Regulation of *in Vitro* Nucleic Acid Strand Annealing Activity of Heterogeneous Nuclear Ribonucleoprotein Protein A1 by Reversible Phosphorylation†

Haitham Idriss,‡ Amalendra Kumar,‡ Jose R. Casas-Finet,§ Hong Guo,‖ Zahi Damuni,‖ and Samuel H. Wilson*,‡

Sealy Center for Molecular Science, The University of Texas Medical Branch, Galveston, Texas 77555, Structural Biochemistry Laboratory, Program Resources Inc./DynCorp, National Cancer Institute—Frederick Cancer Research and Development Center, Frederick, Maryland 21702, and Department of Cellular and Molecular Physiology, State University of Pennsylvania College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033

Received April 13, 1994; Revised Manuscript Received July 8, 1994⊗

ABSTRACT: Phosphorylation *in vivo* of several proteins in the mammalian heterogeneous nuclear ribonucleoprotein complex (hnRNP), including A1, has been observed and proposed as a regulatory step in pre-mRNA splicing [Mayrand, S. H., Dwen, P., & Pederson, T. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 7764–7768]. We examined the ability of recombinant hnRNP protein A1 to act as a substrate for a number of purified Ser/Thr protein kinases *in vitro.* A survey of seven protein kinases showed that A1 was heavily phosphorylated by protein kinase C (PKC) and also was phosphorylated by casein kinase II, protamine kinase, and protein kinase A. In contrast, autophosphorylation-activated protein kinase and two forms of myelin basic protein kinase failed to phosphorylate A1. Proteolysis with trypsin and V8 protease revealed that PKC phosphorylates A1 at three main sites, two in the N-terminal domain (spanning residues 2–196) and one in the C-terminal domain (spanning residues 197–320). Amino acid sequencing revealed that these sites were Ser^{95}, Ser^{192}, and Ser^{199}; phosphorylation at Ser^{192} was more abundant than at Ser^{95} and Ser^{199}. Phosphorylation by PKC inhibited the strand annealing activity of A1. Protein phosphatase 2A, but not protein phosphatase 1, dephosphorylated A1 and reversed the inhibitory effect of PKC phosphorylation on the strand annealing activity. A conformational change in the C-terminal domain of A1 was observed upon PKC phosphorylation, and this was associated with a decrease in A1's affinity for single-stranded polynucleotides. The results are consistent with a role of phosphorylation of A1 in regulating its strand annealing activity *in vivo.*

After pre-mRNA synthesis, nascent RNA is complexed with a set of globular proteins, termed heterogeneous nuclear ribonucleoproteins (hnRNPs).[1] These proteins participate in several processes including pre-mRNA packaging, polyadenylation, splicing, and transport of mRNA to the cytoplasm [Choi *et al.*, 1986; Sierakowsa *et al.*, 1986; reviewed in Dreyfuss *et al.* (1993)]. The hnRNPs include a core of six polypeptides, ranging in molecular mass from 32 000 to 44 000 Da, which are designated as A1, A2, B1, B2, C1, and C2, respectively (Beyer *et al.*, 1977; Kumar *et al.*, 1986, 1987). These proteins are present in an apparently invariant stoichiometry of 3:3:1:1:3:1, respectively (Barnett *et al.*, 1989, 1991; Huang *et al.*, 1994). In addition, groups of 68–120-kDa proteins are also identified as constituents of the hnRNP complex by immunopurification with monoclonal antibodies to core hnRNP proteins (Pinol-Roma *et al.*, 1988).

hnRNP A1 is a 34-kDa protein of 320 amino acids (Cobianchi *et al.*, 1986). The protein is organized in two well-defined domains that can be separated by mild proteolytic cleavage (Cobianchi *et al.*, 1986; Kumar *et al.*, 1986, 1990). The N-terminal domain, composed of 196 amino acids, is a globular protein and is identical to the well-known calf thymus helix destabilizing protein UP1. It consists of two subdomains termed UP1a and UP1b, both of which contain the conserved nucleic acid binding sequences termed RNP-1 (an octapeptide) and RNP-2 [a hexapeptide; Merrill *et al.*, 1988; reviewed in Kenan *et al.* (1991)]. The 124-residue C-terminal domain of A1 is rich in glycine (40%) and has little predicted secondary structure (Cobianchi *et al.*, 1986; Riva *et al.*, 1986; Wilson, 1990). A1 binds to single-stranded nucleic acid with moderate cooperativity, which probably occurs through protein–protein interactions between the C-terminal domains of adjacent nucleic acid-bound A1 molecules (Wilson, 1990; Kumar *et al.*, 1990; Cobinachi *et al.*, 1988).

The precise functional role(s) of each hnRNP protein remains unclear; yet, the fact that pre-mRNA is associated with a unique combination of hnRNP proteins under splicing conditions has suggested the idea that RNA packaging into hnRNPs could play a central role in pre-mRNA processing (Bennett *et al.*, 1992). hnRNP proteins show affinity for pre-mRNA sequences, and it has been reported that A1 and UP1 interact specifically with the 3′ splice site (Swanson & Dreyfuss, 1988; Buvoli *et al.*, 1990). By selection and amplification from pools of random-sequence RNA, a consensus high-affinity binding site, UAGGGA/U, was identified for A1; this site resembles the consensus sequence of vertebrate 5′ and 3′ splice sites (Burd & Dreyfuss, 1994). The essential human splicing factor SF2/ASF and hnRNP A1 regulate alternate pre-mRNA splicing *in vitro* through antagonistic effects on 5′ splice site selection; a higher

† Z.D. was supported, in part, by NSF Grant CMB-9019882.

* Correspondence should be addressed to this author at Sealy Center for Molecular Science, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1068.

‡ The University of Texas Medical Branch.

§ National Cancer Institute.

‖ State University of Pennsylvania College of Medicine.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: ds, double stranded; hnRNP, heterogeneous nuclear ribonucleoprotein; AK, autophosphorylation-activated protein kinase; MBPK, myelin basic protein kinase; CPK, cytosolic protamine kinase; CKII, casein kinase II; PKA, protein kinase A; PKC, protein kinase C; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; poly(εA), poly(1,N^6-etheno-adenylic acid); ss, single stranded.

0006-2960/94/0433-11382$04.50/0

concentration of A1 favors distal over proximal 5′ splice sites (Mayeda & Krainer, 1992; Mayeda *et al.*, 1993). In nuclear extracts, UV-induced cross-linking of hnRNP A1 and C to pre-mRNA is dependent on U1 and U2 SnRNPs (small nuclear ribonucleoproteins), suggesting that specific interactions take place between the hnRNP and SnRNP complexes (Mayrand & Pederson, 1990).

Reversible phosphorylation is utilized by the cell to regulate a variety of cellular processes (Edelman *et al.*, 1987; Hunter, 1987). Several DNA/RNA binding proteins, including a number of transcription factors, are regulated by phosphorylation (Karin & Hunter, 1992; Meisner & Czech, 1991). Although protein phosphorylation occurs on Ser, Thr, or Tyr, over 99% of proteins are phosphorylated on Ser or Thr (Cohen, 1988). Dephosphorylation of proteins occurs by protein phosphatases, of which there are four major types, termed protein phosphatases 1, 2A, 2B, and 2C. Previous studies had shown that hnRNPs may be regulated by phosphorylation. For instance, the C1 and C2 proteins are phosphorylated *in vivo* by a casein kinase II-like activity, and dephosphorylation is required for binding of the C proteins to nucleic acids (Holocomb & Friedman, 1984; Mayrand *et al.*, 1993). Recently, A1 was shown to be a phosphoprotein in HeLa cells, and phosphorylation *in vitro* by PKA was observed in a peptide sequence that contains both Ser^{197} and Ser^{199} (Cobianchi *et al.*, 1993).

