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Developmental Characterization of a *Drosophila* RNA-Binding Protein Homologous to the Human Systemic Lupus Erythematosus-Associated La/SS-B Autoantigen

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Patients with humoral autoimmune diseases such as systemic lupus erythematosus and Sjögren's syndrome contain antibodies in their sera directed against certain normal cellular components such as the La/SS-B autoantigen, an RNA-binding protein believed to function as a putative processor of RNA polymerase III precursor transcripts. We have identified cDNA clones from the fruit fly *Drosophila melanogaster* that encode a protein displaying significant sequence homology with human La/SS-B. The fly protein (which we refer to as D-La) contains a putative ribonucleoprotein 1 (RNP1) and RNP2 RNA-binding domain. D-La also possesses a leucine zipper motif, suggesting that it may interact with itself or other proteins. Using gel retardation analysis, we show that D-La can bind RNA; in addition, we demonstrate the first reported DNA-binding activity associated with a La protein. Northern (RNA) blot analysis revealed a single 1,600-nucleotide transcript expressed throughout embryonic, larval, pupal, and adult development. Surprisingly, whole-mount in situ hybridization experiments revealed that D-La transcripts are not present in all ovarian tissues. In addition, early expression throughout the embryo is followed by a restricted pattern of mesodermal expression that is later confined to the visceral mesoderm, gonads, gut, and salivary glands. These results suggest that D-La may play a more specialized role during fly development as opposed to a rather general role inferred by its homology to La proteins from other organisms.

Human rheumatological autoimmune diseases such as systemic lupus erythematosus and Sjögren's syndrome are associated with abnormal humoral or antibody-mediated immune responses directed against a select group of autoantigens (reviewed in reference 20). It is not known why these proteins are recognized as foreign antigens, but one of the major targets of this abnormal immune response in patients with systemic lupus erythematosus is La/SS-B. La is a nuclear protein normally associated with the 3′ end of small nuclear RNA (snRNA) precursors transcribed by RNA polymerase III (Pol III). Examples include precursors of tRNA, 4.5S RNA, 5S RNA, 7S RNA, and U6 RNA (22, 23), in which cases La specifically binds to a short tract of uridylate residues (17, 21, 28). La does not specifically bind to processed snRNAs. This binding forms a ribonucleoprotein (RNP) complex which is thought to function in the termination, processing, and/or nuclear export of RNA Pol III precursor transcripts. La can also bind to some small viral RNAs (14) as well as U1 (15), which is an RNA polymerase II transcript. It has been suggested that La-RNP complexes, rather than La alone, are most likely the immunogens in vivo.

Basic research on La has focused exclusively on molecular and biochemical analysis of the human and homologous molecules from several animal models. Studying La in the context of an entire organism could provide insight into its basic in vivo function, which may help us understand how this molecule becomes immunogenic. Unfortunately, this has not been possible because the organisms in which La has been identified to date are not appropriate for detailed developmental genetic analysis for technical, practical, and ethical reasons. However, such studies are possible in *Drosophila melanogaster*, an organism amenable to sophisticated developmental genetic analysis, and here we describe the initial phase of such work with identification and developmental characterization of a La homolog in fruit flies.

A *Drosophila* homolog of the human La antigen was isolated in cDNA expression cloning screens designed to identify cDNAs that encode proteins that bind either single-stranded DNA or RNA (29). Here we report two major differences between *Drosophila* and La proteins from other species. In addition to the RNA-binding activity conferred by the conserved RNP1 and RNP2 consensus sequences, the *Drosophila* protein (D-La) can also bind DNA, presumably from a motif absent in La molecules from other organisms consisting of a leucine zipper dimerization domain followed by a basic region. Another important distinguishing feature of D-La is that it is not transcribed in all tissues, suggesting that it may play a more specialized role during fly development as opposed to a more general role inferred by its homology to La homologs. Elucidation of the role played by D-La during *Drosophila* development can provide new information about the basic in vivo function of this family of molecules and may accelerate our understanding of how they cause disease in humans.

