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The Multifunctional RNA-Binding Protein La Is Required for Mouse Development and for the Establishment of Embryonic Stem Cells

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The La protein is a target of autoantibodies in patients suffering from Sjögren's syndrome, systemic lupus erythematosus, and neonatal lupus. Ubiquitous in eukaryotes, La functions as a RNA-binding protein that promotes the maturation of tRNA precursors and other nascent transcripts synthesized by RNA polymerase III as well as other noncoding RNAs. La also associates with a class of mRNAs that encode ribosome subunits and precursors to snoRNAs involved in ribosome biogenesis. Thus, it was surprising that La is dispensable in the yeasts *Saccharomyces cerevisiae and Schizosaccharomyces pombe,* the organisms from which it has been characterized most extensively. To determine whether La is essential in mammals and if so, at which developmental stage it is required, mice were created with a disrupted La gene, and the offspring from $La^{+/-}$ intercrosses were analyzed. $La^{-/-}$ offspring were detected at the expected frequency among blastocysts prior to implantation, whereas no nullizygotes were detected after implantation, indicating that La is required early in development. Blastocysts derived from $La^{+/-}$ intercrosses yielded 38 $La^{+/+}$ and $La^{+/-}$ embryonic stem (ES) cell lines but no $La^{-/-}$ ES cell lines, suggesting that La contributes a critical function toward the establishment or survival of ES cells. Consistent with this, $La^{-/-}$ blastocyst outgrowths revealed loss of the inner cell mass (ICM). The results indicate that in contrast to the situation in yeasts, La is essential in mammals and is one of a limited number of genes required as early as the development of the ICM.

La antigen, also known as Sjögren's syndrome antigen B (SS-B), is a target of autoantibodies in patients suffering from systemic lupus erythematosus, neonatal lupus, and related disorders and exists in cells complexed with various RNAs (20). Homologs of La are present in all of the eukaryote genomes examined, and La proteins have been characterized in ciliates, yeasts, flies, frogs, and mammals (7, 22, 33). While La has been implicated in many RNA-related pathways, its most established role is protecting the UUU-OH 3′ ends of precursor tRNAs and other small RNAs from digestion (21, 23, 29, 33).

Vertebrate La proteins can modulate the translation of mRNAs that contain internal ribosome entry sites, as well as mRNAs that contain 5′-terminal oligopyrimidine motifs that encode ribosome subunits and translation factors (8, 28; reviewed in reference 33). The association of human Mdm2 mRNA with La promotes MDM2 translation with consequent decrease in p53 protein level and leukemia progression (31). La is also found associated with mRNAs in *Saccharomyces cerevisiae*, including mRNAs that encode ribosome subunits (14). Deletion of La from yeasts leads to alterations in the maturation pathways of pre-tRNAs (2, 5, 6, 16, 17, 26, 35) and pre-snoRNAs involved in rRNA biogenesis (14, 21). Thus, it was surprising that La is nonessential in yeasts, except when tRNAs or RNA-associated factors acquire debilitating mutations (21, 29, 33) and upon a conditional induction of the unfolded protein response (14).

The conserved N-terminal domain of La is comprised of a La motif and RNA recognition motif (RRM) that cooperate for high-affinity 3′ UUU-OH binding (1, 9, 18). However, while these motifs constitute the La proteins of yeasts, metazoan La proteins also contain another, atypical RRM in their C termini (18).

In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the organisms in which La has been characterized most extensively, La is dispensable. Small-interfering-RNA-mediated knockdown of La modestly decreased HeLa cell survival but was associated with more significant growth defects in the protozoan *Trypanosoma brucei* (10, 32). A genetic analysis suggested that La is required during a late (larval) stage of development in *Drosophila melanogaster* (3). While the La protein of humans (hLa) differs in length and sequence from those of *D. melanogaster*, *T. brucei*, and yeasts (<34%, <28%, and <24% identity with hLa, respectively), it is most highly homologous to mouse La (mLa) in overall structure and sequence (76% identity) (22). Yet, although much has been learned about hLa using heterologous systems and from in vitro analyses (15, 16, 18, 21, 33), there has been relatively very little analysis of La function in a mammal. A question that remained was whether La is essential in mammals and if so, at what developmental stage. To explore this, we created a disrupted La gene in mice and analyzed its effects on mouse development and the establishment of embryonic stem (ES) cells in vitro. The results demonstrated that, in contrast to yeasts, La is required in mammals as early as blastocyst development.

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MATERIALS AND METHODS

Mice carrying a disrupted La allele. Mice carrying a disrupted La allele were generated by Ingenious Targeting Laboratory (Stony Brook, NY). The targeting vector contained 9.5 kb of genomic DNA upstream of the mouse La exon 1, followed by a neomycin resistance cassette plus 900 bp from intron 2 (see Fig. 2A). The neomycin resistance gene (driven by the phosphoglycerate kinase I promoter in the orientation opposite that of La) replaced La gene exons 1 and 2, the latter of which contained the ATG start codon. NotI-linearized targeting vector was transfected into 129SV/EV embryonic stem cells (Ingenious Targeting Laboratory, Stony Brook, NY). Clones were selected and expanded in G418, and PCR analysis identified ones that had undergone homologous recombination, which were microinjected into C57BL/6J and BALA/cj blastocysts. The resulting chimeric mice were mated with C57BL/6 mice, and those that exhibited germ line transmission of the disrupted La gene were used to produce a founding pair of $La^{+/-}$ mice (LaKO21 and LaKO27), both of which obtained the disrupted mLa allele from the same targeted ES cell clone.