An interesting biochemical activity of hnRNP A1 is that it enhances the renaturation of single-stranded nucleic acids *in vitro* (Pontius & Berg, 1990; Kumar & Wilson, 1990; Munroe & Dong, 1992). The implication of such an activity in splicing is that it may facilitate a variety of intermolecular RNA–RNA interactions occurring in the spliceosome (Guthrie, 1991). In this study, we further characterized the phosphorylation of A1 *in vitro*, with particular emphasis on phosphorylation with PKC. We show that A1 is reversibly phosphorylated with protein kinase C/protein phosphatase 2A and that phosphorylation regulates A1's nucleic acid binding and strand annealing activities.

MATERIALS AND METHODS

Expression and Purification of Recombinant Rat hnRNP Protein A1 and UP1. Recombinant rat hnRNP protein A1 was expressed in *Escherichia coli* and purified essentially as described by Cobinachi *et al.* (1988). UP1 was prepared by partial cleavage of hnRNP protein A1 with trypsin as described by Kumar *et al.* (1986).

Purification of Mammalian Protein Kinases and Phosphatases. Autophosphorylated activated protein kinase (AK) was purified essentially as described by Guo *et al.* (1993) and Guo and Damuni (1993). Myelin basic protein kinases 1 (MBPK1) and 2 (MBPK2) were purified as in Reddy *et al.* (1993). Protamine kinase (CPK) was prepared as in Damuni *et al.* (1989). Purification of casein kinase II (CKII) was described by Damuni (1990). Protein phosphatases 2A (PP2A) and 1 (PP1) were purified as described by Amick *et al.* (1992). Protein kinase A (PKA) was from Sigma, and protein kinase C (PKC) was purchased from Calbiochem, San Diego, CA.

Phosphorylation of hnRNP Protein A1 in Vitro. hnRNP protein A1 was phosphorylated *in vitro* essentially as described by Idriss *et al.* (1994). Typically, *E. coli* recombinant hnRNP A1 was phosphorylated at 30 °C in a reaction mixture (50 μL) containing 50 mM Tris-HCl, pH 7.0, 10% glycerol, 1 mM benzamidine, 14 mM β-mercaptoethanol, 0.1 mM PMSF, 10 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP (150–300 cpm/pmol), 0.1–0.5 mg/mL A1, and a protein kinase. Protein kinase C reaction mixtures also contained 0.5 mM $CaCl_2$ and 0.4 μg/mL phosphatidylserine. Protein kinase was omitted from control reaction mixtures. For fluorometric and CD analysis, phosphorylation was carried out in 10 mM sodium phosphate, pH 7.5, 1 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.2 mM ATP, and a final concentration of 0.5 mg/mL A1. Each kinase was preincubated in the above mixture with unlabeled ATP for 10 min prior to addition of substrate and labeled ATP. At the end of the incubation (30 min), SDS–PAGE sample buffer was added; samples were heated at 100 °C for 4 min, and proteins in the sample were separated by SDS–12.5% PAGE.

Dephosphorylation of hnRNP Protein A1 by Protein Phosphatases. In vitro phosphorylated protein A1 was diluted 10 times in kinase buffer (50 mM Tris-HCl, pH 7.0, 10 % glycerol, 1 mM benzamidine, 14 mM β-mercaptoethanol, 0.1 mM PMSF, and 10 mM $MgCl_2$) and treated with PP2A or PP1 (10 μg/mL) at 30 °C for 1 h. The samples were then either used for strand annealing experiments or processed for SDS–PAGE as described above.

Identification of ^{32}P-Labeled Domain of hnRNP Protein A1. Protein A1 was phosphorylated with PKC as described earlier. ^{32}P-Labeled A1 (8.4 μg, 0.12 μg/μL) was mixed with 4.0 μg (0.2 μg/μL) of unlabeled A1. This mixture was digested separately with trypsin (substrate:enzyme ratio of 750:1) and *Staphylococcus aureus* V8 protease (S:E ratio of 350:1). The digestion was carried out at 25 °C for varying times, and the reaction was stopped by addition of 4× SDS–PAGE sample buffer and heating at 90 °C for 4 min. Samples were subjected to SDS–15% PAGE, and the gel was stained, dried, and subjected to autoradiography.

Isolation of ^{32}P-Labeled Domain of hnRNP Protein A1 and Sequence Analysis of the Phosphorylation Site. Protein A1 (60 μg, 0.35 μg/μL) was phosphorylated with PKC (0.6 μg) for 2 h in a total volume of 170 μL. The protein was then partially digested with V8 protease at an S:E ratio of 350:1 at 25 °C for 2 h. The digest was resolved by use of a Vydac reverse-phase C18 column (2 mm × 15 cm) by high-performance liquid chromatography (HPLC) at a flow rate of 0.1 mL/min with a linear gradient of acetonitrile (10–80% in 70 min) in 0.1% aqueous trifluoroacetic acid (TFA). The column eluent was monitored at 280 and 220 nm, and the peak fractions were counted for radioactivity by a scintillation counter. Peak fractions were also analyzed by SDS–15% PAGE, and fractions containing the C-terminal domain (187–320) were lyophilized for sequencing. ^{32}P-labeled C-terminal domain was applied in 5-μL aliquots to a Sequelon-AA membrane disk placed on a mylar sheet. The membrane was allowed to dry completely between sample applications. The membrane disk was then suspended in 5 μL of carbodiimide solution in an eppendorf tube and incubated at 25 °C overnight. The sample disk was then dried, washed alternately with 1 mL of distilled water followed with 1 mL of methanol, and sequenced using a Milligen ProSequencer. The phenylthiohydantoin amino acid derivatives were identified by HPLC and also analyzed for radioactivity.

Identification of Phosphorylation Sites in UP1. Recombinant UP1 (70 μg) was phosphorylated with PKC (0.6 μg) for 2 h in a total volume of 200 μL. The protein was then completely digested with 4 μg of V8 protease overnight, prior to loading on a C18 column for reverse-phase chromatography as described above except that a gradient of 0–50% acetonitrile was applied. Radioactivity in each peptide peak was determined by Cerenkov counting, and those containing high levels

of radioactivity were selected for sequencing as described above.

DNA Strand Annealing Assay. The 180-base-pair promoter fragment of the human DNA polymerase β gene was 5′-end-labeled with ^{32}P and used as a probe in the assays (Kumar & Wilson, 1990). ssDNA was prepared by boiling the dsDNA for 5 min and then cooling the mixture immediately with vigorous shaking in an ice bath. A typical reaction mixture in a final volume of 25 μL contained 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 80 mM NaCl, 8.5 nM ^{32}P-labeled ssDNA, and increasing amounts of A1. The reaction was carried out at 37 °C for 30 min and samples were processed as described by Casas-Finet *et al.* (1993).

Fluorescence Spectroscopy. Fluorescence data were obtained using a SLM 48000S spectrofluorometer equipped with double monochromators containing holographic gratings with 1500 grooves/mm. Excitation was performed with a 450-W Xe arc lamp. Detection was accomplished with cooled red-sensitive photomultiplier tubes offset to null the dark counts. Readings were collected in the ratio mode using a quantum counter a triangular quartz cell containing 3 g/L rhodamine B solution. Excitation wavelength was at either 280 or 297 nm; emission was collected between 290 and 500 nm. Excitation monochromator slits were set at 1-nm band width, where sample photobleaching was undetectable. Emission monochromator slits were set to 8-nm resolution.