MATERIALS AND METHODS

Library screening, sequence analysis, and alignments. D-La was identified in expression cloning screens of ovarian cDNA libraries designed to identify plaques that encode proteins capable of binding single-stranded DNA (29). Nine clones representing five unique and novel *Drosophila* genes were identified (29, 30); one of these genes (represented by three identical cDNAs) encodes a protein displaying significant

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FIG. 1. cDNA and deduced protein sequences of D-La. The RNP1 and RNP2 consensus sequences of the RNA-binding motif are indicated and underlined. The conserved leucines are boxed within the underlined leucine zipper motif. Possible N-linked glycosylation sites (N-glyc) are enclosed by parentheses. Consensus bipartite nuclear localization sequences (BNLS) are bracketed. The cDNA sequence contains a polyadenylation consensus sequence (underlined) and is flanked by a poly(dA) tail (not shown).

sequence homology to human La/SS-B and is the focus of this report. Double-stranded DNA was prepared from these three plaque-purified cDNAs, digested with *Eco*RI and *Not*I, and subcloned into pBluescript KS+ (Stratagene). The ends of all three clones were sequenced and found to be identical. Sequencing was performed with a Sequenase kit (United States Biochemical), and nested deletions of clone c7a were generated by using exonuclease III and mung bean nuclease. Computer analysis of the DNA and predicted amino acid sequence was conducted with Mac Vector software (IBI-Kodak). Alignments were performed by using sequence data provided by GenBank, retrieved with the program BLAST.

Protein purification and gel retardation assays. The cDNA insert was also subcloned as an *Eco*RI fragment into the pGEX-1 expression vector, and transformation, growth, induction, and affinity purification were performed as described by Smith and Johnson (27) except that a French press instead of sonication was used to break the cells. This glutathione *S*-transferase (GST)–D-La fusion protein (which lacks the 36 C-terminal amino acids of D-La) was used to perform gel retardation analysis (9) with our standard modifications [as described in reference 29, except that we used 240 ng of poly(dI-dC) · poly(dI-dC) per reaction], using both ^{32}P-labeled RNA and ^{32}P-labeled DNA probes. Labeled RNA transcripts of 63 and 65 nucleotides were produced from *Eco*RI-digested pBluescript KS+ (Stratagene) by using [α-^{32}P]GTP plus T3 and T7 RNA polymerases, respectively, and purified on denaturing gels before use in RNA gel shifts. Oligonucleotide A (5′-CTAGACAAAGATGCGGCGCAAAATCGGAAATGG AGGTAC) was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and used as a single-stranded DNA probe in gel retardation experiments, whereas an unlabeled complementary oligonucleotide (oligonucleotide B [5′-CTCCATTTCC GATTTTGCGCCGCATCTTTGT]) was annealed to oligonucleotide A and used as a double-stranded DNA probe.

Northern (RNA) blotting. RNA from wild-type strain Oregon-R was prepared and electrophoresed on 1.4% agarose gels as described by Mariani et al. (16), with the modifications described by Tolias and Kafatos (33). Approximately 10-μg samples of total RNA from embryos, larvae, pupae, adult males, adult females, and ovaries were loaded in the respective lanes. cDNA probes were labeled with α-^{32}P-labeled deoxynucleoside triphosphates by using random hexanucleotide primers and Klenow DNA polymerase I. The blot was probed with a D-La cDNA and then reprobed with a labeled cDNA representing the *rp49* gene as a loading control.

RNA whole-mount in situ hybridization. Ovaries and embryos were obtained from strain Oregon-R. The specific probe used in these experiments was a 1.1-kb *Eco*RI fragment from cDNA clone c7a, which was gel purified and labeled with

