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Mosquito Homolog of the La Autoantigen Binds to Sindbis Virus RNA

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We have isolated a 50-kDa mosquito protein that binds with high affinity to a riboprobe representing the 3′ end of the minus strand of Sindbis virus RNA. The isolated protein has been used to obtain cDNA clones encoding this protein that have been sequenced and used to express the protein in large amounts. Sequence comparisons make clear that this protein is the mosquito homolog of the La autoantigen. The N-terminal half of the protein shares considerable sequence identity with the human La protein, the rat La protein, and the recently identified *Drosophila melanogaster* homolog. There is one stretch of 100 amino acids in the N-terminal domain in which 48 residues are identical in all four proteins. In contrast, the C-terminal domain of the mosquito protein shares little identity with any of the other three proteins. We have also shown that the mosquito protein, the human protein, and a putative chicken homolog of the La protein cross-react immunologically and, thus, all share antigenic epitopes. The mosquito La protein is primarily nuclear in location, but significant amounts are present in the cytoplasm, as is the case for the La proteins of other species. The equilibrium constant for the binding of the expressed mosquito La protein to the Sindbis virus riboprobe is 15.4 nM, and thus the affinity of binding is high enough to be physiologically relevant. Furthermore, the conservation of this protein in the animal kingdom may be significant, because Sindbis virus utilizes mosquitoes, birds, and mammals as hosts. We propose that the interactions we observe between the La protein and a putative promoter in the Sindbis virus genome are significant for Sindbis virus RNA replication.

It is becoming clear that host binding factors are involved in the replication, transcription, or translation of many viruses. Alphaviruses are thought to require cellular proteins as factors for their RNA synthesis, as first suggested by studies of host range mutants of Sindbis virus (11) or of the effects of inhibitors of cellular RNA synthesis upon viral RNA replication (reviewed in reference 30). A role for host proteins in viral RNA replication was also suggested by mutational analyses of viral sequence elements believed to be promoters for RNA replication or transcription, such as the 5′ nontranslated region (NTR) (19), the 51-nucleotide conserved element (20), and the 3′ NTR (12), which demonstrated that many mutations had different effects on RNA replication in different cells. If these elements do in fact bind cellular proteins during RNA replication, these cell factors must be present in both vertebrate and invertebrate cells and in many different tissues, as alphaviruses have a very broad host range (reviewed in reference 31).

Recently, we demonstrated that cellular proteins from chicken embryo fibroblasts and from mosquito cells could bind to the complement of the 5′ NTR of Sindbis virus RNA (22, 23). Using this domain as a probe in binding assays, we first demonstrated that it could interact with chicken proteins of 42/44 kDa (which migrated as a doublet) and 52 kDa. A riboprobe with a deletion of residue 5 (this deletion is lethal for the virus) could form RNA-protein complexes which had a threefold-longer half-life than that of the wild-type complexes. Since the complement of the 5′ NTR is potentially able to form stem-loop structures, the deletion of residue 5 could cause a change in the structure, and it is unclear whether the effect of the deletion signified that the cellular factors recognized nucleotide 5 as a contact residue for binding or whether the altered secondary structure affected binding. We also showed that mosquito cell proteins of 50/52 kDa (called p50 and p52, which migrated as a doublet) could bind to three high-affinity binding sites and one low-affinity binding site within the 3′-terminal 250 residues of the minus-sense Sindbis virus RNA. These proteins were also found to bind specifically to the complement of the 5′ NTR of Semliki Forest and Ross River virus RNAs, demonstrating that binding of these proteins is a general phenomenon in alphavirus infection (22). Several changes in the sequence of the riboprobe resulted in decreased affinity for binding. We hypothesized that the interaction between the mosquito proteins and the complement of the 5′ NTR of Sindbis virus RNA represents an important step in virus replication.

In this report, we extend our studies on these cellular binding factors by the purification, expression, and characterization of the mosquito protein p50. Sequence comparisons demonstrated that this protein is the mosquito homolog of the La autoantigen. Purified mosquito La protein expressed in *Escherichia coli* was characterized for its binding abilities and was found to have a very high affinity for the Sindbis virus 3′ minus-sense probe (equilibrium constant [K_{eq}] = 15.4 nM) but a lower affinity for a 5′ plus-sense NTR probe. Competition experiments using homopolymers of uridylate, which are known to be one high-affinity recognition element for the La protein (at least when present at the 3′ terminus), demonstrated that the mosquito La protein bound the Sindbis virus 3′ minus-sense riboprobe with a much greater affinity than it did oligouridylates. We also showed that the protein is recognized by antibodies directed against the human La protein, indicating that the two proteins share common epitopes, and that, like its human counterpart, the mosquito La protein is localized primarily in the nucleus but is also present in the cytoplasm of the mosquito cell. We hypothesize that the La protein plays an important role in alphavirus replication.

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MATERIALS AND METHODS

Preparation of cell extracts. Monolayers of C6/36 mosquito cells from *Aedes albopictus* (about 10^{10} cells) were grown to confluence in 150-mm-diameter dishes, and S100 mosquito cell extract was prepared as described previously (23). The extract was concentrated eightfold in a stirring magnetic cell (Amicon) according to the manufacturer's protocol.

Protein purification. The concentrated extract was loaded onto a sulfo-propyl fast-flow Sepharose column previously equilibrated in 5% glycerol–2.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5)–1 mM dithiothreitol–12 mM $MgCl_2$–0.1 mM EDTA–50 mM NaCl. The column was extensively washed with the equilibrating buffer, and the binding protein was eluted with a gradient of 0.05 to 1 M NaCl. Each fraction was monitored for the presence of the binding protein by band retardation assay as described previously (23). The active fractions (which eluted between 750 and 950 mM NaCl) were pooled and concentrated on a Centricon 10 device (Amicon) according to the manufacturer's instructions. The sample was then loaded on a preparative 14% polyacrylamide gel containing sodium dodecyl sulfate (SDS). The location of the binding protein was determined by staining a small portion of the gel with Coomassie blue. After excision, the protein was electroeluted in 25 mM *N*-ethylmorpholine–0.1% SDS for 4 h at room temperature. In order to assess its purity, a portion of the electroeluted protein was submitted to reverse (phase) high-performance liquid chromatography (rHPLC), and only a single protein peak was visible. Finally, a portion of the pooled fractions representing the peak was tested for the presence of binding activity by band retardation assay.

Amino acid sequencing. Approximately 1 μg of purified protein was submitted to endopeptidase Lys-C digestion (Boehringer Mannheim Biochemicals) with 0.03 μg of the enzyme in 25 mM Tris HCl (pH 8.5)–1 mM EDTA for 3 h at 37°C. The resulting peptides were purified by rHPLC and subjected to automated Edman degradation in a 477A gas phase sequencer (Applied Biosystems, Inc.).