Genotyping. DNA was purified from tail clippings, typically at 3 weeks of age, using a DNeasy tissue kit (catalog no. 69506; Promega), examined, and quantitated by gel electrophoresis and ethidium staining. Triplex PCR containing 200 ng of DNA and three primers was used to assay for disrupted and wild-type mLa alleles simultaneously; allele-specific primers (wild type [5′-ATAGGCCACAATGGCTGAAAATGGAG] and disrupted [5′-TTATGGGCAATTTCCCCACAGCCA]) together with a common reverse primer (5′-TGCGAGGCCACAGGCCACTT) were used. PCR parameters were as follows: (i) 94°C for 1 min; and (ii) 30 cycles, with 1 cycle consisting of 94°C for 45 s, 67°C for 25 s, and 68°C for 45 s. The expected products from wild-type and disrupted alleles are 700 and 500 bp long, respectively. Genotypes of all mice used for breeding, embryo production, and Southern and Northern blotting were confirmed by two additional tail clips and genotyping.

Preimplantation embryos were flushed from uteri, and individual embryos were incubated overnight at 55°C in 15 μl of embryo lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8, 2 mM $MgCl_2$, 0.45% Nonidet P-40 [NP-40], 0.45% Tween 20, and 0.1 mg/ml proteinase K). The proteinase was then inactivated by incubation at 100°C for 2 min, and 8-μl samples of the lysed embryos were added to 17 μl of PCR master mix containing 10× PCR buffer, primer, deoxynucleoside triphosphates, and *Taq* polymerase (BD Biosciences). PCR mixtures were heated at 94°C for 1 min and then subjected to 35 cycles, with 1 cycle consisting of 94°C for 45 s and 68°C for 2 min.

Southern and Northern blotting. DNA obtained from the livers of $La^{+/+}$ and $La^{+/-}$ mice was digested with AflIII, EcoRI, or HindIII, separated on 0.9% agarose gel, denatured, and transferred to a nylon membrane (GeneScreen Plus; Perkin-Elmer Life Sciences). Probes for Southern analysis were derived from mLa gene intron 2 and the Neo gene as indicated in Fig. 2A. Probes were labeled by random priming with [α-^{32}P]dCTP (Lofstrand Labs, Gaithersburg, MD). Hybridization was in QuikHyb solution processed accordingly (Stratagene). The membrane was exposed to a Fuji phosphorimager screen and analyzed using Image Gauge software.

Adult mouse multiple tissue and mouse embryo Northern blots were obtained from Clontech. Full-length mLa and beta-actin cDNAs were labeled by random priming with [α-^{32}P]dCTP. For $La^{+/+}$ and $La^{+/-}$ samples, total RNA was prepared from tissue dissected from adult littermates using TRIzol (Invitrogen), and 30 μg was electrophoresed and transferred to a charged nylon membrane (Gene Screen Plus; Perkin-Elmer Life Sciences, Inc.). Blots were probed first with a radiolabeled 0.7-kb 3′ fragment of mLa cDNA followed by beta-actin cDNA. Hybridization was performed as described above for Southern blotting.

Cloning, expression, and purification of His-tagged recombinant mLa antigen and affinity purification of anti-mLa Ab. mLa cDNA (30) was modified to encode a C-terminal His_6 tag and cloned into the NcoI-XhoI fragment of pET-28a (Novagen). After 4 h of induction by isopropyl-β-D-thiogalactopyranoside (IPTG), purification was performed by nickel-agarose chromatography (QIAGEN). Anti-mLa serum was obtained from a rabbit immunized with purified recombinant mLa. For affinity purification, a preparative sodium dodecyl sulfate gel containing purified recombinant mLa was electroblotted onto a nitrocellulose strip, and the band corresponding to full-length mLa was cut out and used as an affinity matrix (25). Briefly, the nitrocellulose strips were washed with 100 mM glycine-HCl, pH 2.5, and then with phosphate-buffered saline (PBS) containing 0.05% NP-40, blocked with 5% bovine serum albumin in PBS, and incubated with anti-mLa serum for 3 h at room temperature. The strips were washed, eluted with 100 mM glycine-HCl (pH 2.5), and neutralized with Tris base to pH 7.5. The concentration of the affinity-purified antibody (Ab) was determined by comparison to the concentration of purified rabbit immunoglobulin G (IgG) (Zymed Labs, Inc.) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining.

Immunoblot analysis. Liver and 32D cells were washed once with ice-cold PBS containing 1 mM phenylmethanesulfonyl fluoride (PMSF), and lysed in ice-cold lysis buffer containing 1% NP-40, 0.5 M NaCl, 10 mM HEPES, pH 7.5, 10% glycerol, 1 mM PMSF, and complete protease inhibitor cocktail (Roche). The liver was homogenized on ice with a Brinkmann polytron (Kinematica) and sonicated on ice with an XL-2020 sonicator (Misonix) equipped with a Misonix microtip probe 419 (3.2-mm tip diameter; 16.5-cm length) at 550 W; the liver was sonicated three times for 10 s each time. The samples were then rocked gently for 30 min at 4°C and cleared by centrifugation at 15,000 × g for 10 min. Extraction of 32D cells was the same except without polytron homogenization. Protein quantitation was performed by using Bio-Rad protein reagent. Cleared lysates (50 μg) were separated on a precast 12% Tris-glycine polyacrylamide gel (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was incubated with anti-mLa serum or affinity-purified anti-mLa and visualized with ^{125}I-protein A (Amersham Biosciences).

