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A *Drosophila* single-strand DNA/RNA-binding factor contains a high-mobility-group box and is enriched in the nucleolus

(*Drosophila* nucleolar factor/single-strand binding factor)

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ABSTRACT **We have isolated a *Drosophila melanogaster* cDNA encoding a high-mobility-group (HMG) box-containing protein. This protein shares 50% amino acid identity with the human putative structure-specific recognition protein, hSSRP. The gene encoding the *D. melanogaster* homolog, DssRP, is developmentally regulated and is expressed most abundantly in ovaries (nurse cells in particular). The protein is localized in nuclei and is particularly abundant in the nucleolus. *In vitro* binding studies using DssRP produced in bacteria showed that, despite expectation, the protein does not bind to structured DNA. Instead, it binds to single-stranded DNA and RNA, with highest affinity to nucleotides G and U.**

High mobility group (HMG) proteins were originally defined as a class of ubiquitous nonhistone chromatin proteins that meet certain empirical criteria, such as being extractable in 0.35 M NaCl but soluble in 2–5% perchloric acid (1) and having a high content of charged residues as well as small size (hence the term HMG). As the nucleotide and amino acid sequence information became available, three distinct subgroups showing little sequence similarity were identified; they are exemplified by chromatin proteins HMG-1/-2, HMG-14/-17, and HMG-I, respectively (2).

Recently, it was found that the DNA-binding domain of the HMG-1/-2 subgroup is shared by a number of other DNA-binding proteins (3–5). This domain, termed the HMG box, consists of 70–80 amino acids with a few highly conserved proline, basic, and aromatic residues. The HMG box-containing proteins may or may not be stringent in their DNA sequence requirements for binding; they are thought to recognize certain DNA structural features such as cruciforms, bent double helices, single-stranded regions, poly(A) tracts, etc. These seemingly diverse properties were determined in different laboratories under different assay conditions and with different DNA target preparations; thus, the underlying principle, if any, of the HMG box-mediated DNA binding is difficult to discern. With few exceptions, the biological functions of HMG-box proteins are even less well defined. Because of their apparent affinity for special DNA structural motifs, it is thought that their functions include DNA replication, DNA repair, and arrangements of chromatin structure that may in turn regulate transcription.

In a screen for cDNAs encoding *Drosophila* ovarian DNA-binding proteins, we isolated one that encodes a HMG box-containing factor. Sequence¶ comparisons show that this protein is the homolog of the recently described human structure-specific recognition protein (hSSRP; ref. 6) and the presumed mouse thymus recombination factor T160 (7). Here we report its developmental expression pattern, its cytological localization, and initial characterization of its nucleic acid binding activity. In keeping with the DNA- and RNA-binding activities, we term this gene product a *Drosophila melanogaster* single-strand recognition protein, DssRP.

MATERIALS AND METHODS

Isolation of DssRP cDNA Sequences. A phage λgt11-based *D. melanogaster* stage 10 ovarian expression library was screened as described (8) except for the following modifications. The washing steps after hybridization included one wash for 10 min at 4°C in 1× binding buffer (8) containing 0.25% bovine serum albumin (BSA) and 1 mM dithiothreitol, followed by two washes for 10 min at room temperature in 2× binding buffer (8) containing 0.25% BSA and 1 mM dithiothreitol. The probe used for isolating DssRP cDNA was a *D. melanogaster* chorion gene *s15* promoter fragment encompassing nucleotides −66 to −32 (ref. 9; also see below). Labeled probes at 1.5×10^6 cpm/ml were used in the hybridization solution.

Additional 5′ and 3′ sequences beyond the DssRP clone were obtained by polymerase chain reaction (PCR) amplification of an aliquot of the stage 10 library using internal primers (see Fig. 1) and λgt11 vector primers (New England Biolabs).