Cloning of the mosquito La cDNA. cDNA was synthesized with reverse transcriptase by using total RNA from C6/36 mosquito cells as template and an oligo(dT) primer. Degenerate oligonucleotide primers, 5′-GA(A/G)TA(T/C)TA(T/C)TT(T/C)GGNGA(T/C)GCNAA-3′ and 5′-GT(A/G/T)AT(T/C)TC(T/C)TC(T/C)TC(A/G)TC(A/G/T)AT(T/C)TG-3′, whose sequences were derived from the amino acid sequences of two sequenced peptides from p50, were used to amplify a 775-bp fragment from this cDNA by PCR. The 775-bp fragment was cloned into a T/A cloning vector (Invitrogen) and sequenced. The sequence of the PCR product was used to design two primers that were in turn used to generate a 416-bp fragment by PCR which was then used to probe a C6/36 mosquito cell cDNA library in λzapII (a gift of G. Ludwig, Fort Detrick). About 150 positive clones were found upon screening of 2×10^6 plaques. Three of the positive plaques were picked, and the pBluescript plasmids containing the inserts were excised from the phage by following the manufacturer's instructions (Stratagene) and used to transform a low-copy-number bacterial strain, *E. coli* Able C (Stratagene), because the recombinant plasmids appeared to be toxic for XL1 Blue bacteria (Stratagene). One of the resulting clones, pAB1.1, was entirely sequenced, while the other two (pAB3.7 and pAB7.9) were partially sequenced.

In vitro translation of the mosquito La protein. About 1 μg of pAB1.1 or pAB3.7 DNA was added directly to a coupled transcription-translation system (Promega) containing 25 μl of rabbit reticulocyte lysate, 20 μM (each) essential amino acid except methionine, 40 μCi of [^{35}S]methionine, 50 U of T3 RNA polymerase (Gibco), and 40 U of RNasin in the buffer provided by the manufacturer (diluted to 1×) for a 50-μl final reaction mixture volume. The translation products were analyzed on a 15% polyacrylamide gel containing SDS. The quantity of translated product was measured by trichloroacetic acid precipitation. When [^{35}S]methionine-labeled translation product was used in the binding assay with ^{32}P-labeled probes, a copper screen (1 mm thick) was placed between the gel and the film to stop the radiation from ^{35}S.

Expression of the mosquito La protein. The insert from pAB1.1 was used as a template for PCR. Two primers complementary to the 5′ and 3′ termini of the open reading frame were designed to create an *Nde*I restriction site at the 5′ end that contained the initiation codon and a *Hin*dIII restriction site containing part of the termination codon at the 3′ end. In order to minimize the possibility of misincorporation, PFU of polymerase was used. A PCR product of the expected size was obtained, digested with *Nde*I and *Hin*dIII, and gel purified. The purified fragment was then ligated into the pET 14d expression vector (Novagen) which had been digested with the appropriate restriction enzymes, immediately downstream of a sequence encoding 6 histidine residues, and purified. Recombinant DNA was transformed into BL21(DE3) pLysS *E. coli*. Recombinant fusion protein was induced and purified from bacterial lysates under nondenaturing conditions by using a His · Bind resin column (Novagen) as described by the manufacturer. The purified protein was concentrated on a Centriprep-10 or Centriplus-10 device (Amicon) according to the manufacturer's protocol. The protein concentration was determined by BioRad assay. Cleavage of the histidine tag was performed by digesting 1 mg of the purified fusion protein with 0.5 U of thrombin for 2 h at room temperature. The cleaved protein was purified by chromatography on a His · Bind resin column, and the recovered protein was quantitated.

Transcription of riboprobes and band retardation analysis. Synthesis and purification of radiolabeled RNA transcripts, conditions for the binding reactions, band retardation analysis, UV light cross-linking experiments, and quantitation of the free RNA and of the RNA-protein complexes were performed as described previously (17, 22, 23).

Immunoblotting. Protein samples in an SDS-containing polyacrylamide gel were transferred to nitrocellulose. The membrane was blocked for 1 h in 5% dried milk in Tris-buffered saline (25 mM Tris HCl [pH 7.5], 137 mM NaCl, 2.7 mM KCl) containing 0.02% NaN_3 and incubated with either serum from a patient with lupus erythematosus and mainly directed against the La autoantigen (gift from D. Kenan, Duke University Medical Center), a normal human serum, a serum from a rabbit immunized with the *E. coli*-expressed mosquito La protein (Antisera System; HRP Inc.), or a preimmune rabbit serum (all diluted in blocking buffer) for 1 h at room temperature or 14 h at 4°C. After three washes in Tris-buffered saline, the filter was incubated for 1 h with an alkaline phosphatase-conjugated secondary antibody (Cappel) diluted 1:1,000 in blocking buffer. After another three washes, the membrane was developed colorimetrically by incubation in alkaline phosphatase buffer (100 mM Tris [pH 7.5], 5 mM $MgCl_2$, 100 mM NaCl) containing 0.337 mg of 4-nitroblue tetrazolium chloride per ml and 0.175 mg of 5-bromo-4-chloro-3-indolylphosphate (BCIP) per ml.

Immunofluorescence. Cells were grown in 60-mm petri plates containing two to four Lysine-S (Sigma)-coated coverslips to a density of approximately 2×10^5 C6/36 mosquito or HeLa cells or 10^5 chicken embryo fibroblasts per plate. The cells were incubated with the human anti-human-La or rabbit anti-mosquito-La antiserum in PBS containing 0.5% normal goat serum and 0.02% Triton X-100 for 1 h at room temperature or 14 h at 4°C. After the cells were washed twice in 1% bovine serum albumin in PBS containing 0.4% Triton X-100, fluorescein- or rhodamine-conjugated goat anti-human or anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc.) was added at a dilution of 1:300 in the blocking solution for 1 h at room temperature. After several washes, the coverslips were mounted with glycerol and analyzed with a fluorescence microscope (Axioplan).