Blastocyst outgrowth, immunofluorescence (IF), and genotyping. Blastocysts were recovered at 3.5 days postcoitus and cultured individually in Dulbecco's modified Eagle's medium (Gibco catalog no. 10313-021) supplemented with 15% ES cell-certified fetal bovine serum, 15 mM HEPES buffer, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 100 μM nonessential amino acids, 4.5 mM of L-glutamine, and 100 μM of β-mercaptoethanol on gelatinized chamber slides (Nalge Nunc International) at 37°C in 5% CO_2. The blastocysts were visualized using an Olympus LH50A microscope equipped with a Polaroid DMC Ie digital camera. The chamber slides were incubated in 4% paraformaldehyde in PBS for 30 min and washed in PBS, and the embryos were permeabilized and fixed with 100% methanol for 20 min, washed, and blocked with PBS containing 3% bovine serum albumin and 0.1% Tween 20 for 30 min. The embryos were then washed and incubated with TROMA-I antibody (Developmental Studies Hybridoma Bank) at 1:25 in the blocking solution overnight at 4°C. The embryos were washed and incubated with Texas red-conjugated goat anti-rat IgG (1:200 in blocking solution; Vector Laboratories). The embryos were then washed, incubated in 0.5 μg/ml of purified anti-mLa antibody in blocking solution overnight at 4°C, washed, incubated with FITC-conjugated goat anti-rabbit IgG (1:200 in blocking solution), and washed. The embryos were then mounted on slides in SlowFade Light Antifade medium with 4′,6′-diamidino-2-phenylindole (DAPI) (1.5 μg/ml; Molecular Probes) and observed with a Nikon Eclipse E600 microscope equipped with a Nikon digital camera DXM1200F, using ACT-1 and Adobe software. For genotyping, individual embryos were recovered from the slides, transferred to PCR tubes, and processed as described above.

ES cell lines. Embryonic stage E3.5 (embryonic day 3.5) blastocysts were isolated and cultured on mitomycin C-treated mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (Gibco catalog no. 10313-021) for 4 days as described previously (24, 27). The inner cell mass (ICM) was picked, trypsinized, replated, and cultured for 7 days. Undifferentiated ES cell colonies were picked, trypsinized, and expanded.

RESULTS

Ubiquitous expression of La during mouse development. Northern blots of poly(A)$^+$ RNA samples from adult tissues (Fig. 1A) and embryos of 7 to 17 days gestation (Fig. 1B) revealed that mouse La mRNA was readily detectable in all samples. Microarray analysis revealed the pattern of mLa mRNA expression in preimplantation mouse embryos at various stages of development, including morula and blastocyst (Fig. 1C). La mRNA was detected in the unfertilized egg, declined by the four-cell stage, and increased thereafter (Fig. 1C). Results found using the mRNA synthesis inhibitor α-amanitin suggested that the La mRNA observed prior to the four-cell stage was largely due to maternal stores (data not shown). These results revealed that La mRNA is ubiquitously expressed throughout mouse development and in the adult animal.

To determine whether mLa protein was present in preimplantation embryos, antiserum to recombinant mouse La protein was prepared and tested on immunoblots for reactivity to

