoethanol. Finally, the nuclear envelopes were resuspended in 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 2.1 M sucrose, 1 mM PMSF and stored at –70°C. Nuclei and nuclear envelopes were extracted with Triton X-100 as follows. Pelleted nuclei were resuspended in 25 mM Tris–HCl, pH 7.6, 25 mM KCl, 2.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mM $MnCl_2$, 5.0 mM spermidine–HCl, 250 mM glucose, 5.0 mM 2-mercaptoethanol and Triton X-100 was added to a concentration of 1%. After an incubation for 10 min the demembranated nuclei were removed by centrifugation (1600 *g*, 10 min). Pelleted nuclear envelopes were resuspended in 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$, 250 mM sucrose, 1 mM PMSF and were extracted with 2% Triton X-100 for 10 min. The pore complex laminae were removed by centrifugation (15 000 *g*, 20 min).

### Expression of recombinant La proteins

Two different recombinant human La protein preparations, termed wt1 and wt2, were used (21,22). Preparation wt1 was isolated by two ion-exchange chromatographic steps followed by an immunoaffinity chromatographic step from a bacterial lysate of *Escherichia coli* expressing a La cDNA described previously (23,24). The recombinant human La preparation wt2 and also the recombinant rat La protein were isolated via a single immunoaffinity chromatographic step from bacterial extracts, which were obtained from bacteria expressing either the human (EMBL accession no. X69804) or rat (EMBL accession no. X67859) La cDNAs (21). Bound La protein was eluted as described (19). If not stated otherwise La preparation wt2 was used.

### SDS–polyacrylamide gel electrophoresis and immunoblotting

SDS–PAGE and immunoblotting were performed as described (25). The blocked membrane was incubated with cell culture supernatant of hybridoma cells secreting the anti-La mAb La4B6 (18). Immune complexes were detected with anti-mouse peroxidase conjugate using the ECL-Western blotting detection reagents. After elution of the mouse immune complexes (10) the blot was incubated with monospecific anti-La antibodies isolated from patient serum W10. The immune complexes were visualized with anti-human antibodies covalently linked to alkaline phosphatase using BCIP/NBT as substrate.

### Unwinding assay

Plasmid DNAs were obtained from either pSPT 19, pBluescript II SK(–) and KS(–), or pGEM-4Z. Plasmid DNA was prepared according to a Qiagen midiprep protocol from Diagen (Hilden, Germany) and substrate DNAs were made as follows. In the case of the substrates S1 and S2, pSPT 19 DNA was cut with either *Pvu*II or *Nar*I, in the case of S3 with either *Hin*dIII or *Nar*I, in the case of S4 with either *Pvu*II or *Eco*RI and in the case of S5 with either *Hin*dIII or *Eco*RI. The substrate S6 was prepared from pBluescript II SK(–) by cutting with either *Xho*I or *Not*I. In the case of the substrate S7 the unlabeled strand was prepared from pBluescript II KS(–) after cutting with *Sac*I, the labeled strand was prepared from SK(–) after cutting with *Kpn*I. The substrate S8 was transcribed from pGEM-4Z cut with *Pvu*II. The resulting linear DNAs were transcribed *in vitro* by either SP6-, T7- or T3 RNA polymerase.

*In vitro* transcription was performed according to the established protocols using 1.5 µg of the respective plasmid DNA in a final volume of 20 µl. In order to label a transcript *in vitro* transcription was performed in the presence of 50 µCi [$\alpha$-$^{32}$P]UTP.

Substrates were prepared by annealing two complementary strands (a 2–3-fold molar excess of the unlabeled strand) in hybridization buffer [0.4 M NaCl, 1 mM EDTA, 40 mM PIPES, pH 6.4 and 80%(v/v) formamide] as described (26).

The unwinding of the dsRNA substrate was assayed in a final volume of 20 µl containing 0.5–1.2 µl substrate and 0.1–5 µg La protein in a reaction buffer composed of 30 mM Tris–HCl, pH 7.5, 8 mM $MgCl_2$, 1.5 mM DTT, 30 µg/ml BSA, 0.5 U/µl RNasin

and 2 mM ATP unless stated otherwise. The reaction mixture was incubated for 15 min at 37°C. The reaction was stopped by the addition of SDS and EDTA to final concentrations of 0.3% and 15 mM respectively.