Expression of DssRP in a Bacterial Expression System. The original 2-kilobase (kb) DssRP cDNA fragment (Fig. 1) was subcloned in the pET3b expression vector as described (10). DssRP expression in *Escherichia coli* strain BL21(DE3)pLys was induced essentially as described (10) except that both ampicillin and chloramphenicol were used in growth media and isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 5 mM. The cells were collected by centrifugation, and the cell pellet was resuspended at a density of 1 g of wet cells per 5 ml of lysis buffer (20 mM Tris·HCl, pH 7.5/10 mM NaCl/1 mM $MgCl_2$/1 mM dithiothreitol/0.2% Triton X-100/2 mg of lysozyme per ml/1 mM phenylmethanesulfonyl fluoride). The cell suspension was subjected to three freeze/thaw cycles, bovine pancreas DNase I (Boehringer Mannheim) was added to a final concentration of 5 μg/ml, and the mixture was incubated at 4°C with gentle shaking for 30 min. The insoluble materials containing DssRP were collected by centrifugation at 10,000 × *g* for 30 min. The pellet was resuspended in half of the cell suspension volume in 50 mM Tris·HCl, pH 7.5/10 mM NaCl/0.2% Triton X-100/1 mM EDTA. Of this preparation, 1/300th was used in each lane in the experiment described in Fig. 5. Extracts that were similarly prepared but without IPTG induction served as negative controls.

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Abbreviations: hSSRP, human structure-specific recognition protein; DssRP, *Drosophila melanogaster* single-strand DNA/RNA recognition protein; HMG, high-mobility group; nt, nucleotide units; IPTG, isopropyl β-D-thiogalactoside.

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¶The sequence reported in this paper has been deposited in the GenBank database (accession no. L08825).