RESULTS

Purification of the 50-kDa mosquito binding protein. We showed previously that a 50/52 kDa protein from mosquito cell extracts bound with high affinity to a riboprobe, called 62S(−), that contains the 59 nucleotides at the 3′ end of the Sindbis virus minus-strand RNA (22). The binding protein was purified from mosquito cell extracts by a series of purification steps, using band retardation assays to monitor the binding activity. The cellular extracts were first fractionated on an SP fast-flow Sepharose column; the results from one column are shown in Fig. 1. Complex formation with the 62S(−) probe was observed for the fractions eluting at salt concentrations of 720 to 950 mM (Fig. 1, fractions 30 to 42). Fractions containing the binding activity (fractions 33 to 42 in Fig. 1) were concentrated and subjected to SDS-polyacrylamide gel electrophoresis. A major protein species of about 50 kDa was detected by silver staining, the band was excised from the gel, and the protein was electroeluted and subjected to rHPLC. About 75 ng of homogeneous protein was recovered from the rHPLC fraction and tested for its binding ability by band retardation assay (Fig. 2). Formation of a complex upon incubation with the 62S(−) probe (Fig. 2, lane 5) that was identical in mobility to that formed between this probe and 350 ng of S100 mosquito cell extract was observed (Fig. 2, lane 2). The specificity of the complexes formed between the purified protein and the 62S(−) probe was demonstrated by competition experiments in which the reaction equilibrium was displaced by adding a 50-fold excess of unlabeled specific competitor (Fig. 2, lane 6) but not by adding the same amount of nonspecific competitor (Fig. 2, lane 7). These results were similar to that obtained with the S100 mosquito cell extract (Fig. 2, lanes 3 and 4). The apparent decrease in the ability of the purified protein to form complexes with the 62S(−) probe may be due either to an overestimation of the protein quantity or, more likely, to a loss of binding capacity inherent in the purification process.

Identification of the mosquito homolog of the La autoantigen. A portion of the protein electroeluted from the SDS gel was digested with endopeptidase Lys-C, the resulting peptides were separated by rHPLC, and the amino acid sequences of 5 peptides were determined. A set of 4 degenerate oligonucleotides, with sequences derived from the amino acid sequences of

FIG. 1. Purification of the mosquito homolog of La protein. Concentrated S100 extract (about 400 mg of total protein) was separated on a cation-exchange chromatography column. The presence of the binding protein was assessed by incubating a portion of each fraction (about 30 ng) with 0.3 ng of radiolabeled 62S(−) probe in binding reaction mixtures; this was followed by analysis on a nondenaturing 5% polyacrylamide gel. The fraction number is indicated above each lane. The leftmost lane contained the 62S(−) probe alone. The second lane (Flow through) was also tested for remaining binding activity. The free probe is indicated by an arrow.

two peptides and representing both plus and minus polarities, were used in different combinations as primers in PCR of mosquito cDNA. One pair of primers gave a 775-nucleotide fragment that was cloned and sequenced. An internal region of this fragment was amplified by PCR and used as a probe to isolate full-length cDNA clones from a mosquito cDNA library. Three cDNA clones, pAB1.1, pAB3.7, and pAB7.9, were characterized. pAB1.1 and pAB3.7 contained the same open reading frame and identical 3′ NTRs but possessed 5′ NTRs that differed in size and sequence. pAB7.9 lacked the 5′ NTR as well as the first AUG and the following 29 nucleotides, while the rest of its sequence was identical to that of the two other clones. The primary sequence of clone pAB1.1 is presented in Fig. 3A; the DNA sequence encodes a protein of 383 amino acids with a predicted molecular mass of 42 kDa and has 5′ and 3′ NTRs of 211 and 144 nucleotides, respectively. The encoded protein is closely related to the human La autoantigen. Figure 3B shows an alignment of the mosquito protein with the human and rat La proteins and with the *Drosophila melanogaster* homolog of this protein. The La proteins exhibit two distinct domains. The N-terminal half of the protein is highly conserved in all four species; by way of illustration, note that in one stretch of 100 amino acids in this region, 48 residues are identical in all four proteins. This region also contains two RNA recognition motifs (2), referred to as RNP1 and RNP2. These motifs are both highly conserved in all of the sequenced La proteins, although they are not located within the most highly conserved region of the protein. In contrast, the C-terminal half of the protein exhibits little conservation between invertebrates and vertebrates. This region contains many charged residues, both acidic and basic.

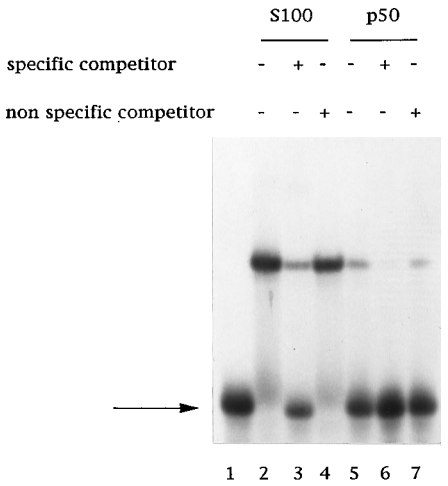

FIG. 2. Characterization of purified mosquito homolog of La protein. About 75 ng of protein separated by rHPLC was added to a binding reaction mixture containing radiolabeled 62S(−) probe (lanes 5 to 7). As a positive control, 350 ng of mosquito cell extract was incubated in parallel with the same probe (lanes 2 to 4). Samples were analyzed on a nondenaturing 5% polyacrylamide gel. Above each lane is indicated the presence or absence of 15 ng of specific or nonspecific competitor. Lane 1 contained the 62S(−) probe alone. The free probe is indicated by an arrow.

Overall, the two vertebrate proteins are 82% identical. The identity between the mosquito and the human La proteins is 35%, and the *Drosophila* and human La proteins exhibit a similar identity, 39%. The mosquito and the *Drosophila* proteins are somewhat more closely related to one another than either is to the vertebrate proteins (45% identity). This greater identity results in part from specific features that are present only in the two invertebrate La proteins. At the N termini of both invertebrate proteins is a stretch of amino acids (30 and 37 for the mosquito and the *Drosophila* La protein, respectively) that is absent from the human and murine species, although this sequence is only poorly conserved between the mosquito and the *Drosophila* La proteins. Another insertion present in the invertebrate proteins relative to the vertebrate proteins includes residues 182 to 186 of the mosquito protein (three of these five inserted residues are identical in the mosquito and *Drosophila* proteins). In addition, there are deletions of 15 and 11 residues, respectively, following residue 232 in the invertebrate proteins relative to the mammalian ones. Conversely, both invertebrate proteins lack about 50 amino acids at the C terminus of their vertebrate counterparts.

Immunoblotting of the mosquito La protein expressed in *E. coli* (see below) demonstrated that antigenic epitopes in the protein are recognized by human anti-La antibodies, further establishing that the mosquito protein is a homolog of the mammalian La protein (data not shown; see below).

RNA binding ability of the mosquito La protein. To verify that the two recombinant plasmids pAB1.1 and pAB3.7 encoded a protein that had an RNA binding activity, we transcribed and translated plasmid DNA in a coupled cell-free transcription-translation system. A translation product with an apparent molecular mass of 50 kDa was produced in both cases, although the calculated size was 42 kDa (data not shown). The presence of multiple lysine residues in the protein might account for its unconventional migration. The translation product from each clone was added to a binding reaction mixture and incubated with the 62S(−) probe (Fig. 4). Complexes that were identical in migration to those formed upon incubation of the probe with an S100 extract were formed (Fig.