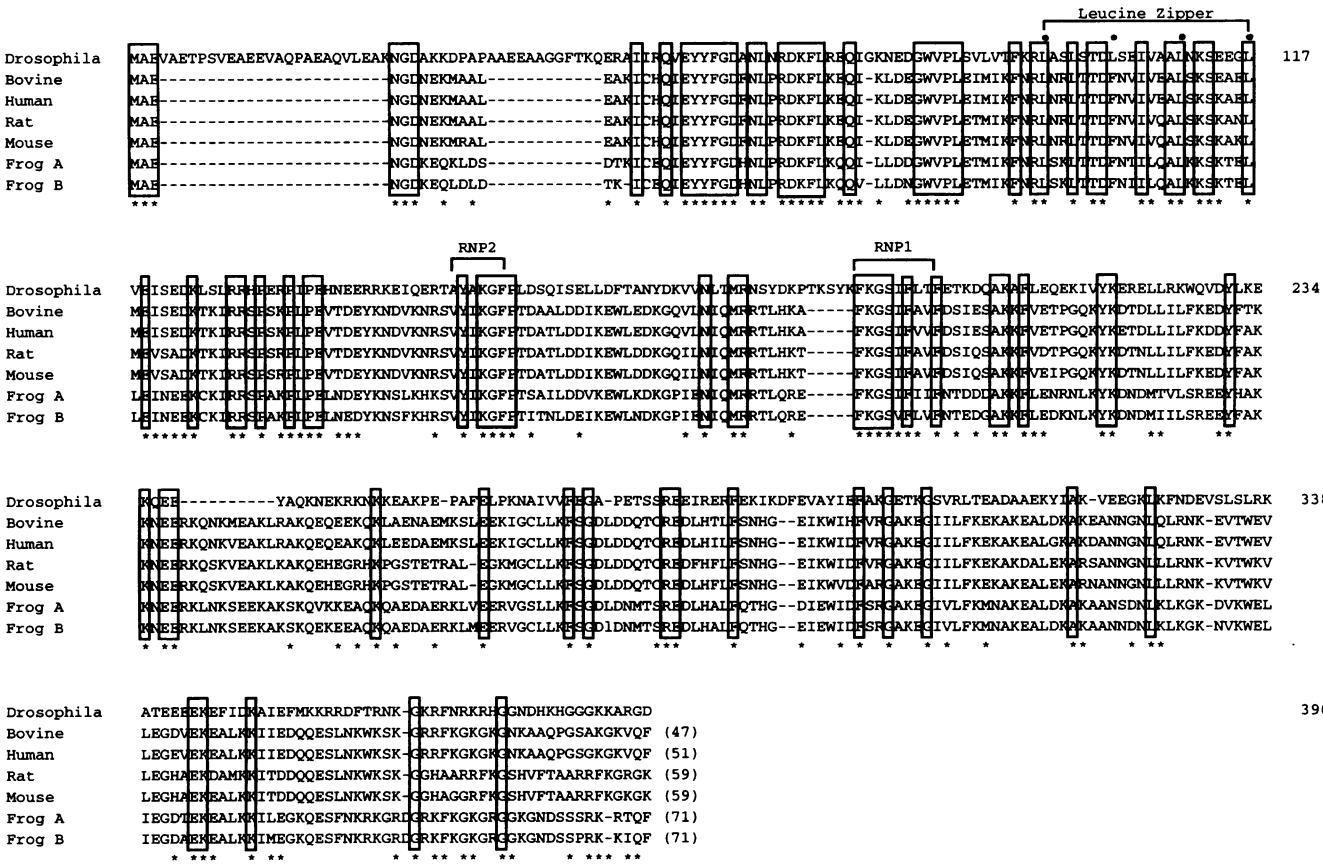

FIG. 2. Alignment of D-La to known homologs from other organisms. Alignments were performed by using sequence data provided by GenBank (sequences were retrieved with the program BLAST). Residues identical in all seven proteins are boxed. Asterisks denote D-La residues that are identical in at least one other molecule. Dashes indicate gaps in protein sequences introduced to achieve optimum alignment. Numbers in parentheses at the end of the protein sequence refer to the number of amino acids not shown at the C terminus. D-La RNP1 and RNP2 consensus sequences are indicated, and the conserved leucines of the leucine zipper are marked with dots.

digoxigenin by using a Genius kit (Boehringer Mannheim). Whole-mount in situ hybridization of ovaries was performed as originally described for embryos (32), with the modifications for ovaries proposed by Suter and Steward (31). Embryonic expression was revealed by using the standard whole-mount in situ hybridization technique (32). Salivary gland expression (shown in Fig. 5i) was revealed by using a digoxigenin-labeled antisense RNA probe from cDNA clone c7a (cloned in pBluescript KS+ [Stratagene]), using a Genius RNA labeling kit (Boehringer Mannheim).

In situ hybridization to polytene chromosomes. Oregon-R larval salivary gland polytene chromosomes were squashed and hybridized (24) by using a D-La cDNA probe that was labeled with biotin.

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been assigned GenBank accession number U07652.

RESULTS

We have conducted molecular screens designed to identify cDNAs that encode polypeptides that bind to single-stranded DNA probes. From these screens, we have isolated clones that encode single-stranded DNA- and RNA-binding proteins expressed during *Drosophila* oogenesis (29, 30). Three of the cDNAs identified in these screens represent the same gene (Fig. 1) and encode a protein displaying significant sequence homology with the human La autoantigen (5) as well as La equivalents from other organisms, including bovine (6), rat (26), mouse (34), and two frog molecules (25), as shown in Fig. 2.

