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Phosphorylation of the Human La Antigen on Serine 366 Can Regulate Recycling of RNA Polymerase III Transcription Complexes

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Summary

The human La antigen is an RNA-binding protein that facilitates transcriptional termination and reinitiation by RNA polymerase III. Native La protein fractionates into transcriptionally active and inactive forms that are unphosphorylated and phosphorylated at serine 366, respectively, as determined by enzymatic and mass spectrometric analyses. Serine 366 comprises a casein kinase II phosphorylation site that resides within a conserved region in the La proteins from several species. RNA synthesis from isolated transcription complexes is inhibited by casein kinase II-mediated phosphorylation of La serine 366 and is reversible by dephosphorylation. This work demonstrates a novel mechanism of transcriptional control at the level of recycling of stable transcription complexes.

Introduction

RNA polymerase (pol) III is responsible for synthesizing tRNAs as well as a variety of other small transcripts (reviewed in Willis, 1993). To accomplish accurate and efficient RNA synthesis, pol III must rely on transcription factors (TFs) that bind to the control elements of RNA genes. Adenovirus-associated (VA) RNA and cellular tRNA genes use internal promoters and 3′ terminators, which bind mammalian TFIIIC2 and TFIIIC1, respectively, to direct transcription by pol III (Yoshinaga et al., 1987; Dean and Berk, 1988; Wang and Roeder, 1996). The resulting DNA–protein complex recruits TFIIIB and subsequently positions pol III for transcription initiation (Wang and Roeder, 1996). Although assembly of the preinitiation complex is rate-limiting for initiation of the first round of RNA synthesis, once formed, the complex is stable and can be efficiently reutilized for multiple rounds of transcription by pol III (Bogenhagen et al., 1982; Lassar et al., 1983; Kassavetis et al., 1990; Dieci and Sentenac, 1996).

Despite remarkable progress in understanding the assembly of the preinitiation complex, relatively little attention has been focused on factors that control the recycling of transcription complexes and pol III (Kovelman and Roeder, 1990; Dieci et al., 1993; Maraia et al., 1994; Wang and Roeder, 1995, 1996; Maraia, 1996). With few exceptions, most in vitro transcription assays have used reconstituted systems that contain at least one partially purified fraction. For example, in mammalian systems TFIIIC2, TFIIIB, and pol III have been highly purified, while TFIIIC1 and TFIIIC1′ represent relatively crude yet essential fractions (Wang and Roeder, 1995, 1996). These results indicate that all the mammalian pol III TFs have not yet been identified. Likewise, in the yeast system, pure or recombinant TFIIIB and TFIIIC function suboptimally and transcription efficiency is increased by an extract fraction, B′′, that contains TFIIIE, or by the TFIIIE fraction itself (Dieci et al., 1993; Kassavetis et al., 1995; Ruth et al., 1996). Indeed, TFIIIE is also present in partially purified IIIB and IIIC fractions and confers its effect not through assembly of transcription complexes per se, but through their efficient utilization by pol III (Dieci et al., 1993).

Accumulating evidence indicates that the oligo(dT) termination signal not only defines the 3′ terminus of the nascent transcript but also mediates assembly and utilization of transcription complexes (Allison and Hall, 1985; Yoshinaga et al., 1987; Kassavetis et al., 1990; Chu et al., 1995; Dieci and Sentenac, 1996; Wang and Roeder, 1996). Although the composition of TFIIIC in yeast and human appears to differ significantly, a component of TFIIIC binds to the terminator in both systems (Kassavetis et al., 1990; reviewed in Wang and Roeder, 1996). Terminator-containing but not terminator-lacking templates can efficiently recycle a fraction of pol III to the preinitiation complex by an unknown mechanism (Dieci and Sentenac, 1996). Mammalian TFIIIC1 binds the terminator and mediates assembly of the preinitiation complex, as well as start-site selection by pol III (Wang and Roeder, 1996). Thus, in yeast and mammals the terminator is essential for efficient utilization of transcription complexes, although the mechanisms that determine recycling efficiency remain unclear (Dieci and Sentenac, 1996; Wang and Roeder, 1996). Understanding basic aspects of termination and reinitiation by pol III will require understanding the mechanisms that control the factors involved.

Human La protein binds to the oligo(U) 3′ termini of nascent pol III transcripts and facilitates termination and reinitiation by pol III (Stefano, 1984; Gottlieb and Steitz, 1989a, 1989b; Maraia et al., 1994; Maraia, 1996). La is a modular protein composed of an N-terminal RNA-binding domain and a phosphoserine-containing C-terminal domain (Pizer et al., 1983; Francoeur et al., 1985; Pfeifle et al., 1987; Chang et al., 1994; Kenan, 1995), the latter of which contains a basic region recently shown to be required for the transcription activity of La (J. L. G. et al., submitted). Phosphorylation of La therefore represents a potential control mechanism for pol III transcription, although the significance and exact site of La phosphorylation had been unknown.

Previous studies of the transcription activity of native La protein were complicated by the fact that HeLa-derived La purified by heparin and polyU chromatography exhibited little activity, and no distinction was made between phosphorylated and unphosphorylated La protein (Gottlieb and Steitz, 1989a, 1989b). Here we show that heparin and polyU chromatography yields a phosphorylated form of La that is inactive for transcription,