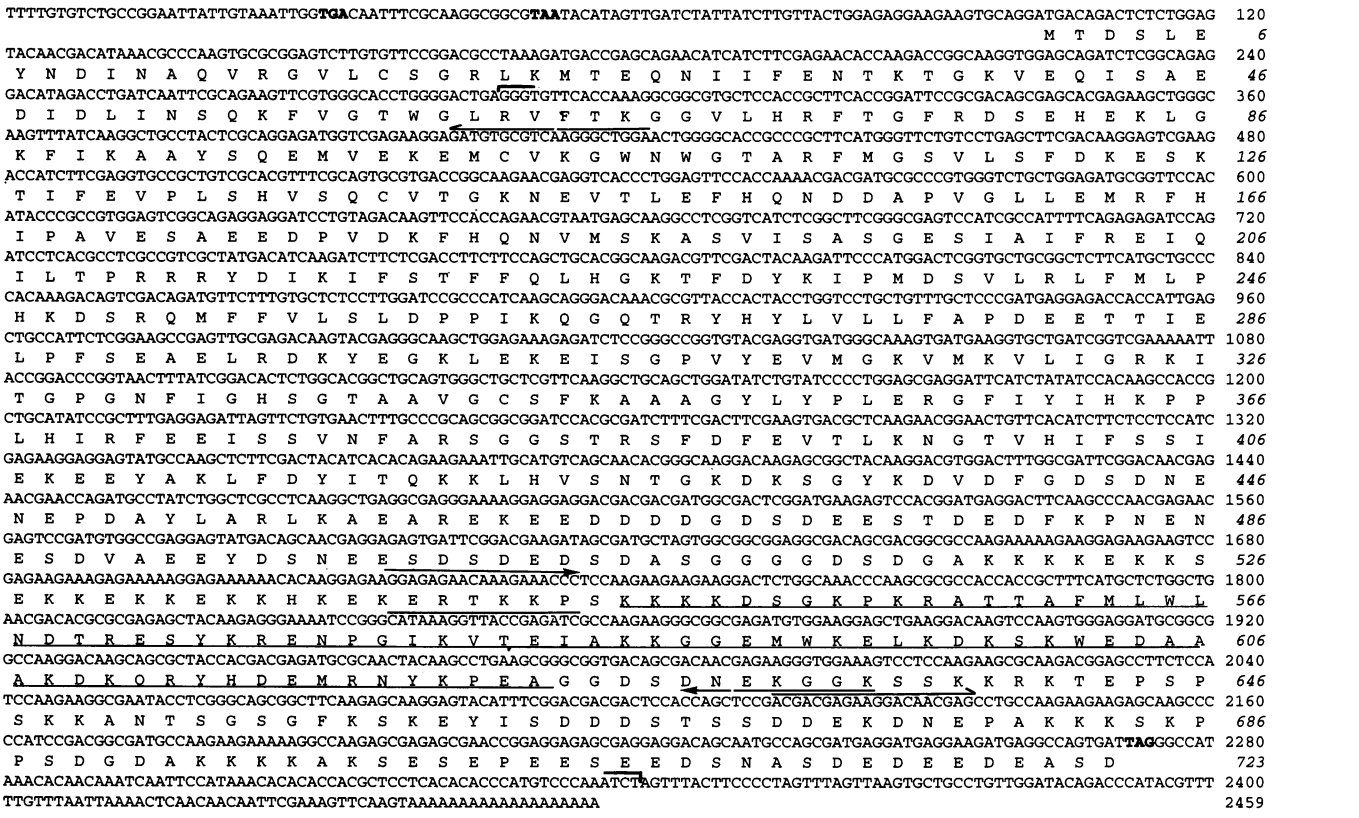

FIG. 1. Nucleotide sequence of DssRP cDNA and the derived amino acid sequence in single-letter code. The ends of the original cDNA clone are indicated with ⌐ and ¬ overbars. The underlined amino acid sequence is the HMG box. Horizontal arrows and lines above the nucleotide sequence indicate oligonucleotide primers used in PCR amplification of additional terminal cDNA sequences (→), in reverse transcription/PCR (→), and in subsequent hybridization for detecting the amplified fragment (—). The in-frame termination codons are in bold letters.

Reverse Transcription/PCR. Total RNAs were prepared from various developmental stages as described (11), and poly(A)-containing RNAs were purified by using the PolyATtract mRNA isolation system (Promega) as described by the supplier. Coupled reverse transcription and PCR with 0.1 μg of the poly(A)$^+$ RNA and DssRP-specific primers (Fig. 1) were carried out as described (12), followed by Southern blotting. The ribosomal protein 49 gene (*rp49*)-specific primers were included in the same reaction to provide an internal control.

Whole-Mount *in Situ* Hybridization. Ovaries were dissected from Canton-S wild-type *Drosophila*. Whole-mount *in situ* hybridization was performed as described (13) with certain modifications. Treatment with proteinase K at 50 μg/ml for 10 min at room temperature was followed by additional fixation with 4% paraformaldehyde. A detection probe was prepared by using the digoxigenin DNA-labeling kit from Boehringer Mannheim according to the supplier's instructions.

Immunostaining of Embryos. The DssRP-specific antiserum was obtained from female Sprague–Dawley rats injected with crushed gel slices containing bacteria-produced DssRP. The staining and laser scanning confocal microscopy were as described (14).

Oligonucleotides Used for Nucleic Acid–Protein Hybridization. A single-stranded 40-mer probe (see Fig. 5*E*) was synthesized, including 31 nucleotides (nt) of the *s15* chorion promoter (−64 to −34) and an extraneous 3′ tail. The double-stranded counterpart was synthesized by extending a primer complementary to the tail. The primer was first 5′-end-labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP and annealed to the template (3:1 molar ratio) before primer extension with the Klenow enzyme. The double-stranded, full-length species was then purified by gel electrophoresis and electroelution. Immediately prior to use in hybridization, this probe was digested with 10 units of mung bean nuclease (New England Biolabs) at 30°C for 30 min in 20 μl of 50 mM sodium acetate, pH 5/30 mM NaCl/1 mM $ZnCl_2$. Another set of single- and double-stranded probes consisting of a 20-nucleotide degenerate sequence flanked by 2 primers of unique sequence (14, 15) were prepared similarly. The nucleotide sequences of the other *s15*-derived single-stranded oligonucleotide probes are described in Fig. 5*C*. The 5′-end-labeled single-stranded probes used in hybridization were purified by passage through Sephadex G-25 spin columns (Boehringer Mannheim) and precipitation with ethanol.

