Detailed Analysis of the Phosphorylation of the Human La (SS-B) Autoantigen. (De)phosphorylation Does Not Affect Its Subcellular Distribution†

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ABSTRACT: The La (SS-B) autoantigen is an evolutionarily conserved phosphoprotein which plays an important role, most likely as an RNA chaperone, in various processes, such as the biosynthesis and maturation of RNA polymerase III transcripts in the cell nucleus and (internal) initiation of translation in the cytoplasm. In this study, the phosphorylation state of this protein from human HeLa and HEp-2 cells was characterized by high-resolution two-dimensional IEF/SDS−PAGE analysis, and phosphorylation sites were mapped by nanoelectrospray mass spectrometry. Furthermore, the effect of phosphorylation at the sites identified on the subcellular distribution of the protein was studied by site-directed mutagenesis. At least 14 isoelectric isoforms were discerned on 2-D gels with La protein from both types of cells. Metabolic labeling in combination with alkaline phosphatase treatment revealed that only a limited number of these isoforms could be attributed to phosphorylation. Four phosphorylation sites, Thr-302, Ser-325, Thr-362, and Ser-366, were mapped by mass spectrometric analysis of the isolated La protein from HeLa cells or the carboxy-terminal half of this protein. The analysis of mutants of La, in which the respective phosphorylated residues were replaced by either a neutral (alanine) or an acidic (aspartate) residue, by microinjection into *Xenopus laevis* oocytes on the one hand and transfection of HEp-2 cells on the other hand revealed that the subcellular distribution of this protein was not affected by these amino acid substitutions. These results strongly suggest that the signals that determine the subcellular distribution of this protein are not regulated by (de)phosphorylation of the target residues examined.

Sera from patients with rheumatic diseases such as systemic lupus erythematosus or Sjögren's syndrome frequently contain antibodies to cellular proteins. One class of autoantibodies is directed to a 47 kDa protein, called La or SS-B (*1, 2*). In cells, the La protein associates with various small RNA molecules to form La ribonucleoprotein particles (La RNPs)[1] (*3*).

The RNA component of a La RNP is in most cases produced by RNA polymerase III, like, for example, (the precursor of) 7S RNAs, 5S rRNA, tRNA, U6 RNA, and Y RNA. In addition, some virally encoded RNAs, such as adenovirus VA RNAs and Epstein−Barr virus EBER RNAs, have been reported to be associated with the La protein (*2*). The primary binding site for the La protein is the 3′-oligouridine stretch, common to all RNA polymerase III transcripts (*4*−*6*). La associates with most RNAs only transiently, because the 3′-oligouridine stretch is removed during maturation of these RNAs. However, the Y RNAs and the virally encoded VA and EBER RNAs retain the La binding site, and La therefore binds these RNAs in a more stable fashion.

Consistent with the binding of La to all newly synthesized RNA polymerase III transcripts, the La protein has been shown to be involved in the synthesis of these RNAs. The La protein is necessary for correct and efficient termination of RNA polymerase III transcription (*7*−*9*) and has recently been identified as an RNA polymerase III initiation factor as well (*10*). Other roles for La in RNA/RNP biogenesis have been demonstrated by the groups of Wolin and Maraia, who showed that La can modulate both 5′- and 3′-end metabolism of pre-tRNA as well as U6 snRNP assembly (*11*−*14*). Also a role in histone mRNA stabilization has been reported (*15*). Potentially related to these activities, purified La has been shown to contain ATP-dependent unwinding activity for DNA−RNA hybrids and double-stranded RNA (dsRNA) (*16*−*18*). In addition, the results of in vitro studies showed that La may also be involved in internal initiation of translation of poliovirus RNA (*19, 20*) and hepatitis C virus

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[1] Abbreviations: AP, alkaline phosphatase; CK-I, casein kinase I; CK-II, casein kinase II; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; IEF/SDS−PAGE, isoelectric focusing/sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Nano ES-MS, nanoelectrospray mass spectrometry; NLS, nuclear localization signal; PKA, protein kinase A; PKC, protein kinase C; RNP, ribonucleoprotein particle.

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RNA (*21*) or in alleviation of translation repression by the leader sequence of HIV-1 mRNA (*22, 23*). An RNA chaperone activity for La, which might be consistent with all of its reported activities, has recently been proposed (*14, 24*).

La is a highly phosphorylated protein with multiple isoelectric forms (p*I* ranging from 6 to 7.5) (*25*−*27*). The phosphorylated residues, which were shown to be mainly serine(s) and to a lesser extent threonine(s) (*27*), are only present in the C-terminal half of the protein, as revealed by the phosphorylation state of the most stable degradation products of the protein (*28*). Recently, the phosphorylation state of Ser-366 was shown to be important for the activity of the protein in RNA polymerase III transcription (*29*).

Complementary DNA clones encoding the complete human La protein have been isolated by several groups (*25, 30*−*32*). The human La protein is composed of 408 amino acids with a predicted molecular mass of 46.7 kDa. In its primary sequence, several structural/functional motifs can be discerned like two RNP motifs, three PEST regions and a potential ATP binding site (*1, 33, 34*). The La protein is well conserved during evolution as revealed by a high degree of amino acid sequence conservation between human, bovine, murine, rat, and *Xenopus laevis* La cDNA clones (*31, 34*−*37*). In addition, La homologues from insects and yeasts have been described (*13, 37*−*40*). Most immunofluorescence studies using anti-La antibodies show predominantly nuclear staining which is consistent with the role of La in Pol III transcription. Other techniques also indicate that the majority of La indeed is present in the nucleus (*41*−*43*). In agreement with the nuclear localization, La has been shown to contain a nuclear localization signal (NLS) at the extreme C-terminus of the molecule (*44*). The nuclear localization is not restricted to the nucleoplasm, since La has been found in the nucleolus as well (*45*). In contrast to the nuclear immunofluorescence, cytoplasmic staining by anti-La antibodies has been reported under certain stress conditions, such as viral infection (*19, 46*−*49*), UV irradiation (*50*−*52*), and inhibition of mRNA synthesis (*53*). Based on these observations, La was proposed to shuttle between the nucleus and cytoplasm and to be involved in nucleocytoplasmic transport of RNA polymerase III transcripts (*54*). However, La was shown not to be involved in the nuclear export of 5S rRNA and hY1 RNA in *Xenopus laevis* oocytes (*55, 56*).

Nanoelectrospray mass spectrometry (Nano ES-MS) has been demonstrated to be a very powerful and sensitive technique to obtain sequence information of polypeptides isolated from polyacrylamide gels (*57, 58*). In the present study, we have mapped several phosphorylation sites in the C-terminal half of the human La protein by this technique. We also show that the recombinant La protein can be phosphorylated in vitro by several kinases such as casein kinase II (CK-II), PKA, and PKC. Two-dimensional IEF/SDS−PAGE analysis of the human La protein reveals that the multiple isoforms cannot all be attributed to different phosphorylation states of the protein. Using phosphorylation site mutants of the La protein, the effect of (the lack of) phosphorylation on its subcellular distribution was investigated by microinjection in *Xenopus laevis* oocytes and by transfection in cultured mammalian cells.