Fluorometric equilibrium binding isotherms for the binding of untreated or phosphorylated A1 or UP1 proteins to poly-(1,N^6-ethenoadenylic acid), poly(ϵA), were obtained at 25 °C in 10 mM sodium phosphate, pH 7.0, containing 0.1 mM EDTA. Stock solutions of A1 and UP1 were 13.6 and 11.2 μM, respectively. In untreated samples either ATP or protein kinase C were omitted. The starting concentration of poly-(ϵA) was 1.8 μM in nucleotide bases. Excitation was at 315 nm with 1-nm band width, and the poly(ϵA) emission was measured at 410 nm with 8-nm band width. Data acquisition was performed by averaging 16 intensity readings with 16 acquisitions/reading. Samples were held in a dual path length (0.2 × 1.0 cm) Suprasil quartz cuvette in a thermostated cell holder, with the narrow path length facing the excitation beam. Samples (200 μL) were used in a capped cell. A minimum of 8–10 aliquots of protein was added and the poly(ϵA) fluorescence emission intensity was monitored; background fluorescence was subtracted and readings were corrected for dilution effects. Inner filter effects due to protein absorbance were negligible at the concentrations used. Data were processed by an IBM-PC/XT computer. Titrations were plotted as relative fluorescence (F/F_0) *vs* [protein]/[oligo] ratio, where F_0 and F are the fluorescence emission intensity (in arbitrary units) at the beginning or during the titration, respectively. Relative binding affinities were calculated from the fractional saturation (θ) at the stoichiometric point.

Circular Dichroism Spectroscopy. Circular dichroism experiments were carried out at 25 °C on a Jasco J-720 spectropolarimeter over the range extending from 260 to 180 nm, using a 1-nm band width, a 16-s time constant, and a data density of 10 points/nm. Strain-free demountable Suprasil quartz cells, closed at both ends, with a path length of 20 μm (Uvonic) were used to minimize the solvent contribution to the CD spectra. Spectra were collected in a single pass without smoothing; triplicate acquisitions were performed for samples and blanks in the same cell and with identical sample geometry. Spectra were nearly superimposable except for the presence of noise features (spikes) below 190 nm. Net spectra were obtained by averaging and background subtraction.

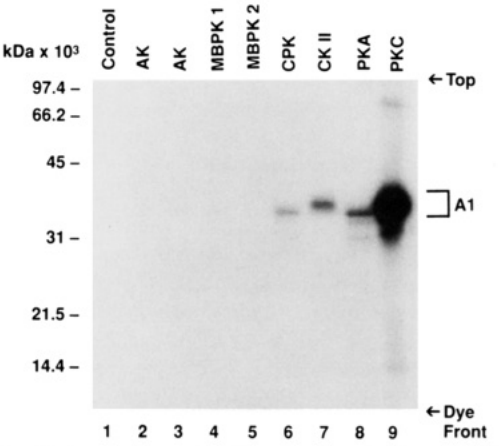


FIGURE 1: Autoradiogram showing phosphorylation of bacterially expressed hnRNP protein A1 *in vitro*. Protein A1 was phosphorylated *in vitro* with seven Ser/Thr kinases as described in Materials and Methods using [γ-^{32}P]ATP; ^{32}P incorporation was determined by SDS–12.5% PAGE and autoradiography. Amount of each protein kinase added to 50-μL reaction mixture is as follows: AK (lane 2), 0.3 μg; AK (land 3), 0.2 μg; MBPK1, 1.0 μg; MBPK2, 0.3 μg; CPK, 0.5 μg; CKII, 0.5 μg; PKA, 0.3 μg; and PKC, 0.03 μg. Lane 1 represents a control which contain all the components except kinase. Lanes 2–9 are for protein A1 phosphorylated with two different preparations of AK, MBPK1, MBPK2, CPK, CKII, PKA and PKC, respectively. The position of molecular weight markers is shown on the left and the migration position of A1 is indicated on the right.

RESULTS

hnRNP Protein A1 Is a Substrate for Purified Protein Kinases and Protein Phosphatase 2A. Inspection of the amino acid sequence of A1 (Cobianchi *et al.*, 1986) shows that it contains 32 Ser and 12 Thr residues. Of these, eight are in the casein kinase II consensus phosphorylation sequence (S/T XX E/D), 11 are in the PKC/PKA consensus sequence (R/K X S/T, R/K XX S/T), seven are in the PKC consensus sequence (S/T X R/K), and one is in the proline-directed kinase consensus sequence (S P K) (Pearson & Kemp, 1991). Therefore, 13 Ser and eight Thr residues of A1 are in potential phosphorylation sites for the kinases mentioned above. A survey of A1 phosphorylation *in vitro* with seven different Ser/Thr protein kinases showed that four of the enzymes are able to phosphorylate A1 (CPK, CKII, PKA, and PKC), whereas three are not (AK, MBPK1, and MBPK2) (Figure 1). Phosphorylation was much more extensive with PKC than with the other kinases tested and occurred at multiple sites (see below). Phosphorylation with CKII generated a slower migrating species of A1 (Figure 1), not observed with the other kinases. These results indicate that PKC strongly phosphorylates A1 and that A1 is a substrate for three of the other kinases tested.

To investigate dephosphorylation of A1, we used protein phosphatase 2A. *In vitro* phosphorylation of A1 with each of the four protein kinases could be reversed through the action of purified mammalian PP2A, which was capable of complete dephosphorylation of A1 (Figure 2). Under the conditions used, PP2A completely dephosphorylated A1 phosphorylated by CPK, CKII, and pKA and dephosphorylation was partial in the case of PKC (Figure 2, lane 8); however, when more PP2A was added to the latter reaction mixture, dephosphorylation of A1 was complete (Figure 3a, lane 1). We also examined the ability of protein phosphatase 1 to dephosphorylate A1. PP1-treated A1 retained the same radioactive signal as the control (untreated) (Figure 3a, lanes 2 and 3), indicating lack of activity by PP1. We confirmed that our PP1 was an enzymatically active preparation (data not shown)

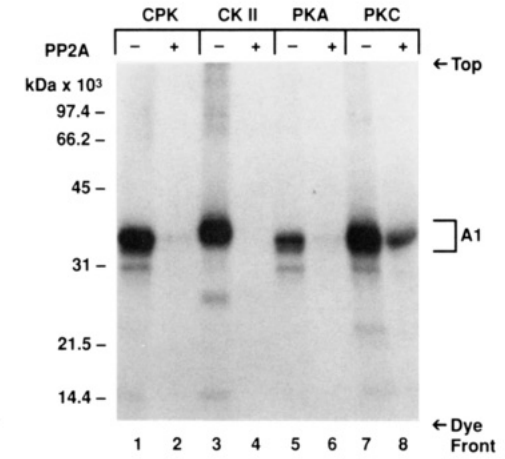


FIGURE 2: Photograph of an autoradiogram showing dephosphorylation of ^{32}P-labeled hnRNP protein A1 with PP2A *in vitro*. Protein A1 was phosphorylated *in vitro* with CKII, CPK, PKA, or PKC (lanes 1, 3, 5, and 7) as described under Materials and methods and using the amount of kinases indicated in Figure 1. The phosphorylated A1 was dephosphorylated with PP2A (lanes 2, 4, 6, and 8) for 30 min as described in Materials and Methods. Samples were subjected to SDS–12.5% PAGE and autoradiography. The positions of moleculoar weight markers and protein A1 are indicated.

