Phosphorylation of Human and Bovine Prothymosin α in Vivo

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Received September 10, 1992; Revised Manuscript Received December 8, 1992

ABSTRACT: Prothymosin α is post-translationally modified. When human myeloma cells were metabolically labeled with [^{32}P]orthophosphoric acid, they synthesized [^{32}P]prothymosin α. The incorporated radioactivity was resistant to DNase and RNases A, T1, and T2, but could be completely removed by alkaline phosphatase. No evidence was found for an RNA adduct as postulated by Vartapetian et al. [Vartapetian, A., Makarova, T., Koonin, E. V., Agol, V. I., & Bogdanov, A. (1988) *FEBS Lett. 232*, 35–38]. Thin-layer electrophoresis of partially hydrolyzed [^{32}P]prothymosin α indicated that serine residues were phosphorylated. Analysis of peptides derived from bovine prothymosin α and human [^{32}P]prothymosin α by treatment with endoproteinase Lys-C revealed that the amino-terminal 14-mer, with serine residues at positions 1, 8, and 9, was phosphorylated at a single position. Approximately 2% of the peptide in each case contained phosphate. Further digestion of the phosphopeptide with Asp-N followed by C18 reversed-phase column chromatography produced two peptides: a phosphate-free 9-mer containing amino acids 6–14 and a labeled peptide migrating slightly faster than the N-terminal 5-mer derived from the unmodified 14-mer. Positive identification of the phosphorylated amino acid was obtained by colliding the 14-residue phosphopeptide with helium in the mass spectrometer and finding phosphate only in a nested set of phosphorylated fragments composed of the first three, four, and five amino acids. The results prove that prothymosin α contains N-terminal acetylserine phosphate. In a synchronized population of human myeloma cells, phosphorylation occurred throughout the cell cycle. Furthermore, prothymosin α appeared to be stable, with a half-life slightly shorter than the generation time. Although prothymosin α is known to be essential for cell division, the constancy of both the amount of the protein and the degree of its phosphorylation suggests that prothymosin α does not directly govern mitosis.

Prothymosin α is a small, highly acidic polypeptide (Haritos et al., 1985; Sburlati et al., 1990) found in the nuclei of virtually all mammalian cells (Manrow et al., 1991; Palvimo & Linnala-Kankkunen, 1990; Watts et al., 1989; Clinton et al., 1991). The protein is abundant in cultured cells only under conditions of rapid growth and appears at high levels only in proliferating tissues (Sburlati et al., 1991; Haritos et al., 1984b; Economou et al., 1988; Clinton et al., 1989; Frillingos et al., 1991). In biological terms, there is one prothymosin α molecule in the nucleus for every one to two nucleosomes, an amount equivalent to that of histone cores. The polypeptide is essential for proliferation; upon uptake of antisense oligodeoxyribonucleotides complementary to prothymosin α mRNA, cells become deficient in the protein and cease to divide (Sburlati et al., 1991). The effect is reversible. In a synchronous population of myeloma cells, intracellular degradation of the oligonucleotides restores a normal cell cycle. Recently, it has been reported that transcription of the prothymosin α gene increases in the presence of active c-myc protein (Eilers et al., 1991), an observation which appears to corroborate the importance of prothymosin α in cell division.

Prothymosin α is an atypical protein. The most abundant form of the mature human protein, after cleavage of the amino-terminal formylmethionine, has a molecular mass of 12 kDa and contains 109 amino acids (Eschenfeldt & Berger, 1986; Pan et al., 1986), but unlike other highly acidic nuclear proteins such as nucleoplasmin (Burglin et al., 1987; Dingwall et al., 1987) or nucleophosmin (Chan et al., 1989), it is deficient in many amino acid residues. Prothymosin α is devoid of tyrosine, tryptophan, phenylalanine, histidine, methionine, and cysteine and possesses single isoleucine, leucine, and proline residues, but it is extremely rich in acidic amino acids, bearing a total of 53 carboxyl groups. The organization of the primary structure is also unusual: the ten basic amino acids occur at the extremities in clusters of five. The basic cluster at the carboxyl terminus has been identified as a potent nuclear translocation signal, whereas the cluster near the amino terminus is ineffective (Manrow et al., 1991; Watts et al., 1990). Ten potential phosphorylation sites in the human protein (nine in other mammals) also reside near the termini, six within the first thirteen amino acids and the remainder in close proximity to the nuclear translocation sequence. Between them lies a central region which is composed predominantly of acidic residues interspersed with occasional Gly, Asn, Gln, and Ala residues. An acidic subset in this region bears a marked resemblance to the presumed histone binding site of nucleoplasmin (R. E. Manrow and S. L. Berger, unpublished; Burglin et al., 1987; Dingwall et al., 1987), a *Xenopus* protein believed to be an assembly factor responsible for histone deposition onto and removal from DNA during S phase and mitosis, respectively (Earnshaw et al., 1980; Dilworth et al., 1987; Kleinschmidt et al., 1990).

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The explicit role of prothymosin α in processes leading to mitosis is unknown. Bogdanov and co-workers (Vartapetian et al., 1988; Makarova et al., 1989) have proposed that prothymosin α is covalently bound to a small RNA much like the VPg's linked to the 5′-termini of certain viral genomic RNAs. Just how such an RNA might perform was beyond the scope of their study.

To arrive at a more precise function, we undertook a study of the structure of the human protein, its stability, and its putative post-translational modifications. We found no evidence for a covalent attachment to RNA. Furthermore, we have been unsuccessful in demonstrating affinity of prothymosin α for DNA or RNA (R. E. Manrow and S. L. Berger, unpublished), an unlikely trait in view of the protein's extreme acidity. Instead, we found that prothymosin α is phosphorylated at the amino-terminal acetylserine residue, that a minority species bears the phosphate, and that the level of the phosphorylated form appears to remain constant throughout the cell cycle in a synchronized population. We also measured the half-life of prothymosin α in rapidly growing cells; the protein remained stable, with a half-life almost equivalent to the doubling time. On the basis of these findings, we postulate a role(s) exerted throughout several phases of the cell cycle which, when interrupted, abrogates normal entry into M phase.