EXPERIMENTAL PROCEDURES

cDNA Constructs. A cDNA clone encoding human La, which has been described previously by Pruijn et al. (*1*), was inserted into the *Eco*RI site of pGEM-3Zf(+) lacking the unique *Bam*HI site (*59*). Amino acid substitution mutants, in which the codons for two or three amino acids were changed (La151/2, La266/9, La338/40, La344/6, La355/6, La362/3, La366/7, La383/4, La388/9), have been described previously (*44*). The numbers indicate which amino acids of the human La sequence were mutated (La151/2: aa 151−152 KG replaced by ID; La266/9: aa 266−269 RGAK replaced by IDAE; La338/40: aa 338−340 GKG replaced by VID; La344/6: aa 344−346 KAA replaced by IDD; La355/6: aa 355−356 VQ replace by GS; La362/3: aa 362−363 TK replaced by ID; La366/7: aa 366−367 SD replaced by GS; La383/4: aa 383−384 KR replaced by GS; La388/9: aa 388−389 ET replaced by ID). Point mutants LaT302A (threonine at position 302 replaced by an alanine), LaT302D, LaS325A, LaS325D, LaT362A, LaT362D, LaS366A, and LaS366D were made using the QuikChange site-directed mutagenesis kit (Stratagene). The integrity of the mutant cDNAs was checked by DNA sequencing.

Enzymes. Casein kinase I (CK-I) and casein kinase II (CK-II) isolated from rat liver, cAMP-dependent protein kinase catalytic subunit (PKA) isolated from bovine heart, and protein kinase C (PKC) isolated from rat brain were obtained from Promega. Trypsin purified from bovine pancreas was obtained from Boehringer Mannheim. Calf intestinal phosphatase (AP) was obtained from Boehringer Mannheim.

Cyanogen Bromide Cleavage. Approximately 2 μg of La protein immunopurified from HeLa cell extracts was incubated overnight at room temperature with 50 mg of cyanogen bromide in 0.5 mL of 70% formic acid. Subsequently, solvents were evaporated in a vacuum centrifuge (1 h), and the remaining material was dissolved in 20 μL of SDS-sample buffer and neutralized by the addition of 5 M NaOH.

In Vivo Labeling of HeLa Cells and HEp-2 Cells. HeLa and HEp-2 cells were labeled for 20 h with [^{32}P]orthophosphate (1 mCi/20 mL of medium) in phosphate-free DMEM (Dulbecco's modified Eagle's medium) supplemented with 5% (by volume) dialyzed fetal calf serum. Cells were washed with PBS, and extracts were prepared by lysis in a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5% NP-40, 1 mM EDTA, 1 mM dithioerythritol, 0.5 mM PMSF. Insoluble material was removed by centrifugation for 15 min at 12000*g*.

Immunoprecipitation/Immunoaffinity Chromatography. Protein A−agarose beads were coated with monoclonal anti-La antibodies [SW5 (60)] by rotation at 20 °C for at least 1 h in IPP_{500} (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% NP-40). The coated beads were incubated with the (^{32}P-labeled) cell extract in 500 μL of IPP_{150} (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40), for 2 h at 4 °C. The precipitated proteins were solubilized in SDS-sample buffer or 2D-sample buffer and analyzed by one- and two-dimensional gel electrophoresis. Large-scale isolations were performed by immunoaffinity chromatography. Briefly, SW5 was produced in protein-free hybridoma medium (PFHM II, Gibco-BRL), isolated from the medium by Protein A−Sepharose selection, and coupled to CNBr-activated Sepharose. La-containing peak fractions from a DEAE-Sepharose col-

umn were loaded on the SW5 affinity column, and after extensive washing with a 1 M NaCl containing buffer, La protein was eluted from the column by 0.1 M glycine, pH 2.5, and the fractions were neutralized immediately after elution.

Dephosphorylation of Immunoprecipitated La. Soluble AP was added to immunoprecipitated La (still bound to protein A−agarose beads), and the mixture was incubated at 37 °C for 2 h. After removal of AP by extensive washing, proteins were eluted with 2D-sample buffer and analyzed by two-dimensional gel electrophoresis.

In Vitro Phosphorylation Assay. In vitro phosphorylation was performed in 20 μL reactions containing 0.5 μg of recombinant wild-type La protein, or mutants thereof, 20 units of kinase (CK-I, CK-II, PKA, PKC), 10 μCi of [γ-^{32}P]-ATP, 10 mM $MgCl_2$, 20 mM Hepes−KOH, pH 7.4, and 0.1% β-mercaptoethanol. After incubation at 30 °C for 30 min, SDS-sample buffer was added, and the proteins were analyzed by 10% SDS−PAGE followed by autoradiography.

In-Gel Digestion. Proteins separated by 10% SDS−PAGE were stained with Coomassie and the bands excised from the gel. After reduction and alkylation of the protein, it was digested with trypsin or Lys-C protease as previously described (*57*, *58*). The resulting peptides were extracted and dried down in a vacuum centrifuge.

Nanoelectrospray Mass Spectrometry and Sample Desalting. The peptide mixture was reconstitued in 10 μL of 5% formic acid solution and applied to a 0.5% formic acid equilibrated microcolumn (Poros oligoR3 sorbent, Perseptive Biosystems, Farmingham, MA). After the column was washed, the peptides were eluted in a stepwise fashion (with 20% methanol, 50% methanol, and 50% methanol/5% ammonia) directly into the spraying needle of the nanoelectrospray ion source, as previously described (*61*). Each fraction was analyzed individually.

All mass spectrometric experiments were carried out on a triple quadrupole mass spectrometer (API III, Perkin-Elmer Sciex, Ontario, Canada), equipped with a nanoelectrospray ion source with a flow rate of 20−40 nL/min (*62*). All experiments were carried out as previously described (*62*, *63*). For Q1 scans, the stepwidth was 0.1 amu and the dwell time 1 ms at unit resolution. For parent and daughter ion scans, the stepwidth was 0.2 amu, the dwell time was 3 ms, and the resolution was set to allow transmission of 3−4 amu.

One- and Two-Dimensional Gel Electrophoresis with Immunoblotting. One-dimensional SDS−polyacrylamide gel electrophoresis was performed using standard methods in a BioRad protean II system. Two-dimensional gel electrophoresis was performed by isoelectric focusing in the first dimension (*64*) and 10% SDS−PAGE in the second dimension. Pharmalyte (Pharmacia) ampholenes with pH ranges 3.0−10.0 and 6.0−8.0 were used for IEF gels in a ratio of 1:4. After two-dimensional gel electrophoresis, the proteins were transferred to nitrocellulose and were subsequently visualized by either autoradiography or immunoblotting with anti-La antibodies (either polyclonal patient antibodies or a monoclonal antibody) and peroxidase-conjugated rabbit anti-human or rabbit anti-mouse antibodies. Patient anti-La antibodies, used for immunodetection, were affinity-purified from serum W10 using bacterially expressed human La protein (*65*). Alternatively, the mouse monoclonal anti-La antibody SW5 (*60*) was used.

In Vitro Transcription/Translation. In vitro transcription/translation was carried out as described previously (*59*). The full-length La(mutant) proteins and all LaΔC380 (mutant) proteins were produced after linearization of the template DNA for transcription with *Hin*dIII and *Ava*II, respectively.