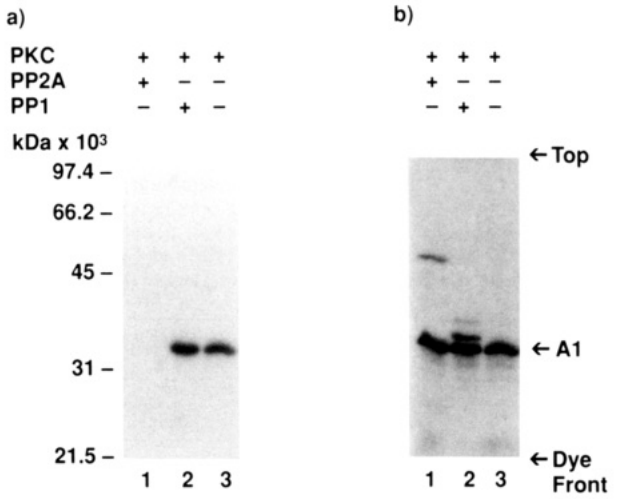


FIGURE 3: Dephosphorylation of ^{32}P-labeled hnRNP protein A1 with PP2A and PP1 *in vitro*. Protein A1 was phosphorylated with PKC as described in Figure 1 and subjected to dephosphorylation by PP2A and PP1 as described in Materials and Methods. The incubation was carried out for 2.5 h at 30 °C. ^{32}P incorporation in the samples was determined by SDS–12.5% PAGE, and the gel was stained with Coomassie blue, destained, dried, and subjected to autoradiography. Panel a represents an autoradiogram, and panel b is a photograph of the Coomassie blue-stained gel. Presence of PKC and protein phosphatases PP2A and PP1 is indicated at the top.

and note that our PP2A and PP1 were highly purified enzyme preparations, as judged by Coomassie blue staining after SDS–PAGE (Figure 3b).

PKC Phosphorylation of A1 Occurs at Multiple Sites. The N-and C-terminal domains of A1 can be readily separated by controlled proteolysis with trypsin or V8 protease (Cobianchi *et al.*, 1986; Kumar *et al.*, 1990). Trypsin and V8 protease generate N-terminal fragments of amino acids 2–196 and 2–185, respectively, and C-terminal fragments of amino acids 197–320 and 186–320, respectively (Kumar *et al.*, 1990). To locate the PKC phosphorylation sites, A1 was phosphorylated *in vitro* to a stoichiometry of 1 mol of phosphate/mol of A1 and then cleaved with trypsin or V8 protease to generate the respective N- and C-terminal domains. The results from such a study (Figure 4) indicate that A1 is phosphorylated at multiple sites by PKC, in both the N- and C-terminal domains.

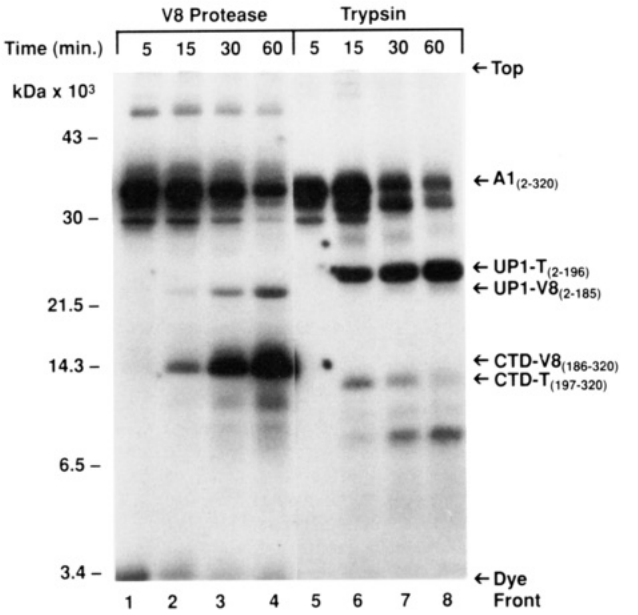


FIGURE 4: Domain mapping of ^{32}P-labeled hnRNP protein A1 using V8 protease and trypsin. A photograph of an autoradiogram is shown. Protein A1 was digested with V8 protease (lanes 1–4) and trypsin (lanes 5–8) for different times (indicated at the top) as described in Materials and Methods. The samples were subjected to SDS–15% PAGE and the radioactive domain fragments were visualized by autoradiography. Molecular weight markers are indicated on the left. Labeling on the right side is as follows: A1 (2–320), hnRNP protein A1; UP1-$T_{(2-196)}$, UP1 generated by trypsin; UP1-$V8_{(2-185)}$, UP1 generated by V8 proteinase; CTD-$V8_{(186-320)}$, C-terminal domain generated by V8; CTD-$T_{(197-320)}$, C-terminal domain generated by trypsin.

For digestion with V8 protease, the radioactive signal of the C-terminal domain was stronger than that of the N-terminal domain. For proteolysis of A1 with trypsin, the radioactive signal of the N-terminal domain was stronger than that of the C-terminal domain. These results demonstrated that at least three sites were phosphorylated: one is located between residues 2 and 186 (N-terminal fragment generated by trypsin or V8 digestion); one between residues 187 and 196 (seen on the N-terminal fragment generated by trypsin and the C-terminal fragment generated by V8 proteolysis), and one between residues 197 and 320 (C-terminal fragment generated by trypsin or V8 treatment).

Identification of the PKC-Phosphorylated Residues. Identification of the residues phosphorylated in the C-terminus of A1 was performed by separating the ^{32}P-labeled C- and N-terminal domains by C18 reverse-phase chromatography, following digestion with V8 protease. The isolated C-terminal domain was then sequenced (up to 80 amino acids), and we also collected the PTH-amino acids from each cycle to measure ^{32}P-amino acid by Cerenkov counting. The results (Figure 5) revealed two major phosphorylation sites, Ser^{192} and Ser^{199}, and minor sites at S^{197}, Ser^{223}, and Ser^{231}.

For phosphorylation in the N-terminal domain, complete V8 protease digestion of phosphorylated UP1 (Figure 6A) generated the peptide DSQRPGAHLTVKKIFVGGIK that contains phosphorylated residues. Sequencing revealed Ser^{95} and Thr^{103}, with Ser^{95} more heavily phosphorylated than Thr^{103} (Figure 6 and Table 1). The same peptide was isolated from a V8 digest of phosphorylated A1 and the same phosphoamino acid sequencing results were obtained, showing that both of these residues also are phosphorylated by PKC in intact A1 (not shown). All of the phosphoamino acids found conform to a PKC consensus sequence. The major sites were confirmed by sequencing of C18-purified peptides generated by complete

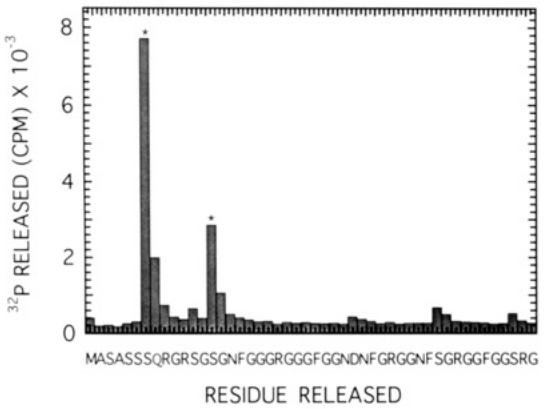
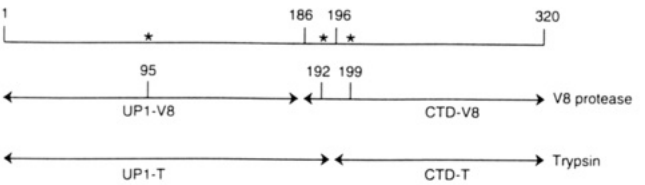


FIGURE 5: Amino acid sequencing of ^{32}P-labeled C-terminal domain derived by purification on a C18 reverse-phase column, following PKC phosphorylation and limited V8 proteolysis, as described in Materials and Methods. Sequencing was from Met^{186} to Asn^{265}, but ^{32}P released during each sequencing cycle is shown for Met^{186}–Gly^{233}. No ^{32}P was released during sequencing of amino acids 234–265 (not shown). Two Ser residues marked with asterisks correspond to Ser^{192} and Ser^{199}.