MATERIALS AND METHODS

Myeloma Cells. Human myeloma cells, type RPMI 8226, were obtained as a gift from Dr. David Nelson (National Cancer Institute) or were provided by the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI 1640 with fetal bovine serum and antibiotics at 37 °C in an atmosphere of 5% CO_2 as described by Sburlati et al. (1991) and Manrow et al. (1991).

Labeling of Myeloma Cells with [^{32}P]Orthophosphoric Acid. Rapidly growing myeloma cells were seeded at 10^5–10^6/mL and labeled for 2–40 h with 10–50 μCi/mL [^{32}P]-orthophosphoric acid (New England Nuclear-Dupont, NEX 053, carrier free) in complete or 80–85% phosphate-depleted medium (Sburlati et al., 1991; Manrow et al., 1991). Analytical experiments were performed in 100 × 15 mm bacteriological Petri dishes (Falcon) in 10 mL to facilitate recovery of a sticky subpopulation of cells.

Labeling of Myeloma Cells with [^{3}H]Glutamic Acid or [^{3}H]Uridine. Rapidly growing myeloma cells (5×10^5/mL) were pulsed-labeled with 100 μCi/mL L-[G-^{3}H]glutamic acid (Amersham TRK.445, 50 Ci/mmol) in complete medium at 37 °C for 3 h and chased at the same concentration in 10% conditioned medium without radioactivity. Myeloma cells (10^6/mL) were also labeled for 30 min at 37 °C with 100 μCi/mL [5-^{3}H]uridine (Amersham TRK.178, 30 Ci/mmol). At the stated times, cells were harvested, and prothymosin α together with nucleic acids was extracted, precipitated, and analyzed electrophoretically.

Synchronization of Myeloma Cells. Cells were synchronized by allowing them to remain in stationary phase for 4–6 days depending on their history. They were collected by centrifugation, resuspended in complete medium, and counted in the presence of 0.5% trypan blue before being seeded at $<10^6$ cells/mL in fresh medium. To ascertain both the degree of synchrony and the accumulation of [^{32}P]prothymosin α, cells were double-labeled with [^{32}P]orthophosphoric acid mixed with [5′-^{3}H]thymidine (Amersham TRK.328, 15.2 Ci/mmol). The nuclei from $\sim 10^8$ such cells in $\sim$4 mL of buffer (10 mM Tris-HCl at pH 7.5, 0.5% sodium dodecyl sulfate, and 0.5% 2-mercaptoethanol) were disrupted by pipetting and subjected to three consecutive extractions with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). DNA was recovered by precipitation essentially as described by Wallace (1987), dissolved in 100 μL of water, sheared, and used for liquid scintillation counting. Prothymosin α was recovered from postnuclear supernatants.

Isolation of Prothymosin α. Human prothymosin α from cultured cells was isolated from the aqueous phase of phenol-extracted postnuclear supernatants as a homogeneous protein using the method of Sburlati et al. (1990). Bovine prothymosin α was obtained from 60 g of pulverized, frozen calf thymus (Pel Freeze) by homogenization in buffer (50 mM Tris-HCl at pH 7.5, 25 mM KCl, 3 mM $MgCl_2$, and 0.4 mM phenylmethanesulfonyl fluoride), filtration through four layers of cheese cloth, and centrifugation at 6000 rpm for 15 min to remove debris. Supernatants were made 0.1% in sodium dodecyl sulfate and 1% in NP40 and phenol extracted. Where noted, 50 mM NaF was included in the lysis buffers. Nucleic acids were digested with 50–300 μg/mL RNase A and 130 units/mL of DNase (bovine samples only); enzymes were removed by additional phenol extractions (Sburlati et al., 1990), and particles were eliminated by centrifugation through Centricon 100 filters. Final purification was carried out either electrophoretically (Sburlati et al., 1990) or by C18 reversed-phase column chromatography as described below. The yield from 60 g of thymus was $\sim$300 μg of prothymosin α.

Electrophoretic Techniques. Proteins were analyzed in 15% polyacrylamide–SDS gels (Sburlati et al., 1990) or in the Novex system (San Diego, CA) at 100 V for 2.5 h using precast 18% polyacrylamide Tris–glycine gels, which acquire SDS from the running buffer. Coomassie brillant blue R-250 (0.05%) in 0.05% $CuSO_4$, 10% acetic acid, and 50% methanol was used to stain gels, whereas 10% acetic acid with either 10 or 40% methanol was used for destaining. Gels containing tritium were fixed, treated with fluor, and dried, whereas gels containing ^{32}P as the sole source of radioactivity were simply dried before exposure to X-ray film (Manrow et al., 1991).

For half-life measurements, purified [^{3}H]prothymosin α was quantified by scanning fluorographs of gels with a Molecular Dynamics 300A computing densitometer.

Chromatographic Techniques. High-pressure liquid chromatography was carried out with a Waters 625 liquid chromatography system using either reversed-phase C4 or C18 columns (Vydac, 5 μm, 4.6 × 250 mm) which were loaded with aqueous samples, washed for 5 min with 5% acetonitrile in 0.1% trifluoroacetic acid, and developed during an interval of 35 min (or 70 min in the expanded format) with a gradient consisting of 5 to 45% acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/min. Fractions of 250 μL were collected.

Generation of Synthetic Prothymosins. Synthetic prothymosin α (M_r 12 kDa) and a pseudogenomic protein containing nine additional amino acids at the carboxyl terminus (PT-MAP4) were prepared in vitro by translating synthetic, capped mRNAs in the wheat germ system with [^{3}H]glutamic acid as the sole radioactive precursor (Manrow et al., 1991, 1992).