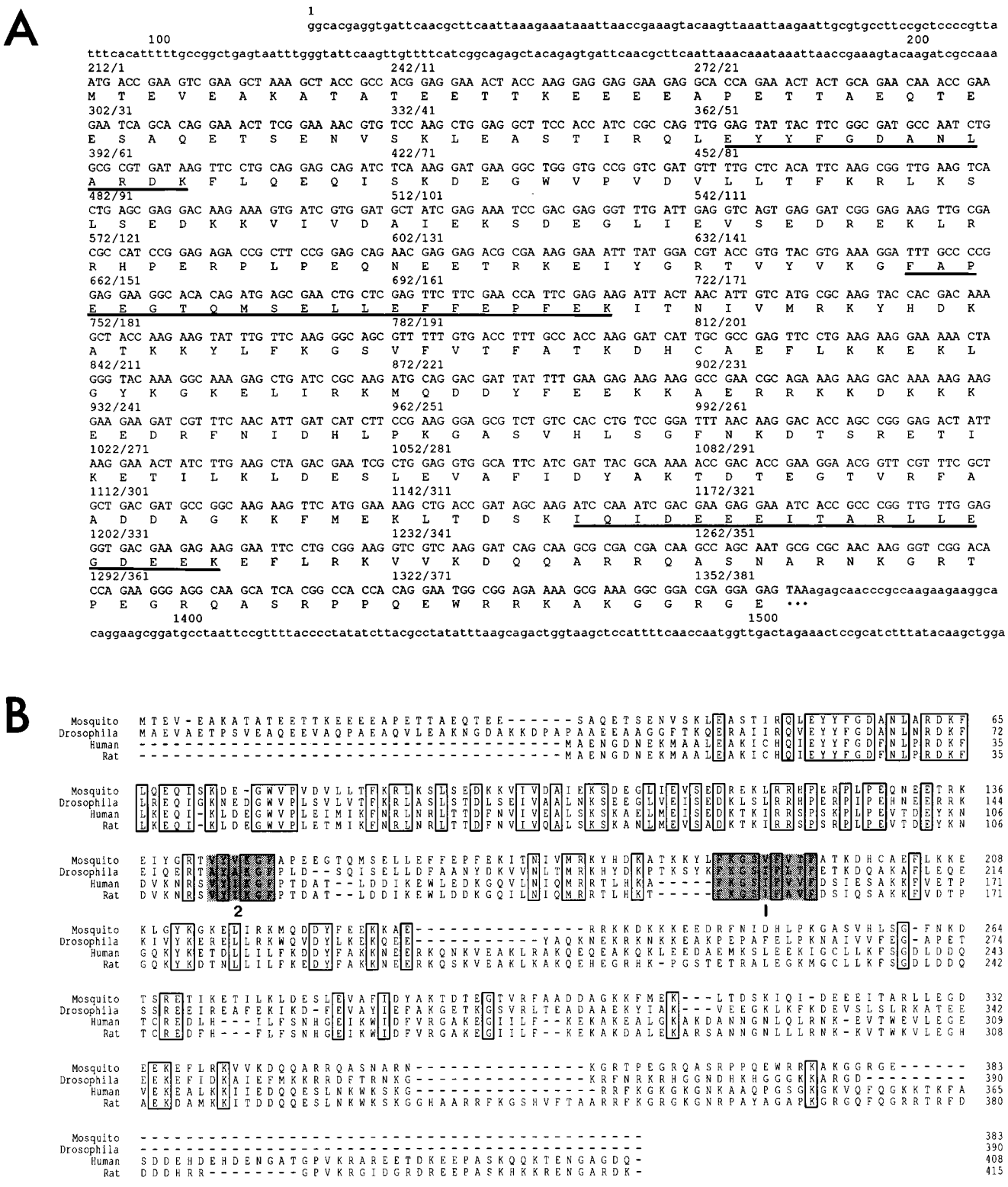

FIG. 3. The mosquito La protein. (A) Nucleotide and predicted amino acid sequences of mosquito La cDNA. Sequences obtained by microsequencing of peptides from the purified protein are underlined. (B) Alignment of La proteins from different animals. Mosquito, *Drosophila* (34), human (4), and rat La protein sequences were aligned by using the Genetics Computer Group software Pileup program; dashes indicate alignment gaps. Amino acids that are identical in all four proteins are boxed. The RNP1 and RNP2 motifs (designated 1 and 2, respectively) are shaded.

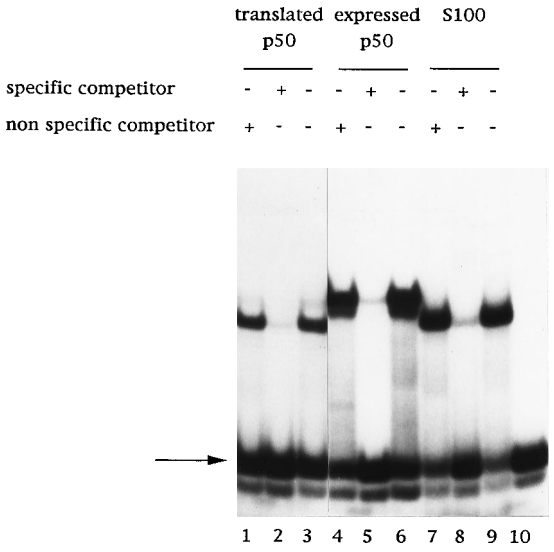

FIG. 4. Comparison of complexes formed with mosquito La protein translated in vitro, expressed in *E. coli*, or present in mosquito cell extracts. Ten nanograms of mosquito La protein translated in reticulocyte lysates (determined from the radioactivity in the translated La protein and the specific activity of the incorporated [^{35}S]methionine) (lanes 1 to 3), 10 ng of mosquito La protein expressed in *E. coli* (determined by colorimetric assay for protein) (lanes 4 to 6), or 350 ng of mosquito cell extract (lanes 7 to 9) was incubated with radiolabeled 62S(−) probe in binding assays. The sample in lane 10 is the 62S(−) probe alone. The samples were analyzed on a nondenaturing 5% polyacrylamide gel. Above each lane is indicated the presence or absence of 30 ng of specific (unlabeled 62S(−) probe) or nonspecific (short SP6 transcript of pBluescript II) competitor. The free probe is indicated by an arrow.

4, lanes 3 and 9, respectively). Competition experiments to examine the specificity of complex formation gave identical results for the translation product and the S100 extract; unlabeled Sindbis virus (specific) probe outcompeted the 62S(−) probe (Fig. 4, lanes 2 and 8), whereas the nonspecific probe did not (Fig. 4, lanes 1 and 7). A second band migrating slightly more slowly was also observed (Fig. 4, lane 3) but was also present in a control in which the transcription-translation mix alone was added to the binding reaction mixture, and it seems likely that this band represents complexes that are formed between an unknown protein in the reticulocyte lysate and the 62S(−) probe. This complex was present in much smaller amounts when the probe was incubated in the presence of the translated mosquito La protein (as shown in Fig. 4) than when incubated with the mix alone, indicating a lower affinity of the reticulocyte lysate protein for the Sindbis virus probe compared with that of the mosquito La protein (data not shown).