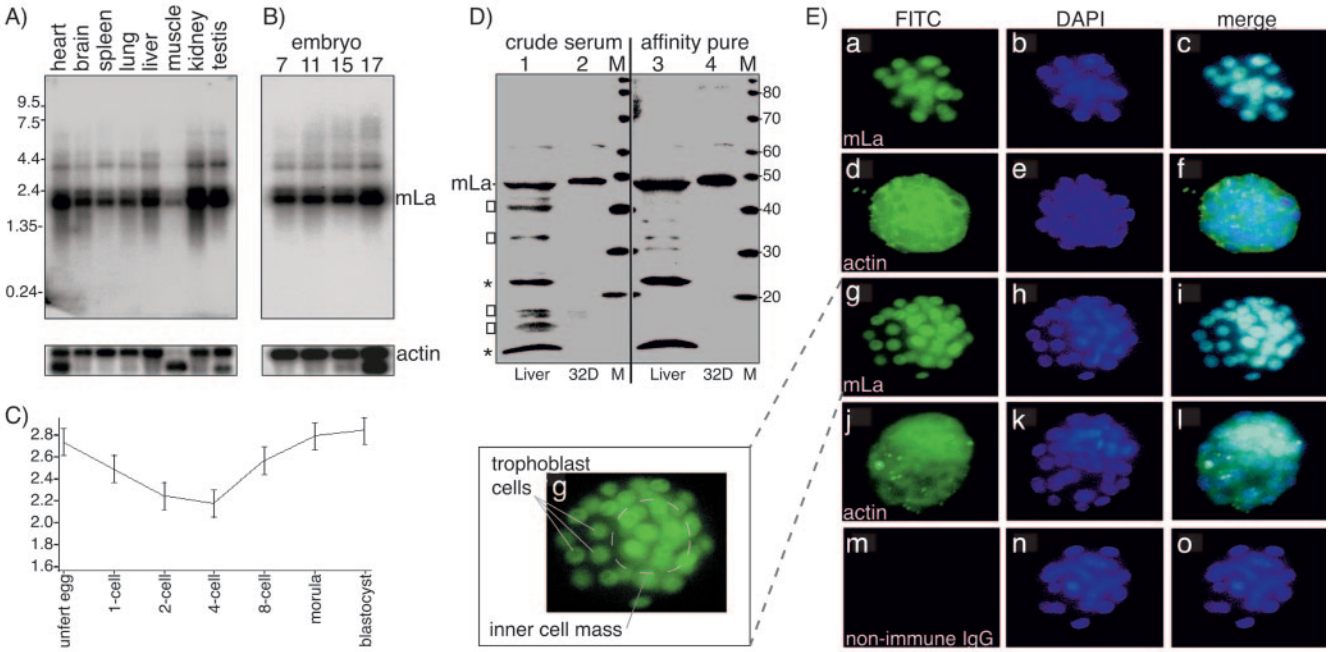

FIG. 1. mLa is ubiquitously expressed throughout development and in adult mice. Northern blots of poly(A)$^+$ RNA from (A) adult tissue and (B) 7- to 17-day-old embryos, probed with mLa cDNA. Size markers are indicated in kilobases on the left. The same blots were probed with beta-actin cDNA (bottom blots). (C) Expression pattern of La mRNA during preimplantation stages of early development as measured by microarray analysis as described previously (12). Morphological stages are indicated along the *x* axis (unfert egg, unfertilized egg). Values are means ± standard errors (error bars) from at least three independent hybridization results obtained for pools of 500 embryos for each stage of preimplantation development. RNAs were extracted from batches of 500 embryos at each stage of development. A universal mouse reference RNA was used for all hybridizations so that all stages could be compared to the same reference points. The *y* axis shows logarithm of signal intensity in arbitrary units relative to the control reference RNA (12). (D) Immunoblotting was used to characterize the Ab used for immunofluorescence shown in panel E. Lanes 1 and 3 contain adult mouse liver extract, and lanes 2 and 4 contain extract from mouse 32D cultured cells, which have previously been shown to express mLa protein (31). The blot on the left was incubated with crude anti-mLa serum, and the blot on the right was incubated with Ab that was affinity purified using highly purified mLa protein as the affinity matrix. Bands indicated by asterisks and brackets (>) are discussed in Results. The positions of molecular mass markers (lanes M) (in kilodaltons) are indicated to the right of the blot. (E) Immunofluorescence using affinity-purified anti-mLa Ab (a to c and g to i), antiactin (d to f and j to l), and nonimmune rabbit IgG (m to o). Primary Ab was visualized with FITC-conjugated anti-rabbit IgG. The samples were also stained with DAPI, and the FITC and DAPI images were merged as indicated.

mouse liver extract as well as extract from a cultured mouse cell line (32D), which served as a positive control (Fig. 1D, lanes 1 and 2) (31). Ab that was affinity purified using immobilized recombinant mLa was also tested (Fig. 1D, lanes 3 and 4). Immunoblotting with the crude anti-mLa serum revealed a major band of approximately 50 kDa representing native mLa protein as well as bands of lower molecular mass, whereas the 32D cultured cells revealed only the major band corresponding to native mLa. The lower bands from the liver extract indicated by asterisks in Fig. 1D appeared to be mLa related, since they were recognized by the affinity-purified Ab, whereas the nonspecific bands indicated by brackets were not (compare lanes 1 and 3). The bands indicated by asterisks presumably reflect sensitivity of La to endogenous proteolysis, as documented previously (11, 13). Since the bands indicated by brackets in lane 1 were diminished in lane 3 relative to the mLa band, we concluded that the affinity-purified Ab was highly specific for mLa. The affinity-purified anti-mLa Ab was then used for immunofluorescence of preimplantation embryos (Fig. 1E).

Several preimplantation embryos collected at E3.5 appeared at the morula and blastocyst stages, as represented in Fig. 1E. In Fig. 1E, panels a to c and g to i reveal immunofluorescence using affinity-purified anti-mLa, while panels d to f and j to l used antiactin as a control. mLa was readily detected in the samples shown and in all other preimplantation embryos examined (not shown). mLa was predominantly nuclear as expected, although cytoplasmic staining also seemed apparent, albeit to a lesser degree, most notably in cells of the ICM (Fig. 1E, panel g). These results confirmed that embryo stages in which mLa mRNA was detected by microarray also produced mLa protein.

Disrupting the La gene in mice. A vector targeting homologous recombination was introduced into mice in which the first two exons of the mLa gene (the second of which contained the initiation AUG) were replaced by a neomycin resistance cassette (Fig. 2A). Germ line transmission of the disrupted allele was confirmed by PCR genotyping of offspring (Fig. 2B). The presence of the PCR band of the expected size from the disrupted allele reflects correct targeting, since one of the primers was outside the targeting vector.