Xenopus laevis Oocyte Microinjection. Oocytes were released from the ovary by treatment with collagenase B (2.5 mg/mL) in Barth-medium (*66*) without Ca^{2+}. In vitro translated proteins were injected into the cytoplasm (40 nL per oocyte) and nucleus (20 nL per oocyte). Oocytes were incubated in Barth-medium supplemented with cycloheximide (100 μg/mL) to inhibit incorporation of [^{35}S]methionine into endogenous proteins. After 18 h of incubation at 18 or 0 °C, the oocytes were manually dissected in J-buffer (20 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 7 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM dithiothreitol, 10% glycerol). Isolated nuclei (N) were immediately precipitated in ethanol, whereas the 'total' oocyte (T) and cytoplasmic (C) fractions were first homogenized in 300 μL of $TN_{150}E$ (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). After removal of part of the yolk and insoluble components by centrifugation, proteins were precipitated by adding 5 volumes of acetone. Pellets were resuspended in SDS-sample buffer, and portions of 6 oocyte equiv were analyzed by 10% SDS−PAGE. To control nuclear injection, samples were mixed with dextran blue (Serva Biochemica) (10 mg/mL). After dissection, only oocytes with blue nuclei were used for analysis.

GFP−La Fusion Protein Constructs. To create a transfection plasmid encoding a GFP−La fusion protein, the human La cDNA was inserted into the *Eco*RI site of vector pCI-neo (Promega, Leiden, The Netherlands). Subsequently, the (enhanced-)GFP open reading frame was isolated from vector pEGFP-C1 (Clontech, Leusden, The Netherlands) by *Nhe*I−*Sal*I digestion and inserted into the *Nhe*I−*Xho*I sites of the La/pCI-neo clone. This resulted in an open reading frame with GFP at the N-terminus followed by a 43 amino acid linker sequence derived from vector and La cDNA 5′ UTR sequences and La at the C-terminus. Similar constructs for the amino acid substitution mutants were obtained by replacing a *Kpn*I−*Kpn*I fragment of the wild-type GFP/La/pCI-neo construct with the corresponding mutant *Kpn*I−*Kpn*I fragment isolated from the mutant cDNA constructs in pGEM-3Zf(+).

Transfection of HEp-2 Cells. Logarithmically growing HEp-2 cells, cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, were transfected with the GFP−La fusion protein transfection plasmids, or the GFP alone construct (pEGFP-C1) as a control, by means of electroporation. Per transfection, 3×10^6 cells were electroporated with 10 μg of plasmid DNA in 0.8 mL of medium at 276 V and a capacity of 950 μF using a Gene Pulser II (BioRad). After transfection, cells were resuspended in 10 mL of culture medium and grown on coverslips. Cells were analyzed by fluorescence microscopy 24 h after transfection.

RESULTS

Two-Dimensional IEF/SDS−PAGE Analysis of the La Protein Isolated from HeLa Cells. Previously, convincing evidence has been obtained that the La protein is phospho-

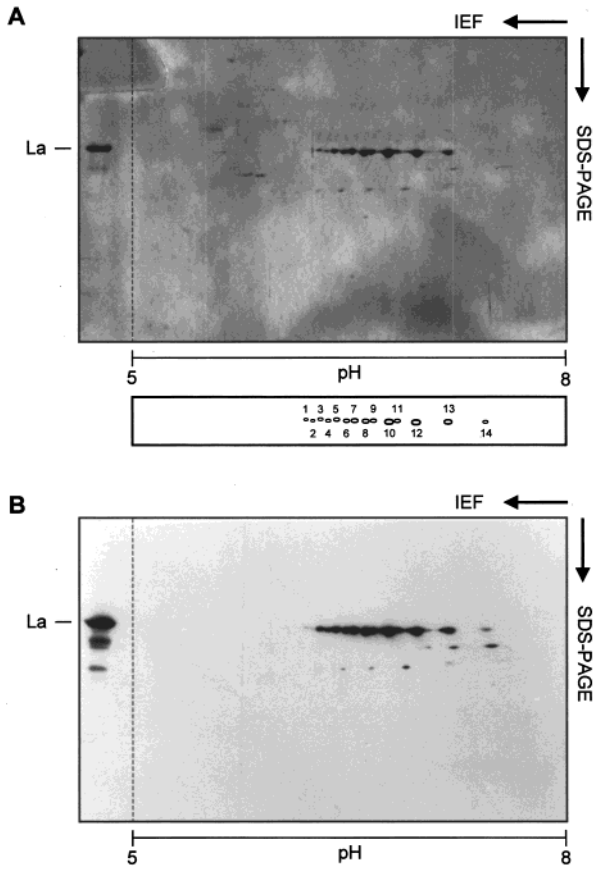

FIGURE 1: Two-dimensional IEF/SDS−PAGE analysis of La protein isolated from HeLa cells. The La protein was isolated from a HeLa cell extract by immunoaffinity purification with monoclonal antibody SW5. The resulting material was fractionated by isoelectric focusing using a linear gradient from pH 5.0 to pH 8.0, followed by SDS−PAGE in the second dimension. The La protein was visualized either by silver-staining (A) or by Western blotting using a patient anti-La serum (B). A schematic representation of the 2D spots including their numbering is given below panel A. On the left, a nonisoelectrically focused sample of the La protein was run in parallel on the SDS−PAGE gel. The position of the full-length La protein is indicated on the left. The lower molecular mass species presumably represent degradation products of La, since these were also reactive with SW5 (results not shown).

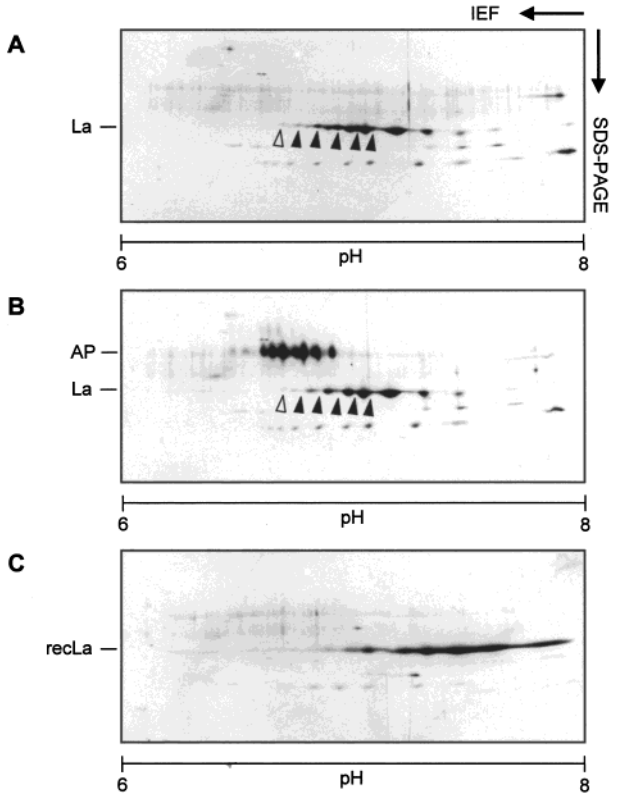

FIGURE 2: 2D IEF/SDS−PAGE analysis of dephosphorylated HeLa La and recombinant La. Immunoaffinity-purified La from HeLa cells was incubated either in the absence (A) or in the presence (B) of alkaline phosphatase and subsequently fractionated by 2D IEF/SDS−PAGE using a linear pH gradient from pH 6.0 to pH 8.0. In parallel, biochemically purified, bacterially expressed recombinant human La protein (C) was fractionated by the same procedure. Proteins were visualized by silver-staining. The positions of HeLa and recombinant (rec) La as well as that of alkaline phosphatase (AP; panel B) are indicated on the left. The closed arrowheads mark those spots that disappeared after treatment with alkaline phosphatase. The open arrowhead indicates the position of a nonabundant isoform that also disappears by alkaline phosphatase treatment (see Figure 3), which is, however, not evident by silver staining.

rylated in the C-terminal half of the protein (*28*). Nevertheless, hardly anything is known on the exact number and location of phosphorylated residues in the protein sequence.