After unwinding RNAs were separated by SDS–PAGE using 10 or 11.5% gels lacking a stacking gel and the reaction products were visualized by autoradiography.

## RESULTS

During the last few years assay systems have been developed to test for unwinding activity of putative helicases (26,27). In such an assay we have analyzed the unwinding of dsRNA substrates consisting of a hybrid of a radioactively labeled strand and an unlabeled complementary strand, both transcribed *in vitro* from a variety of plasmids (see Materials and Methods). Unwinding of such a (partially) double-stranded RNA substrate can be detected because the released labeled strand will usually migrate faster than the dsRNA in an SDS–polyacrylamide gel.

### dsRNA unwinding activity of the La protein

The dsRNA substrate S1 consists of an unlabeled ssRNA molecule of 357 nucleotides (nt) and a labeled ssRNA of 107 nt, which, after hybridization, form a substrate with 3′-overhangs of 285 and 35 nt respectively, and a dsRNA region of 72 base pairs (bp) (A in Fig. 1A). Unwinding of substrate S1 releases the radiolabeled strand of 107 nt (B in Fig. 1A). S1 was unwound by recombinant La protein preparations (Fig. 1B, lanes c, d and j) as well as by the native La protein purified to homogeneity (Fig. 1B, lane i) when using 0.1 µg La protein/assay. No unwinding activity was detected in the absence of La protein (Fig. 1B, lane b) nor with a bacterial protein control (Fig. 1B, lane h). Concentrations of La protein below 0.1 µg/assay were less effective (lanes e, f and g), while higher concentrations of La led to more unwinding (see e.g. Fig. 3A, lane b). The dsRNA unwinding activity was found for two different recombinant human La protein preparations termed wt1 (Fig. 1B, lane c) and wt2 (Fig. 1B, lane d) as well as for a recombinant rat La protein preparation (Fig. 1B, lane j). The recombinant human La protein preparations were isolated from total bacterial extracts by different procedures and expressed by La cDNAs from two different laboratories (see Materials and Methods). Independent of the isolation procedure equivalent amounts of recombinant La proteins exhibited similar activities. The La protein preparations used were essentially free of contaminating proteins. This became especially evident when the purified La protein fractions were biotinylated and analyzed by SDS–PAGE and Western blotting (data not shown).

Double-stranded RNA unwinding by the La protein required ATP (Fig. 1C) as only a weak if any activity was found when ATP was omitted (lane d), or replaced by GTP (lane b) or the non-hydrolyzable methylene ATP analog (lane c).

With 0.5 µg recombinant human La protein unwinding of substrate S1 was almost complete after 20 min of incubation at 37°C (Fig. 2).

### Inhibition of dsRNA unwinding activity of the La protein by anti-La antibodies

To confirm that the dsRNA unwinding activity was an inherent activity of the La protein a monospecific polyclonal anti-La antibody preparation was added to the unwinding assay. As shown in Figure 3A the affinity-purified anti-La antibody preparation (W10) inhibits the unwinding activity of the recombinant human La protein (1 µg/assay) in a dose-dependent manner (lanes b, c and d). The specificity of the anti-La antibodies used is illustrated by the Western blot in Figure 3B, which was prepared using total extracts obtained from either human or mouse cultured cells. The specificity of W10 antibodies (Fig. 3B,