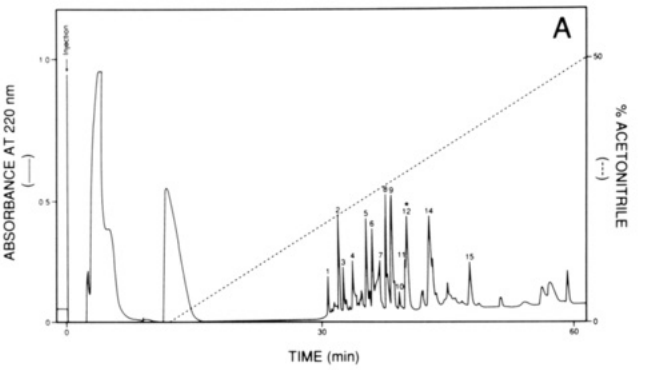
tryptic digestion of labeled protein A1 (not shown). In summary, A1 residues Ser^{95}, Ser^{192}, and Ser^{199} are the three major phosphorylation sites by PKC *in vitro*, as shown here:



Phosphorylation of hnRNP Protein A1 by PKC in Vitro Inhibits Its Strand Annealing Activity. PKC-phosphorylated A1 was tested for its ability to promote interstrand annealing of ^{32}P-labeled single-stranded DNA. A1 and its C-terminal domain fragment are capable of potent strand annealing activity for complementary single-stranded polynucleotides of both RNA and DNA (Kumar & Wilson, 1990). Since we have not observed differences with these two nucleic acid lattices, we used complementary single-stranded DNA as a probe in the present study. The experiment in Figure 7a shows that strand annealing activity was completely lost after A1 was phosphorylated with PKC. Control experiments where A1 was not phosphorylated but was incubated under PKC reaction conditions failed to show any effect on strand annealing.

The strand annealing activity of A1 was regenerated by dephosphorylation of A1 with PP2A, which was able to completely restore normal A1 activity (Figure 7b). Interestingly, PP1, which does not dephosphorylate ^{32}P-labeled A1 *in vitro* (Figure 3, lane 3), failed to revive interstrand annealing of A1. This suggests that PP2A may be responsible for A1 dephosphorylation *in vivo* (see discussion). Protamine kinase- or casein kinase II-phosphorylated A1 was also tested for strand annealing activity. Strand annealing was not completely inhibited as for PKC-phosphorylated A1, but modest inhibition was observed compare to unphosphorylated A1 (not shown).

Effect of Phosphorylation on the Secondary Structure of hnRNP Protein A1. Structural implications of phosphorylation on A1 were assessed by fluorescence spectroscopy, by CD analysis, and by fluorescence enhancement upon binding to poly(ϵA).



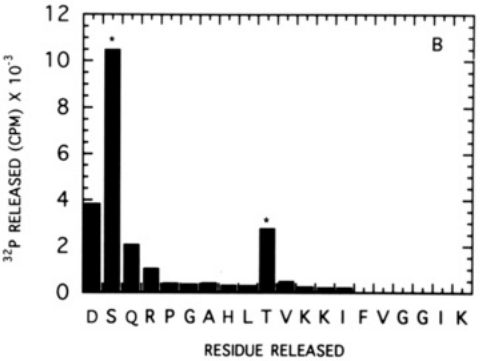


FIGURE 6: Sequence analysis of ^{32}P-labeled N-terminal domain. Panel A, separation of UP1 peptides, generated by complete V8 proteolytic cleavage, by C18 reverse-phase chromatography, following phosphorylation of UP1 with PKC and [γ-^{32}P]ATP. The radioactivity in each of the major peaks (labeled 1–15) was determined by Cerenkov counting (Table 1). Panel B, the peptide in peak 12 was selected for sequencing since it contained the bulk of the radioactivity. Sequence analysis was carried out from Asp^{94} to Lys^{113} and ^{32}P release was shown up to Ile^{107}. Ser^{95} and Thr^{103} are marked with asterisks.

Table 1: Radioactivity Content of ^{32}P-Labeled Peptides from Total V8 Protease Digest of Phosphorylated UP1

peptide peak (from C18 reverse-phase column)[a]	extent of ^{32}P labeling (cpm)
1	18265
2	10124
3	6527
4	16386
5	4761
6	3580
7	1686
8	5069
9	2718
10	4222
11	19987
12[b]	198645
13	5575
14	7845
15	3932

[a] Elution profile from the C18 reverse-phase column is given in Figure 6A. [b] Peptide peak corresponding to the peptide DSQRPGAHLTVKK-IFVGGIK.

Fluorescence Spectroscopy of Phosphorylated A1 and UP1 Proteins. Changes in the microenvironment of the aromatic residues of UP1 (1 Trp, 4 Tyr) and A1 (1 Trp, 12 Tyr) occurring as a result of phosphorylation were monitored spectrofluorometrically. Note that Phe residues are not excited under our experimental conditions. No change in wavelength maximum was detected upon phosphorylation of UP1 or A1, monitoring either emission of the Trp chromophore only or overall emission. The Trp emission intensity of UP1 was marginally affected by PKC phosphorylation, resulting in *ca.*

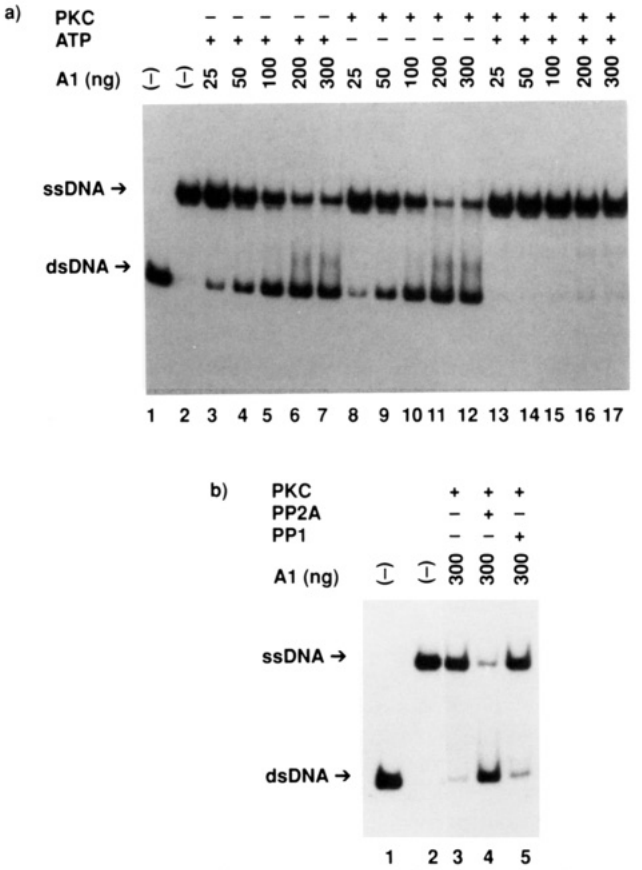


FIGURE 7: DNA strand annealing activity of phosphorylated protein A1 (panel a) and dephosphorylated protein A1 (panel b). Photographs of autoradiograms are shown. The denatured 180-base-pair DNA was incubated with protein A1 as indicated the top of panels a and b and as described in Materials and Methods. ssDNA and dsDNA were separated by a nondenaturing 10% polyacrylamide gel and the gel was subjected to autoradiography. Positions of dsDNA and ssDNA are marked.