Since phosphorylation of proteins alters their isoelectric points, we first determined the pattern of isoelectric isoforms by two-dimensional isoelectric focusing/SDS−polyacrylamide gelelectrophoresis (2D IEF/SDS−PAGE). The La protein was isolated from a HeLa cell extract by immunoprecipitation or immunoaffinity chromatography with a monoclonal anti-La antibody (SW5) and subjected to 2D IEF/SDS−PAGE. Note that the epitope recognized by SW5 is located in the N-terminal half of the protein (*67*), diminishing the chance that phosphorylation influences the isolation of the protein. The isolated La protein was separated into multiple isoforms with p*I* values ranging from 6.0 to 7.5. The results in Figure 1A show that at least 14 spots were discernible by silver-staining of the 2D gel. To confirm that all of these spots represented La isoforms, the La protein in the 2D gel was visualized by Western blotting using either the monoclonal antibody SW5 or anti-La antibodies affinity-purified from a patient serum. A pattern virtually identical to that observed by silver-staining was obtained with both types of antibodies (Figure 1B and results not shown).

To investigate whether the appearance of isoelectric isoforms could be attributed to phosphorylation, three experiments were performed. First, the isolated La protein from HeLa cells was treated with calf intestine alkaline phosphatase (AP) prior to 2D IEF/SDS−PAGE analysis. Like the untreated La protein, the AP-treated La focused into several spots, some of which were found in both the untreated and AP-treated protein preparation (Figure 2A,B). At least eight spots could be discerned on a silver-stained 2D gel for AP-treated HeLa La. Assuming that La does not contain any phosphate after dephosphorylation by AP, this might indicate that not all isoforms of the La protein can be attributed to differences in the phosphorylation state of the protein. Alternatively, AP might not be able to remove all phosphates from the protein. The isoforms that clearly disappeared by AP treatment (spots numbered 3, 5, 7, 9, and 11 in Figure 1A) each seemed to be shifted toward the most proximal, more basic spot on the 2D gel (spots 4, 6, 8, 10, and 12, respectively). Second, a preparation of bacterially expressed recombinant human La protein, which is supposed to be completely unphosphorylated, was subjected to 2D IEF/

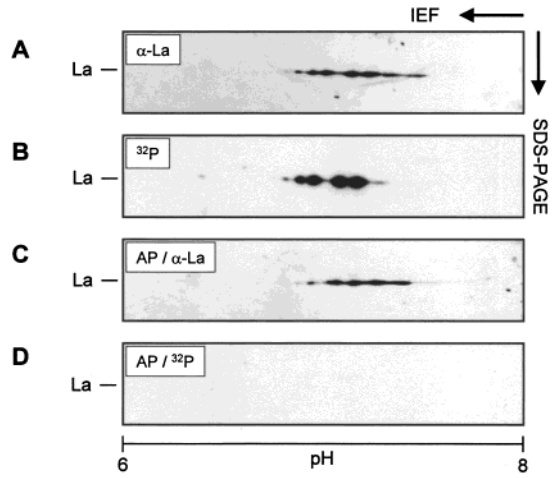

FIGURE 3: 2D IEF/SDS−PAGE analysis of metabolically ^{32}P-labeled HeLa La protein. La protein immunoaffinity purified from extracts of HeLa cells grown overnight in the presence of [^{32}P]-orthophosphate was fractionated by 2D IEF/SDS PAGE. (Radiolabeled) La was visualized by immunoblotting with a polyclonal anti-La antiserum (A and C) or by autoradiography (B and D). The autoradiograms shown in panels B and D were obtained using the blots shown in panels A and C, respectively. The material analyzed in panels C and D was treated with alkaline phosphatase prior to 2D electrophoresis.

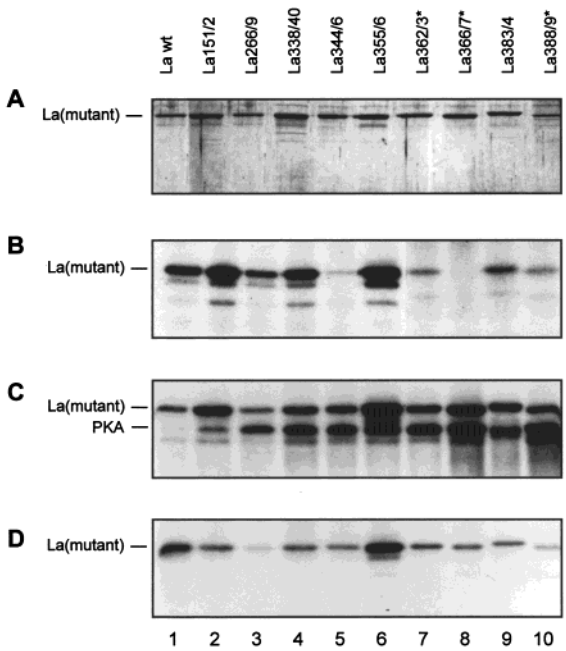

FIGURE 4: In vitro phosphorylation of recombinant human La protein. Bacterially expressed, recombinant human La protein and mutants thereof were biochemically purified by ion exchange chromatography. These proteins were either directly analyzed by SDS−PAGE (Coomassie Brilliant Blue staining) (A) or incubated with CK-II (B), PKA (C), or PKC (D) in the presence of [γ-^{32}P]-ATP prior to SDS−PAGE and autoradiography (B−D). The positions of (mutant) La protein and the 40 kDa catalytic subunit of PKA (panel C, due to autophosphorylation) are indicated on the left. In lanes 1 the wild-type La protein was analyzed, while in lanes 2 through 10 the following mutants were analyzed: La151/2, La266/9, La338/40, La344/6, La355/6, La362/3, La366/7, La383/4, and La388/9, respectively.

SDS−PAGE analysis. Although also this protein was separated into several (at least six) isoelectric isoforms, these were focused in a more basic region of the 2D gel ($7.0 < pI < 8.0$) than the HeLa isoforms (Figure 2C). Possibly the four most acidic isoforms of bacterially expressed La might correspond to the HeLa isoforms focusing in spots numbered 10, 12, 13, and 14, respectively. Third, to unambiguously show which of the isoelectric isoforms of La from HeLa cells is phosphorylated, HeLa cells were metabolically labeled with [^{32}P]orthophosphate, followed by isolation of La and 2D IEF/SDS−PAGE analysis. The results in Figure 3B show that the vast majority of radioactive phosphate was found in four isoelectric isoforms, corresponding to the spots numbered 3, 5, 7, and 9 in Figure 1A. In addition, some radioactive phosphate was found in two isoforms, which most likely correspond to spots 1 and 11. Treatment of this material with AP led to undetectable levels of radioactive La protein (Figure 3D), strongly suggesting that this phosphatase removes virtually all phosphates from the protein. To visualize the La protein in both the untreated and AP-treated ^{32}P-labeled material, the blots were stained by immunostaining with a polyclonal anti-La antibody (Figure 3A,C). The same metabolic labeling experiment was performed with La from HEp-2 cells, which gave identical results (data not shown).