Restriction analysis by Southern blotting with a probe derived from intron 2 of the mLa gene confirmed the targeted locus in $La^{+/-}$ offspring (Fig. 2C). Since the mLa intron probe used is fully represented in the targeting vector, the absence of

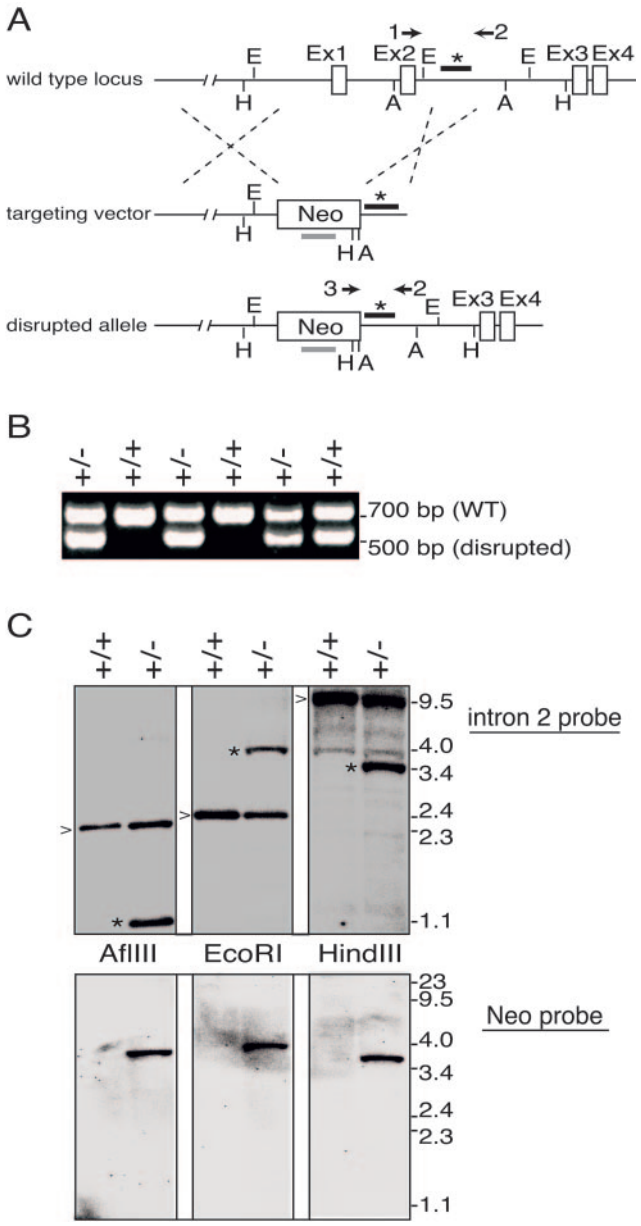

FIG. 2. Creation of a disrupted mLa gene. (A) Schematic showing the structures of the wild-type genomic mLa locus, targeting vector, and disrupted allele. The first 4 of the 12 exons of the mouse La gene are shown. Exon 2 (Ex2) contains the ATG and encodes the first 22 amino acids of mLa. A neomycin resistance gene (Neo) replaced exons 1 and 2 in the targeting vector, which also contained 9.5 kb of genomic DNA upstream of the mLa gene and 0.9 kb corresponding to intron 2. Transcription of Neo is in the direction opposite that of the mLa gene. For the disrupted allele, AflIII and EcoRI sites were replaced by HindIII and AflIII sites, distinguishing it from the wild-type allele (A, AflIII; E, EcoRI; H, HindIII). Arrows numbered 1, 2, and 3 indicate primers used for PCR-based genotyping; primer 2 is common to both alleles (Materials and Methods). (B) Results of triplex PCR genotyping assay performed on DNA from offspring; wild-type (WT) and disrupted alleles are reflected by 700-bp and 500-bp products, respectively; La genotypes are designated above the lanes. (C) Southern blot of genomic DNA isolated from livers of $La^{+/+}$ and $La^{+/-}$ sibling mice. Purified DNA digested with AflIII, EcoRI, and HindIII was probed with a 0.7-kb DNA fragment derived from intron 2, corresponding to the horizontal black bar with an asterisk in the wild-type locus in panel A. The blot was then stripped and reprobed with a probe derived from the Neo cassette. Bands derived from wild-type and disrupted alleles are indicated by arrowheads and asterisks, respectively. The positions of molecular size markers (in kilobases) are indicated to the right of the blots.

TABLE 1. Genotypes of progeny of $mLa^{+/-}$ intercrosses

Developmental stage	No. of progeny with genotype:		
	$La^{+/+}$	$La^{+/-}$	$La^{-/-}$
Live pups	218	394	0
Embryos			
E6.5–E10[a]	20	55	0
E3.5[b]	16	41	23
ES cell lines[c]	14	24	0

[a] Postimplantation embryos.
[b] Preimplantation embryos.
[c] From 22 independent blastocysts.

unexpected additional bands in the $La^{+/-}$ offspring provides evidence that the targeting vector was limited to insertion at the La locus only (Fig. 2C, top panels). Stripping followed by reprobing of the same blot using a probe derived from the Neo cassette revealed only bands of the size predicted for insertion at the La locus with no evidence of additional insertions (Fig. 2C). The PCR and Southern blot data allow the conclusion that the mLa gene was correctly targeted as indicated in Fig. 2A.