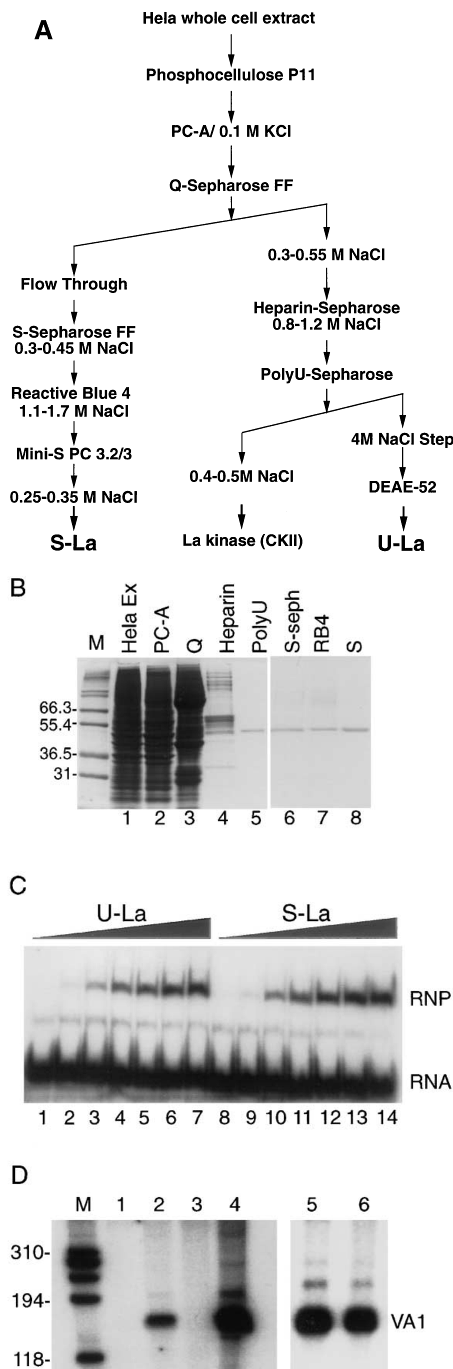

Figure 1. Purification and Characterization of Two Forms of La

(A) Purification schemes for S-La, U-La, and La kinase (see text) from HeLa extract.

(B) SDS–PAGE (12%) and Coomassie staining of native La protein at various stages of purification. Identities of the eluted fractions as indicated above the lanes correspond to the scheme in (A).

(C) RNA-mediated electrophoretic mobility shift assay (EMSA). Zero to thirty nanograms of U-La (lanes 1–7) and S-La (lanes 8–14) were incubated with *hY4* ^{32}P-RNA and analyzed on 6% native polyacrylamide gel. Positions of the free RNA and the RNA–protein (RNP) are indicated to the right.

(D) Pol III transcription activity of purified La proteins. Preassembled immobilized *VA1* transcription complexes were employed in a La-dependent assay as described (Maraia, 1996). Buffer alone (lane 1) or 20 pmol of various La preparations (lanes 2–6) were analyzed for their ability to support *VA1* ^{32}P-RNA synthesis from isolated transcription complexes. Lanes: 2, recombinant La; 3, U-La; 4, S-La; 5, S-La plus buffer alone; 6, S-La plus U-La. After synthesis, ^{32}P-RNA products were purified and analyzed by denaturing PAGE and autoradiography. The position of *VA1* RNA is indicated to the right. The positions of ^{32}P-labeled markers are indicated to the left in nucleotides.

and we describe a protocol that yields unphosphorylated active La protein. Mass spectrometry and other methods revealed that inactive La is phosphorylated at a single site, serine (Ser) 366. The fact that Ser-366 can be specifically phosphorylated in vitro by casein kinase II (CKII) has allowed us to investigate the role of phosphorylation and dephosphorylation in transcription. La phosphorylated at Ser-366 binds RNA efficiently but does not support efficient transcription by pol III. Most importantly, La phosphoprotein can be reactivated for transcription by dephosphorylation.

Results

Separation of Transcriptionally Active and Inactive Forms of La

Although some La antigen was found in each of the standard P11 phosphocellulose (PC) -A, -B, and -C fractions used for pol III transcription, the majority was in the PC-A fraction (data not shown). As outlined in Figure 1A, PC-A La can be separated into two forms by Q-Sepharose chromatography. La that bound Q-Sepharose was purified by heparin, polyU, and DEAE chromatography, and designated U-La (Figure 1A, right). La that flowed through Q-Sepharose was purified by S-Sepharose, Reactive blue 4, and mini-S chromatography, and designated S-La (Figure 1A, left). Both U-La and S-La were highly purified, as demonstrated by SDS–PAGE and Coomassie blue staining (Figure 1B, lanes 5 and 8). U-La and S-La exhibited similar activity in RNA-binding assays (Figure 1C), indicating that both represent native active proteins. It is therefore significant that U-La was inactive for transcription (Figure 1D, lane 3), while an equivalent amount of S-La was active (Figure 1D, lane 4). S-La (Figure 1D, lane 4) was more active than recombinant La (rLa) (Figure 1D, lane 2) and remained active in reactions that contained U-La, indicating that U-La did not contain a general inhibitor of transcription (Figure 1D, lanes 5 and 6).

CKII Copurifies with Inactive La and Phosphorylates La Ser-366

In a pilot investigation, we found that La purified by a conventional approach that employed anion exchange, followed by heparin and polyU chromatography (Stefano, 1984; Gottlieb and Steitz, 1989a, 1989b), contained a robust activity that phosphorylated rLa. This kinase activity was subsequently found to copurify with U-La through the Q and heparin chromatography steps and to separate from La upon gradient elution from polyU-Sepharose (data not shown; see Figure 1A). Biochemical characteristics of this kinase, which was suspected to be CKII on the basis of target site preference (below), are shown in lanes 1–12 of Figure 2A. Although the kinase could not be inhibited by the CKII-insensitive inhibitors staurosporine, K252b, or DMAP (Figure 2A, lanes

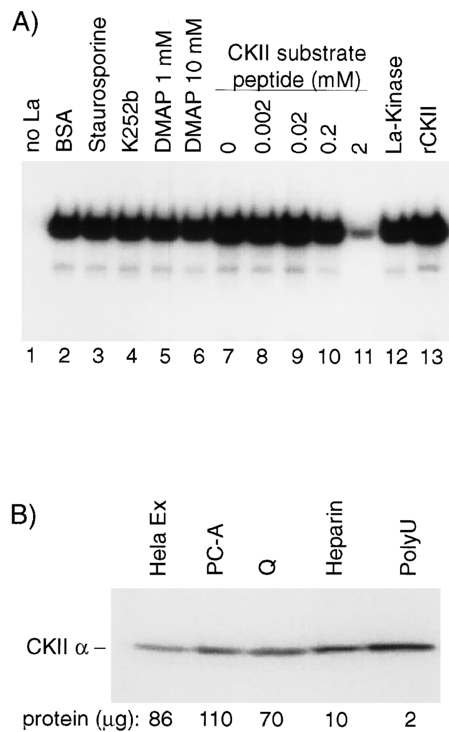

Figure 2. HeLa-Derived La Kinase Is Casein Kinase II

(A) Characterization of La kinase (lanes 1–12): 3 μl of purified La-kinase was incubated with buffer alone (lane 1) or 10 pmol of rLa (lanes 2–13) in the presence of BSA (lane 2), various kinase inhibitors to which CKII is insensitive (lanes 3–6), or synthetic CKII-specific peptide substrate (lanes 7–11). Reactions in lanes 1–11 contained [^{32}P-γ]ATP as phosphate donor while lanes 12 and 13 contained [^{32}P-γ]GTP. Recombinant (r)CKII (0.5 U) was used in lane 13 as a control. Products were analyzed by SDS–PAGE and autoradiography.