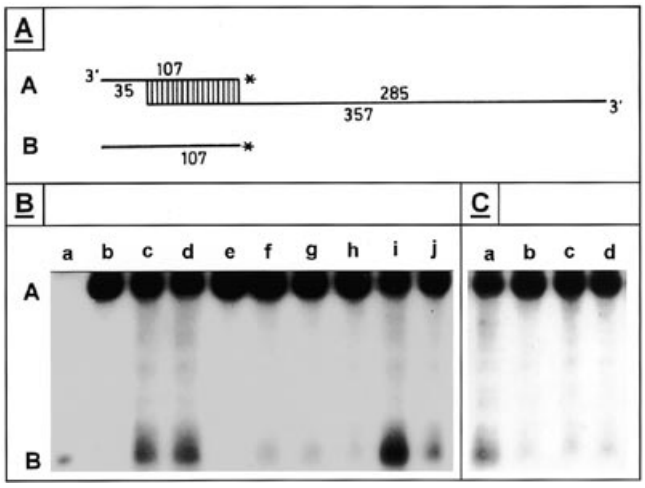


**Figure 1.** Unwinding of dsRNA by the La protein. **(A)** Schematic structure of substrate S1 (A) and radiolabeled product of unwinding of S1 (B). The numbers indicate the lengths in nucleotides. A star marks the radiolabeled strand. **(B)** Unwinding of substrate S1 by different La protein preparations. Bands A and B correspond to structures A and B in (**A**) respectively. Both RNA strands were transcribed from pSPT 19 DNA. After incubation of substrate S1 in the presence or absence of La protein preparations, substrate and products were analysed by SDS–PAGE. The control lanes show the ssRNA, released by heat-denaturation of substrate S1 (lane a; the amount of denatured substrate loaded was significantly smaller then the amount used in the other lanes) and the dsRNA substrate S1 incubated in the absence of any protein preparation (lane b). In lanes c–j the following protein preparations were used: lane c, recombinant human La protein (wt1), 0.1 µg; lane d, recombinant human La protein (wt2), 0.1 µg; lanes e, f and g, recombinant human La protein (wt1), 0.01, 0.02 and 0.05 µg respectively; lane h, control immunoaffinity purified fraction from bacteria transformed with the expression vector without La cDNA insert; lane i, purified rat La protein, 0.1 µg; lane j, recombinant rat La protein, 0.1 µg. **(C)** ATP requirement of unwinding by La. Unwinding activity of 0.5 µg recombinant human La protein wt2 was assayed after addition of 2 mM ATP (lane a), 2 mM GTP (lane b), 2 mM non-hydrolyzable ATP analog (lane c), and in the absence of any NTP (lane d).

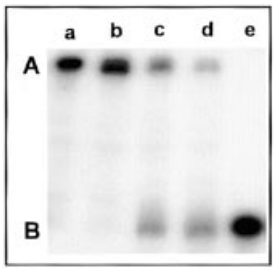


**Figure 2.** Time-course of dsRNA unwinding by the La protein. Substrate S1 was incubated with 0.5 µg recombinant human La protein wt2 for 0 min (lane b; reaction stopped immediately after addition of the La protein), 10 min (lane c) or 20 min (lane d). Lane a contains the dsRNA substrate and in lane e the dsRNA substrate was heat denatured before gel electrophoresis.

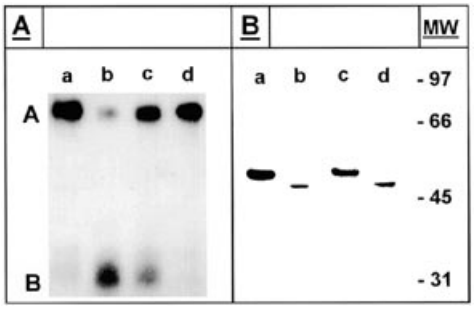


**Figure 3.** Inhibition of dsRNA unwinding activity by anti-La antibodies. **(A)** Anti-La antibodies inhibit the unwinding of substrate S1 by La. The unwinding of substrate S1 by 1 µg recombinant human La protein wt2 was analyzed in the presence of increasing amounts (lane b, 0.1 µg; lane c, 1 µg; lane d, 5 µg) of purified, monospecific human anti-La antibodies from patient serum W10. Lane a shows the products from a control incubation in the absence of La protein. **(B)** Specificity of anti-La antibodies. Total extracts from cultured human (lanes a and c) and mouse (lanes b and d) cells were fractionated by SDS–PAGE and transfered to membranes by Western blotting to analyze the specificity of the affinity-purified human W10 anti-La antibodies (lanes c and d) and anti-La monoclonal antibody La4B6 (lanes a and b) as a control. The position of molecular weight markers is indicated on the right (MW; in kDa).