Taken together, these results strongly suggest that only a limited number of La isoforms are due to differences in the phosphorylation state of the protein and that only a subset of La molecules from HeLa cells, i.e., only the molecules focusing in spots 1, 3, 5, 7, 9, and 11, are phosphorylated.

In Vitro Phosphorylation of Recombinant La Protein. In the amino acid sequence of the human La protein, several potential phosphorylation sites are found, such as a casein kinase I (CK-I) site at position 389, casein kinase II (CK-II) sites at positions 225 and 366, and protein kinase C (PKC) sites at positions 331 and 362. The availability of recombinant La protein on the one hand and number of kinases on the other hand allowed in vitro phosphorylation assays to investigate whether some of these sites indeed can be phosphorylated by the respective kinases. Incubation of the recombinant La protein with CK-I, CK-II, cAMP-dependent protein kinase (PKA), and PKC in the presence of radiolabeled ATP showed that La was efficiently phosphorylated by CK-II, PKA ,and PKC (Figure 4A−D, lanes 1), while La appeared to be a poor substrate for CK-I. Hardly any phosphorylation was observed with the latter kinase (results not shown). In addition to the wild-type protein, also several amino acid substitution mutants (*44*) were assayed for in vitro phosphorylation by the same set of kinases. The results are also shown in Figure 4. Interestingly, CK-II, which efficiently phosphorylated the wild-type La protein (Figure 4B, lane 1), did not detectably phosphorylate mutant La366/7, in which the amino acids Ser-Asp at positions 366−367 were replaced by Gly-Ala, while a number of mutants carrying substitutions in the neighborhood of aa 366−367 (e.g., La344/6, La362/3, La383/4 and La388/9) seemed to be phosphorylated with reduced efficiencies. These results strongly suggest that Ser-366 is the only functional CK-II phosphorylation site in the La protein. Although one should be cautious to interpret these data quantitatively, the reduced efficiencies of the phosphorylation of some of the mutants by CK-II might be due to conformational changes that affect the accessibility of the Ser-366 phosphorylation site. In contrast, both PKA and PKC were able to phosphorylate all La mutants analyzed. This suggests either that the phosphorylation site used by these kinases was not destroyed

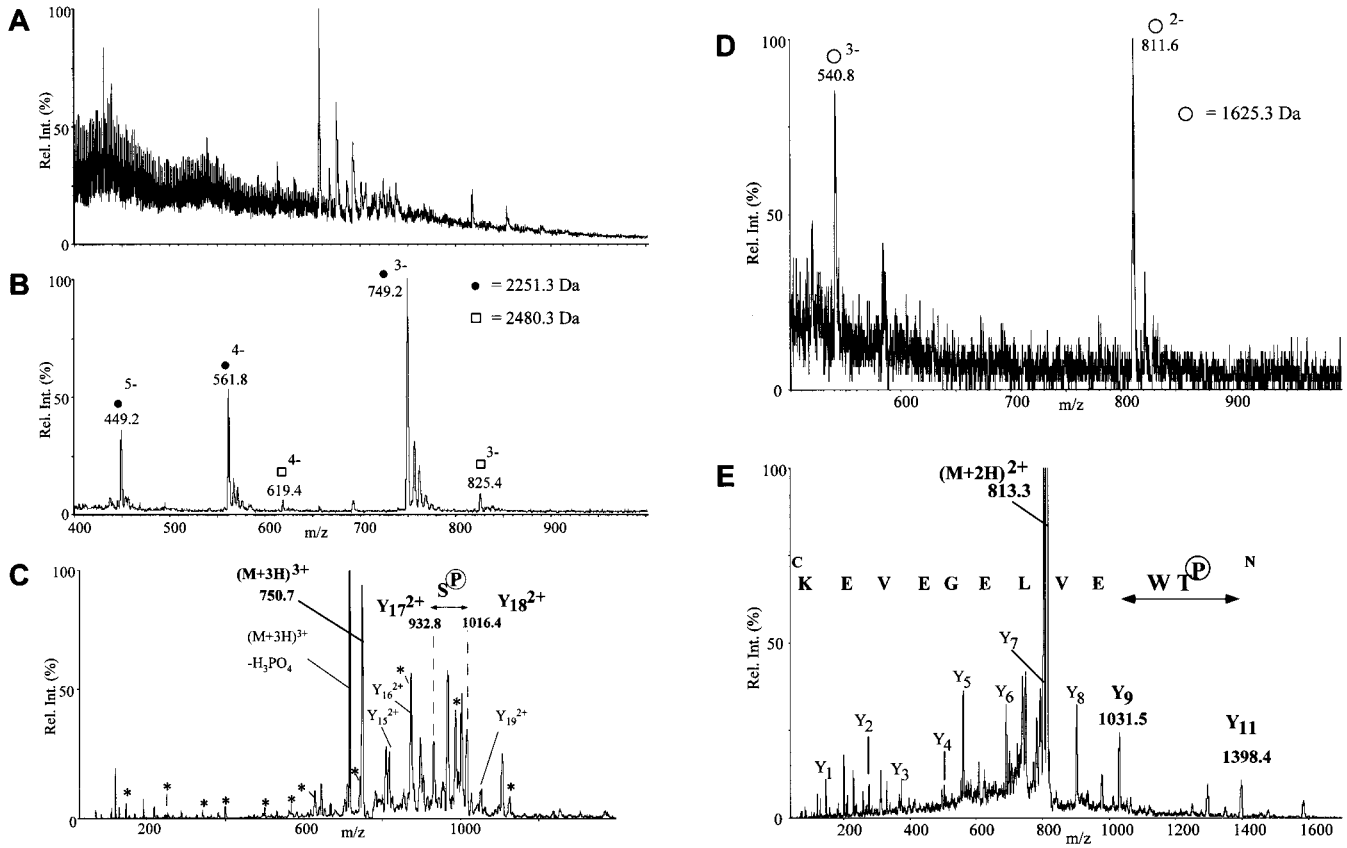

FIGURE 5: Identification of the phosphorylation sites of the C-terminal fragment of the La protein generated by CNBr cleavage. The 20 kDa band separated by SDS−PAGE was digested with Lys-C protease; the peptides were extracted from the gel and purified using a Poros oligoR3 desalting column. Three fractions were eluted with different percentages of methanol (20% and 50%), the last elution with basic instead of neutral pH (50% methanol/5% ammonia). The eluates were analyzed individually. No phosphopeptides were detected in the last eluate (data not shown). (A) Q1 scan in negative ion mode of the first fraction of peptides eluted in 20% methanol from the desalting column. In this scan mode, all peptides are detected. The phosphopeptides (shown clearly in panel B) are not visible above the chemical noise. (B) Parent ion scan for *m*/*z* 79 (PO_3^-) of the same analyte solution. Only those ions are detected which produce an indicative fragment of *m*/*z* 79 (PO_3^-). Marked with bullets are the differently charged ions of peptide 364−383 (2251.3 Da). Marked with squares are the peaks corresponding to peptide 362−383 (2480.3 Da), a partially uncleaved variant of peptide 364−383. (C) Product ion scan of peptide 364−383 (sequence: FAS^{P}DDEHDEHDENGATGPVK). In the first quadrupole, the *m*/*z* value of 750.7 amu (the triply charged ion of peptide 2251.3 Da) was selected, and upon collision with argon atoms in the collision cell, a nested set of fragments is produced. These fragments are separated by the third quadrupole and detected. The mass difference between the doubly charged Y ions Y_{17}^{2+} and Y_{18}^{2+} corresponds to the mass of a serine residue carrying a phosphate moiety. Marked with asterisks are the singly charged Y-ion series, additionally confirming the sequence of the peptide. (D) Parent ion scan for *m*/*z* 79 of the peptide fraction eluted with 50% methanol. The doubly and triply charged states of peptide 300−312 (1625.3 Da) are detected. (E) Product ion scan of the doubly charged peptide ion in the positive ion mode. The Y-ion series allows the determination of the sequence of the peptide. The mass difference between Y_9 and Y_{11} corresponds to the residue masses of a tryptophan plus a phosphorylated threonine, locating the phosphate group to T302.

in any of these mutants or that more than one amino acid in the La protein can be phosphorylated by these kinases. The efficiency of phosphorylation by CK-I was too low to draw any conclusion on phosphorylation of the mutants by this kinase.