(B) Western blot analysis with anti-CKIIα was used to monitor purification of CKII. Identities of the samples are indicated above the lanes and correspond to the purification scheme in Figure 1A; e.g., the polyU lane contains material from the 0.4–0.5 M NaCl fraction eluted from polyU-Sepharose. The amount of protein from each fraction loaded on the gel is indicated below the lanes.

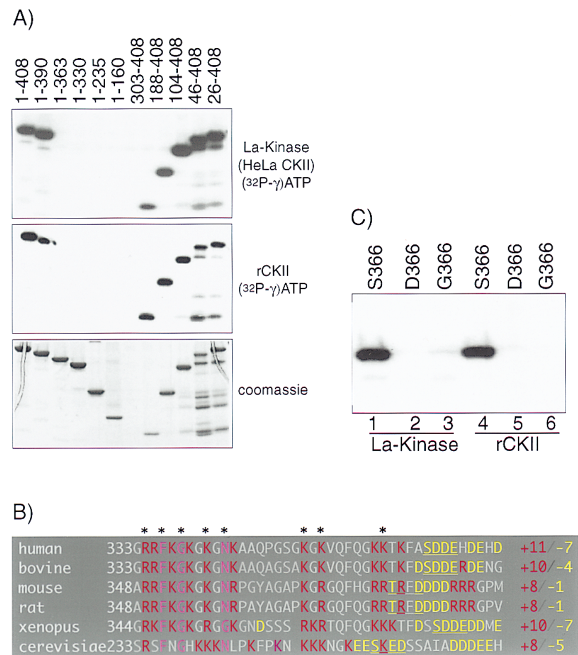

Figure 3. HeLa-Derived Kinase and rCKII Phosphorylate La on Ser-366

(A) Ten picomoles of the La protein indicated was incubated with either 3 μl of HeLa-derived La kinase (upper panel) or 0.5 U rCKII (middle panel) and [^{32}P-γ]ATP under standard kinase reaction conditions, separated by 12% SDS–PAGE, and analyzed by autoradiography (upper and middle), or loaded directly and stained with Coomassie R-250 (lower). Identities of the constructs are listed above the lanes and correspond to the span of residues present in that protein.

(B) Alignment of the region around the human La Ser-366 CKII site with the C-terminal domains of La proteins from other species. The CKII consensus sites (S/T)XX(D/E) are underlined (Bairoch et al., 1996). Numbers at the left of the sequence correspond to amino acid numbering. Basic residues are shaded red, acidic residues including the predicted phosphorylated residue are shaded yellow, and highly conserved residues are shown in pink. Asterisks indicate highly conserved residues N-terminal to the CKII site. Numbers to the right indicate net charge of the regions N-terminal and C-terminal to the phosphorylated residue in the potential CKII sites. Full-length S. cerevisiae La-homologous protein was aligned with full-length human La using Bestfit (GCG package, Wisconsin), which aligned the C-termini as indicated except for the single gap that was introduced upon alignmemt with Xenopus La. Bestfit alignments of the two available insect La homologs with human La revealed that both terminated within the region compared here and are not shown.

(C) Purified wild-type La S366 and point mutants D366 and G366, as indicated above the lanes, were incubated with either HeLa-derived La kinase (lanes 1–3) or rCKII (lanes 4–6) and [^{32}P-γ]ATP under standard kinase reaction conditions, separated by 12% SDS–PAGE, and analyzed by autoradiography.

3–6), it could be inhibited by two CKII-specific inhibitors, heparin (data not shown) and a concentration of peptide substrate known to be inhibitory of CKII activity (K_m = 0.5 mM; compare Figure 2A, lanes 10 and 11) (Kuenzel and Krebs, 1985). Another distinguishing feature of CKII is its ability to use either ATP or GTP as a phosphate donor (Edelman et al., 1987). The HeLa-derived La kinase can use either [^{32}P-γ]ATP (Figure 2A, lanes 2–11) or [^{32}P-γ]GTP (Figure 2A, lane 12) as a phosphate donor, as can rCKII (Figure 2A, lane 13). Although the 0.4–0.5 M NaCl fraction that eluted from polyU-Sepharose contained many Coomassie blue-staining bands (data not shown), Western blotting with anti-CKIIα demonstrated that this antigen copurified (more than 500-fold) with the La kinase activity (Figure 2B).

To identify the region of La phosphylated in vitro, we treated a panel of N- and C-terminal truncated His-tagged purified La proteins with both HeLa-derived kinase and rCKII, and [^{32}P-γ]ATP. Figure 3A shows that the HeLa-derived kinase (Figure 3A, upper panel) and rCKII (Figure 3A, middle panel) are limited in substrate recognition to a region between amino acids 364 and 390 of La. This region contains a CKII phosphorylation site at Ser-366 as identified by the program PROSITE, which searches for the CKII consensus site (S/T)XX (D/E) (Bairoch et al., 1996).

The C-termini of La proteins from several species contain CKII consensus sites, suggesting that this site may be of physiological significance. The residues around the potential CKII site also revealed a high degree of conservation, including basic residues N-terminal to it

and acidic patches C-terminal to it, as shown in Figure 3B.

We made substitution mutations in full-length La that changed Ser-366 to either glycine (La G366) or aspartic acid (La D366). The His-tagged proteins were purified using nickel agarose and tested for CKII-substrate activity (Figure 3C). Wild-type La S366 was readily phosphorylated by HeLa kinase and rCKII (Figure 3C, lanes 1 and 4), while neither La D366 nor La G366 were good substrates for either kinase (Figure 3C, lanes 2, 3, 5, and 6). Longer exposure of this and other gels revealed that La D366 and La G366 were labeled at a low level by CKII (probably due to inefficient phosphorylation of threonine 389; see Discussion). These results demonstrated that Ser-366 is highly favored over all other potential CKII sites in La, of which six are predicted by PROSITE (data not shown).