7% enhancement of its integrated emission, relative to the untreated protein; no change of spectral line width was detected. It is worth noting that, because of the low level of UP1 phosphorylation attained under our experimental conditions (about 0.3 mol of phosphate/mol of protein), a maximal effect of up to 3 times as large could result from complete reaction of the single phosphorylation site present in UP1. Nevertheless, the effect of phosphorylation on the UP1 Trp quantum yield is of moderate magnitude, as expected from the absence of a phosphorylation site in the protein subdomain carrying the sole Trp residue of UP1 and the probable distal location of the phosphorylation site from the chromophore. In the case of A1, an 18% increase in Trp emission intensity was observed upon phosphorylation, with no change in line shape. The level of phosphorylation was 1 mol of phosphate/mol of protein. Since A1 carries three major phosphorylation sites, complete phosphorylation could potentially exhibit a somewhat larger effect.

A1 exhibits a significant increase in its Trp emission upon phosphorylation. Note, however, that the C-terminal domain of A1 is known to participate in homologous protein–protein associations (Kumar *et al.*, 1990; Casas-Finet *et al.*, 1993). It is conceivable that a major conformational change in this region of the protein could affect A1 oligomerization and, indirectly, the quantum yield of the partially solvent-exposed Trp chromophore (Casas-Finet *et al.*, 1991). Support for this hypothesis is presented below.

The total fluorescence emission of UP1 (Trp + Tyr) was altered upon phosphorylation, resulting in a 19% reduction of

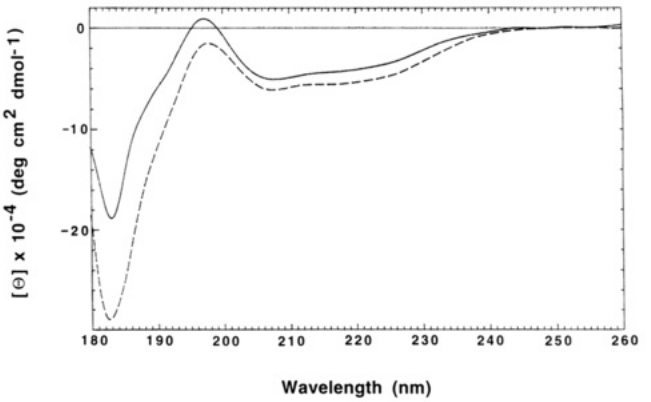


FIGURE 8: Circular dichroism spectra of unphosphorylated (solid line) and phosphorylated (dashed line) hnRNP protein A1. Phosphorylation of protein A1 was with PKC, at a final substrate concentration of 0.5 mg/mL, for 120 min as described in Materials and Methods.

the integrated fluorescence intensity. Thus, a significantly larger effect can be ascribed to the Tyr population, versus the single Trp chromophore. Our calculations suggest a major reduction of Tyr fluorescence intensity upon complete phosphorylation of UP1.

In contrast, the total emission of A1 protein *increased* by *ca.* 34% upon phosphorylation. Given that, following phosphorylation, the Trp emission of A1 was enhanced only by 18% and the Trp contribution to the overall fluorescence emission of A1 is about 47% (Casas-Finet *et al.*, 1991), our results indicate a dramatic effect of phosphorylation on Tyr quantum yield. This effect would be localized to the C-terminal domain of A1 (where eight of its 12 Tyr side chains reside), since a *reduction* in Tyr quantum yield was observed upon phosphorylation of the N-terminal domain. It is known that, in spite of the significantly higher Tyr to Trp ratio of A1, relative to UP1, the contribution of its Tyr population to the overall emission is much smaller than might be expected (Casas-Finet *et al.*, 1991). We estimate the increase in the Tyr quantum yield of the C-terminal subdomain at about 50%. An effect of this magnitude on such a large number of Tyr residues is unlikely to stem from alteration of the local environment of a few of these side chains. Instead, we suggest that a global change in the conformation of the C-terminal domain occurs following phosphorylation. As the C-terminal region of the protein has been predicted and shown to lack secondary structure (Kumar *et al.*, 1990; Casas-Finet *et al.*, 1991), a likely explanation for our observations would be a decrease in collisional quenching that would occur as a result of a more ordered structure.

Circular Dichroism of Phosphorylated A1 and UP1 Proteins. The circular dichroism spectrum of UP1 is dominated by extrema of negative ellipticity at 208 and 218 nm, characteristic of α-helical and β-structure [see Figure 1 of Casas-Finet *et al.* (1993)]. Upon phosphorylation, the magnitude of the negative ellipticity band increased by *ca.* 29% and resulted in a slight blue shift of the peak to 206 nm and a less pronounced shoulder at 218 nm (Figure 8). Taken together, these results suggest an increase in the fraction of α-helical structure from 32% (Casas-Finet *et al.*, 1993) to 41%.

The far-UV CD spectrum of untreated A1 protein also showed a significant content of α-helical and β-structure, as revealed by the extrema of negative ellipticity at 208 and 218 nm, respectively. Upon phosphorylation, a small band of positive ellipticity disappeared, and the magnitude of all the negative ellipticity bands became larger. This is indication

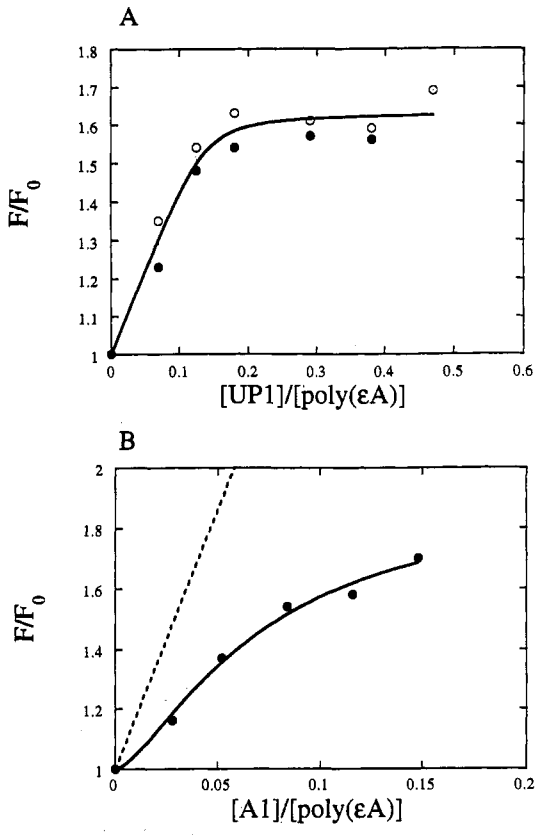


FIGURE 9: Binding to poly(ϵA) by phosphorylated and unphosphorylated N-terminal domain or UP1 (panel A) and A1 (panel B). Binding analysis was conducted as described under Materials and Methods in a solution of 10 mM sodium phosphate, pH 7.0, and 0.1 mM EDTA. (A) Fluorometric titration curves of poly(ϵA) with untreated (open circles) or phosphorylated (solid circles) UP1. (B) Fluorometric titration curves of poly(ϵA) with phosphorylated (full circles) A1. The dashed line is a curve taken from known A1 binding parameters, as reported by Cobianchi *et al.* (1988); the curve was verified in this experiment with data points at multiple A1 concentrations.

of an increase in ordered structure in A1 following phosphorylation. Since the CD spectrum of phosphorylated UP1 indicates that the sum of the α-helical and β-structure components constitute a large fraction (>60%) of its secondary structure, it is likely that the effect observed following phosphorylation is a result of a conformational change on the largely disordered C-terminal fragment (Casas-Finet *et al.*, 1993).