To further explore the binding activity of the mosquito La protein, cDNA encoding this protein was subcloned into a pET expression vector, in which 6 histidine residues are positioned at the N terminus of the protein. Expression of the protein was induced by IPTG (isopropyl-β-D-thiogalactopyranoside), and the fusion protein was purified by affinity chromatography on a Ni^{2+} column. After concentration, the ability of the fusion protein to bind to the 62S(−) probe was assessed by gel retardation analysis (Fig. 4). The complexes formed were slightly larger than those obtained upon incubation with the S100 mosquito extract, probably because of the presence of the histidine tag (Fig. 4, compare lanes 6 and 9). A second band migrating slightly faster, which might represent complexes formed with either misfolded La protein molecules or shorter forms of the protein, was also present. The specificity of the complexes present in both bands was confirmed by competition experiments similar to that performed with the complexes formed upon incubation with the S100 extract, in which the unlabeled 62S(−) probe outcompeted the complex formed with the labeled Sindbis virus probe (Fig. 4, lane 5) but the nonspecific probe did not (Fig. 4, lane 4). Removal of the histidine tag by treatment with thrombin resulted in a complex that now had the same mobility as that produced in the S100 extract, and competition experiments again demonstrated that the complexes formed were specific (data not shown).

Complexes formed between the cleaved form of the mosquito La protein and the labeled Sindbis virus probe were cross-linked with UV light and treated with RNase, and the labeled proteins were displayed on acrylamide gels (22, 23). The labeled proteins migrated as a 50/52 kDa doublet that was identical to that formed with the S100 extract (data not shown). Thus the 50- and 52-kDa doublet proteins both derive from a single protein, p50. It is possible that the cross-linking induced by UV light leads to conformational changes in a fraction of the protein molecules that results in altered migration.

Antibodies raised in rabbits against the mosquito protein expressed in *E. coli* recognized the 50/52 kDa proteins from mosquito cell extract (data not shown).

Effect of homopolymers on La protein binding. Previous experiments have demonstrated that 3′-terminal oligouridylates are a recognition element for the La protein (29). Other studies have suggested that structural context and internal features also contribute to complex formation between RNAs and the La protein (26, 27). We performed competition experiments to study the relative affinity of the mosquito La protein for polynucleotides. Poly(U) with an average size (determined by the manufacturer) of 5S, comparable to that of the Sindbis virus probes we used in the binding reactions, competed very poorly with the Sindbis virus probe. When 25 μg of poly(U) was added to the binding reaction mixture, complex formation with the Sindbis virus probe (present at 0.3 ng) was reduced by only 60% (Fig. 5, lane 10); 250 μg of poly(U) was required for complete competition (Fig. 5, lane 11). In contrast, only 30 ng of the unlabeled specific probe was necessary for complete competition (Fig. 5, lanes 12), and the specific probe is thus >1,000-fold more effective as a competitor. The addition of 25 or 250 μg of poly(A) was essentially without effect (Fig. 5, lanes 8 and 9, respectively).

Binding affinity of the mosquito La protein for different Sindbis virus probes. In previous experiments, we found that the ability of the sequence complementary to that of the 62S(−) probe (i.e., the 5′ plus-sense end of Sindbis virus RNA), called 62S(+), to form complexes with proteins from mosquito S100 extracts was very poor (21). Since a small quantity of complexes was consistently observed, however, we performed binding experiments with the purified La protein (Fig. 5). The 62S(+) probe was able to form complexes with the La protein (Fig. 5, lane 1) that were identical to those formed with the 62S(−) probe (Fig. 5, lane 7); these complexes were specific, since 30 ng of unlabeled competitor 62S(+) could outcompete complex formation, while 25 μg of nonspecific poly(A) competitor could not (Fig. 5, lanes 6 and 2, respectively). However, 25 μg of poly(U) completely abolished the specific complex formation, as did 250 μg of poly(U) or poly(A) (Fig. 5, lanes 4, 5, and 3, respectively), whereas 250 μg of poly(U) was necessary to completely abolish complex formation with the 62S(−) probe (Fig. 5, lane 11) and 250 μg of poly(A) had no effect. These results indicate that the 62S(+) probe has a lower affinity for the mosquito La protein than the 62S(−) probe.

In order to quantitate the difference in binding affinities of

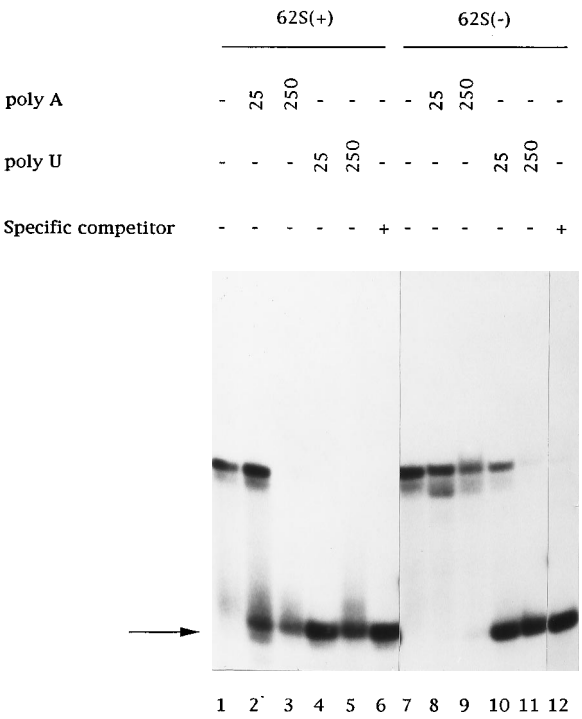

FIG. 5. Effect of polynucleotides on complex formation. Binding reaction mixtures containing 100 ng of mosquito La protein expressed in *E. coli* and either the 62S(+) (lanes 1 to 6) or 62S(−) (lanes 7 to 12) probe were incubated with either poly(A) (lanes 2, 3, 8, and 9), poly(U) (lanes 4, 5, 10, and 11), or unlabeled probe as a specific competitor (lanes 6 and 12). The complexes were resolved on a nondenaturing 5% polyacrylamide gel. Above each lane is indicated the presence or absence of the various competitors, and in the case of the polynucleotides, the quantity is expressed in micrograms. The specific competitor was used at 30 ng. The free probe is indicated by an arrow.

the two probes, increasing amounts of the protein were added to binding reaction mixtures containing the same amount of either probe. After band retardation analysis, the proportion of free and complexed probes was quantitated, and the average values from four independent binding experiments for each probe are plotted in Fig. 6. The values for the equilibrium constant (K_{eq}), defined as the concentration of La protein that bound 50% of the probe, were 15.4 nM for the 62S(−) probe and 27 nM for the 62S(+) probe (Fig. 6A). This confirms that the mosquito La protein has a higher affinity for the minus-sense probe than for the plus-sense probe. Adjustment for the best-fitting curve (realized from the Levenberg-Marquardt algorithm) suggested that two protein molecules bind the 62S(−) probe (Fig. 6B).