La is essential in mice, and haploinsufficiency leads to reduced La mRNA levels. PCR genotyping of pups from $La^{+/-}$ intercrosses revealed no $La^{-/-}$ offspring detected (Table 1). Thorough pathological examination of several $La^{+/-}$ heterozygotes revealed no abnormalities compared to a control $La^{+/+}$ group (data not shown). Although the $La^{+/-}$ mice exhibited no overt phenotype, their mRNA levels were examined nevertheless. RNA isolated from three different tissues from each of five $La^{+/-}$ and $La^{+/+}$ mice were examined by Northern blotting, quantitated, and normalized to beta-actin mRNA (Fig. 3A). La mRNA levels in $La^{+/-}$ mice were reduced to 60 to 70% of the level in $La^{+/+}$ mice (Fig. 3B).

La is required for the early embryonic development of the mouse. To determine the stage at which $La^{-/-}$ embryos could be detected, embryos from $La^{+/-}$ intercrosses were examined at E6.0 to E10.5. No $La^{-/-}$ embryos were identified (Table 1) with no evidence of resorption (not shown), suggesting that $La^{-/-}$ embryos died prior to implantation.

We next analyzed preimplantation embryos from $La^{+/-}$ intercrosses. The PCR genotyping data obtained from a small sample of preimplantation embryos is shown in Fig. 4A. Among 80 preimplantation morulae and blastocysts examined, 23 were found to be $La^{-/-}$, reflecting the expected Mendelian inheritance pattern (Table 1). These data indicate that $La^{-/-}$ embryos did not survive beyond the blastocyst stage.

Nullizygous La blastocysts fail to produce ES cell lines in culture. To determine whether La is required for ES cell development, blastocysts from $La^{+/-}$ intercrosses were processed to establish ES cell lines (19, 24, 27). The PCR genotyping data from a small sample of ES cell lines is shown in Fig. 4B. Thirty-eight ES cell lines were developed from 22 blastocysts; 14 and 24 of the ES cell lines were found to be $La^{+/+}$ and

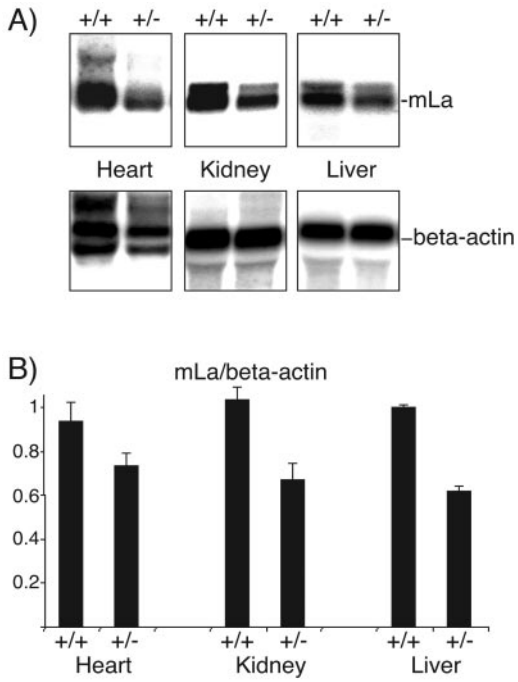

FIG. 3. mLa mRNA levels are decreased in $La^{+/-}$ mice relative to $La^{+/+}$ mice. (A) Examples of Northern blots of total RNA from heart, kidney, and liver tissue samples from $La^{+/-}$ and $La^{+/+}$ mice, using mLa cDNA and beta-actin cDNA probes as indicated. (B) Graphic representation of La mRNA levels normalized to beta-actin mRNA in the same gel lanes. Values are means ± standard errors (error bars) for samples derived from five different mice.