Mapping of Phosphorylation Sites of La Protein by Nanoelectrospray Mass Spectrometry. For the identification of the phosphorylation sites of native La protein isolated from HeLa cells, proteolytic digests of the protein were analyzed by nanoelectrospray tandem mass spectrometry. La was isolated from HeLa cell extract by immunoprecipitation with monoclonal anti-La antibody (SW5) and separated from potential contaminants by SDS−PAGE. The La band was excised from the gel and digested with either trypsin or Lys-C protease. In one experiment, the C-terminal part of La was isolated by cleavage with cyanogen bromide prior to electrophoresis, resulting in a 20 kDa and a 30 kDa band on a 12% SDS−polyacrylamide gel. These bands were also excised and subjected to Lys-C digestion. After the peptides were extracted from the gel, they were desalted and eluted in a stepwise fashion. Each fraction was analyzed individually by nanoelectrospray mass spectrometry (for example, see Figure 5). The phosphopeptides were identified using parent ion scanning, which allows the specific detection of a class of molecules which produce a 'reporter' ion upon collision with argon atoms in the collision cell of the mass spectrometer (for example, see Figure 5B,D). In the case of phosphopeptides, the reporter ion is PO_3^- (*m*/*z* 79). Once the phosphopeptides were identified, partial sequences were determined in the same experiment by product ion scans in the positive ion mode, confirming the identity of the peptide and localizing the phosphorylated residue (Figure 5C,E). Using this approach, nine phosphopeptides were detected, corresponding to four different phosphorylation sites (Table 1). All of these sites are located in the C-terminal region of the protein. In some cases (peptides 318−328, 317−328, 313−330, 355−363), it was not possible to sequence the peptide due to overlaying of other peptides or to low

Table 1: Phosphopeptides Observed by Parent Ion Scans after Trypsin or Lys-C Digestion

La peptide	measured molecular mass (Da)	calculated molecular mass (Da)	sequence	modified amino acid
364−383	2250.9	2250.2	FA**S^{P}**DDEHDEHDENGATGPVK	S366[b]
364−384	2406.1	2406.3	FA**S^{P}**DDEHDEHDENGATGPVKR[a]	S366
364−386	2634.4	2633.6	FA**S^{P}**DDEHDEHDENGATGPVKRAR[a]	S366
362−383	2480.3	2479.4	TKFA**S^{P}**DDEHDEHDENGATGPVK[a]	S366
300−312	1625.3	1625.8	EV**T^{P}**WEVLEGEVEK	T302[b]
318−328	1396.2	1395.8	IIEDQQE**S^{P}**LNK	S325
317−328	1523.5	1523.8	KIIEDQQE**S^{P}**LNK[a]	S325
313−330	2281.7	2280.5	EALKKIIEDQQE**S^{P}**LNKWK[a]	S325
355−363	1143.2	1142.6	VQFQGKK**T^{P}**K	T362

[a] The detection of more than one phosphopeptide containing the same phosphorylated residue by incomplete digestion is further proof of the identity of the peptide. [b] Peptides were sequenced by tandem mass spectrometry.

ionization efficiency in the positive ion mode. However, since there is only a single serine or threonine present in these peptides (S325 and T362, respectively), and no other predicted phosphopeptide masses of La match with the observed ones, these two residues are most likely modified. Interestingly, two of the sites (represented by peptides 318−328, 317−328, 313−330, and 355−363) were only observed in a preparation of the intact La protein, which could reflect a low level of phosphorylation at these sites. This could also be the case for peptide 300−312, which was only identified and mapped to residue T302 when analyzing the 20 and 30 kDa bands obtained after cyanogen bromide cleavage (Figure 5D,E). The predominant phosphorylation site was found to be at S366, the site that was uniquely phophorylated by CK-II in vitro and recently reported by Fan et al. (*29*).

Influence of La (De)phosphorylation on Subcellular Localization. It has been shown before that nuclear import of the La protein is energy-dependent and is governed by a nuclear localization signal (NLS) located in the extreme C-terminal part of the protein. In addition, using mutants lacking a functional NLS, regions involved in nuclear retention and nuclear export have been mapped also in the C-terminal part of the protein. The localization of the phosphorylation sites identified within or close to these regions prompted us to investigate whether phosphorylation at a certain position affects the subcellular localization of the protein. Therefore, a number of new amino acid substitution mutants were made, in which the phosphorylated amino acids were individually replaced by either a neutral residue, alanine, or an acidic residue, aspartate, which physicochemically resembles a phosphorylated residue and therefore might be considered a constitutively phosphorylated molecule. The subcellular localization of these mutants and, as a control, the wild-type La protein was studied in two distinct systems, *Xenopus laevis* oocytes (microinjection) and transfection of a human cell line with La(mutant)−green fluorescent protein fusion constructs. For oocyte microinjection, the La(mutant) cDNA clones were transcribed and translated in vitro in the presence of ^{35}S-labeled methionine. The radiolabeled La protein was first injected into the cytoplasm of *Xenopus laevis* oocytes, and, after an overnight incubation, the intracellular distribution of the La protein was determined by dissecting oocytes into cytoplasmic and nuclear fractions, followed by SDS−PAGE analysis. As observed before (*44*), an efficient nuclear accumulation of wild-type La protein was obtained in this system, while a characteristic degradation product of La representing the N-terminal half of the protein (lacking the NLS) failed to enter the nucleus (Figure 6A). A similar nuclear accumulation was observed with all serine/threonine substitution mutants, LaT302A, LaT302D, LaS325A, LaS325D, LaT362A, LaT362D, LaS366A, and LaS366D (Figure 6A), indicating that the function of the nuclear localization signal is not abrogated by (de)phosphorylation at one of these positions. In a second experiment, the same set of La(mutant) proteins, but now produced using a template RNA terminating 5′ from the NLS-encoding sequence leading to C-terminally truncated proteins (C-terminus at position 380) lacking the NLS, were microinjected into the oocyte nuclei. This approach allows us to study the effect of the mutations on nuclear retention of the protein (*44*). If such molecules would leave the nucleus, they will not be re-imported due to the absence of a functional NLS. Also in this case all La(mutant) proteins analyzed remained in the nucleus during an overnight incubation (Figure 6B), strongly suggesting that also nuclear retention of La is not modulated by (de)phosphorylation of these residues.