Localization of the La protein in mosquito cells. The mosquito La protein expressed in bacteria was used to prepare rabbit antibodies to the protein. A polyclonal antiserum that reacted with the expressed protein was obtained, and this antiserum was used to examine the distribution of the La protein in the mosquito cell with an immunofluorescence assay. Cells were permeabilized, treated with the rabbit antiserum, and rhodamine-coupled secondary antibodies were added (Fig. 7). The La protein is localized mainly in the nucleus, but a portion of it is found in the cytoplasm (Fig. 7D). A similar localization has been found for the La protein in vertebrate cells, in which the La protein is present primarily in the nucleus (10) but appears to shuttle between nucleus and cytoplasm (1). The localization of the mosquito La protein as well as its partition between nucleus and cytoplasm of the mosquito cell remained unchanged upon infection by Sindbis virus, at least within the sensitivity of our immunofluorescence assay (data not shown).

Cross-reactivity of different La proteins. The existence of common epitopes between the human and mosquito La proteins was confirmed by immunoblotting of *E. coli*-expressed mosquito La protein with human anti-La serum (data not shown). The human anti-La antibodies also reacted with a protein present in chicken embryo fibroblast extracts, suggest-

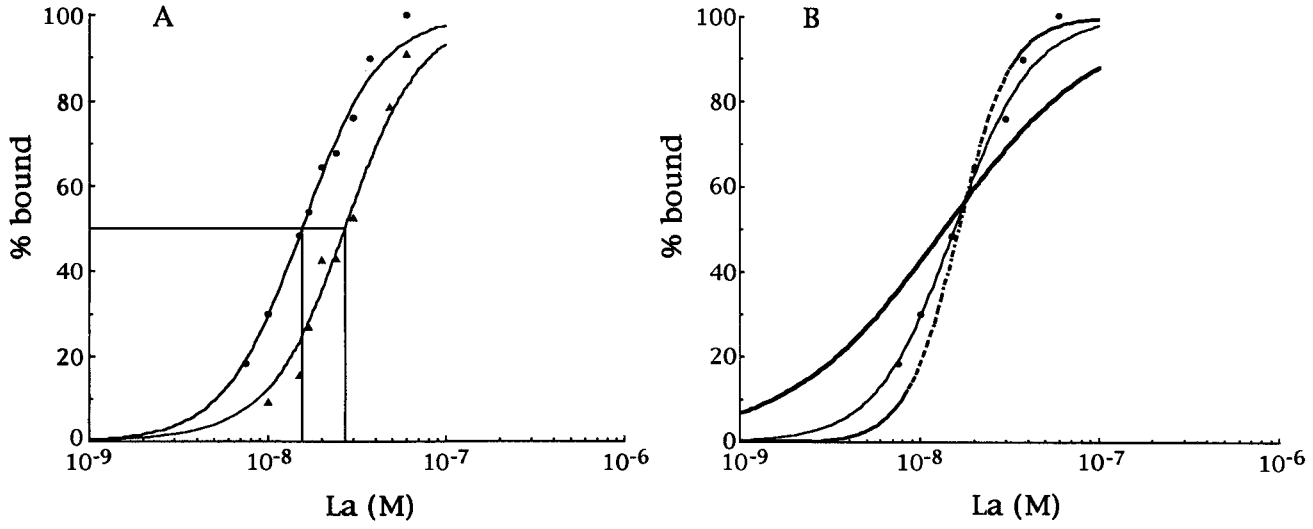

FIG. 6. Quantitation of the binding of the mosquito La protein to the 62S(−) and 62S(+) probes. The probes were transcribed and labeled in vitro. The probes (about 0.3 ng of each), having the same specific activities, were used in binding reaction mixtures containing increasing amounts of La protein. The complexes were resolved on nondenaturing 5% polyacrylamide gels, and the amounts of radioactivity present in the free RNA and RNA-protein complexes were quantitated after excision of the corresponding bands from the gel and counting in a scintillation counter. The concentration of La protein was plotted against the percentage of bound probe. (A) Best-fit curves for the two probes are shown as plain lines. The datum points for the 62S(−) and 62S(+) probes are represented by circles and triangles, respectively. K_{eq}, derived from the fitted curves, was defined as the concentration of La protein that bound 50% of the probe. (B) Theoretical curves, calculated from the Levenberg-Marquardt algorithm, are shown for the cooperative binding of one (thick line), two (thin line), or three (dotted line) protein molecules to the 62S(−) probe. The actual datum points are shown by circles and best fit the curve for binding by two protein molecules.