Analysis of Phosphoamino Acids. Gel-purified [^{32}P]-prothymosin α (50 μg) obtained from $\sim 3 \times 10^8$ myeloma cells labeled for 40 h with 10 μCi/mL [^{32}P]orthophosphoric acid in phosphate-depleted medium was hydrolyzed for 2 h at 100 °C in 6 N HCl. Aliquots of hydrolyzed prothymosin α (5–10 μg) were mixed with 2 μL of a mixture of phosphoserine and phosphothreonine, each at 7.5 mg/mL in 1 mM HCl, and resolved by means of a thin-layer electrophoretic technique

(Kelley & Adelstein, 1990). Briefly, phosphoamino acids were separated from partially hydrolyzed protein in acid solution using Whatman 20 × 20 cm silica gel (60 Å, 250 μm) thin-layer chromatography plates; phosphoamino acid standards were visualized by staining with ninhydrin; and the labeled phosphoamino acids derived from prothymosin α were located by autoradiography.

Enzymatic Treatment of [^{32}P]Prothymosin α. Partially purified [^{32}P]prothymosin α was incubated for 1 h at 37 °C with the following enzymes and buffers in the volumes noted: 5 units of DNase I in 50 μL of 0.5× transcription buffer supplied with the Gemini transcription system (Promega); 90 units of RNase T2(Gibco-BRL) in 50 μL of 30 mM sodium acetate at pH 4.8; 125 units of RNase T1 (Gibco-BRL) in 50 μL of 1 mM Tris-HCl and 0.1 mM EDTA at pH 8; 100 μg of pancreatic RNase A (Sigma) in 50 μL of 1 mM Tris-HCl and 0.1 mM EDTA at pH 8; and 100 μg of proteinase K (Gibco-BRL) in 50 μL of 1 mM Tris-HCl and 0.1 mM EDTA at pH 8. These prothymosin α samples were the dissolved pellets obtained from ethanol precipitating the aqueous phases of phenol extractions. Gel-purified [^{32}P]prothymosin α (~2 μg) was treated at 37 °C for 1 h in a volume of 100 μL with 750 units of bacterial alkaline phosphatase in 0.1 M Tris-HCl at pH 9 or 120 units of calf alkaline phosphatase in buffer supplied by the manufacturer. Owing to a major difference in the unit definitions, a unit of the calf enzyme could be as much as 1000-fold more active than a unit of the bacterial enzyme.

Digestion of Prothymosin α with Proteases. Prothymosin α (~30–200 μg at 1–1.5 μg/mL) was digested for 6–17 h at 37 °C with endoproteinase Lys-C (Boehringer Mannheim) in buffer (50 mM Tris-HCl at pH 8.5, 0.025% sodium dodecyl sulfate, and 5% acetonitrile) using a ratio of 1 part enzyme to 20 parts protein on a weight basis. N-terminal peptides were treated with endoproteinase Asp-N (Boehringer Mannheim) for 3–6 h at 37 °C in 10 μL of 100 mM NH_4HCO_3 made 5% in acetonitrile. Enzyme:peptide ratios ranged from 1:10 to 1:50. When the radioactive, phosphorylated peptide from human prothymosin α was digested, a small amount of unlabeled, unphosphorylated peptide was included as carrier and as an aid to monitoring the digestion and subsequent chromatography.

Search for Phosphatases. Proteases were tested for phosphatase activity by monitoring the hydrolysis of 0.4 mM *p*-nitrophenyl phosphate at 410 nm for 18 h in a total volume of 0.5 mL. Hydrolysis of *p*-nitrophenyl phosphate by 150 units of bacterial alkaline phosphatase served as a positive control.

Amino Acid Analysis. Methods for hydrolyzing proteins and peptides and for determining the amino acid composition have been described (Sburlati et al., 1990).

Analysis of Prothymosin α and Its Peptides with the Mass Spectrometer. Determination of the mass of the intact protein and peptides derived from it was performed with a JEOL Model SX102 mass spectrometer. Prothymosin α (~100 pmol in 50 μL of acetic acid/hexafluoro-2-propanol/H_2O, 1:49:50) was introduced at 0.4 μL/min into the instrument using an Analytica electrospray source. The apparatus was configured to collect positive ions; a needle-to-capillary voltage of –5 kV was maintained while the source was opearated at 5 kV. Peptides were characterized with fast atom bombardment. To determine the spectra of positive or negative ions, the sample (~250 pmol or less in 1 μL) was desorbed from glycerol (1 μL) at 6 kV; when positive ions were selected, 1 μL of 0.1% trifluoroacetic acid was sometimes included. The methods are reviewed in Carr et al. (1991).

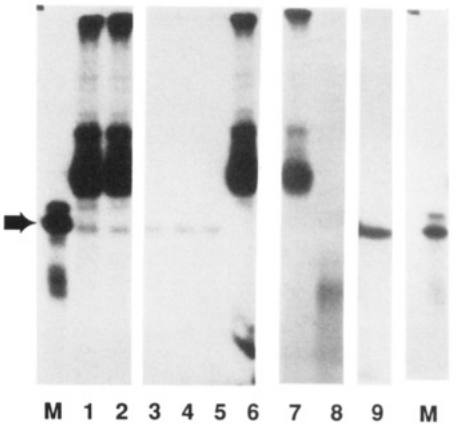

FIGURE 1: Proteins and nucleic acids recovered from phenol-extracted postnuclear supernatants. Cells labeled with [^{32}P]orthophosphoric acid (lanes 1–6), [^{3}H]uridine (lanes 7–8), or [^{3}H]glutamic acid (lane 9) were phenol-extracted to obtain prothymosin α as follows: [^{32}P]-Prothymosin α was isolated from ~2 × 10^7 cells labeled with 50 μCi/mL [^{32}P]orthophosphoric acid for 2 h. Partially purified materal recovered from the aqueous phase was passed through two consecutive spun G-25 Sephadex columns. An aliquot representing 10% of the total was treated with each of the following enzymes: lane 1, none; lane 2, DNase I; lane 3, RNase T2; lane 4, RNase T1; lane 5, pancreatic RNase A; lane 6, proteinase K. Tritium-labeled molecules were isolated similarly from 3 × 10^6 cells treated with 100 μCi/mL [^{3}H]uridine, and half of that material appears in lane 7; lane 8 contains the remainder treated with RNase T2. Prothymosin α was also obtained by the same technique from 10^7 cells labeled with [^{3}H]-glutamic acid (lane 9). The lanes labeled M contain ^{3}H-labeled synthetic protein derived from pseudogene (PTMAP) 4 (band above prothymosin α), ^{3}H-labeled synthetic prothymosin α (designated with the arrow), and a smudge representing degradation products. Macromolecules were resolved in 15% polyacrylamide gels, which were subsequently fixed, dried, and exposed to film for 1–3 days or, for glutamic acid-labeled prothymosin α, 4 weeks.