La is Phosphorylated on Ser-366 In Vivo

S-La was an excellent substrate in our Ser-366-specific kinase assay (Figure 4A, lane 1), and pretreatment with phosphatase had little effect on its phosphorylatability (Figure 4A, lane 2), suggesting that S-La contains a high content of nonphosphorylated Ser-366. By contrast, U-La exhibited very little substrate activity (Figure 4A, lane 3) but could be readily phosphorylated if pretreated with phosphatase (Figure 4A, lane 4). By this assay, S-La was at least as good a substrate as His-tagged rLa (compare Figure 4A, lanes 1 and 5). The substrate activity of rLa was not affected by CIP (Figure 4A, lane 6), suggesting that Ser-366 is not phosphorylated in E. coli. A control mixing experiment demonstrated that U-La did not inhibit phosphorylation of S-La (Figure 4A, lanes 7–9). Thus, U-La does not contain an inhibitor of the kinase; rather, the data indicate that U-La is a poor substrate because it is saturated with phosphate on Ser-366.

La isolated from HeLa cells separates into multiple isoforms (Pizer et al., 1983; Francoeur et al., 1985). We examined La using a one-dimensional vertical slab isoelectric focusing (VSIEF) polyacrylamide gel/Western blot assay (Figure 4B; Maurides et al., 1989). Both U-La and S-La focused into multiple bands, some of which were found in both protein preparations. The distribution of U-La (Figure 4B, lane 1) was shifted toward the acidic end of the gel as compared to S-La. Even in the part of the gel where there was overlap, S-La and U-La exhibited significant differences in the composition of doublet bands (Figure 4B, lane 1, arrows). S-La lacked bands present in U-La and contained bands missing from U-La. Moreover, the most basic forms of S-La disappeared upon treatment with CKII and [^{32}P-γ]ATP (Figure 4B, lanes 3 and 3* [see legend]) as they were converted to more acidic forms found specifically in U-La (Figure 4B, compare lanes 1–3).

It was previously demonstrated by the high resolution phosphopeptide mapping approach of Hunter and colleagues that in vitro phosphorylation of La with CKII yields the same tryptic phosphopeptide as La labeled in vivo (Beemon and Hunter, 1978; Pfeifle et al., 1987). We labeled HeLa cells with ^{32}P-orthophosphate, immunoprecipitated La, digested it with cyanogen bromide

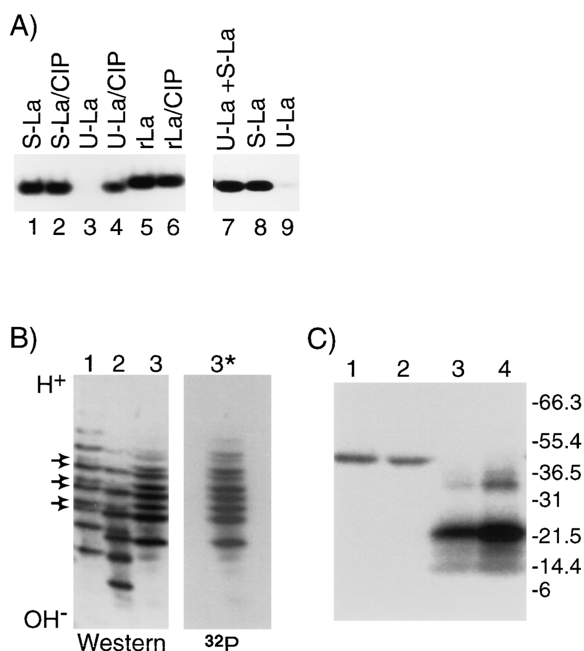

Figure 4. Native La Is Phosphorylated on Ser-366

(A) Substrate activity of La proteins. S-La, U-La, and rLa were subjected to CKII and [^{32}P-γ]ATP in either untreated form (lanes 1, 3, and 5) or after pretreatment with immobilized CIP (lanes 2, 4, and 6), and subsequently analyzed by 12% SDS–PAGE and autoradiography. rLa migrates slower than native La owing to its His-6 tag. A control mixing experiment is shown in lanes 7–9; U-La alone (lane 7), S-La alone (lane 8), and U-La together with S-La (lane 9) were assayed as above.

(B) Vertical slab isoelectric focusing (VSIEF) gel electrophoresis of various forms of La. Equal amounts (0.35 μg) of U-La (lane 1), S-La (lane 2), and S-La that was pretreated with CKII and [^{32}P-γ]ATP (lane 3) were subjected to VSIEF and Western blotting. Lane 3* shows an autoradiogram of the nitrocellulose-containing lane 3 that was exposed to X-ray film prior to incubation with the antiserum. Thus, the bands in lane 3* represent CKII-phosphorylated ^{32}P-La, the substrate of which was native S-La. A control CKII phosphorylation of S-La with nonradioactive ATP produced the same pattern by Western analysis as that shown in lanes 3 and 3* (data not shown).

(C) La protein labeled either in vivo or by CKII in vitro were compared after digestion with CnBr. The undigested La proteins are shown in lanes 1 and 2 and the CnBr-digested proteins are shown in lanes 3 and 4. Lanes 1 and 3 represent CKII-labeled ^{32}P-La and lanes 2 and 4 represent in vivo-labeled ^{32}P-La. Positions of size markers coelectrophoresed in the same gel are indicated to the right in kilodaltons.

(CnBr), and resolved the fragments by SDS–PAGE. Comparative analysis revealed that in vivo-labeled La exhibited the same digestion pattern as did S-La phosphorylated in vitro by rCKII (Figure 4C).

As a more direct approach toward identifying and localizing covalent modifications, mass spectrometry (MS) was performed on our purified La proteins. In the mass spectra of both rCKII-phosphorylated rLa and U-La, two peptides with masses of 2406.5 kDa and 2635.8 kDa, along with many other peptides, were observed (Experimental Procedures). These masses correspond precisely and specifically to the tryptic fragments La 364–384 and La 362–384, each with one additional phosphate group (calculated masses for these phosphopeptides are 2406.3 kDa and 2635.6 kDa). No peptides corresponding to the unphosphorylated form of

these peptides were observed in U-La or CKII-phosphorylated rLa. This establishes that La is phosphorylated in the region of 364–384 both in vitro by CKII and in vivo. Since phosphoserine is the only phospho-amino acid observed in vivo (Pizer et al., 1983; Pfeifle et al., 1987), and since Ser-366 is the only serine in this region, we concluded that Ser-366 is the phosphorylation site in vivo.