RNA Homopolymers Used for Nucleic Acid–Protein Hybridization. RNA homopolymers (300–1000 nt long; not shown) were purchased from Sigma, dephosphorylated with alkaline phosphatase (Boehringer Mannheim), and 5′-end-labeled with T4 DNA kinase and [γ-^{32}P]ATP. The labeled mixes were passed through Sephadex G-50 columns and heat-denatured just before hybridization.

Nucleic Acid–Protein Binding Assays. The protein suspensions described above were solubilized and resolved by SDS/gel electrophoresis. Fractionated proteins were blotted onto nitrocellulose membrane and monitored by staining with Ponceau S (Sigma) to ensure that all lanes used in subsequent hybridization contained similar levels of protein. The nucleic acid–protein hybridization was performed as for library screening (see above).

RESULTS

Isolation and Primary Structure of DssRP cDNA. A cDNA clone was isolated from a *D. melanogaster* stage 10 ovarian expression library based on binding of the encoded protein to a chorion gene *s15* promoter fragment (*Materials and Meth-*

ods; see also below). Subsequently, we completed the sequence by using internal primers to amplify from the same library additional 5′ and 3′ cDNA sequences by PCR.

The combined cDNA sequences contain a complete open reading frame of 723 amino acids and a putative poly(A) tail (Fig. 1). Their length [2440 nt excluding the poly(A) tail] is in agreement with a single 2.6-kb poly(A)-containing transcript detected in Northern (RNA blot) analyses (not shown). Computer analysis using the BLAST program indicated that the 81-kDa conceptual protein encoded by this clone is the *D. melanogaster* homolog of the hSSRP (ref. 6) and the presumed mouse recombination factor T160 (7). The overall amino acid identity between the fly and human homologs is nearly 50% (Fig. 2). The protein is highly charged in the C-terminal half (residues 407–723), which includes 30% acidic and 23% basic residues. The HMG box resides in this region, and the *Drosophila* protein also contains an "acidic tail" at the extreme C terminus (where 15 of 24 residues are either glutamate or aspartate). Acidic tails are found in other HMG box proteins such as vertebrate HMG-1 and transcription factor UBF (3) and are thought to mediate protein–protein interactions (17, 18). The acidic tail is much less prominent in hSSRP (Fig. 2*B*). In fact, the most conserved region of the *Drosophila* SSRP relative to hSSRP is in the N-terminal half (residues 1–406, showing 60% identity; Fig. 2*A*). A few stretches of over 10 amino acids are completely conserved (Fig. 2*B*).