Peptides dissolved in 50% methanol and 0.5% acetic acid were also characterized using electrospray ionization performed with a Finnigan Model TSQ-700 triple quadrupole mass spectrometer. Daughter ion spectra were obtained by fragmenting the doubly charged parent ion at –10 eV using helium as the collision gas (reviewed in Smith et al., 1990).

RESULTS

Labeling of Prothymosin α with [^{32}P]Orthophosphoric Acid. The peculiar amino acid composition of prothymosin α makes possible a one-step procedure for removing contaminant proteins. Unlike virtually all proteins, prothymosin α partitions to the aqueous phase of a phenol extraction together with RNA, DNA, and carbohydrates (Sburlati et al., 1990). Furthermore, it leaks quantitatively out of the nuclei of detergent-lysed cells, a property which facilitates its isolation, particularly from DNA. Putative post-translational alterations to prothymosin α were studied by analyzing human myeloma cells metabolically labeled with [^{32}P]orthophosphate. As demonstrated in Figure 1 (lane 1), the aqueous phase of phenol-extracted postnuclear supernatants contained a host of radioactive species, one band of which migrated with the same mobility as synthetic prothymosin α (arrow in Figure 1, lane M). When the ^{32}P-labeled sample was treated with proteinase K (Figure 1, lane 6), that band disappeared, whereas all other radioactive material was unchanged either in intensity or in mobility. This result is consistent with the data in lane 9 (Figure 1), which show that using our technique only prothymosin α, among proteins, can be extracted from cells metabolically labeled with [^{3}H]glutamic acid. The identity of this protein has been established by amino acid analysis (Sburlati et al., 1990).

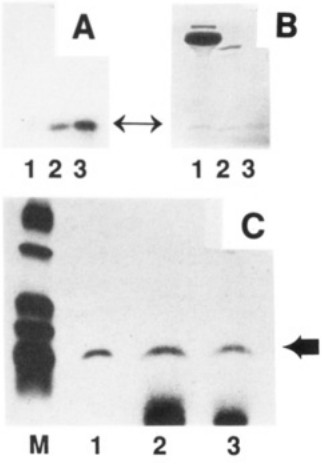

FIGURE 2: Treatment of [^{32}P]prothymosin α with alkaline phosphatases and isolation of [^{32}P]prothymosin α in the presence of a phosphatase inhibitor. (A) Gel-purified [^{32}P]prothymosin α obtained from 10^7 cells labeled for 40 h with 20 μCi/mL [^{32}P]orthophosphoric acid were treated with calf intestine alkaline phosphatase, lane 1, or bacterial alkaline phosphatase, lane 2, or were untreated, lane 3. The 18% polyacrylamide gel was exposed to film for 12 h. (B) The gel in (A) was photographed after it was stained with Coomassie blue. All lanes contain ~2 μg of prothymosin α. The higher molecular weight phosphatases appear in lanes 1 and 2. (C) Prothymosin α was obtained from 1.6×10^7 cells which had been labeled for 36 h with 10 μCi/mL [^{32}P]orthophosphoric acid and phenol-extracted in the absence, lane 2, or the presence, lane 3, of 50 mM sodium fluoride. Both partially purified samples were subjected to pancreatic RNase A before electrophoretic analysis. Synthetic [^{3}H]prothymosin α appears in lane 1; ^{14}C-labeled molecular mass markers in the lane designated M are as follows: ovalbumin, 43 kDa; carbonic anhydrase, 30 KDa; β lactoglobulin, 18.4 KDa; lysozyme, 14.3 KDa; bovine trypsin inhibitor, 6.2 KDa; and insulin A and B chains, 2.3 and 3.4 KDa. Samples were analyzed in an 18% polyacrylamide gel, which was exposed to film for ~20 h. The arrows indicate prothymosin α.

The nature of the labeled protein was investigated further by treating the partially purified sample with nucleases. Prothymosin α was unaffected by incubation with DNase I (Figure 1, lane 2), suggesting that the labeled phosphate did not reside in DNA. That the DNase was active was established by digesting end-labeled commercial markers made by restricting λ with *Hin*dIII (data not shown). The labeled protein was also digested with a series of ribonucleases, including RNases T2 and T1 and pancreatic RNase A (Figure 1, lanes 3–5, respectively). In each case, the ribonuclease degraded all of the phenol-extracted labeled molecules with the sole exception of prothymosin α. Because the nucleases as a group exhibit broad specificity toward RNA, we concluded that an RNA or DNA covalently attached to prothymosin α was unlikely, regardless of its sequence. Furthermore, we were unable to label prothymosin α during cell growth with radioactive uridine (Figure 1, lane 7) despite intense labeling of molecules susceptible to RNase T2 (Figure 1, lane 8). Analysis of [^{32}P]prothymosin α in two-dimensional gels corroborated the evidence for the absence of a large charged adduct; the metabolically labeled protein comigrated with synthetic [^{3}H]prothymosin α (data not shown). Thus, we have been unable to confirm the results of the Bogdanov group (Vartapetian et al., 1988; Makarova et al., 1989), who proposed that prothymosin α is covalently attached to an RNA containing ~20 bases.

To identify the phosphate-labeled moiety, gel-purified [^{32}P]-prothymosin α was treated with either bacterial or calf intestinal alkaline phosphatase. As indicated in Figure 2A, each of the phosphatases digested the protein, with complete disappearance of radioactive prothymosin α in the presence of the enzyme from calf intestine. Thus, all phosphate covalently linked to prothymosin α is apparently sensitive to alkaline phosphatase. Figure 2B shows that the loss of ^{32}P did not occur as a consequence of protease contamination in the enzymes; when the gel shown in Figure 2A was stained with Coomassie blue rather than visualized autoradiographically, neither the amount of the protein nor its mobility had changed. These experiments suggest that prothymosin α is phosphorylated and, because the molecular weight under denaturing conditions remained unaffected, that few residues are modified.