The rest of the peptides observed by MS can all be assigned to other sequences in the La protein, and none of those were phosphorylated. Thus, we found evidence for a single phosphorylated peptide in U-La, while the remaining tryptic peptides (with exception of the N-terminal 16 residues, which were not observed) were detected with no evidence of modification. In contrast to U-La and as expected, the unphosphorylated peptide containing Ser-366 was readily detected in S-La. Although a phosphorylated peptide containing Ser-366 was also detectable in S-La, the amount of this relative to the unphosphorylated peptide cannot be determined by MS. Therefore, S-La contains a mixture of unphosphorylated and phosphorylated Ser-366-containing peptide, while the rest of the peptides were unmodified (with the possible exception of the N-terminal 16 residue peptide, which was not observed). In addition to supporting our conclusion that Ser-366 is phosphorylated in U-La and unphosphorylated in S-La, MS suggested that Ser-366 is the only modified residue in La in logarythmically growing HeLa cells (Discussion).

Inhibition and Reactivation of Transcription by Phosphorylation and Dephosphorylation of La

Attempts to detect transcription after dephosphorylation of U-La were unsuccessful, owing to the potent inhibitory effects of phosphatase on pol III transcription (data not shown). Experimental constraints owing to this problem and a limited supply of U-La were circumvented by using affinity-purified His-tagged La. We treated His-tagged La singly or sequentially with CKII plus nonradioactive ATP and/or calf intestinal phosphatase (CIP), purified the products by nickel chromatography, and used equal amounts in transcription assays (Figure 5A). Although mock and CIP treatments of La led to some decrease in transcription (Figure 5A, compare lanes 2, 3, and 5), CKII-phosphorylation inhibited ~80% of the transcription activity of mock-treated La (Figure 5A, lanes 3 and 4). Most significantly, the inhibition mediated by CKII was reversed by CIP treatment (Figure 5A, compare lanes 4 and 6). CIP treatment had little if any effect on nonphosphorylated La (Figure 5A, compare lanes 3 and 5). Assays that monitored CKII and CIP activities from aliquots of these reactions revealed that these enzymes indeed added and removed ^{32}P as expected (data not shown). Figure 5A provides direct evidence that phosphorylation inhibits La activity in the pol III transcription assay (Figure 5A, lane 4), and more importantly, that the resulting phosphoprotein can be reactivated by dephosphorylation (Figure 5A, compare lanes 4 and 6).

We were unable to inactivate La by phosphorylation completely, despite several attempts. Kinetic analysis revealed that the transcription activity of La decreased with CKII treatment but that some activity remained even after 4 hr of exposure to CKII and ATP (Figure 5B). We found that the La products of these CKII reactions could be further phosphorylated upon exposure to a subsequent CKII reaction that monitored phosphorylatability with [^{32}P-γ]ATP. As expected, La recovered after short periods of exposure to the initial CKII reaction could be phosphorylated to a greater extent than La recovered after longer periods of exposure to CKII (data not shown). Thus, this analysis demonstrated that both phosphorylation and transcription inhibition were incomplete, providing a correlation between the extents

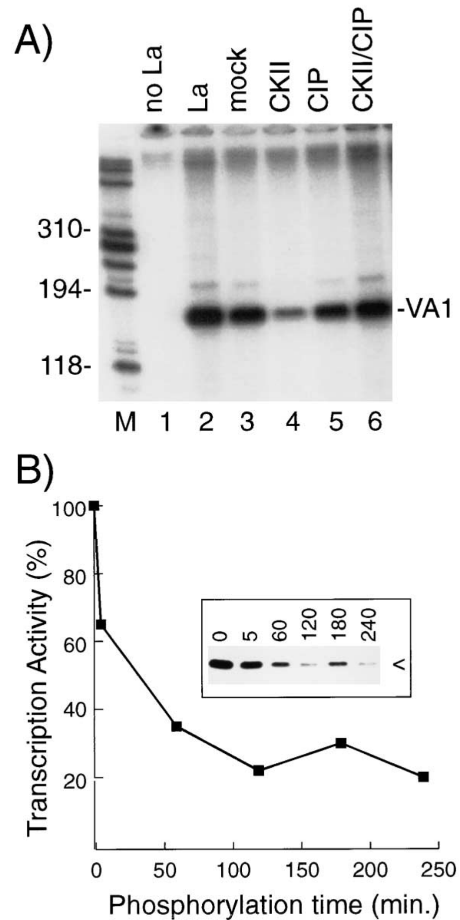

Figure 5. Phosphorylation and Dephosphorylation Inhibits and Reactivates La

(A) Recombinant La proteins (16 pmol each) that were pretreated with CKII/ATP, CIP, CKII/ATP followed by CIP, or mock-treated were used in the pol III transcription assay. Lanes: 1, no La protein added; 2, untreated La; 3, mock-treated La; 4, CKII/ATP-treated La; 5, CIP-treated La; 6, CKII/ATP treatment followed by CIP. Lane M contains denatured markers; sizes in nucleotides are indicated to the left. The position of *VA1* ^{32}P-RNA is indicated to the right.

(B) Kinetics of phosphorylation-mediated transcription inhibition. His-tagged La was incubated with rCKII and nonradioactive ATP and aliquots were removed at the indicated times and subsequently purified by nickel chromatography. Sixteen picomoles of La from each time point were then incubated with *VA1* transcription complexes and pol III for 45 min in the pol III transcription assay, and *VA1* ^{32}P-RNA was analyzed as above. Radioactivity counts in the *VA1* ^{32}P-RNA bands (inset) were quantitated and plotted as percentage transcription activity (amount at 0 min = 100%) against phosphorylation time. Numbers above the lanes in the inset indicate extent of phosphorylation reaction in minutes.

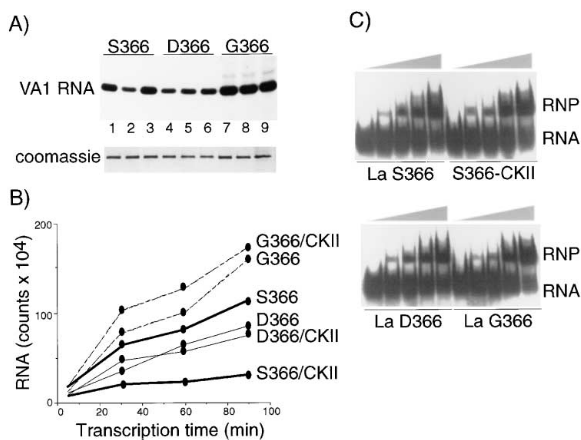

Figure 6. Phosphorylation-Dependent Inhibition of Pol III Transcription Activity of La Is Mediated through Ser-366

(A) Purified wild-type La S366 and point mutants La D366 and G366 were analyzed for pol III transcription activity after no treatment (lanes 1, 4, and 7), CKII/ATP treatment (lanes 2, 5, and 8), and CKII/ATP treatment followed by CIP treatment (lanes 3, 6, and 9). The upper panel shows *VA1* ^{32}P-RNA products of in vitro transcription, and the lower panel shows SDS–PAGE-Coomassie staining of the relative amounts of each purified protein used.