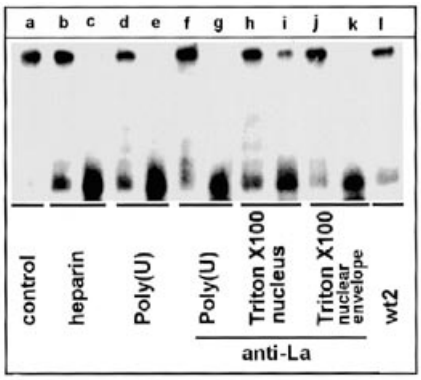


**Figure 4.** Copurification of the dsRNA unwinding activity with La. The La protein was purified from rat liver by the procedure described in the Materials and Methods section. Various fractions of (partially) purified La were assayed for unwinding of substrate S1. Lane a shows the dsRNA substrate incubated in the absence of any La protein. In lanes c, e, g, i and k, a 3-fold amount of the same La fraction was used as in lanes b, d, f, h and j respectively. Substrate S1 was incubated with the following: lanes b and c, La isolated from a rat liver extract by ammonium sulphate precipitation, Sephadex G-150 gel filtration, anti-Ro depletion and heparin chromatography; lanes d and e, La isolated from a rat liver extract as described for lanes b and c but further purified by poly(U)–Sepharose chromatography; lanes f and g: La isolated from a rat liver extract as described for lanes d and e but further purified by anti-La immunoaffinity chromatography; lanes h and i, La isolated by immunoaffinity chromatography from a rat liver nuclei preparation obtained by solubilization of isolated nuclei with Triton X-100; lanes j and k, La isolated by immunoaffinity chromatography from a rat liver nuclear envelope preparation obtained by solubilization of isolated nuclear envelopes with Triton X-100. In lane l recombinant human La preparation wt2 was used as a control.

lanes c and d) was indistinguishable from that of the monoclonal anti-La antibody La4B6 (Fig. 3B, lanes a and b). Both stain a single protein band with a molecular weight of ~50 kDa in the human cell extract and of ~47 kDa in the mouse cell extract, in accordance with the apparent molecular weight of the La protein from these species.

## Copurification of the unwinding activity with La from rat liver extract

The results described above indicated that also the rat La protein displayed dsRNA unwinding activity. To substantiate these data and to investigate whether the unwinding activity indeed represented an endogenous activity of the rat La protein, the protein was extensively purified from rat liver. Rat liver extract was fractionated by ammonium sulphate precipitation, Sephadex G-150 gel filtration, depletion by anti-Ro antibodies (to remove Ro RNPs which are known to be stably associated with a subset of La molecules) and finally heparin chromatography. The La containing fractions of the heparin column were further fractionated by poly(U)–Sepharose chromatography followed by anti-La immunoaffinity chromatography. The presence of the La protein in all fractions was assayed by Western blotting using monoclonal and patient anti-La antibodies. When La containing fractions from the last three columns were analysed in the dsRNA unwinding assay, all were able to unwind dsRNA (Fig. 4, lanes b–g). Also La protein isolated by immunoaffinity chromatography from lysed rat liver nuclei or nuclear envelopes was able to unwind substrate S1 (Fig. 4, lanes h–k).

From the combined results described above it was concluded that the unwinding activity is an endogenous activity of the La protein.

## Substrate specificity of dsRNA unwinding by La

As depicted before substrate S1 was composed of a double-stranded region of 72 bp bordered by two 3′-overhangs. When the 357 nt strand was radiolabeled (substrate S2) instead of the shorter 107 nt strand as in S1, unwinding could not be assessed due to the fact that the relatively long radiolabeled strand comigrated with the substrate in the gel (results not shown).

To learn more about the substrate specificity of unwinding by the La protein, a number of other substrates were prepared. Substrate S3 consisted of a hybrid between an unlabeled ssRNA of 357 nt and a labeled ssRNA of 61 nt, which was completely complementary to a region of the longer strand. This resulted in a substrate containing a double-stranded region of 61 bp and both a 3′- and a 5′-overhang (Fig. 5A, band A). The La protein was able to release the small labeled ssRNA from this substrate (Fig. 5A, lane b, band B).

Substrate S4 was identical to substrate S3 except for the fact that the other strand (357 nt) was radiolabeled. As observed for S2, substrate S4 and its unwinding product displayed similar mobilities (results not shown), implying that unwinding of S4 could not be assessed.