Although prothymosin α can be metabolically labeled with [^{32}P]orthophosphate, it was possible that some of the incorporated radioactivity had been removed during the isolation procedure. To reduce the effect of putative endogenous phosphatases, postnuclear supernatants were phenol-extracted in the presence (Figure 2C, lane 3) or absence (Figure 2C, lane 2) of sodium fluoride, a known phosphatase inhibitor (Brautigan & Shriner, 1988); the inclusion of the inhibitor did not change the intensity of the labeled prothymosin α band, nor did it result in the production of labeled protein at higher molecular weight. It was also conceivable that prothymosin α had become phosphorylated during the purification procedure. Such an artifact was ruled out by lysing twice the number of unlabeled cells in the presence of 5-fold more radioactivity (50 μCi/mL [γ-^{32}P]ATP), relative to the samples in Figure 2C, without producing a definitive signal (data not shown).

Identification of the Phosphorylated Amino Acid(s). There are ten residues in human prothymosin α which could be stably phosphorylated: four serines and six threonines. To distinguish which broad category was phosphorylated, [^{32}P]-prothymosin α was treated with pancreatic ribonuclease to degrade contaminating RNAs as shown in Figure 1, gel-purified, excised from the gel, and passed through two spun G-50 Sephadex columns. Approximately 50 μg of purified [^{32}P]prothymosin α was hydrolyzed in acid for only 2 h to prevent destruction of phosphothreonine and, to a greater extent, phosphoserine during the hydrolysis step. The resultant mixture, still containing large amounts of partially hydrolyzed protein, was mixed with phosphoamino acid standards and analyzed by thin-layer electrophoresis. The standards were stained with ninhydrin (Figure 3A), whereas radioactive materials, including peptides and phosphoamino acids, became evident upon autoradiography (Figure 3B). For ease in interpretation, Figure 3A actually shows the stained plate with the autoradiograph superimposed. In this configuration, the origin and labeled peptides, but not labeled amino acids, could be discerned. By comparing panels A and B of Figure 3, it can be seen that radioactivity comigrated with phosphoserine, but not with phosphothreonine. The conclusion was drawn that prothymosin α is phosphorylated on serine.

Identification of a Phosphorylated Serine Residue at the Amino Terminus of Prothymosin α. [^{32}P]Prothymosin α was obtained from human myeloma cells and purified further by reversed-phase column chromatography. A limited series of peptides was generated by digestion with Lys-C, which attacks the eight peptide bonds indicated by the arrows in Chart I (Wingard et al., 1972; Goodall et al., 1986; Eschenfeldt & Berger, 1986). The peptides were fractionated on a C4 reversed-phase column. As shown in Figure 4A, the elution pattern consisted of three major peaks reflecting the three peptides large enough to absorb significantly at 214 nm; the remainder chromatographed near the front. The absorbance peaks at 17.5, 23, and 25 min, respectively, were identified as

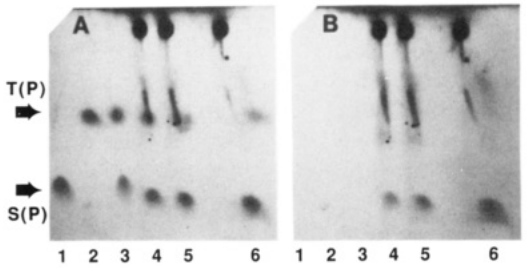

FIGURE 3: Thin-layer electrophoresis of metabolically labeled, acid-hydrolyzed [^{32}P]prothymosin α. Acid-hydrolyzed [^{32}P]prothymosin α was mixed with unlabeled phosphothreonine and phosphoserine and analyzed by thin-layer electrophoresis. The standards were visualized with ninhydrin; the labeled amino acids, by means of autoradiography. Results are displayed with the origin at the top and the anode at the bottom. (A) The photograph was generated by overlaying the ninhydrin-stained TLC plate with the autoradiograph. The ninhydrin spots for 15 μg of phosphoserine [S(P)] (lane 1), 15 μg of phosphothreonine [T(P)] (lane 2), and a mixture of the two (lane 3) are noted. Lanes 4, 5, and 6 contain respectively 5, 7.5, and 10 μg of hydrolyzed prothymosin α, each mixed with the two standards. Dark regions other than phosphoserine and phosphothreonine are large amounts of radioactive material at the origin and streaking radioactive acidic peptides. The ninhydrin color obscures radioactivity comigrating with phosphoamino acids. (B) The identical autoradiograph, produced in a 4-day exposure, appears alone. The same lane numbers have been used. Note that a smudge of radioactivity located approximately midway between the origin and phosphoserine does not comigrate with phosphothreonine.

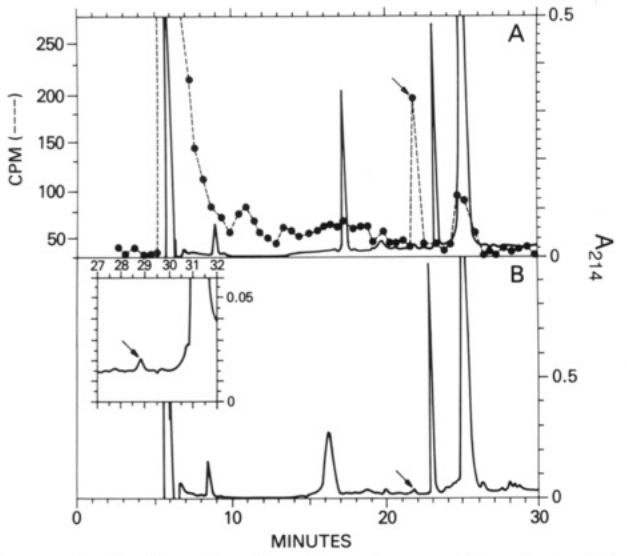

FIGURE 4: Fractionation of prothymosin α peptides obtained by digestion with endoproteinase Lys-C. Peptides from ^{32}P-labeled and unlabeled human prothymosin α (~30 μg of each) (A) or from 100 μg of bovine prothymosin α (B) were resolved by C4 reversed-phase column chromatography. Lys-C-digested bovine prothymosin α (220 μg) was also subjected to C4 column chromatography using an expanded gradient (inset). Peptides that elute between 21.5 and 23.5 min under standard conditions are displayed in the expanded format. Arrows mark the phosphorylated 14-mer.