Fluorometric Titrations of A1 and UP1 Proteins. Equilibrium binding isotherms were constructed by monitoring enhancement of poly(ϵA) fluorescence induced by UP1 or A1 binding. The UP1 titration curve showed a hyperbolic shape characteristic of noncooperative binding, as found earlier (Casas-Finet *et al.*, 1993). Titrations carried out with phosphorylated UP1 were qualitatively similar but exhibited a slightly lower limiting fluorescence enhancement and fractional saturation of the polynucleotide lattice at a given [UP1]/[poly(ϵA)] ratio (Figure 9A). Analysis of these titration curves suggested only a slight decrease (<20%) in UP1 affinity for poly(ϵA) following phosphorylation.

A1 titration curves (Figure 9B) exhibited a larger occluded binding site size and reached a higher limiting fluorescence enhancement of poly(ϵA) fluorescence relative to UP1, as observed previously (Casas-Finet *et al.*, 1991). Phosphorylation of A1 resulted in a titration curve with a sigmoidal shape (Figure 9B). This sigmoidal character is diagnostic of cooperative A1 binding to polynucleotides mediated by its C-terminal domain, as has been shown in previous studies (Cobinachi *et al.*, 1988; Kumar *et al.*, 1990; Nadler *et al.*, 1991; Casas-Finet *et al.*, 1993). Such an inflection in the curve was not apparent in unphosphorylated A1, which, due to its higher nucleic acid binding affinity, exhibits near-stoichiometric binding throughout the binding isotherm in this experiment. Analysis of the titration curves suggest a ~3-fold decrease in the affinity of A1 for poly(ϵA), following phosphorylation. Given that the sample used had only partial saturation of the three major A1 phosphorylation sites, our data suggest the maximal decrease in binding affinity could be greater than in Figure 9B.

It is noted that the sigmoidal character of the titration with phosphorylated A1 indicates that a measure of cooperativity still remains in phosphorylated A1. Nevertheless, the decrease in affinity following phosphorylation must stem mainly from a lower cooperativity parameter. This is evident because A1 and the N-terminal domain possess identical intrinsic binding affinities (i.e., binding excluding cooperativity; Nadler *et al.*, 1991) and N-terminal domain affinity is affected only minimally by phosphorylation.

DISCUSSION

We have studied the phosphorylation of the hnRNP protein A1 *in vitro* and found it to be a substrate for a number of serine/threonine protein kinases. Particularly, A1 was a good substrate for protein kinase C, which phosphorylated the protein at three major sites. Domain mapping and sequence analysis demonstrated that one of these sites was located around the junction of the N- and C-terminal domains, one in the C-terminal domain, and one in the N-terminal domain. The PKC-phosphorylated protein was a good substrate for the phosphatase 2A, which was capable of almost completely dephosphorylating A1. Phosphorylation of A1 inhibited its interstrand annealing activity, which could be regenerated by treatment with phosphatase 2A but not phosphatase 1.

The ability of A1 to be phosphorylated by a number of kinases (CPK, CKII, PKA, and PKC) offers a potential mechanism for regulation of the multiple activities of the protein *in vivo*, through differential phosphorylation by various kinases. Control of A1 activity *in vivo* through phosphorylation/dephosphorylation would appear to be more flexible, if A1 is a substrate for a number of protein kinase. For instance, PKA has been recently shown to phosphorylate A1 at a single site, most probably Ser^{199} (Cobianchi *et al.*, 1993). This single-site phosphorylation is able to inhibit the interstrand annealing activity of A1. CKII has also been reported to phosphorylate A1 at the same site, although our results suggest other sites are also phosphorylated with CKII, as this causes a mobility shift not observed with PKA phosphorylation at Ser^{199} (Figure 1).

Protein A1 *in vivo* is methylated, containing 3.1 mol of methyl groups/mol of protein (Kumar *et al.*, 1986). One of the methylated sites, R^{194}, is located between S^{192} and S^{199}, which are heavily phosphorylated by PKC. It is interesting to note that trypsin cleaves A1 at residue R^{196}. Thus, modification(s) in this region may play a role in cleavage at R^{196} by trypsin-like proteases present in nuclei (Pandolfo *et al.*, 1985). PKC phosphorylation of A1 was more abundant at site S^{192} and this site is in a PKC consensus site (S/T X R/K; S^{192} Q R^{194}). Dimethylarginine at residue R^{194} may affect phosphorylation and, in turn, phosphorylation at residue S^{192} may affect methylation of residue R^{194}.

Kinases that phosphorylate A1 are present in the cytoplasm and nucleus and thus may be able to modulate A1 function

at different steps. Some of the kinases for A1 phosphorylation are found in hnRNP particles (Wilk *et al.*, 1985; Holocomb & Friedman, 1984); these can phosphorylate A1 and affect the nucleic acid binding and strand annealing activity. Phosphorylation and dephosphorylation of A1 could affect its protein–protein interactions with other hnRNP proteins and thus may be a regulatory step for assembly/disassembly of the hnRNP complex. It has been demonstrated that A1 shuttles between the nucleus and cytoplasm during nucleocytoplasmic transport of mRNA (Pinol-Roma & Dreyfuss, 1992), and phosphorylation of A1 by kinases present in the cytoplasm could mediate this process.

PKC phosphorylates A1 at three major sites (one of which is Ser^{199}) and at least three other sites. Yet, the observed inhibition of strand annealing activity could be accounted for by Ser^{199} phosphorylation alone. CD spectroscopic analysis showed a change in secondary structure of A1 upon phosphorylation, which probably occurs through generation of a more ordered structure in the C-terminal domain. Whether such a structural change is associated with the block in strand annealing activity of A1 is not clear at the moment, as the mechanism of strand annealing is largely unknown, although it clearly is contributed by the C-terminal domain (Kumar & Wilson, 1990). Phosphorylation of A1 with PKC also inhibits A1's binding to nucleic acid, and it is reasonable to expect that nucleic acid binding is involved in the strand annealing effect of A1. We note that the C-terminal domain Ser residues that are PKC-phosphorylated are preceeded by the recently identified RNA binding sequence RGG (Kiledjan & Dreyfuss, 1992).

Spectrofluorometric and spectropolarimetric characterization of phosphorylated and untreated A1 and N-terminal domain proteins strongly suggests that N-terminal domain secondary structure is not significantly affected by phosphorylation, whereas intact A1 exhibits significant changes of its Tyr quantum yield and molar ellipticity, pointing to a structural alteration of its C-terminal domain. This largely disordered domain is responsible of conferring nucleic acid binding cooperativity to A1 protein and is required for A1 to show strand annealing activity. The larger decrease in A1 nucleic acid binding affinity observed upon phosphorylation, relative to that of the N-terminal domain, is additional evidence pointing to the C-terminal domain as the primary functional target of PKC phosphorylation.

Our demonstration that hnRNP A1 is a substrate for PP2A is interesting in view of the recent finding that the hnRNP C protein is also a substrate for this phosphatase (Mayrand *et al.*, 1993); the activity of phosphatase 2A is essential for one of the steps of pre-mRNA splicing (Mermoud *et al.*, 1992). A1 is not recovered in a phosphorylated state from cells cultured in labeled phosphate, but the finding that A1 is a phosphoprotein in HeLa cells in the presence of okadaic acid (Cobianchi *et al.*, 1993), a potent inhibitor of PP2A and PP1, and our results indicating that A1 is a substrate for PP2A, but not for PP1, point to A1 as a physiological substrate for PP2A *in vivo*. Since a role for A1 in pre-mRNA splicing has been recently proposed (Mayeda & Krainer, 1992; Mayeda *et al.*, 1993; Sun *et al.*, 1993) and as PP2A is required for step 2 of pre-mRNA splicing (cleavage of the 3′ splice site and exon ligation; Mermoud *et al.*, 1992), it is possible that PP2A dephosphorylation of A1 facilitates this process. Experiments are currently underway to test the ability of phosphorylated A1 to promote splicing of pre-mRNA *in vitro*.