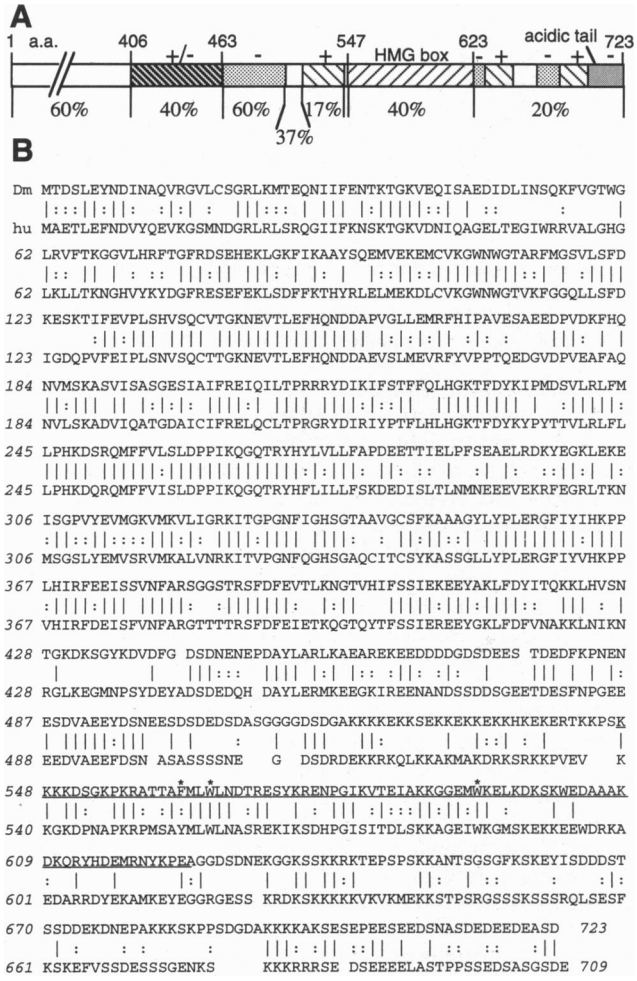

FIG. 2. Charged domains of DssRP (*A*) and sequence comparison with human SSRP (*B*). (*A*) Charged domains in the C-terminal half of DssRP. Note the HMG box, the acidic tail, and the differently shaded boxes indicating regions rich in basic residues (+), acidic residues (−), or both (+/−). The percent amino acid identities with the human homolog (hSSRP) are indicated in designated regions. (*B*) Amino acid sequence comparison between the *Drosophila* (Dm) and human (hu) homologs. Underlined residues indicate the HMG box, and asterisks mark the conserved aromatic residues. Vertical bars identify identical residues, and colons represent residues with similar characteristics. Spaces indicate gaps introduced for alignment as described by Altschul *et al.* (16).

Expression of DssRP RNA. In the Northern analyses, we detected a single DssRP mRNA species of 2.6 kb only in ovarian and embryonic samples (not shown). Reverse transcription/PCR provided a more sensitive assay for the presence of DssRP in other developmental stages (Fig. 3). We caution that this method, although sensitive to low-level expression, tends to decrease the actual quantitative differences between samples. It appears that in adults, DssRP transcripts are mainly found in ovaries; there are only low levels of expression in female carcasses devoid of ovaries and in males. The transcripts are abundant throughout oogenesis, although less so in the prechoriogenic period (stages 1–9). Their level remains high throughout embryogenesis but drops during larval stages. It rises again in pupae, perhaps reflecting early ovary development. *In situ* RNA hybridization on whole-mounts of follicles (egg chambers) shows that the transcripts are found mainly in the nurse cells (Fig. 4), which provide most of the proteins and RNAs needed for oogenesis (19). Nurse cell expression intensifies at stage 10 and persists through at least stage 12 (presence in the oocyte cannot be assayed by this procedure). The intracellular location of DssRP was elucidated by immunocytochemistry and confocal microscopy by using polyclonal anti-DssRP serum. In Western blots of embryonic extract, this antiserum stains a cluster of three polypeptides, the largest and most abundant of which is of the expected size (data not shown). The immunostainable antigen is largely nuclear, and is intensely concentrated in the nucleolus, both in embryonic cells and in the polytene salivary gland nuclei (Fig. 4).

DNA-Binding Activity. The bacteria-produced partial DssRP (Fig. 5*A*) aggregates readily in solution, and soluble preparations are only possible in high salt (e.g., 0.4 M NaCl) or at very low concentrations (e.g., <20 μg of total bacterial proteins per ml), conditions unfavorable for binding assays. Nevertheless, when the insoluble protein fraction is denatured, electrophoretically separated, and immobilized on nitrocellulose membrane, DssRP still exhibits satisfactory DNA-binding activity (see below).

Experiments with purified probes showed that the protein only binds to single-stranded material (Fig. 5*B*). When the double-stranded probe was gel purified after primer extension and digested with mung bean nuclease to remove residual single-stranded DNA without affecting the integrity of the

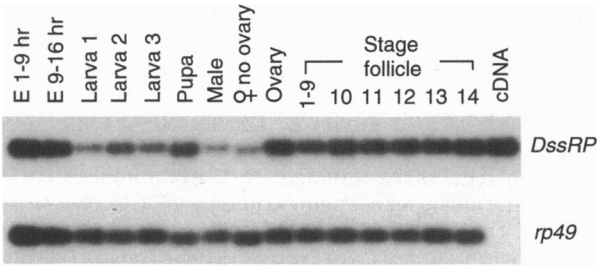

FIG. 3. Developmental expression of DssRP RNA. Transcript encoding the HMG box (408 bp) and fragments of the *rp49* open reading frame (259 bp) were amplified, fractionated, and Southern blotted. The DssRP oligonucleotide probe shown in Fig. 1 and the *rp49* amplification primers (12) were used to detect DssRP and *rp49* sequences, respectively. The sources of poly(A)-containing RNA are indicated above the lanes. E, embryo; Larva 1, 2, and 3, 1st, 2nd, and 3rd instar larva, respectively. Follicle (egg chamber) stages are defined as described by King (19).