(B) Effect of La phosphorylation on kinetics of transcription complex recycling. Six transcription reactions were set up containing La S366, D366, and G366 with and without pretreatment with CKII/ATP. Aliquots were removed from each reaction at various times thereafter, and the *VA1* ^{32}P-RNA products were purified, electrophoresed, quantitated (counts $\times 10^{-4}$), and plotted against transcription time. Thick solid lines indicate La S366, thin solid lines indicate La D366, and dashed lines indicate La G366. All ^{32}P-RNA products were generated in parallel reactions and quantitated by PhosphorImager analysis of the same exposure.

(C) One to thirty nanograms of the La proteins indicated were incubated with *hY1* ^{32}P-RNA and analyzed in parallel on native 6% polyacrylamide gels. Upper left, unphosphorylated La S366; upper right, CKII-phosphorylated La S366; lower left, La D366; lower right, La G366. Positions of free RNA and RNA–protein (RNP) are indicated.

of phosphorylation and inhibition of La activity. In the next section, we show that the CKII-dependent inhibitory effect on transcription is mediated through La Ser-366.

The Phosphorylation State of La Ser-366 Controls Efficiency of Pol III Transcription

Next, we examined La S366, La G366, and La D366 for transcription activity before and after phosphorylation by CKII and subsequent dephosphorylation by CIP. The La proteins were treated with CKII + ATP or CKII + ATP then CIP, purified by nickel agarose, and used in the pol III transcription assay (Figure 6A). Transcription activity of La S366 (Figure 6A, lane 1) was inhibited by phosphorylation (Figure 6A, lane 2). More importantly, CKII-phosphorylated La could be reactivated by dephosphorylation with CIP (Figure 6A, lane 3). Comparison of the amounts of La protein used in these reactions suggested that G366 exhibits higher activity than S366 and D366 (Figure 6A, lower panel, compare lanes 1, 4, and 7). As expected, CKII and CIP treatments had little effect on the intrinsic activity of La D366 (Figure 6A, lanes 4–6) and La G366 (Figure 6A, lanes 7–9), which lacked the preferred CKII target site. This experiment allowed the conclusion that La Ser-366 mediates the inhibitory effects of CKII phosphorylation on pol III transcription.

Efficient recycling occurs when isolated transcription complexes are supplemented with exogenous La and pol III (Maraia, 1996). We examined the effects of La phosphorylation on the kinetics of recycling, and also examined the La 366 mutants as controls (Figure 6B). La S366 supported efficient recycling, while CKII-phosphorylated La S366 was significantly compromised. In contrast to the inhibitory effects of CKII on La S366, La D366 and La G366 were relatively unaffected by CKII (Figure 6B). Nonphosphorylated and phosphorylated La S366 bound to RNA efficiently (Figure 6C), indicating that CKII treatment did not lead to a general inactivation of La protein. La D366 and La G366 also bound RNA well (Figure 6C). These data support the important conclusion made from the results with S-La and U-La that the transcription activity of La is not attributable solely to RNA-binding activity. We conclude that phosphorylation of La Ser-366 impairs the ability to support efficient recycling of pol III transcription complexes.

Discussion

In this report, we demonstrated that the human La antigen is phosphorylated on Ser-366, and that this modification inhibits the pol III transcription factor activity of La in vitro. Ser-366 is part of a conserved CKII consensus site that resides in a larger conserved region adjacent to a basic motif that was recently shown to be essential for the transcription activity of La (J. L. G. et al., submitted). These results demonstrate that phosphorylation and dephosphorylation of La can be used to regulate pol III transcription in vitro, and suggest that this mode of regulation may be used in vivo. Specificity of CKII for La Ser-366, copurification of CKII and La, and conservation of the CKII consensus site suggest that CKII phosphorylates La in vivo, although this remains to be determined.

The large difference in transcription activity exhibited by S-La and U-La is not attributable solely to RNA binding, since both proteins exhibit similar RNA-binding activity (Figure 1C). The fact that U-La is transcriptionally inactive reconciles the low activity of the La protein previously recovered from HeLa cells by Gottlieb and Steitz (1989b). That study used heparin and polyU chromatography to purify a fraction equivalent to U-La. It is not surprising, therefore, that the La protein used in those studies exhibited little transcription activity when added to anti-La-depleted extract (Gottlieb and Steitz, 1989a, 1989b).

Under certain conditions, La can be hyperphosphorylated by CKII to a form that contains phosphothreonine (Pfeifle et al., 1987). This form of electrophoretically retarded La was never observed in native HeLa La from any source (data not shown). Using mild conditions, CKII-phosphorylated La is nearly indistinguishable from in vivo-labeled La in phosphoserine content, SDS–PAGE mobility, and 2D-IEF pattern, the latter of which revealed a single major phosphopeptide (Pfeifle et al., 1987). Likewise, under these conditions, the major CKII-phosphorylated residue is La Ser-366 (Figure 3B).

Transcription inhibition mediated by CKII was not as complete as that observed for U-La. Although the reason for this is unknown, the following two points should be considered. Data not shown revealed that La phosphorylated with nonradioactive ATP and used for transcription assays was a better substrate in analytical CKII assays that employed radioactive ATP than was U-La (data not shown; see text above describing Figure 5B). This suggests that CKII-phosphorylated La is hypophosphorylated relative to U-La. Thus, although MS detected no unphosphorylated peptide in CKII-phosphorylated La, the analytical kinase assay appears to be more sensitive to the phosphorylation state of La Ser-366 than is MS, and provides both evidence that CKII-phosphorylated La is indeed hypophosphorylated relative to U-La and an explanation for why CKII-phosphorylated La is more active than U-La. In addition, phosphorylation of Ser-366 by CKII in vitro is accompanied by partial phosphorylation of sites that are not phosphorylated in U-La. Analyses not shown, including direct detection by MS, indicate that a fraction of CKII-phosphorylated La is phosphorylated on threonine 389. It is therefore plausible that phosphothreonine 389 partially abrogates the negative influence of phosphoserine 366. We wish to emphasize, however, that threonine phosphorylation does not occur in vivo and therefore appears to be due to an in vitro artifact of CKII (Pizer et al., 1983; Pfeifle et al., 1987). We could nonetheless demonstrate the specific inhibitory effects of phosphoserine 366 with the mutants La D366 and La G366 (Figure 6).