The sequence of a 1,471-bp D-La cDNA is displayed along with the sequence of its deduced 390-amino-acid protein in Fig. 1. Computer analysis of this sequence predicts a helical amphipathic protein with a theoretical molecular mass of 44.9 kDa and an estimated pI of 6.7. The deduced protein lacks cysteine but has high glutamic acid (14.6%) and lysine (12.6%) contents. The cDNA sequence most probably encodes all of the D-La protein. An excellent consensus sequence (13 of 15 nucleotides) for translation initiation in *Drosophila* genes (4) encompasses the first AUG start codon. This open reading frame extends until a UAA stop codon is encountered, which is preferred by *Drosophila* genes more than 50% of the time. The consensus sequence (AATAAA) for polyadenylation begins 25 bp before the 3′ end of the cDNA.

D-La contains putative RNP1 and RNP2 consensus sequences which are also present and confer the ability to bind RNA in homologous molecules from other species (6). In addition, D-La contains a leucine zipper dimerization motif (12) located between amino acids 96 and 117 which may allow it to form homo- or heterodimers. This motif is preceded by and partially contained within a hydrophobic segment (amino

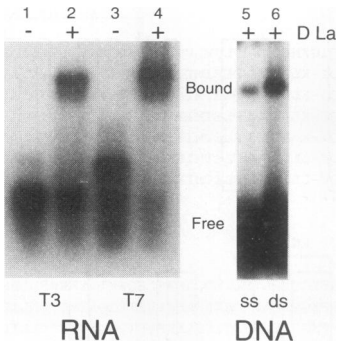

FIG. 3. Gel retardation analysis of D-La RNA- and DNA-binding activity. Lanes 1 to 4 represent reactions performed with ^{32}P-labeled RNAs as substrates which consist of a 63-nucleotide T3 transcript (lanes 1 and 2) and a 65-nucleotide T7 transcript (lanes 3 and 4). The D-La fusion protein was not included in the reactions in lanes 1 and 3 (−), whereas 100 ng of this protein was included in all other reactions (+). The positions of free and bound labeled probes are indicated. Lanes 5 and 6 display identical reactions performed with ^{32}P-labeled single-stranded (ss) and double-stranded (ds) DNAs, respectively, as substrates. See Materials and Methods for compositions of RNA and DNA substrates.

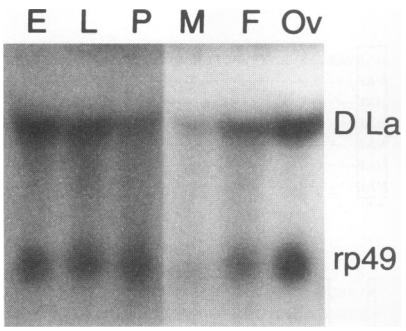

FIG. 4. Developmental Northern blot analysis of the D-La transcripts probed with a labeled cDNA. Total RNAs from each of the following stages of *D. melanogaster* development were assayed: embryos (E), larvae (L), pupae (P), male flies (M), female flies (F), and ovaries (Ov). The blot was reprobed with a cDNA representing the *rp49* gene of *D. melanogaster* to control for loading.

acids 85 to 110) and followed by a region of 31 residues (between amino acids 119 and 149) composed of 10 basic and 9 acidic amino acids. Finally, as many as three possible bipartite nuclear localization consensus signals (7) can be identified between amino acids 128 and 144, 357 and 373, and 372 and 388.

An alignment between D-La and homologous proteins from other organisms is presented in Fig. 2. These proteins are aligned in order (from top to bottom) of greatest homology to D-La. Identical residues among the seven molecules are boxed; 87 of the 390 amino acids of D-La are identical in all seven proteins. Asterisks denote 148 D-La residues that are identical in at least one other molecule. The greatest conservation is centered within the D-La leucine zipper motif and its flanking sequences as well as the RNP1 and RNP2 consensus sites. D-La appears to be slightly longer at the N terminus and shorter at the C terminus. The human and fly proteins are 33% identical. This homology increases to 45% when conservative amino acid substitutions are included. With respect to evolution of human La, identities range from 94% in bovine to 62% in frog and 33% in fly proteins.