amino acids 88–101, the N-terminal 14-mer composed of amino acids 1–14, and the 67-mer representing amino acids 21–87. There were also three peaks of radioactivity: one at the front representing a small amount of highly labeled RNA which copurified with the protein and suffered further degradation, a peak at 22 min, and a peak eluting slightly ahead of the 67-mer. The distribution of radioactivity in the peptide peaks varied, with as much as 80% eluting at 22 min. The radioactivity peak at 25 min represents either an incompletely digested amino-terminal peptide or incorporation of phosphate at serine 83. As this was the minority species, it was not pursued. It is clear that the major radioactive peak did not comigrate with any of the peptides, suggesting immediately that only a small fraction of prothymosin α was phosphorylated.

To obtain sufficient material for further characterization, bovine prothymosin α from calf thymus was isolated; the molecular mass was confirmed in the mass spectrometer by electrospray ionization, and the amino acid composition was determined to establish purity. The bovine protein is almost indistinguishable from the human, differing at only two locations: D at position 31 becomes E, and S at position 83 becomes A (Panneerselvam et al., 1988). The latter substitution also occurs in the pig (Economou et al., 1988), goat (Frillingos et al., 1991), mouse (Schmidt & Werner, 1991), and rat (Haritos et al., 1985), making it unlikely that the serine residue at position 83 of humans is required for activity. Given the degree of conservation, it was not surprising that the pattern of peptides from bovine prothymosin α, after Lys-C digestion (Figure 4B), was almost identical to that from humans. However, in the bovine system, there was sufficient material eluting at 22 min, the location of the labeled human peptide, for evaluation in the mass spectrometer. Using fast atom bombardment in the positive ion mode, two peaks were detected: a large peak at m/z 1466 (MH^+) and a much smaller peak at m/z 1546 (data not shown). The former is the N-terminal 14-mer also found in much greater quantity at 23 min; the latter is precisely the correct mass for a 14-mer bearing a single phosphate group. It also elutes at a lower acetonitrile concentration than its unphosphorylated counterpart does, in agreement with the predictions of Cohen et al. (1991). A larger amount of bovine prothymosin α was prepared and digested with Lys-C to obtain a fraction sufficiently enriched in the phosphorylated 14-mer to allow further characterization. As shown in Figure 4 (inset), a shallower gradient was used to resolve the products. The peak at 29 min (arrow) gave rise to four major species in the mass spectrometer located at m/z 1546 (MH^+), 1568 (MNa^+), 1584 (MK^+), and 1590 ($MNa_2 - H)^+$ (Figure 5A), all of which corroborate the presence of a phosphorylated 14-mer. The extensive sodiation is characteristic of highly negatively charged, hence phosphorylated, species. A small amount of the unphosphorylated 14-mer, m/z 1466 (MH^+), and its sodium adduct, m/z 1488 (MNa^+), persisted as contaminants. Together with the results from the labeled human peptide, the data support phosphorylation of one serine residue within the amino-terminal peptide. It should be noted that this peptide contains all of the serine residues of bovine prothymosin α. The results also provide a means for quantifying phosphorylated prothymosin α molecules. When the absorbance peak for each phosphorylated peptide (arrows in Figure 4) and its corresponding 14-mer were integrated in these and other experiments, the percentage phosphorylated was found to be 1.7–3% of the total N-terminal peptide regardless of whether the parent protein was isolated from human myeloma cells or bovine thymus.

Chart I

acSDAAVDTSSEITTK↓DLK↓EK↓K↓EVVEEAENGRDAPANGNANEENGEQEADNEVDE-

EEEEGGEEEEEEEEGDGEEEDGDEDEEAESATGK↓RAAEDDEDDDVDTK↓K↓QK↓TDEDD

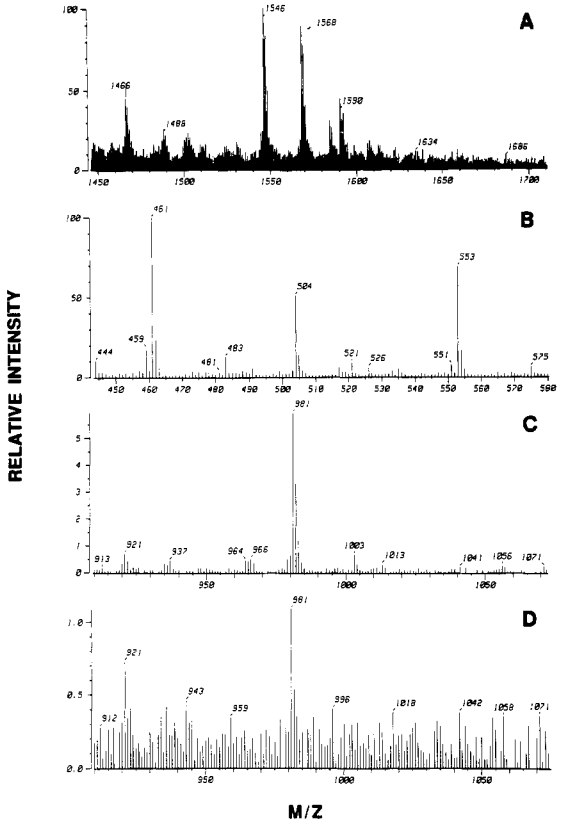

FIGURE 5: Identification of prothymosin α peptides by mass spectrometry. The spectra of positive ions generated by fast atom bombardment are shown as follows: (A) bovine peptide indicated by the arrow in the inset of Figure 4 (identified as the phosphorylated 14-mer); (B) peak 2 in Figure 6 (identified as amino acids 1–5); (C) peak 3 in Figure 6 (identified as amino acids 6–14); (D) peak 4 in Figure 6 (also identified as amino acids 6–14).