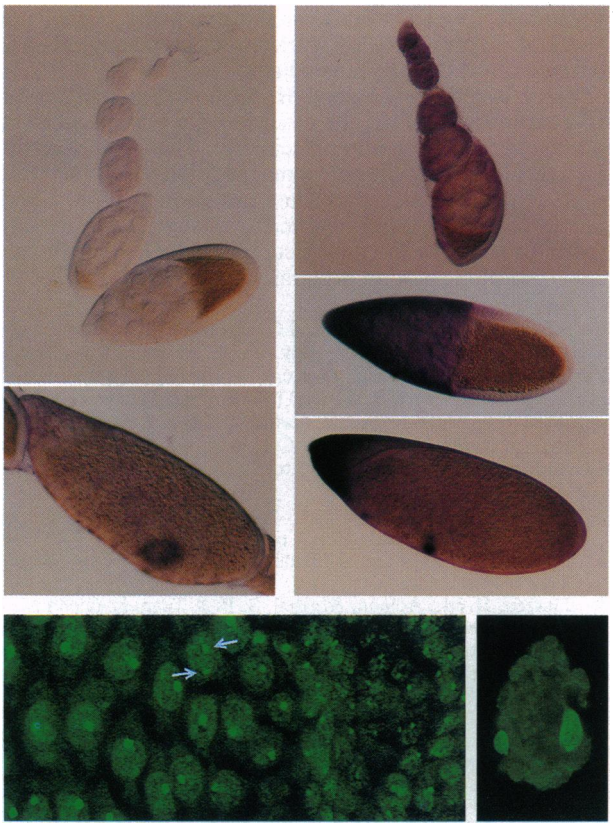

FIG. 4. Expression of DssRP RNA in ovaries and nuclear localization of DssRP protein. (*Upper Left*) Egg chambers (from top to bottom, early to stage 10a and stage 12) that are hybridized with digoxigenin-labeled pBR328 plasmid DNA as the negative control. (*Upper Right*) Egg chambers (early to stage 9, stage 10b, and stage 12) hybridized with the similarly labeled 2-kb DssRP cDNA fragment (Fig. 1). (*Lower Left*) Stage 12 embryonic nuclei from both amnioserosal and epidermal cells (large and small nuclei, respectively) stained with DssRP-specific antiserum. Note the intense staining of the nucleoli (two of which are seen in some cells; arrows) and less intense staining throughout the nucleus. (*Lower Right*) Staining of the polytene chromosomes and especially the nucleoli of a lightly squashed salivary gland cell.

double-stranded material (results not shown), DssRP could no longer bind (lanes 2 and 6); binding to single-stranded DNA was unaffected by gel purification and ethanol precipitation (lanes 3 and 7). The single-stranded DNAs with which binding was documented included a 40-nt fragment encompassing part of the *s15* chorion promoter (lane 7) and a 20-mer degenerate oligonucleotide (lane 3) flanked by PCR primers of defined sequence that do not bind strongly to DssRP (data not shown).