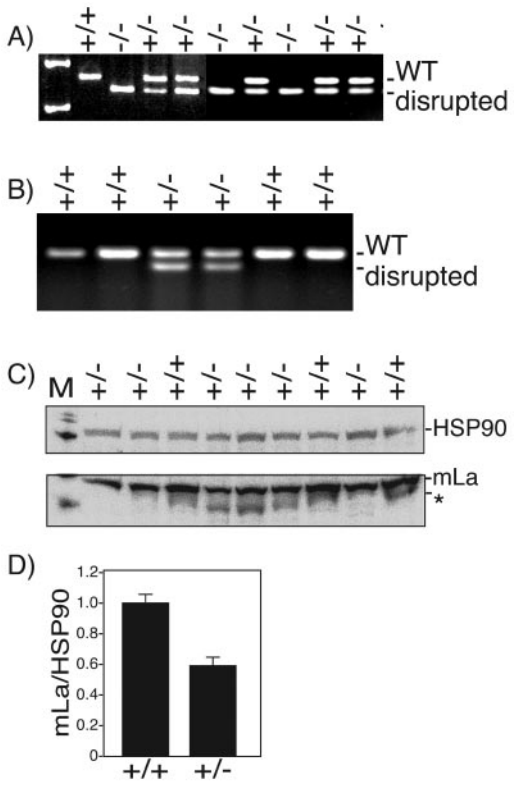

FIG. 4. La protein levels are decreased in $La^{+/-}$ ES cell lines relative to $La^{+/+}$ ES cell lines. (A) Example of PCR genotype data obtained for isolated preimplantation blastocysts. The positions of the bands representing the wild-type (WT) and disrupted mLa genes are shown to the right of the gel. (B) Example of PCR genotype data obtained for ES cells derived from $La^{+/-}$ intercrosses. (C) Immunoblot of extracts made from nine ES cell lines. The La genotypes of the cell lines are indicated above the lanes. The blot was sequentially probed with the antibody for mLa and HSP90 and detected by ^{125}I-protein A. A variable band indicated by the asterisk was detected with the mLa antibodies in both the $La^{+/+}$ and $La^{+/-}$ samples, presumably reflecting the sensitivity of La to endogenous proteolysis irrespective of genotype (11, 13). Lane M contains molecular size markers. (D) mLa protein levels normalized to HSP90 in the same lanes. Values are means ± standard deviations (error bars) for the three $La^{+/+}$ samples or six $La^{+/-}$ samples examined.

$La^{+/-}$, respectively. No $La^{-/-}$ ES cell lines could be established (Table 1).

The $La^{+/-}$ and $La^{+/+}$ ES cell lines were examined for mLa expression. Whole-cell extracts from several of the ES lines, each derived from an independent blastocyst, were examined by immunoblotting and compared to HSP90 (and other controls, not shown) on the same blot (Fig. 4C). A variable amount of a minor band that migrated below the major La band that presumably represents a degradation product was visible in both the $La^{+/+}$ and $La^{+/-}$ ES cell extracts (Fig. 4C and data not shown). $La^{+/-}$ cells expressed about 60% as much mLa protein as $La^{+/+}$ cells did (Fig. 4C and D). These data indicate that La is expressed in ES cells in a gene dose-dependent manner and is required for ES cell development.

mLa is required for maintenance of the inner cell mass in vitro. The requirement of La protein for ES cell development suggested that La was required for maintenance of the ICM. To test this hypothesis, blastocysts isolated at E3.5 were cultured in plastic dishes in vitro, subjected to IF, and genotyped. All of the blastocysts hatched from the zona pellucida and attached to the substratum, their ICMs began to expand, and outgrowths of migrating trophoblast cells started to form by day 3, as exemplified by the three samples shown in Fig. 5A. In contrast to the $La^{+/+}$ and $La^{+/-}$ blastocysts, the ICMs of the $La^{-/-}$ blastocysts began to decrease in mass after day 3 (Fig. 5A and B). ICM loss from the nullizygotes became manifest over the next 3 days, whereas the trophoblast cells as evidenced by their cell type-specific protein TROMA-I (4) persisted (Fig. 5A to D and data not shown). Moreover, nuclei in trophoblast giant cells derived from $La^{-/-}$ blastocysts appeared consistently smaller than those derived from $La^{+/+}$ and $La^{+/-}$ blastocysts. $La^{-/-}$ blastocysts appeared to degenerate by 10 days in culture (Fig. 5C).

When the $La^{-/-}$ blastocyst outgrowths were subjected to IF using anti-mLa, they were clearly reduced compared to those of $La^{+/+}$ and $La^{+/-}$ blastocysts, although a significant amount of mLa protein persisted in the $La^{-/-}$ outgrowths even up to 6 days in culture, presumably reflecting maternally derived protein (Fig. 5B). In contrast to the reduction in intensity observed for mLa by IF and despite the overall decrease in outgrowth mass observed for $La^{-/-}$ blastocysts, TROMA-I was stained with similar intensity in all of the outgrowths (Fig. 5B and D). Figure 5E shows the PCR genotype assay of the blastocyst outgrowths. Taken together, the data described here