Gel retardation analysis of the D-La nucleic acid-binding properties by using an affinity-purified GST–D-La fusion protein has allowed us to confirm its ability to bind ^{32}P-labeled RNA in vitro (Fig. 3). Two unrelated and randomly chosen transcripts, approximately 65 nucleotides in length, were bound nonspecifically by our D-La fusion protein (lanes 2 and 4). In addition, we observed that this protein could nonspecifically bind both single-stranded (lanes 5) and double-stranded (lane 6) DNAs. Similar results were obtained with other unrelated single and double-stranded DNA probes (data not shown).

We then examined the expression of D-La mRNA throughout fly development by Northern blot analysis using total RNA obtained from *D. melanogaster* embryos, larvae, pupae, adult males, adult females, and adult female ovaries. Expression of a single 1,600-nucleotide D-La transcript was observed in all stages of development (Fig. 4).

D-La mRNA expression was visualized by whole-mount in situ hybridization using a digoxigenin-labeled cDNA probe. Figures 5a to c display the pattern of D-La mRNA expression in the adult ovary, where egg chambers differentiate through 14 distinct morphological developmental stages (13). Though nurse cells abundantly express D-La mRNA throughout oogenesis (Fig. 5a and b), the somatically derived follicular epithelial cells do not express D-La transcripts after stage 9 (Fig. 5b). Likewise, the oocyte of stage 10–14 egg chambers accumulated barely detectable levels of D-La mRNA (Fig. 5b and c). Finally, D-La transcripts are not expressed in the remaining somatic cells that make up the ovary. Expression was restricted to a subset of cells that make up the peritoneal sheath near the posterior end of the ovary (Fig. 5c).

The details of D-La mRNA expressing during embryogenesis were also visualized by whole-mount in situ hybridization. *Drosophila* embryos have been described to develop through 17 distinct morphological stages (3). Early zygotic expression was observed throughout the entire embryo (data not shown). However, restricted mesodermal expression was observed as early as stage 7 and became quite evident by stage 10 (Fig. 5d and e). Later, expression was further confined to the visceral mesoderm and gonads (Fig. 5f and h). Gonadal expression was no longer observed in late stage 16 embryos, but staining was still observed in the gut (Fig. 5h). The salivary glands were the only structures that abundantly expressed D-La transcripts in late stage 17 embryos (Fig. 5i).

We have mapped the location of the gene that encodes D-La by in situ hybridization of larval salivary gland polytene chromosomes to cytological position 38A. Genetic mapping studies by others have already identified five lethal complementation groups between 37F5 and 38A1 and two between 38A6 and 38C1. Hence, it is possible that D-La is encoded by one of these loci.

DISCUSSION

Detailed biochemical studies with human La/SS-B have revealed its ability to selectively bind certain viral RNAs and snRNAs (including RNA Pol III precursor transcripts). Though the sequence specificity of this recognition is limited to the terminal uridylate residues (17, 21, 28), La has also been reported to bind nonspecifically with low affinity to other RNA molecules (5). These interactions are mediated by a conserved RNA-binding motif which is present in homologous molecules from other species, including D-La. We show that D-La can bind nonspecifically to small, randomly selected RNA mole-