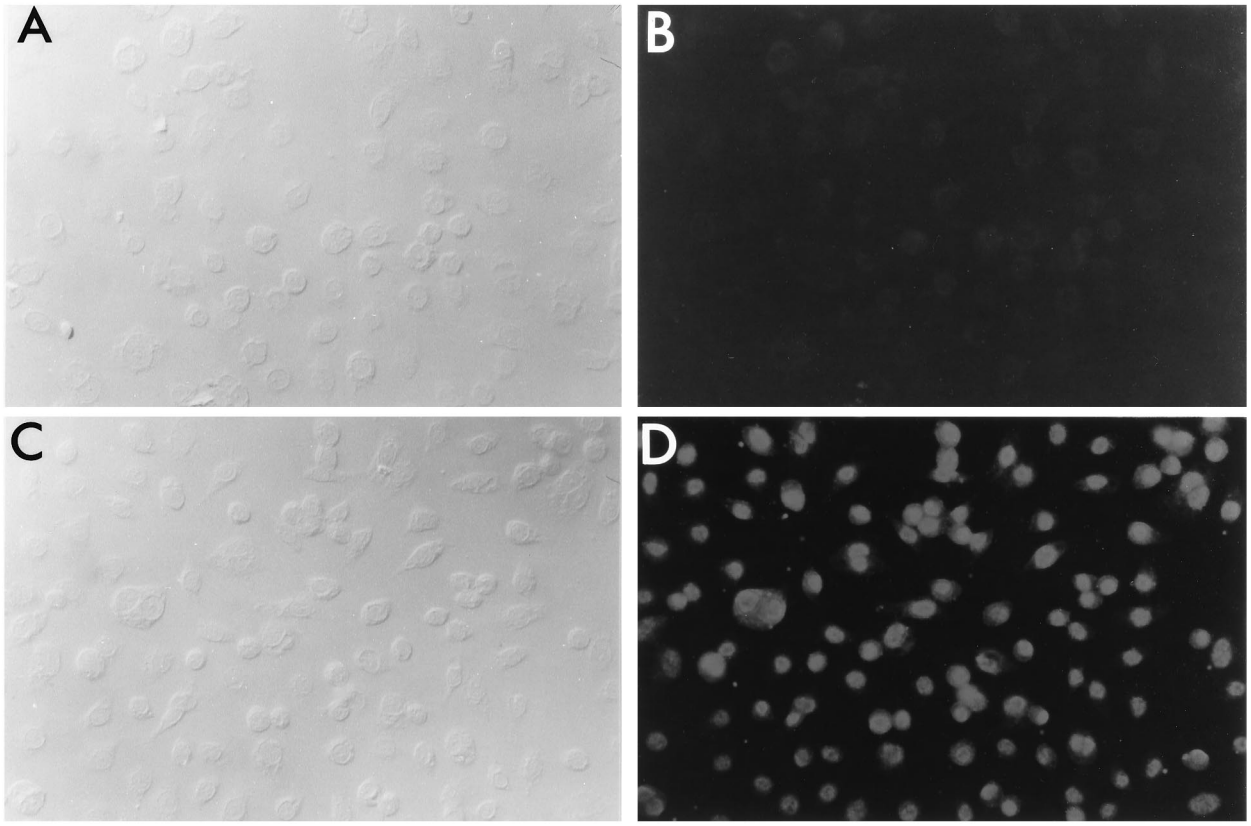

FIG. 7. Immunofluorescence assay for the La protein in mosquito cells. C6/36 mosquito cells were grown on coverslips, fixed, and examined by phase microscopy (A and C) or by indirect immunofluorescence (B and D). Cells were incubated in the presence (C and D) or in the absence (A and B) of rabbit anti-mosquito-La serum. The secondary antibody was rhodamine-conjugated goat anti-rabbit immunoglobulin G. Photomicrographs were taken with Kodacolor film at a magnification of ×400.

ing the existence of an avian homolog for the La protein with epitopes common to both mammals and invertebrates (data not shown). Cross-reactivity in chicken cells was further confirmed by immunofluorescence assays using both human anti-human-La and rabbit-anti-mosquito-La sera (Fig. 8). A protein localized primarily in the nucleus but present in the cytoplasm as well reacted with both antisera (Fig. 8B and D). It is of note that the cross-reactivity appears to be more pronounced in the case of the human antiserum, as suggested by the intensity of the cross-reaction (compare Fig. 8B and D), suggesting that the chicken protein is more closely related to the human La protein than to the mosquito La protein, as would be expected. The immunological cross-reactivity that we observed between mosquito, chicken, and human La proteins is especially interesting in light of a previous report that found no immunological cross-reaction between the *Drosophila* La protein and the human La protein (34).

In a previous study, we reported that one or more chicken proteins that migrated as a doublet of 42/44 kDa after UV cross-linking could form complexes with the 62S(−) probe (23). These proteins were found to comigrate with two chicken Proteins detected by immunoblotting with the human La antiserum (data not shown), suggesting that the 42/44 kDa proteins are probably the chicken homolog of the La protein.

DISCUSSION

In this study, we have purified and characterized a mosquito protein that binds to a domain of Sindbis virus RNA that has been proposed to be a viral promoter, namely, the 3′ end of the minus strand. Through sequence comparisons and examination of cross-reactivity with different antisera, we demonstrated that this protein is the mosquito homolog of the vertebrate La autoantigen. A *Drosophila* homolog of the La protein has also been described recently (34). Interestingly, the two insect proteins, while sharing features that are specific to invertebrates, are only slightly more closely related to each other than they are to the human or murine La protein, which is perhaps not unexpected in view of the fact that these organisms last shared a common ancestor hundreds of millions of years ago.

La is known to bind to the 3′ end of RNA polymerase III transcripts that contain three or more 3′-terminal U residues (16, 29). In so doing, it regulates termination and facilitates multiple rounds of transcription reinitiation (8, 9, 14). Whether it has other functions is not known, but work with viral RNAs, discussed in more detail below, makes it clear that La is able to bind to certain internal regions in RNAs, thought to be structural elements (5), with a higher affinity than it possesses for binding to 3′ poly(U), and it seems likely that La has multiple functions in the eukaryotic cell.

The 383-amino-acid mosquito La protein expressed in *E. coli* was able to form complexes with riboprobes derived from the Sindbis virus sequence. The value of K_{eq} that we determined for the binding of this protein to the 62(−) probe, 15.4 nM, shows that the binding affinity is very high and is about the same as that found for the binding of the human La protein to the *trans* activation response element of the human immunodeficiency virus type 1 (5). As described above, the human La