To determine which of the serine residues contained phosphate, it was necessary to digest the amino-terminal peptides into smaller fragments. Asp-N, with the ability to hydrolyze peptide bonds on the amino-terminal side of aspartic acid residues (Drapeau, 1980), was the enzyme of choice. The expected products were a 9-mer containing two adjacent serine residues (see Chart I) and either a 5-mer or a monomer and a tetramer, depending on whether Asp 2 as well as Asp 6 was a target for hydrolysis. When the unphosphorylated 14-mer was used as a test case, two peptides were produced (Figure 6, peaks 2 and 3), both of which were examined in the mass spectrometer. As illustrated in Figure 5B, peak 2 at m/z 504 (MH^+) is precisely the expected size for the 5-mer consisting of AcSer-Asp-Ala-Ala-Val. Peak 3 (Figure 5C) is the predicted 9-mer, with m/z 981 (MH^+). Apparently the peptide bond between residues 1 and 2 of the 14-mer was not attacked by Asp-N.

Both the labeled human phosphopeptide (Figure 4A, arrow) and the unlabeled bovine counterpart (Figure 4, arrow in inset) were subjected to digestion with Asp-N. Because there is but one phosphorylated amino acid, the products should consist of two peptides: one different from either of the peptides generated from the unphosphorylated 14-mer and one identical to either peak 2 or peak 3. Figure 6 displays the results: the labeled phosphorylated 14-mer from human prothymosin α gave rise to one major peak of radioactivity (peak 1) which eluted ahead of the 5-mer (peak 2) in the correct location for a phosphorylated version, whereas the bovine phosphorylated 14-mer resulted in one identifiable peptide (peak 4) which coeluted with peak 3, the 9-mer. Furthermore, when peak 4 was characterized in the mass spectrometer, a signal appeared at m/z 981 (Figure 5D), which is the correct size for the 9-mer. It should be pointed out that the yield of 9-mer from the phosphorylated 14-mer based on absorbance at 214 nm is ~100%, a finding which eliminates contaminants as the source of peak 4. The data are consistent with phosphoserine at position 1.

Positive identification of the serine residue which was phosphorylated was obtained by fragmenting both the doubly charged 14-mer and the doubly charged phosphorylated 14-mer in the mass spectrometer under electrospray ionization conditions and comparing the daughter ions. If the N-terminal serine is phosphorylated, as suggested by the mobility of the Asp-N peptides, fragments containing the first amino acid should be shifted upward by 80 mass units, relative to the phosphate-free peptide, but fragments derived from any subset of amino acids 2–14 should not shift regardless of which parent peptide is used. With the doubly charged 14-mer (m/z 734) as the parent (Figure 7A), a series of peaks was produced almost all of which were identified as either B or Y ions (definitions appear in the caption to Figure 7.) Other prominent ions included an A ion (see below) and a peak at m/z 725 representing a dehydrated species with two positive charges. The doubly charged phosphorylated 14-mer with m/z 774 (Figure 7B), owing to its scarcity, produced fewer discernible daughter ions, but several were diagnostic for phosphorylation: ions at m/z 368 (A-ion trimer), 467 (B-ion tetramer), and 566 (B-ion pentamer) are located 80 mass units upstream from the corresponding ions derived from the 14-mer, which are located at m/z 288, 387, and 486, respectively (Figure 7A). It is highly significant that the A and B ions noted in the phosphopeptide spectrum were absent from the spectrum of the 14-mer and vice versa. The C-terminal, Y-ion fragments left as remainders from the tetramer and the pentamer, namely, the 10-mer at m/z 1080 and the 9-mer at m/z 981, respectively, were observed in both spectra but, as expected, did not shift. We also observed a phosphorylated 11-mer at m/z 1198 (Figure 7B) with its unphosphorylated counterpart located at m/z 1118 (Figure 7A). A major peak at m/z 725 represents loss of phosphoric acid from the doubly charged parent (Figure 7B). The results prove that phosphorylation occurs within the N-terminal trimer and not at or C-terminal to position 5. Therefore, the phosphate is located on the N-terminal acetylserine residue.

Regulation of Prothymosin α. The activity of cell cycle regulatory proteins depends on the amount of the protein and the degree of phosphorylation. Both aspects of prothymosin α were investigated. If prothymosin α were to be down-regulated at any point in the cell cycle, the event would be reflected in the half-life; as each cohort of a nonsynchronized population entered the phase of the cell cycle at which regulation occurred, prothymosin α levels would plummet. Such behavior averaged over all cells would allow calculation of the half-life. Furthermore, if prothymosin α were to turn over at all stages of growth, the half-life would still be short. To measure protein stability, growing cells were pulse-labeled with [^{3}H]glutamic acid and chased for varying periods of time. Radioactivity in bulk cytoplasmic protein and in gel-purified prothymosin α was ascertained. It is clear from the data in Figure 8A that prothymosin α decayed with a half-life of ~20 h. This value was then compared to the turnover of bulk labeled protein under the identical conditions. The data in Figure 8B show that the disappearance of trichloroacetic acid precipitable [^{3}H]protein followed first-order kinetics, with a half-life of ~27 h. The latter determination should be