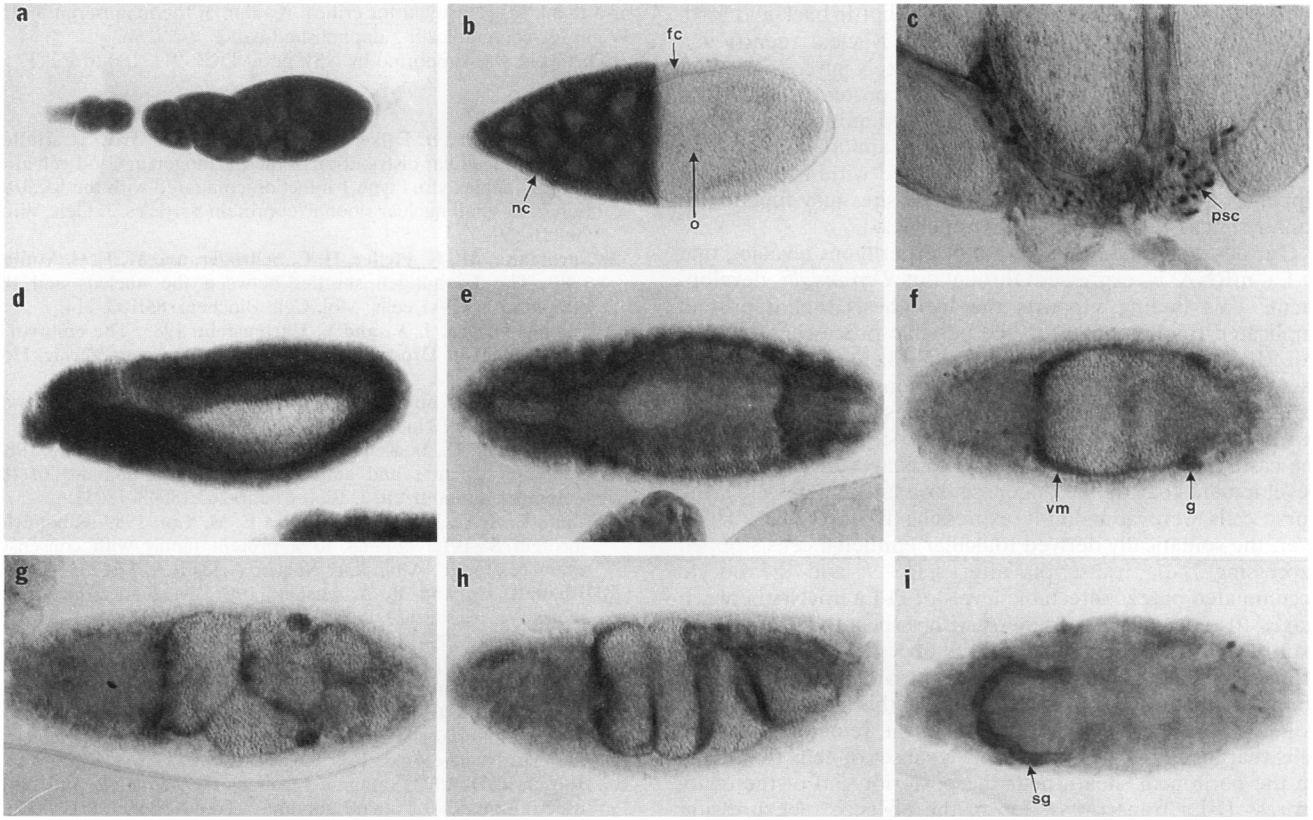

FIG. 5. Expression of D-La transcripts during oogenesis and embryogenesis. Whole-mount in situ hybridization using digoxigenin-labeled D-La probes was performed as described in Materials and Methods. All photographs were taken with Kodak PHR Ektar 25 Professional film; magnification is ×200. Egg chambers (a to c) are oriented with the anterior end on the left except for panel c, in which the anterior end is up. (a) Staining throughout an entire ovariole until stage 9 (see reference 13 for staging). (b) Stage 10b egg chamber. Follicle cells (fc), nurse cells (nc), and the oocyte (o) are indicated by arrows. (c) Posterior end of an ovary. The arrow points to a peritoneal sheath cell (psc). Embryos (d to i) are oriented with the anterior end on the left and viewed from the dorsal side except in panels d and h, in which they are viewed laterally (i.e., the dorsal side is up). The morphological stages of embryonic development are described by Campos-Ortega and Hartenstein (3). (d) Stage 10 embryo undergoing germ band elongation. (e) Stage 12 embryo undergoing germ band retraction. (f to h) Germ band-retracted embryos staining primarily in the visceral mesoderm (vm) and gonads (g). (i) Late stage 17 embryo staining primarily in the salivary glands (sg).

cules that are unrelated to each other. We do not have information regarding the identity of potential specific D-La targets, but possible candidates include RNA molecules similar to those specifically recognized by human La (discussed above).

D-La also contains a leucine zipper dimerization motif (12), suggesting that this protein may form dimers with itself or heterodimers with other molecules. This is the first report that such a structure may exist in a La homolog. In D-La, this motif consists of four leucines each separated by six variant amino acids, but it is apparently absent in other La molecules because the second of the four leucines is missing in all six organisms (Fig. 2). However, since this change results in substitution of the same uncharged nonpolar amino acid in all six cases, it may be possible that a zipper-like dimerization motif can nevertheless be generated. If this were the case, it is intriguing to speculate on possible heterodimeric associations between human La and other proteins that may participate in snRNA processing, effectively increasing its functional repertoire, and under appropriate circumstances, may even initiate the disease state (if, for example, one of the La partners becomes defective).