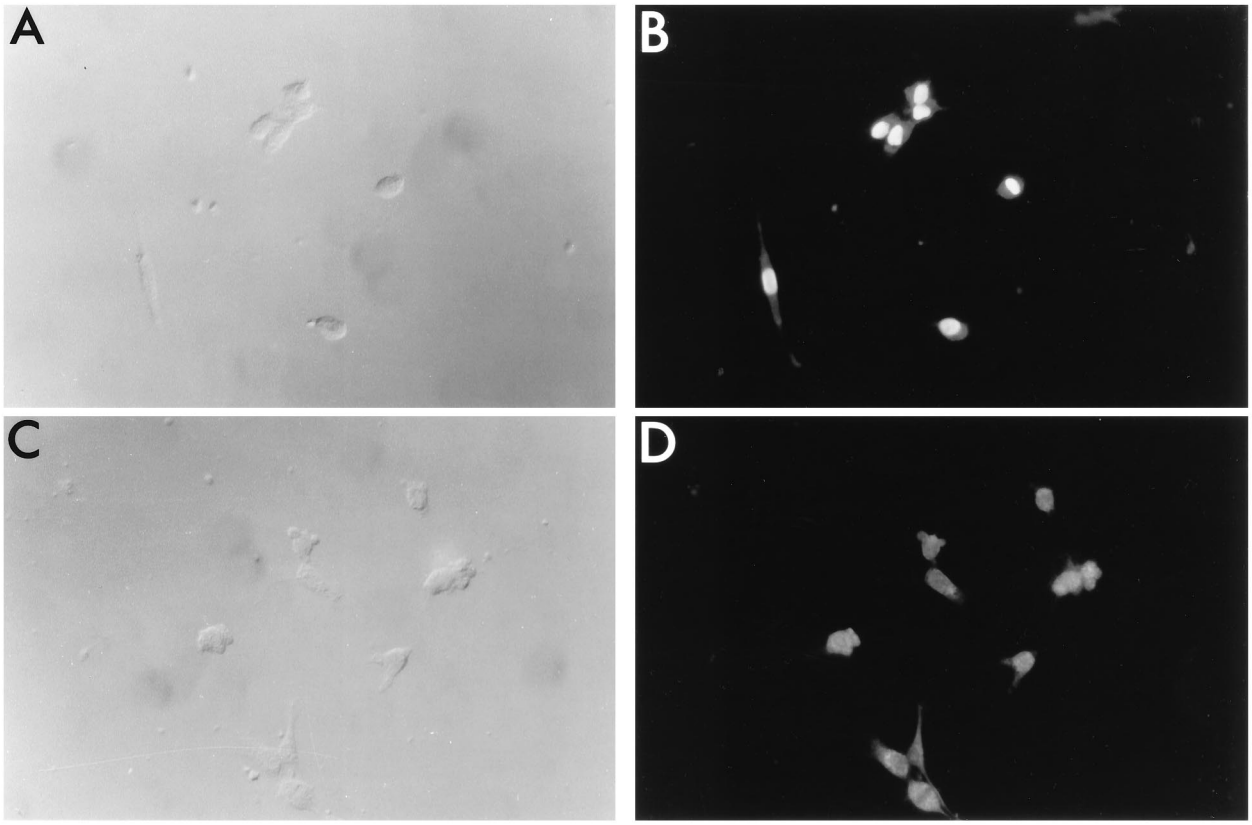

FIG. 8. Immunofluorescence assay for a possible chicken homolog of the La protein. Chicken embryo fibroblasts were grown on coverslips, fixed, and examined by phase microscopy (A and C) or by indirect immunofluorescence (B and D). Cells were incubated with rabbit anti-mosquito La serum (C and D) or with human anti-human-La serum (A and B). The secondary antibody was fluorescein-conjugated goat anti-rabbit (C and D) or anti-human (A and B) immunoglobulin G. Photomicrographs were taken with Kodacolor film at a magnification of ×400.

protein has an affinity for 3′ oligouridylates. Our results show that the mosquito La protein recognizes a structural domain in the Sindbis virus probe p62(−), which is devoid of 3′ uridylates, much more efficiently than it recognizes oligo(U). The human La protein has also been shown to recognize internal structural elements in human immunodeficiency virus leader RNA (5) and in poliovirus RNA (18), leading to high-affinity binding. Interestingly, the La protein in the mosquito cell can display different binding abilities, depending on the secondary structure of the RNA probe, as demonstrated by our previous experiments using probes bearing deletions (22).

The human La protein has been shown to associate with different viral RNAs and appears to play various roles in different host-virus systems. In the case of adenovirus, VA-1 RNA, which plays a role in maintaining efficient translation of viral mRNAs (15), may mediate that function in association with the La protein as a ribonucleoprotein (10). Recently, the La protein has been shown to be involved in the regulation of translation of human immunodeficiency virus leader RNA (5, 33) and of poliovirus RNA (18, 32). In the case of the vesicular stomatitis virus, the binding of the La protein to viral leaders has been demonstrated, although the functional consequences of complex formation are unknown (13). Interestingly, such complexes appear at the same time as shutoff of cellular protein synthesis, suggesting again an involvement of the La protein in the control of translation. Moreover, since a possible role for the leader RNA is to regulate a switch from transcription to replication (3), the La protein, which is found associated with it, might act as a cofactor for viral transcription and replication. It is of note that the La protein has been found to also bind the 3′ end of the minus-strand leader RNA. Thus, it appears that a number of viruses have evolved to take advantage of the binding properties of the La protein for a variety of purposes during virus replication.

Other interesting features of the La protein are its ubiquitous presence in both the nucleus and cytoplasm and its ability to shuttle between these compartments during viral infections, as demonstrated for herpesvirus (1), vesicular stomatitis virus (13), and poliovirus (18). We found the mosquito La protein to be present mainly in the nucleus, but significant quantities were also present in the cytoplasm of mosquito cells. We did not observe changes in this partition upon infection by Sindbis virus, at least within the sensitivity of our assay, as has been found for other viruses.

We have demonstrated in this paper that purified mosquito La protein binds to a probe containing the 3′ end of Sindbis virus minus-strand RNA with a K_{eq} of 15.4 nM and that the interaction appears to involve the binding of two La molecules. This result suggests that the La protein might be involved in the initiation of replication to produce plus-strand copies of the minus-strand RNA. The La protein also binds to the 5′ end of the plus-strand Sindbis virus RNA but with a K_{eq} of only 27 nM. Whether this binding to the plus-strand RNA is significant for virus replication is unknown, but it is noteworthy that the twofold difference in K_{eq}s leads to qualitative and easily observable differences in binding assays that use cellular extracts rather than purified La protein. In cell extracts, binding of the La protein to the minus-strand probe is readily detected and

the measured affinity is high (references 22 and 23 and this paper), whereas binding to the plus-strand probe is weak and difficult to detect (21). Similarly, preliminary results have shown that changes in binding affinity induced by different deletions in the probes are different when using purified La protein than when using mosquito extract proteins (21). It seems probable that other proteins present in the extract but not in purified preparations of La protein might modulate complex formation. In addition, the La protein has been found to be phosphorylated in mammalian cells (6, 24, 25), and increasing phosphorylation reduces its binding affinity for poly(U); the (mosquito) protein expressed in *E. coli* is presumably not phosphorylated, and its activity may differ from that of La protein found in mammalian or mosquito cells. Thus protein-protein or protein-RNA interactions that occur in the mosquito cell extract, and presumably within the infected cell, and that are possibly influenced by the state of phosphorylation of the protein may be responsible for the very different activity of the plus-strand probe with La protein in cell extracts versus assays with purified La. Such interactions could be important for modulation of the binding of the La protein to Sindbis virus RNA in the infected cell.

The RNA binding properties of the La protein make it a likely candidate for playing a regulatory role in virus replication processes. For viruses with a wide host range, such as alphaviruses, which can replicate in a broad range of hosts, including invertebrates, birds, and mammals, it is also a good target as a cellular cofactor because it is an abundant component present in all eukaryotic cells, including yeast cells (references 4, 7, 28, and 34 and this paper). As we have demonstrated, a homolog of the mammalian La protein is present both in mosquito cells and in chicken cells that can bind Sindbis virus RNA with high affinity. While we have no direct evidence that this host factor-virus interaction is involved in the Sindbis virus replication process, the value of K_{eq} that we determined is in the range of that described for biologically significant phenomena. Future studies will focus on involvement of the La protein in Sindbis virus RNA replication in infected cells and on its role in the alphavirus life cycle.

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