ACKNOWLEDGMENT

We thank Mr. Stefan Serabyn for protein microsequence analysis, Ms. Ling Zhang for technical assistance, and Ms. Brenda Romero for typing the manuscript.

REFERENCES

Amick, G. D., Reddy, S. A. G., & Damuni, Z. (1992) *Biochem. J. 287*, 1019–1022.
Barnett, S. F., Friedman, D. L., & LeStourgeon, W. M. (1989) *Mol. Cell. Biol. 9*, 492–498.
Barnett, S. F., Theiry, T. A., & LeStourgeon, W. M. (1991) *Mol. Cell. Biol. 11*, 864–871.
Bennett, M., Pinol-Roma, S., Staknis, D., Dreyfuss, G., & Reed, R. (1992) *Mol. Cell. Biol. 12*, 3165–3175.
Beyer, A. L., Christensen, M. E., Walker, B. W., & LeStourgeon, W. M. (1977) *Cell 11*, 127–138.
Burd, C. G., & Dreyfuss, G. (1994) *EMBO J. 13*, 1197–1204.
Buvoli, M., Cobianchi, F., Biamonti, G., & Riva, S. (1990) *Nucleic Acids Res. 18*, 6595–6600.
Casas-Finet, J. R., Karpel, R. L., Maki, A. H., Kumar, A., & Wilson, S. H. (1991) *J. Mol. Biol. 221*, 693–709.
Casas-Finet, J. R., Smith, J. D., Kumar, A., Kim, J. G., Wilson, S. H., & Karpel, R. L. (1993) *J. Mol. Biol. 229*, 873–889.
Choi, Y. D., Grabowski, P. J., Sharp, P. A., & Dreyfuss, G. (1986) *Science 231*, 1534–1539.
Cobianchi, F., SenGupta, D. N., Zmudzka, B. Z., & Wilson, S. H. (1986) *J. Biol. Chem. 261*, 3536–3543.
Cobianchi, F., Karpel, R. L., Williams, K. R., Notario, V., & Wilson, S. H. (1988) *J. Biol. Chem. 263*, 1063–1071.
Cibianchi, F., Calvio, C., Stoppini, M., Buvoli, M., & Riva, S. (1993) *Nucleic Acids Res. 21*, 949–955.
Cohen, P. (1988) *Proc. R. Soc. London 234*, 115–144.
Damuni, Z. (1990) *Biochem. Biophys. Res. Commun. 166*, 449–456.
Damuni, Z., Amick, G. D., & Sneed, T. R. (1989) *J. Biol. Chem. 264*, 6412–6416.
Dreyfuss, G., Matunis, M. J., Pinol-Roma, S., & Burd, C. G. (1993) *Annu. Rev. Biochem. 62*, 289–321.
Edelman, A. M., Blumenthal, D. K., & Krebs, E. G. (1987) *Annu. Rev. Biochem. 56*, 567–613.
Guo, H., & Damuni, Z. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2500–2504.
Guo, H., Reddy, S. A. G., & Damuni, Z. (1993) *J. Biol. Chem. 268*, 11193–11198.
Guthrie, C. (1991) *Science 253*, 157–163.
Holocomb, E. R., & Friedman, D. L. (1984) *J. Biol. Chem. 259*, 31–40.
Huang, M., Rech, J. E., Northington, S. J., Flicker, P. F., Mayeda, A., Krainer, A. R., & LeStourgeon, W. M. (1994) *Mol. Cell. Biol. 14*, 518–533.
Hunter, T. (1987) *Cell 50*, 823–829.
Idriss, H., Kawa, S., Damuni, Z., Guo, H., Thompson, E. B., & Wilson, S. H. (1994) *Biochem. Biophys. Res. Commun.* (submitted for publication).
Karin, M., & Hunter, T. (1992) *Cell 70*, 375–387.
Kenan, D. J., Query, C. C., & Keene, J. D. (1991) *Trends Biochem. Sci. 16*, 214–220.
Kiledjian, M., & Dreyfuss, G. (1992) *EMBO J. 11*, 2655–2664.
Kumar, A., Sierakowska, H., & Szer, W. (1987) *J. Biol. Chem. 262*, 17126–17137.
Kumar, A., & Wilson, S. H. (1990) *Biochemistry 29*, 10717–10722.
Kumar, A., Williams, K. R., & Szer, W. (1986) *J. Biol. Chem. 261*, 11266–11273.
Kumar, A., Casas-Finet, J. R., Luneau, C. J., Karpel, R. L., Merrill, B. M., Williams, K. R., & Wilson, S. H. (1990) *J. Biol. Chem. 265*, 17094–17100.
Mayeda, A., & Krainer, A. R. (1992) *Cell 68*, 365–375.
Mayeda, A., Helfman, D. M., & Krainer, A. R. (1993) *Mol. Cell. Biol. 13*, 2993–3001.

Mayrand, S. H., & Pederson, T. (1990) *Nucleic Acids Res. 18*, 3307–3318.
Mayrand, S. H., Dwen, P., & Pederson, T. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7764–7768.
Meisner, H., & Czech, M. P. (1991) *Curr. Opin. Cell Biol. 3*, 474–483.
Mermoud, T. E., Cohen, P., & Lamond, A. I. (1992) *Nucleic Acids Res. 20*, 5263–5269.
Merrill, B. M., Stone, K. L., Cobianchi, F., Wilson, S. H., & Williams, K. R. (1988) *J. Biol. Chem. 263*, 3307–3313.
Munroe, S. H., & Dong, X. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 895–899.
Nadler, S. G., Merrill, B. M., Roberts, W. J., Keating, K. M., Lisbin, M. J., Barnett, S. F., Wilson, S. H., & Williams, K. R. (1991) *Biochemistry 30*, 2968–2976.
Pandolfo, M., Valentini, O., Biomonti, G., Morandi, C., & Riva, S. (1985) *Nucleic Acids Res. 13*, 6577–6590.
Pearson, R. B., & Kemp, B. E. (1991) *Methods Enzymol. 200*, 61–82.
Pinol-Roma, S., & Dreyfuss, G. (1992) *Nature 355*, 730–732.
Pinol-Roma, S., Choi, Y. D., Matunis, M. J., & Dreyfuss, G. (1988) *Genes Dev. 2*, 215–227.
Pontius, B. W., & Berg, P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8403–8407.
Reddy, S. A. G., Guo, H., Tarun, S. Z., Jr., & Damuni, Z. (1993) *J. Biol. Chem. 268*, 15298–15304.
Riva, S., Morandi, C., Tsoulfas, P., Pandolfo, M., Biamonti, G., Merrill, B., Williams, K. R., Mulhaup, G., Beyreuther, K., Werr, H., Henrich, B., & Schafer, K. P. (1986) *EMBO J. 5*, 2267–2273.
Sierakowska, H., Szer, W., Furdon, P. J., & Kole, R. (1986) *Nucleic Acids Res. 14*, 5241–5254.
Sun, Q., Mayeda, A., Hampson, R. K., Krainer, A. R., & Rottman, F. M. (1993) *Genes Dev. 7*, 5241–5254.
Swanson, M. S., & Dreyfuss, G. (1988) *EMBO J. 11*, 3519–3529.
Wilk, H. E., Werr, H., Friedrich, D., Kiltz, H. H., & Schafer, K. P. (1985) *Eur. J. Biochem. 146*, 71–81.
Wilson, S. H. (1990) in *Cancer Biology and Biosynthesis*, pp 55–89, *CRC Press*, Boca Raton, FL.