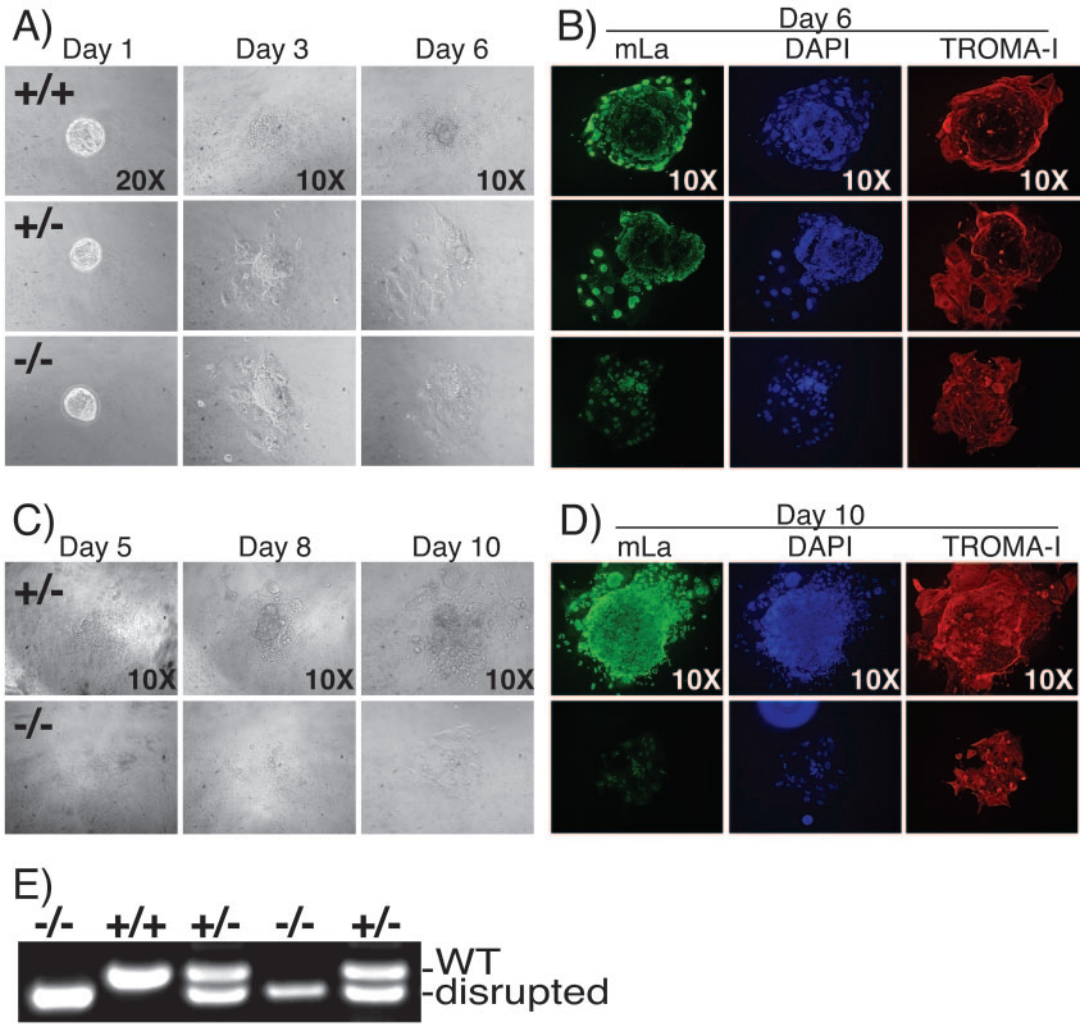

FIG. 5. mLa is required for normal maintenance of the inner cell mass of the blastocyst in vitro. (A to D) Microscopic analysis including immunofluorescence of blastocyst outgrowths cultured in vitro. Panels A and C are visualized by phase-contrast microscopy; panels B and D reveal IF of the day 6 and day 10 panels in panels A and C, respectively, using affinity-purified anti-mLa, DAPI, and anti-TROMA-I, as indicated. The La genotypes are shown as +/+, +/−, and −/− in the leftmost panels of panels A and C. (E) PCR-mediated genotyping of the blastocyst outgrowths shown in panels A to D. The positions of the bands representing the wild-type (WT) and disrupted mLa genes are shown to the right of the gel.

and above show that La is required for development and maintenance of the embryonic stem cells in the blastocyst.

DISCUSSION

The major conclusion that can be drawn from this study is that La is required at the beginning of mammalian development for the production of healthy blastocysts. This is in contrast to the nonessentiality of the yeast La homologs in yeasts (see introduction). This is also in contrast to *Drosophila melanogaster* in which La appeared to become essential, much later, at the midgut level of development (3).

On the basis of the expression profile for La during mouse development, which reflects activation between the four- and eight-cell stage (Fig. 1C), La can be assigned to the mid-preimplantation gene activation group of early embryonic expressed genes, which is consistent with the absence of an effect by alpha-amanitin (12). Microarray data (not shown) revealed that cycloheximide significantly increased the expression of La mRNA. This suggests that the degradation of maternally stored La mRNA is facilitated by a protein that is synthesized after fertilization, similar to other genes showing this expression pattern (12).

As with any targeting strategy, the possibility that another chromosomal locus was disrupted existed. However, since the probe used for Southern blot analysis is fully represented in the targeting vector, the absence of unexpected additional bands in the $La^{+/-}$ offspring provides evidence that the targeting vector was limited to the La locus (Fig. 2C). Likewise, a probe derived from the Neo cassette did not reveal insertions other than at a single La locus (Fig. 2C). Thus, as best as can be determined, the targeting vector appears not to have inserted anywhere other than at the La locus, as designed.

The $La^{+/-}$ mice developed normally although they contained less mLa mRNA than $La^{+/+}$ mice. We examined small

RNAs that are known to be La associated in the $La^{+/-}$ and $La^{+/+}$ ES cells. No significant differences were detected in the levels of the stable RNA polymerase III transcripts of mY3 RNA in the $La^{+/+}$ and $La^{+/-}$ cells (not shown). Preliminary data suggest differences in the metabolism of certain pre-tRNAs in the $La^{+/+}$ and $La^{+/-}$ ES cells (M. W. Bruinsma and R. J. Maraia, unpublished observations), although the significance of this remains to be determined. Future studies will also include characterization of mRNA differences in the $La^{+/+}$ and $La^{+/-}$ ES cells.

Prior data provided evidence that La may be required for development in *Drosophila melanogaster* (3), albeit at a much later stage than observed in mice. As discussed in the introduction, yeast La is nonessential but is conditionally required for the unfolded protein response as well as when essential tRNA genes acquire debilitating mutations. The requirement for La during early mouse development may reflect the fact that metazoan La proteins contain an RRM in their C-terminal domain that is absent in yeasts (22, 33, 34).

In summary, we have shown that nullizygous disruption of the mouse La gene leads to early embryonic lethality, consistent with the observed critical defect in the ICM of the blastocyst observed during blastocyst outgrowth.

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