Next three different substrates were prepared with 5′-overhangs at both ends. Substrate S5 consisted of an unlabeled ssRNA of 62 nt and a labeled ssRNA of 61 nt, which after hybridization resulted in a double-stranded region of 51 bp and two 5′-overhangs of 11 and 10 nt (Fig. 5B, band A). Substrate S6 consisted of an unlabeled ssRNA of 101 nt and a labeled ssRNA of 104 nt, which after hybridization resulted in a double-stranded region of 72 bp and two 5′-overhangs of 29 and 32 nt (Fig. 5C, band A). Substrate S7 consisted of an unlabeled ssRNA of 117 nt and a labeled ssRNA of also 117 nt, which after hybridization resulted in a double-stranded region of 102 bp and two 5′-overhangs of 15 nt (Fig. 5D, band A). Neither S5, nor S6, nor S7 were unwound

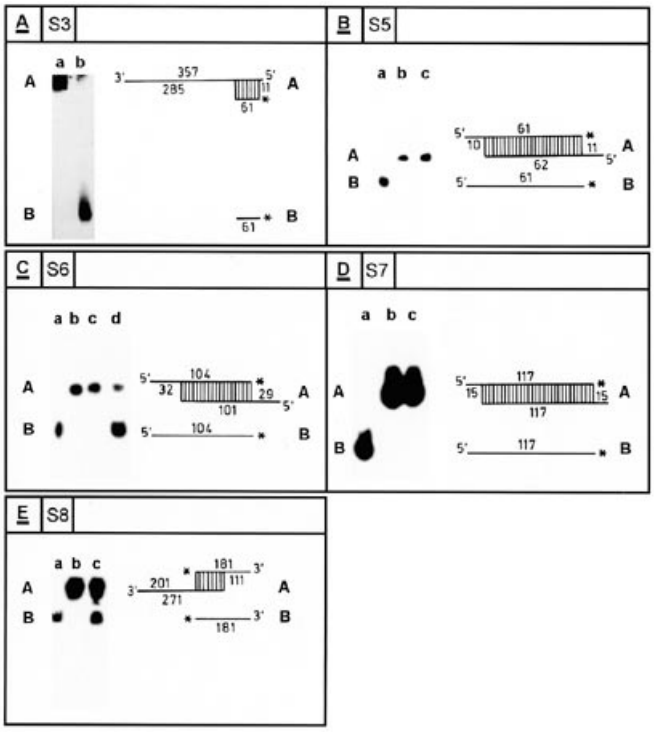


**Figure 5.** Substrate specificity of dsRNA unwinding by La. Various substrates were incubated with 2 µg (unless stated otherwise) of purified La preparation wt2 and analyzed as described in Materials and Methods. **(A)** Substrate S3 (band A) consisted of an unlabeled 357 nt ssRNA and a labeled 61 nt ssRNA resulting in a substrate with both 3′- and 5′-overhangs. The position of the labeled product of the unwinding reaction (band B) is indicated. Lanes a and b contain material from incubations in the absence and presence of La protein, respectively. **(B)** Substrate S5 (band A) consisted of an unlabeled 62 nt ssRNA and a labeled 61 nt ssRNA resulting in a substrate with two 5′-overhangs. The position of the radiolabeled ssRNA was determined by heat denaturation of substrate S5 (lane a) and is indicated by B. Lanes b and c contain material from incubations in the absence and presence of La protein, respectively. **(C)** Substrate S6 (band A) consisted of an unlabeled 101 nt ssRNA and a labeled 104 nt ssRNA resulting in a substrate with two 5′-overhangs. The position of the radiolabeled ssRNA was determined by heat denaturation of substrate S6 (lane a) and is indicated by B. Lanes b and c contain material from incubations in the absence and presence of La protein, respectively. **(D)** Substrate S7 (band A) consisted of an unlabeled 117 nt ssRNA and a labeled 117 nt ssRNA resulting in a substrate with two 5′-overhangs. The position of the radiolabeled ssRNA was determined by heat denaturation of substrate S7 (lane a) and is indicated by B. Lanes b and c contain material from incubations in the absence and presence of La protein, respectively. **(E)** Substrate S8 (band A) was created from a labeled 181 nt ssRNA and an unlabeled 271 nt ssRNA and contained two 3′-overhangs of 111 and 201 nt respectively. The position of the radiolabeled ssRNA was determined by heat denaturation of substrate S8 (lane a) and is indicated by B. Lanes b and c contain material from incubations in the absence and presence of (0.5 µg) La protein, respectively.

by the recombinant La protein (Fig. 5B–D) strongly suggesting that at least one 3′-overhang was required for unwinding by La.