The *s15* promoter probe used in the binding assays contains short and imperfect palindromic sequences (Fig. 5*E*). To determine whether the affinity of DssRP toward this probe is mediated through the formation of a hairpin loop structure or through the single-stranded region, four single-stranded 20-mers were synthesized and tested (Fig. 5*C*): (probe a) a wild-type promoter sequence that includes a palindrome capable of forming an imperfect hairpin loop with a bulge in the stem, (probe b) a 1-nt substitution resulting in a perfect palindrome and a stem–loop with no bulge, (probe c) a 5-nt substitution resulting in a perfectly self-annealing hairpin structure, and (probe d) a 2-nt substitution resulting in a linear probe incapable of forming hairpin structures. The electrophoretic mobilities of these probes on a nondenaturing polyacrylamide gel varied, consistent with their predicted conformation. Surprisingly, only the linear probe d proved

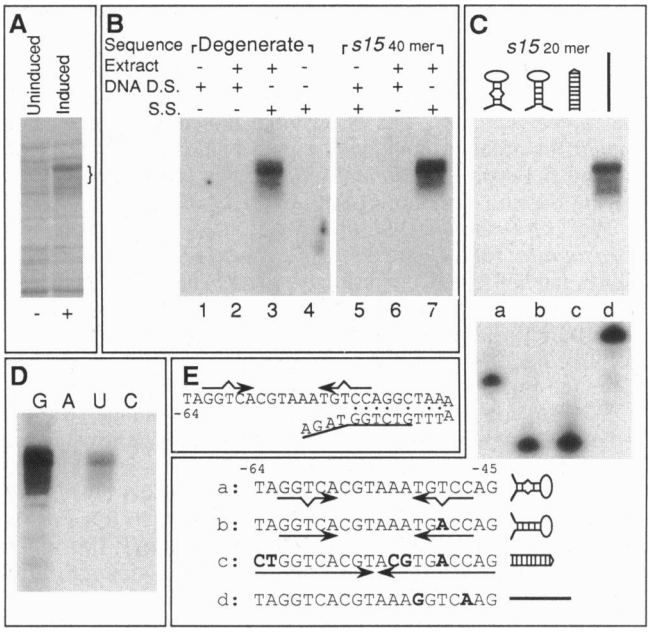

FIG. 5. DNA and RNA binding by DssRP. (*A*) Coomassie blue staining of proteins from IPTG-induced (+) and uninduced (−) *E. coli* cultures carrying DssRP cDNA in the expression plasmid vector. DssRP and partial degradation products are marked with a brace. (*B*) Single-stranded DNA binding. D.S., double stranded; S.S., single stranded. Oligonucleotide probes used are indicated on the top and are described in *Materials and Methods* and in *E*. (*C*) Binding to single-stranded DNA but not to stem or hairpin loop structures. Probes a, b, c, and d (at about 3 pM) were boiled for 5 min and incubated sequentially at 65°C, 37°C, and 25°C for 30 min each before being used in site-specific DNA-protein hybridization (*C Top*). An aliquot of the self-annealed probes was fractionated through a 10% acrylamide gel containing 1× TBE buffer (0.09 M Tris borate/pH 8.3, 2 mM EDTA) and 10% (vol/vol) glycerol (*C Middle*), confirming differences in probe structure. Nucleotide sequences of the four probes (*C Bottom*) are shown with opposing arrows marking palindromic sequences and bold letters indicating nucleotide substitutions; −64 and −45 mark the corresponding nucleotide positions in the *s15* promoter. (*D*) Binding to RNA homopolymers. The lane headings indicate the probes used, which are 5′-end-labeled poly(G), poly(A), poly(U), and poly(C), respectively. (*E*) Nucleotide sequence and possible secondary structure of the *s15* 40-mer probe used in *B*. The extraneous sequence complementary to the primer used for synthesizing the double-stranded counterpart is underlined. Note the long single-stranded 5′ tail, which explains the binding seen in *B*, lane 7.

capable of binding to DssRP. The common single-stranded region between the *s15* 40-mer and probe d is −64 to −52, which is sequestered by stem–loop structures in probes a, b, and c but is released in the 40-mer because of an alternative, more extensive palindrome involving the extraneous primer (Fig. 5*E*). This common region may constitute a DssRP binding domain.

The binding specificity of DssRP toward single-stranded DNA raises the possibility of its ability to bind RNA as well. Although no RNA targets have been reported previously for HMG box proteins, we tested if DssRP has affinity toward RNA homopolymers. Indeed, as shown in Fig. 5*D*, DssRP has strong affinity for poly(G) and also binds to poly(U) but has almost no affinity for poly(A) or poly(C).