Although a phosphate-containing peptide was detectable in S-La by MS, the fraction of phosphorylated peptide can not be determined by this method. Our Ser-366-specific assay indicates that the great majority of S-La is unphosphorylated at this residue (Figure 4A). In any case, S-La contains basic forms that are not present in U-La, and the presence of these correlates with transcriptional activity. Likewise, U-La contains no unphosphorylated La and this accounts for its lack of transcriptional activity.

Recombinant La resolves into nearly as many IEF bands as native HeLa La, arguing that La exhibits intrinsic propensity for charge heterogeneity. Treatment of HeLa La with CIP shifts the most acidic of eight bands to four basic bands while reducing the doublet band pattern to singlets (data not shown). Phosphorylation under conditions that label Ser-366 specifically produces multiple ^{32}P-labeled bands (Figure 4B, lane 3′). Thus, it appears that phosphorylation on a single site can lead to multiple phosphorylated forms of La. Phosphorylation of Ser-366 can account for most if not all of the La phosphorylation in vivo.

Some transcription factors are activated and some are inactivated by CKII (for example, see Lin et al., 1992; Voit et al., 1992). Loss of CKII activity has been reported to lead to decreased transcription by yeast pol III (Hockman and Schultz, 1996). Although the target of CKII was not identified in that study, it was suggested that it was most likely required for the assembly of yeast pol III transcription complexes and/or initiation (Hockman and Schultz, 1996). Indeed, phosphorylation of TFs appears to be a mechanism to control the assembly of pol III transcription complexes (Gottesfeld et al., 1994; White et al., 1995; McBryant et al., 1996). However, our study is to be distinguished from these, since we examined the utilization of preassembled transcription complexes, not their assembly. The cumulative data indicate that utilization of transcription complexes can be regulated independently of assembly. Phosphorylation and dephosphorylation of La can afford the cell the ability to regulate RNA synthesis without having to disassemble and reassemble entire transcription complexes.

Yeast pol III has been shown to undergo facilitated recycling on the same template (Dieci and Sentenac, 1996). Limiting amounts of mammalian pol III are recycled onto stable transcription complexes at least 5-fold more efficiently in the presence of La than in its absence (Maraia et al., 1994). A role for La in clearing the template of both newly synthesized RNA and polymerase that is paused at the terminator would be expected to increase pol III recycling (Gottlieb and Steitz, 1989a, 1989b). Although recent evidence suggests that La may also be capable of directing pol III to the preinitiation complex, it is unknown whether La is involved in facilitated recycling as described for yeast pol III (Maraia, 1996). Yeast TFIIIE appears to function somewhat analogously to La, since it increases the utilization of stable preinitiation complexes (Dieci et al., 1993; also, see Kassavetis et al., 1995; Ruth et al., 1996). Whether TFIIIE contains a La homologous protein (lhp1) must await further characterization. Unlike other pol III transcription factors, lhp1 is dispensable in S. cerevisiae, suggesting that La plays a regulatory rather than an essential role in RNA biogenesis in yeast and/or that another protein can substitute for lhp1 in this function (Yoo and Wolin, 1994). It will therefore be of interest to determine if lhp1 and/or TFIIIE is phosphorylated in yeast and, if so, under what conditions this modification can regulate pol III transcription. In any case, it seems that high levels of pol III transcription in yeast and mammals requires efficient reinitiation at preassembled initiation complexes, and in both systems, specific factors appear to be involved in controlling the efficiency of reinitiation.

Experimental Procedures

Antibodies, Enzymes, and Peptides

Human recombinant (r)CKII was obtained from Calbiochem (La Jolla, CA); soluble calf intestinal phosphatase (CIP) was from Boehringer Mannheim (Indianapolis, IN); immobilized CIP was from Sigma (St. Louis, MO); CKII peptide substrate RRRDDDTDDD was from GIBCO–BRL (Rockville, MD); anti-CKIIα was from Upstate Biotech, Inc.; and anti-La was from the Centers for Disease Control (Atlanta, GA). Pol III was purified from HeLa extract as described with minor modifications (Sklar and Roeder, 1976).

Fractionation of HeLa Extract

HeLa-S3 cells were grown in suspension in Joklik's MEM. One hundred twenty liters of cells (1.2×10^{11}) yielded 700 ml of whole cell extract (equivalent to S-100 plus nuclear extract); this was loaded onto a 500 ml phosphocellulose (Whatman, P11) column equilibrated with buffer A (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) containing 0.1 M KCl. Purification of La was monitored by following La antigen in Western blots. Material that flowed through P11 (PC-A) was used for purification of the two forms of La. Five hundred milliliters of PC-A was loaded onto a 270 ml Q-Sepharose Fast Flow (Pharmacia) column, and the flowthrough was routed to a 125 ml S-Sepharose Fast Flow (Pharmacia) column. La that flowed through Q-Sepharose was not due to overloading

the Q column (data not shown). Both Q and S columns were preequilibrated with La buffer (LB; 25 mM Tris-Cl [pH 8.0], 20% glycerol, 0.1 mM EDTA, 0.5 mM DTT) containing 0.1 M NaCl. A linear gradient of LB containing 0.1 to 1.0 M NaCl eluted the Q column; La eluted between 0.33 and 0.55 M NaCl and was loaded directly onto a 25 ml Heparin-Sepharose (Pharmacia) column, and a linear gradient of 0.4–2.5 M NaCl in LB was applied. La eluted from Heparin-Sepharose between 0.8 and 1.2 M NaCl, and was dialyzed and loaded onto a 16 ml polyU-Sepharose (Pharmacia) column. After washing, a linear gradient of 0.1–2.5 M NaCl was applied and La kinase activity (monitored as described below) eluted between 0.4 and 0.5 mM NaCl. After washing with 2.5 M NaCl in LB, La antigen was step eluted from polyU-Sepharose with 4 M NaCl in LB. After desalting, this was loaded on a 2 ml DEAE-Cellulose 52 (Whatman) column to remove polynucleotides that leeched off the polyU column (data not shown).

La that flowed through Q-Sepharose bound to S-Sepharose with no change of buffer and eluted between 0.3–0.45 M NaCl in LB. La was then bound to a Reactive blue 4 Sepharose (Sigma) column and eluted between 1.1 and 1.7 M NaCl. This solution was concentrated, desalted, and bound to a Mini-S column (Smart system, Pharmacia), and eluted between 0.25 and 0.35 M NaCl. RNA contamination, as monitored by spectrophotometric scanning at 260/280 nm, revealed that U- and S-La were indistinguishable and contained little if any nucleic acid (data not shown).