To exclude the possibility that the unwinding of S1 and S3 by La was dependent on a particular sequence element that might be shared by these substrates in view of the fact that they were derived from the same plasmid, substrate S8 was prepared using a different plasmid. This substrate consisted of an unlabeled strand of 271 nt and a labeled strand of 181 nt, which after hybridization resulted in a double-stranded region of 70 bp and two 3′-overhangs of 201 and 111 nt, respectively. Since S8 was unwound by the La protein (Fig. 5E, lane c) we conclude that the presence of a 3′-overhang rather than a particular sequence element is required for dsRNA unwinding by La.

## DISCUSSION

In this report we describe an intrinsic enzymatic property of the La protein, namely an ATP-dependent dsRNA unwinding activity. We further present evidence that only dsRNA substrates with a 3′-overhang are unwound.

It is obvious that these results are only of interest when it can be shown convincingly that this unwinding activity is displayed by the La protein and not due to some minor contamination of the La preparation used. The following arguments support the notion that dsRNA unwinding is indeed exerted by the La protein.

(i) The unwinding activity was displayed by recombinant (human, rat) and native (rat liver) La protein purified from different organisms (*E.coli*, rat). Various purification protocols including ammonium sulphate precipitation followed by gel filtration, heparin chromatography and various affinity chromatography steps were unable to separate or remove the unwinding activity from the La protein. Although SDS–PAGE analyses of the various preparations (not shown) revealed almost identical protein profiles [full length La protein and its well-known degradation products (6,7,18)] such protein staining patterns can of course not exclude the possibility that some minor contamination of *E.coli* or rat liver proteins is present.

(ii) Affinity-purified patient anti-La antibodies are able to inhibit the unwinding activity. This result further corroborates our conclusion that the unwinding activity is an intrinsic property of the La protein, because it is very unlikely that the antibodies would also bind to a possible contamination.

(iii) Some mutant La proteins are unable to unwind the dsRNA substrate (unpublished observations). These mutants were purified from *E.coli* in the same way as the active wt La proteins, and as a consequence would be expected to contain the same set of contaminating proteins.

(iv) Purified and active La preparations are able to unwind dsRNAs with a 3′-overhang to completion but are unable to unwind similar substrates with merely 5′-overhangs.

Taken together we feel confident to conclude that the La protein is not only able to melt DNA–RNA hybrids, as has been reported before (10), but is also an ATP-dependent dsRNA unwinding enzyme. While this work was in progress dsRNA unwinding by La has also been reported in relation with the ability of La to inhibit activation of the dsRNA dependent protein kinase PKR (13).

Presently, the mechanism by which La unwinds dsRNA is unknown. The observation that a 3′-overhang is required might suggest that unwinding is initiated at the border of double-stranded and single-stranded RNA in the substrate. In the *in vitro* unwinding assay the concentration of recombinant La was in the micromolar range and the concentration of substrate RNA in the nanomolar range implying that the single-stranded RNA binding activity of La may act to change the equilibrium between dsRNA and its single-stranded components. Although we cannot exclude yet that La acts in a stoichiometric rather than a catalytic fashion, preliminary unwinding data obtained with mutants of La suggest that in addition to the RNA binding domain at the N-terminus of La also elements in the C-terminal part are required for unwinding (unpublished observations). In addition, it is not known which percentage of the recombinant La protein mol-

ecules is biologically active and thus the effective concentration of recombinant La in the unwinding assay might be much lower than the total concentration of La protein. Based upon these considerations and the fact that ATP is required for unwinding, we favour a mechanism in which La acts in a catalytic fashion, but additional experiments are required to confirm this.