The leucine zipper is also found in many DNA-binding proteins. Some possess additional noncontiguous DNA contact motifs that are not located near the dimerization surface (12). Others, like the bZIP class, contain clusters of basic residues that are immediately followed by the leucine zipper (35). D-La also has a region containing clusters of basic amino acids in close proximity but located C terminal to the leucine zipper (residues 119 to 149). In addition, this same region contains clusters of acidic amino acids. Hence, D-La cannot be classified as a bZIP protein. However, we speculate that the nonspecific D-La DNA-binding activity that we demonstrate with gel retardation analysis may be conferred by this region of clustered basic and acidic amino acids located adjacent to the leucine zipper. Though the specificity of this interaction is unknown, to our knowledge, this is the first report of a DNA-binding activity associated with a La homolog. The ability of our D-La fusion protein to bind a variety of nucleic acid substrates is not an artifact conferred by the GST portion of the fused protein since several different GST-fused versions of other RNA- and DNA-binding proteins used in this assay have displayed distinct binding properties (reference 29 and unpublished data). Future studies will address the functional significance of D-La's ability to bind RNA and DNA.

The presence of three putative bipartite nuclear localization consensus sequences (7) suggests that D-La is probably a nuclear protein. The subcellular distribution of the human La

protein in tissue culture cells appears to shuttle back and forth between the nucleus and the cytoplasm. Nuclear reentry is a process that is dependent on the direct N glycosylation of either La or a putative La transporter protein (1, 2). It is interesting to note the presence of two possible N-linked glycosylation sites (19) in D-La located at amino acid positions 111 and 177. The first of these is located within the leucine zipper. N glycosylation of D-La at these sites may function to confer similar nuclear shuttling properties.

Our developmental Northern blot experiment revealed that D-La mRNA is expressed throughout *Drosophila* development. This finding supports the hypothesis that a protein implicated (by homology) in the possible processing of RNA Pol III precursor transcripts is most likely encoded by an essential gene. Since RNA Pol III functions in all cells, we also expected to find D-La transcripts present in all cells of particular developmental stages. To our surprise, this was not the case. During the 14 morphological stages of egg chamber development that define oogenesis in adult females (13), the nurse cells were abundantly expressing D-La mRNA. However, the somatically derived follicular epithelial cells stopped expressing D-La transcripts after stage 9, and the oocyte accumulated barely detectable levels of D-La mRNA between stages 10 and 14. This asymmetry with respect to D-La mRNA expression within the nurse cells and oocyte is unusual considering that the former are actively providing the egg with abundant maternal components during this period. In addition, D-La mRNA is not expressed in the remaining somatic cells that make up the ovary. Only a subset of cells that make up the peritoneal sheath near the posterior end of the ovary express D-La transcripts. Hence, the absence of expression from certain ovarian cell types suggests that D-La may have a more specialized role during *Drosophila* oogenesis.

Embryonic expression was also surprising. Following an initial period when D-La transcripts accumulate throughout the embryo, a more refined pattern is encountered initially in the mesoderm and later in the visceral mesoderm, gonads, gut, and salivary glands. This skewed expression pattern again suggests that D-La may play a specialized, developmentally regulated role in the differentiation of these structures rather than a more general role that one might expect from a putative processor of RNA Pol III transcripts. If a La-like activity is truly required in all *Drosophila* cell types, we speculate that other molecules substitute this function in cells that do not express D-La.

The objective of this study was to initiate experiments addressing the basic functions of La in the context of a living organism that is feasibly amenable to genetic manipulation using *D. melanogaster* as a model. Future studies will focus on a genetic identification and analysis of the phenotypes associated with different D-La mutations. Such studies have not been initiated previously even though La homologs have been identified in other higher organisms. In addition, the surprising similarity between vertebrate innate immunity (18) and insect immunity (8, 10) and the conserved mechanism by which these systems are induced within analogous tissues (11, 18) further support the use of *D. melanogaster* as a model organism for La genetic studies. The genetic analysis of the gene encoding D-La may enhance our understanding of systemic lupus erythematosus because La is a normal component of cells yet is also the cornerstone against which a severe humoral response is mounted in this disease.

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