Recombinant La Proteins

cDNAs encoding wild-type or truncated La followed by a His-6 tag were expressed in pET28a (Novagen). La D366 and La G366 were generated by oligonucleotide-mediated mutagenesis using full-length His-La cDNA as template. Purification was achieved by nickel-affinity resin according to the manufacturer's instructions (Qiagen), and for Las S366, D366, and G366 this was followed by S-Sepharose chromatography. Concentrations of La proteins were determined by comparison to a standard La (generous gift of D. Kenan, Duke University) following SDS–PAGE and Coomassie staining.

Standard Kinase Assay

In vitro analytical kinase assays were performed in 10 μl reactions containing a premixed pool of 0.2 mM ATP, [^{32}P-γ]ATP (NEN, 3000 Ci/mmol; equivalent to 0.2 μl per reaction), 10 mM $MgCl_2$, 10 mM Tris-Cl (pH 7.5), 1–3 μl of each HeLa fraction to be assayed depending on the stage of purity, or rCKII, and 10 pmol of La protein. After 45 min at 30°C, reactions were stopped by addition of 10 μl of 2× SDS-loading buffer, heated to 100°C for 3 min, and analyzed by 12% SDS–PAGE and autoradiography. For the kinase assay shown in Figure 4A, U-La, S-La, and rLa were incubated with 2 U of prewashed agarose-immobilized CIP in a 40 μl reaction at 37°C for 60 min. CIP-agarose was removed by microfiltration and the soluble La proteins were recovered and used in the kinase assay.

Phosphorylation and Dephosphorylation of rLa with CKII and CIP

Preparative in vitro phosphorylation was performed in 200 μl reactions containing 20 μg of His-tagged La S366, D366, or G366, 500 U of rCKII, 0.2 mM ATP, 10 mM $MgCl_2$, 50 mM NaCl, and 20 mM Tris-Cl (pH 7.5). After incubation at 30°C for 2 hr, the His-tagged proteins were purified by nickel agarose and washed. Seventy-five units of soluble CIP or buffer alone was then added and the mixture incubated an additional 3 hr. After removal of CIP and extensive washing, La proteins were eluted from the nickel agarose, dialyzed, concentrated by microcon-10 ultrafiltration (Amicon), quantitated, and used for subsequent assays.

RNA Binding

hY RNAs are natural ligands for La in vivo and were used here in the electrophoretic mobility shift assay (EMSA). RNA was synthesized by T7 RNA polymerase in the presence of [^{32}P-α]GTP and was gel purified. The templates were designed to generate *hY* RNA that contained native 5′ and 3′ termini. The ^{32}P-RNA was incubated with varying amounts of La protein in LB and poly(C) at 21°C for 20 min and analyzed by electrophoresis on 6% native polyacrylamide gels, followed by autoradiography.

Peptide Mapping

HeLa cells were incubated with ^{32}P-orthophosphate for 10 hr (Pizer et al., 1983). Cell monolayers were washed with PBS and whole cell extract was prepared. Recombinant La labeled in vitro with ^{32}P by CKII and the in vivo ^{32}P-labeled extract were immunoprecipitated with anti-La IgG immobilized on protein A-Sepharose (Pharmacia). The immunoprecipitates were treated with RNase to degrade labeled RNA. The ∼50 kDa ^{32}P-La bands were readily identified by SDS–PAGE and excised, and the gel slices containing the bands were crushed, equilibrated with 70% formic acid, subjected to 50 mg/ml of CnBr (Sigma) in 70% formic acid, and analyzed by SDS–PAGE (10%–27% polyacrylamide) (Barsh and Byers, 1981).

La-Dependent Pol III Transcription

Preinitiation complexes preassembled on the *VA1* RNA gene were isolated as described and aliquotted to multiple tubes (Maraia, 1996). Pol III (20 U) and La (16 pmol) were added in a total volume of 25 μl and incubated for 45 min, or these reactions were scaled up and aliquots were removed at various times, and RNA prepared. Quantitation was performed using a PhosphorImager equipped with ImageQuant software (Molecular Dynamics).

Vertical Slab Isoelectric Focusing and Western Blotting

The procedure described by Maurides et al. (1989) was used with slight modifications. Pharmalytes (pH 5–8 and pH 3–10) (1:1, Pharmacia) were used at a concentration of 6% in the gel and 2% in the overlay and sample buffers. A 17 cm × 1 mm gel was electrophoresed for 21 hr at 2.5 mA with a 1000 V limit. A DSG-200 electrophoresis unit (CBS Scientific, Del Mar, CA) was used with a circulating pump set at 17°C. The upper reservoir contained the anode and 0.01 N phosphoric acid, and the lower chamber contained the cathode and 0.02 M NaOH. The VSIEF gel was electroblotted to Protran nitrocellulose (Schleicher and Scheull, Keene, NH) in 1× Tris–glycine–SDS buffer (Biorad) containing 20% methanol at 100 V for 1.5 hr at 10°C. The blot was incubated with anti-La and processed using ^{125}I-protein A (Amersham).

Mass Spectrometry

A matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Chait and Kent, 1992) (model Voyager-DE, PerSeptive Biosystems, Framingham, MA) was used. One hundred nanograms of sequencing–grade modified trypsin (Boehringer Mannheim) was added to 10 μl of rLa or rCKII-phosphorylated rLa, both at 0.12 μg/μl in 20 mM HEPES (pH 7.9), 20% glycerol, and 0.2 M NaCl. This was incubated at 37°C for 2 hr, after which an equal volume of acetonitrile was added. One microliter of the resulting mixture was combined with 1 μl of working matrix solution (2-fold dilution of 2,5-dihydroxybenzoic acid in 1:1 water:acetonitrile) on the sample plate of the spectrometer. The sample plate was dried in ambient air, inserted into the spectrometer, and an MS spectrum of the tryptic peptide mixture was obtained. Similar trypsin digestions were carried out using 5 μl of U-La (0.25 μg/μl in LB containing 0.2 M NaCl) with 100 ng of trypsin and 5 μl of 50 mM ammonium bicarbonate (pH 8). One microliter of S-La (0.8 μg/μl in LB containing 0.4 M NaCl) was digested with 50 ng of trypsin and 3 μl of 50 mM ammonium bicarbonate. The MS spectra of the resulting tryptic peptides are described in the text.

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