### The helicase domain of La

During the last few years a rapidly growing protein family of putative RNA helicases with the common amino acid motif DEAD or DEXH have been described (29). Sequence comparisons of these putative unwinding enzymes resulted in the identification of up to eight common elements (27,29) including the so-called A or Walker motif, representing the ATP binding site, and the B-motif also known as DEAD/DEXH box. For only a few of the DEAD box proteins, e.g. eIF-4A and the protein p68 (30,31), an ATP-dependent helicase activity has been demonstrated. In some other cases, for example the SrmB protein and the prp16 protein, only an RNA-dependent ATPase activity was established (32,33). An RNA-dependent ATPase activity associated with the capability to melt DNA–RNA hybrids has been described for the procaryotic transcription termination factor rho (34) and for the autoantigen La (SS-B) (10). For the latter protein also a dsRNA unwinding activity was proposed and has now been firmly established (13, this paper).

The question arises whether the La protein contains amino acid sequences related to the A- and the B-motifs or to other conserved regions of DEAD/DEXH box proteins. Recently Topfer and co-workers (28) pointed out that the La protein contains the evolutionarily conserved (36) sequence motif A/GXXXXGKG (amino acids 333 to 340 in human La). This sequence represents the major part of the A-motif with only one difference, the final amino acid T of the A motif A/GXXXXGKT is replaced by a G. A similar deviation has been found in the ATP-binding site of some other ATP-binding proteins as for example adenylate kinase and the H(+)-ATPase of *E.coli* (35). Most recently, a monoclonal anti-La antibody (La4B6) directed to this putative ATP-binding site was generated. It could subsequently be shown that ATP abolished the binding of La4B6 to the La protein (18), indicating that this site indeed interacts with ATP. Although the La protein does not belong to the DEAD or DEXH families of RNA helicases, a sequence sharing some similarity to the B-motif can be discerned in the human La protein: DDEHDEHDENG, starting at position 367. Further experiments are required to investigate whether this element is required for the unwinding activity.

Finally, Clemens (37) indicated that the La protein shares some sequence homologies with the interferon induced dsRNA dependent protein kinase PKR (also known as DAI), primarily in regions reminiscent of a dsRNA binding motif (38). Interestingly, most of the amino acids suggested to be conserved in these PKR related motifs appear to be conserved in La from different species. Moreover, the B-motif related cluster of acidic amino acids flanks the PKR dsRNA binding motif-like element at the C-terminal side. Experiments are in progress to investigate whether (some of) these elements are involved in the unwinding activity.

### Possible function of the dsRNA unwinding activity

Members of the DEAD/DEXH protein families are known, or suggested, to be involved in diverse cellular functions including translation initiation, splicing, ribosome assembly, cell growth and division, embryogenesis and spermatogenesis (39). Also the La protein can be expected to be involved in a number of cellular processes. Its function in the nucleus as an RNA polymerase III transcription termination factor has been firmly established (11,12,40), but cytoplasmic functions of La cannot be ruled out. It has been suggested previously that La might shuttle between nucleus and cytoplasm in dependence on transcription (15,16) or infection with various viruses such as herpes-, adeno- and poliovirus (9,41). Indeed, Peek and co-workers provided further evidence for a partially cytoplasmic localization of the La protein using cell-enucleation as fractionation method (14), which is substantiated by the recent demonstration that a subset of La molecules is associated with small ribosomal subunits (23). In line with these findings, it has been suggested that the La protein could be involved in the internal initiation of poliovirus mRNA translation (9,42). In this respect an interesting observation of the present study is that a 3′-overhang in the RNA is required for unwinding. Secondary structures in 5′-UTRs of mRNAs and in internal ribosomal entry sites will always be accompanied by '3′-overhangs' and therefore it is tempting to speculate that the dsRNA unwinding activity of the La protein melts such secondary structures thereby stimulating initiation of translation. Internal initiation may not only occur on viral RNAs but could also be used by other cellular mRNAs, such as protooncogene mRNAs and mRNAs of growth factors or in translation of proteins involved in the regulation of mitotic events (9). It is conceivable that the dsRNA unwinding activity of La is involved in the internal initiation process.

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