To study the effect of the mutations in a mammalian system, fusion proteins of the La(mutant) protein and GFP were expressed in transfected HEp-2 cells, and their localization was examined 24 h after transfection. Also in these cells the distribution of all of the mutants was indistinguishable from that of the wild-type protein (Figure 7 and results not shown), in agreement with the lack of effect of any of the mutations on nuclear import or retention.

DISCUSSION

We demonstrated that the La protein isolated from HeLa cells is phosphorylated at positions T302, S325, T362, and S366. Two of these sites were confirmed by tandem mass spectrometric sequencing (T302 and S366), while the other two sites are inferred by the masses of the phosphopeptides only (S325 and T362). Since there is only one residue which can be phosphorylated in each of the peptides, these two residues are the most likely to be modified.

In the La protein isolated from logarithmically growing HeLa cells, marked differences seem to occur in the level of phosphorylation at these four sites, S366 being by far the most efficiently phosphorylated residue. Despite the location of all four modified residues in a region of the molecule that has previously been shown to harbor subcellular localization signals, phosphorylation at these positions did

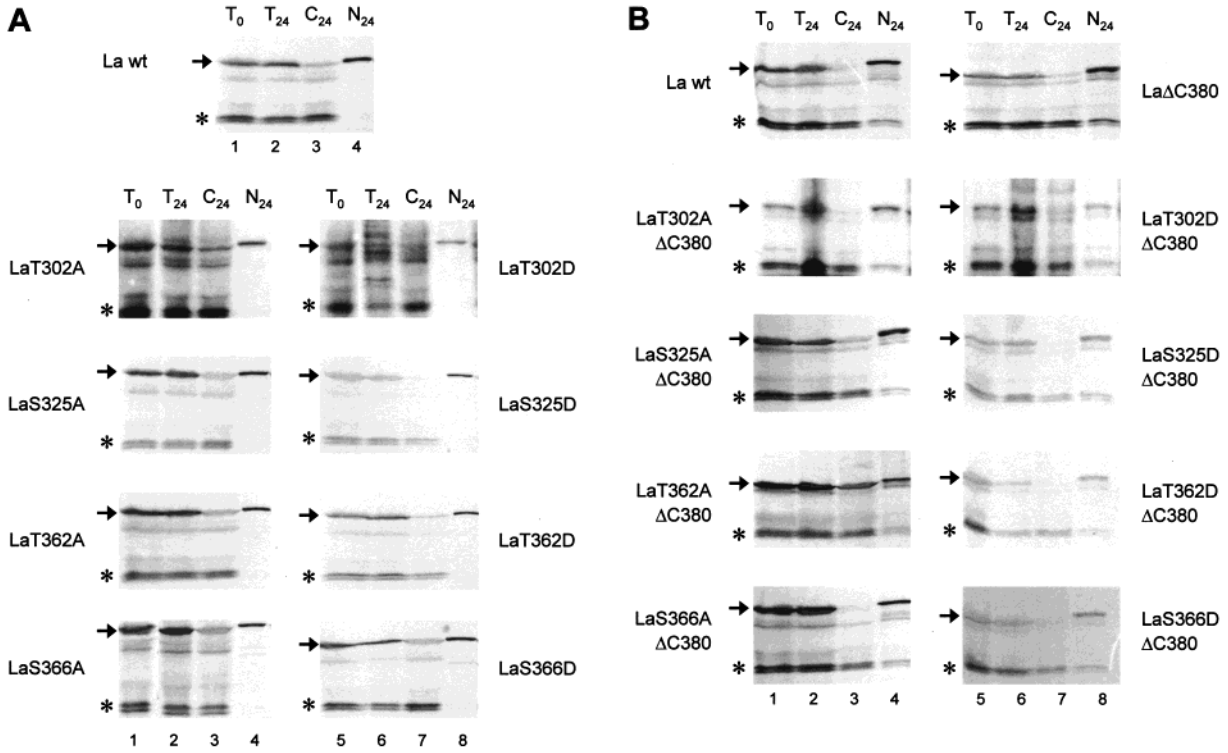

FIGURE 6: Subcellular localization of phosphorylation site mutants in *Xenopus laevis* oocytes. (A) In vitro translated wild-type La protein (La wt) or La mutants in which the phosphorylated amino acids were individually replaced by either an alanine (LaT302A, LaS325A, LaT362A, LaS366A) or an aspartate (LaT302D, LaS325D, LaT362D, LaS366D) were microinjected into the cytoplasm of *Xenopus laevis* oocytes. After a 24 h incubation at room temperature, the oocytes were dissected into cytoplasm and nucleus, and the distribution of the radiolabeled proteins was analyzed by SDS−PAGE and autoradiography. T_0: material from total oocytes immediately after injection; T_{24}, C_{24}, N_{24}: material from total oocytes, cytoplasm, and nuclei, respectively, after a 24 h incubation. The arrows mark the positions of full-length La wt and La mutants. The asterisks mark the positions of the characteristic degradation products of La, which represent the N-terminal half of the molecule lacking a functional nuclear localization signal (*44*). (B) In vitro translated wild-type La protein (La wt), a La mutant lacking the C-terminal 28 amino acids (LaΔC380), or similarly C-terminally truncated La mutants in which the phosphorylated amino acids were individually replaced by either an alanine (LaT302A-ΔC380, LaS325A-ΔC380, LaT362A-ΔC380, LaS366A-ΔC380) or an aspartate (LaT302D-ΔC380, LaS325D-ΔC380, LaT362D-ΔC380, LaS366D-ΔC380) were microinjected into the nucleus of *Xenopus laevis* oocytes. After a 24 h incubation at room temperature, the oocytes were dissected into cytoplasm and nucleus, and the distribution of the radiolabeled proteins was analyzed by SDS−PAGE and autoradiography. T_0: material from total oocytes immediately after injection; T_{24}, C_{24}, N_{24}: material from total oocytes, cytoplasm, and nuclei, respectively, after a 24 h incubation. The arrows mark the positions of La wt, LaΔC380, and the various La mutants. The asterisks mark the positions of the characteristic degradation products of La, which represent the N-terminal half of the molecule lacking nuclear entry and nuclear retention signals (*44*).

not seem to regulate the activity of these signals, since neither neutral (alanine) nor acidic (aspartate) amino acid substitutions resulted in altered subcellular localizations as analyzed in two different systems, *Xenopus laevis* oocytes and transfected HEp-2 cells.

In vitro phosphorylation experiments showed that the bacterially expressed recombinant human La protein can be phosphorylated by CK-II, PKA, and PKC, but not or only very inefficiently by CK-I. A similar analysis of a series of amino acid substitution mutants of La revealed that some of the mutations might reduce the efficiency of La phosphorylation by these enzymes, which is possibly caused by conformational changes in the molecules due to the mutations that affect the accessibility of the phosphorylation site(s). A striking example of such a mutant is La344/6, which is only scarcely phosphorylated by CK-II. Note that in the latter mutant neither a Ser nor a Thr residue is changed. Most interestingly, one of the mutants analyzed, La366/7, was not at all phosphorylated by CK-II, strongly suggesting that Ser-366 is the only functional CK-II phosphorylation site in the human La protein. Recently, CK-II has been reported to copurify with a subset of La molecules, and in the latter study, CK-II was also demonstrated to be able to phosphorylate the recombinant La protein on Ser-366 (*29*), which is part of a CK-II consensus sequence.