DISCUSSION

We have isolated a cDNA from *D. melanogaster*, encoding a protein that shares nearly 50% identity with hSSRP and the presumed mouse recombination factor T160. The protein is highly charged, with blocks of acidic and basic residues,

notably in a basic region just N-terminal to an HMG box and in an acidic tail at the C terminus (Figs. 1 and 2*A*). The basic region probably interacts with the nucleic acid backbone, and the acidic tail with other chromatin proteins and/or enzymatic machinery (17, 18). The N-terminal half is not significantly similar to other proteins in the data bank but is highly conserved between *Drosophila* and humans (60% identity), implying the conservation of important functions.

We have shown that under our assay conditions, this *Drosophila* factor specifically recognizes single-stranded DNA and RNA, and accordingly we have named it DssR. The observed binding is (*i*) specific to DssRP, since it is not seen with a control bacterial extract, and (*ii*) independent of bacterial cofactors of different molecular weight, since it is detected by site-specific DNA–protein hybridization.

We do not know the degree to which single-stranded binding is sequence-specific. The protein does show binding to a degenerate oligonucleotide probe, and thus its specificity can be explored by PCR selection experiments (14). Interestingly, it shows nucleotide-specific affinity to RNAs (Fig. 5*D*). The strong affinity to poly(G) and poly(U) but not to poly(A) and poly(C) indicates that DssRP may recognize the oxyl groups at C-6 of guanine and C-4 of uracil.

The DssRP does not bind to double-stranded DNA or secondary structures such as stem–loops (Figs. 5 *B* and *C*). Preferential binding to single-stranded DNA by HMG-1 has been reported previously (20). In fact, a combination of basic and aromatic amino acid residues similar to the motif found in the HMG box has been shown to be important for single-stranded DNA binding (21). These properties may be pertinent to the as yet not fully explored DNA-binding properties of hSSRP and mouse T160. hSSRP was isolated as a cellular factor binding to DNA modified by the anticancer drug cisplatin, which crosslinks adjacent guanosine-guanosine and adenosine-guanosine pairs (22). Such intrastrand purine crosslinks can lead to melting of the double helix (23), and thus hSSRP may in fact recognize the single-stranded region generated around cisplatin-purine adducts. Mouse T160 was isolated as a protein factor that binds to the recombination signal sequence (RSS) in the *V–(D)–J* joining region of the immunoglobulin heavy chain genes. One notable feature of the RSS is a poly(A) motif that may separate from its complementary poly(T) and form stacking interactions on itself, thus generating a local single-stranded region (24).

What are the functions of this type of protein? The mouse T160 is thought to be part of the recombination machinery in cells of the immune system but is ubiquitous in all cell types examined (7). Human SSRP is reported to modulate the cytotoxicity of DNA damaged by anticancer drugs, but to be most abundant in cytosol (25). Our observations indicate that DssRP is almost exclusively nuclear and is strongly concentrated in the nucleolus. The nucleolar localization is worth noting because of the strong and nucleotide-specific affinity of DssRP to RNA. Interestingly, a motif reminiscent of certain RNA binding proteins (26), as exemplified below, is encountered in the best conserved part of the DssRP HMG box. The aromatic residues of this motif are thought to bind to RNA by stacking with the bases.

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                                        *  *  *
human U1snRNP-70 kDa   140 SGKP RG YAFIEY
                           |||| |: :||: :
DssRP                  551 SGKPKRATTAFMLW
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It is also worth noting that another HMG box protein, UBF, has been shown to be a transcription factor of RNA polymerase I (3, 18). Therefore, it is equally possible that DssRP is involved in rRNA transcription through a single-stranded DNA binding mechanism.

DssRP is only the second HMG box-containing protein reported in *Drosophila*, the other being a HMG-1-like small and ubiquitous chromatin protein (27). The gene maps by *in situ* hybridization to chromosomal position 60A1-4 on the 2R polytene chromosome arm (data not shown). The superior genetic tools of *Drosophila* make this gene an attractive model for studying the biological functions and biochemical properties of HMG box proteins in general.

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