Previously, 2D IEF/SDS−PAGE analyses of La have been reported by several investigators. Pizer and colleagues compared the 2D patterns obtained with immunoprecipitated La from metabolically ^{32}P-labeled, uninfected or adenovirus type 5 infected KB cells and concluded that the isolelectric points of the phosphorylated species varied from 7.0 to 7.5, and that additional phosphate residues are added to unoccupied positions on the polypeptide chain after adenovirus infection (*25*). Phosphoamino acid analysis revealed the presence of predominantly phosphoserine and in addition some phosphothreonine. Higher resolution 2D analysis of La isolated from a [^{35}S]methionine-labeled HeLa cell extract led to the identification of eight isoelectric forms with p*I* values ranging from 6 to 7, most of which were also detected when the cells were labeled with radioactive phosphate instead of [^{35}S]methionine (*26*). The presence of phosphothreonine in addition to phosphoserine in the La protein from human cell lines has also been reported by Thomas and collaborators (*68*) and by Pfeifle and colleagues (*27*), although it should be noted that in the latter study La was hyperphosphorylated in vitro by CK-II. Taken together, these

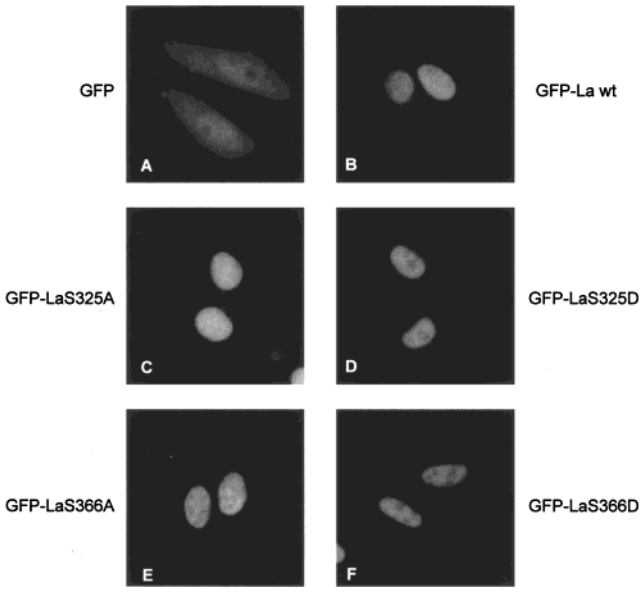

FIGURE 7: Subcellular localization of phosphorylation site mutants expressed as a GFP-fusion protein in HEp-2 cells. Fusion proteins of the La(mutant) protein and GFP were expressed in transfected HEp-2 cells, and their localization was examined 24 h after transfection by fluorescence microscopy. (A) GFP without fusion partner; (B) fusion protein of GFP with La wt; (C−F) fusion proteins of GFP with LaS325A, LaS325D, LaS366A, and LaS366D, respectively.

data are consistent with our results, which demonstrated an even higher number of isoelectric forms with p*I* values ranging from 6 to 7.5 and identified both phosphoserine and phosphothreonine residues in La from human epithelial cell lines. The increased number of isoelectric forms is most likely due to the resolution of the 2D gels, which allowed the separation of spots 2 and 3, 4 and 5, etc. In combination with the observed effects of alkaline phosphatase treatment, these 2D results might correspond to the one-dimensional isoelectric focusing data obtained with two biochemically separated subpopulations of La molecules (*29*), which also displayed multiple isoforms, some of which were only detected in the phosphorylated subpopulation. Interestingly, in vitro phosphorylation of the more basic subpopulation using CK-II converted it to the more acidic species. Altogether, these data indicate that Ser-366 is the major phosphorylated residue in the La protein from logarithmically growing human cell lines and that phosphorylation at this position accounts for the appearance of several isoelectric forms. It is not unlikely that the isoforms numbered 1, 3, 5, 7, 9, and 11 represent the position 366 phosphorylated counterparts of isoforms 2, 4, 6, 8, 10, and 12, respectively. If this is true, this would mean that about one-fourth to one-third of the La molecules from HeLa cells contain a phosphorylated Ser-366, as estimated using the results shown in Figure 1. The level of phosphorylation at the other positions is probably at least 10-fold lower, which implies that no more than a few percent of the La molecules will carry a phosphate at these positions. The results shown in Figures 2 and 3 demonstrate that dephosphorylation of native HeLa La does not reduce the isoelectric complexity of La to a single form. Still at least eight isoforms can be discerned in this material, suggesting that in addition to phosphorylation La might be posttranslationally modified by at least one other type of modification. Interestingly, also recombinant La resolved in a number of isoelectric forms, which are, however, shifted to a more basic region in comparison with dephosphorylated HeLa La. These data suggest not only that La exhibits an intrinsic propensity for charge heterogeneity, as suggested before (*29*), but also that the nature of the yet unknown modification renders the protein more acidic.

A comparison of the primary structure of the La protein from a variety of species reveals that the four (putative) phosphorylation sites are evolutionarily well conserved (*1*). While T302 is found in all known mammalian sequences at an equivalent position, S325 and T362 occur also in *Xenopus laevis* La. Surprisingly, the major phosphorylated residue S366 seems to be lost in the rodent protein, although it is found in *Xenopus laevis*. The residues around these phosphorylation sites also display a high degree of conservation, which in two of the four cases match with consensus protein kinase phosphorylation sequences: a protein kinase C site at position 362 and a casein kinase II site at position 366. The context of T302 in the human protein resembles a cAMP-dependent protein kinase site, and even better matches the consensus sequence in the rodent protein, where an additional lysine is found at the −2 position.

In view of the close proximity of the identified phosphorylation sites to the signals/elements that are involved in the regulation of the subcellular localization of La, it is somewhat surprising that phosphorylation at none of these positions seems to affect the activity of the localization determining signals. So what might be the biological function of these phosphorylations? Recently, evidence has been published that phosphorylation−dephosphorylation of the human La protein on Ser-366 allows the coordination of transcriptional and early posttranscriptional stages of RNA biogenesis (*11, 69*). Such a role is supported by the requirement for La in transcription termination and subsequent reinitiation by RNA polymerase III studied in mammalian cell extracts (*8, 10*) and by the modulation of both 5′ and 3′ processing of Pol III transcripts, such as tRNA and U6 snRNA, by La in both mammalian and yeast systems (*11*−*14, 24*). While the transcriptional activity appeared to be inhibited by phosphorylation of Ser-366, the association of dephosphorylated La with pre-tRNA appeared to attenuate 5′ processing of the transcript (*11*). Since the other phosphorylation sites are all located within or close to a recently identified basic region of La, including residues 329−363, which is required for Pol III transcription activity, it would be very interesting to study the effect of (de)phosphorylation at these positions on Pol III transcription and processing of the transcripts as well. Recent studies of the transcriptional properties of La in a *Xenopus laevis* system led to the conclusion that La does not act as a specific transcription factor, but that the observed activities are best explained by an RNA chaperone function for La. Such a function would also more easily explain the other functional activities reported for La, such as a role in (internal) initiation of translation of (viral) mRNAs, histone mRNA stabilization, nuclear import, and retention of RNA and dsRNA unwinding (*15, 17*−*19, 23, 56, 70*−*72*). Clearly, phosphorylation may modulate such a functional activity of the protein.

Very recently, we observed that upon induction of apoptosis the La protein is rapidly and efficiently dephosphorylated on Ser-366 (*73*). It is tempting to speculate that dephosphorylation abrogates the putative RNA chaperone activity of

this protein, thereby affecting all processes La has been shown or proposed to play a role in.

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