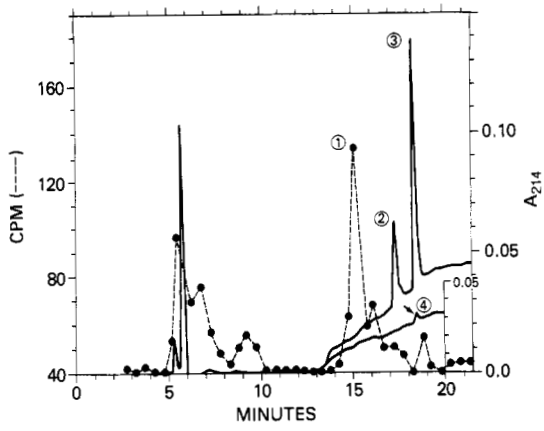

FIGURE 6: Fractionation of the peptides obtained by digesting the amino-terminal 14-mer of prothymosin α with endoproteinase Asp-N. The diagram displays three superimposed elution profiles obtained by C18 reversed-phase column chromatography. The profile of radioactivity (dashed line) was generated using all of the labeled peptide indicated by the arrow in Figure 4A. The absorbance profile plotted against the 0.15 scale reflects the products derived from ~3 μg of the unphosphorylated 14-mer eluting at 31–31.5 min in the inset of Figure 4, and the absorbance profile plotted against the 0.05 scale represents the products obtained from all of the unlabeled peptide indicated by the arrow in the inset of Figure 4. Circled numbers denote peaks discussed in the text.

roughly equivalent to the generation time of the cells because stable polypeptides dominate the cellular average. In these particular experiments, cells doubled in ~24–30 h, rather than in <24 h, owing to their exposure to radioactivity, and washing and handling (data not shown). The results suggest that prothymosin α is only slightly less stable than bulk protein and, more importantly, that it does not vary significantly over the cell cycle.

Cell Cycle Dependence of Phosphorylation. There are many examples in which the activity of a protein is regulated by cell cycle-dependent phosphorylation [reviewed in Cyert and Thorner (1989)]. Because prothymosin α is a nuclear phosphoprotein required for cell division, experiments were designed to examine the phosphorylation process in more detail. A synchronized population was pulse-labeled with [^{32}P]-orthophosphate to search for an interval in which the cells became heavily phosphorylated or, alternatively, a period in which they were refractory. We began by determining the growth characteristics of these cells. As illustrated in Figure 9A, the cell concentration remained constant throughout each 4-h interval until 20–24 h, at which time it doubled, indicating that the majority of cells had progressed synchronously through the cell cycle and divided. This view was confirmed by measuring nuclear DNA synthesis with 4-h pulses of [^{3}H]-thymidine; S phase apparently occurred in most of these cells between 12 and 16 h. With synchronized cells in hand, it was possible to determine when in the cell cycle prothymosin α could acquire phosphate. The cells, which were pulse-labeled with thymidine, were simultaneously pulse-labeled with [^{32}P]-orthophosphate, and prothymosin α was isolated, treated with RNase A, and analyzed electrophoretically. As shown in Figure 9B, phosphorylation occurred throughout the cell cycle, with equivalent amounts of [^{32}P]prothymosin α produced in each 4-h interval. Minor variations in phosphorylation occurred in all experiments, but there was no sustained change at any stage. We also measured the total amount of prothymosin α in synchronized cells at various times by visualizing the protein with Coomassie blue (Figure 9C). The latter data are consistent with and extend previous information about the behavior of prothymosin α in cycling myeloma cells. Whereas the mRNA increased 4–6 h after growth stimulation (Sburlati et al., 1991), prothymosin α was induced between 8 and 10 h, and remained as an abundant protein thereafter. In these specific cultures, however, a combination of the large starting cell number, the small volume, and the geometry of the vessel suppressed rapid growth beyond 18 h. Considering all of the data in Figures 8 and 9, the results suggest that prothymosin α becomes phosphorylated and remains abundant at all phases of cell growth.

DISCUSSION

Structure. Prothymosin α is a phosphorylated, nuclear protein which is essential for cell division. It is also highly acidic and found in quantities equivalent to those of histones. Its function is unknown. Previously, Bogdanov and co-workers claimed that prothymosin α was covalently linked to the 5′-end of an RNA of about 20 bases (Vartapetian et al., 1988; Makarova et al., 1989). We have not confirmed this finding despite the fact that our method for phenol-extracting the protein is almost identical to theirs. Apparently, owing to its unusual amino acid composition, prothymosin α partitions to the aqueous phase despite the absence of covalently bound nucleic acids or carbohydrates (Sburlati, 1990). In our experiments, natural prothymosin α, isolated from myeloma cells, comigrated with a synthetic protein in both one- and two-dimensional gels, behavior which is not consistent with a large negatively charged adduct acquired in vivo.

It is also important that our experiments utilized prothymosin α, both tagged metabolically with [^{32}P]orthophosphate and without isotopic replacement. We found that [^{32}P]-prothymosin α was unaffected by digestion with a host of nucleases, including RNase T2, the enzyme employed by the Bogdanov group. However, it *was* rendered nonradioactive by alkaline phosphatase without a change in apparent molecular weight. These methods are quite unlike those of Vartapetian et al. (1988), who employed extremely small amounts of material visualized with Bolton–Hunter reagent, a tool for iodinating protein and not for labeling RNA. In our laboratory, when RNA was labeled intracellularly, prothymosin α remained free of [^{3}H]uridine (Figure 1). We conclude that RNA is not covalently attached to prothymosin α.

Function. Cell division depends on prothymosin α (Sburlati et al., 1991). When levels of this protein are insufficient, owing to the presence of intracellular antisense oligomers, cells cannot divide. It is also clear that high concentrations of prothymosin α and its mRNA are indicative of rapid growth, whereas low concentrations coincide with the stationary, starving, or resting state (Figure 9; Eschenfeldt & Berger, 1986; Gomez-Marquez et al., 1989; Sburlati et al., 1991; Bustelo et al., 1991; Haritos et al., 1984b; Economou et al., 1988; Clinton et al., 1989; Frillingos et al., 1991). In the present study, we show that prothymosin α remained abundant during proliferation of human myeloma cells, with a half-life slightly shorter than the doubling time of the cells. Thus, if the activity of the protein were to be adjusted during the cell cycle, post-translational modification would have to be invoked. The possibility for such regulation was heightened by the discovery of a phosphorylated serine residue at the N-terminus of prothymosin α. However, since our data also indicate that the protein was phosphorylated throughout the cell cycle, we conclude, instead, that the activity of prothymosin α does not vary and that the protein does not *directly* govern cell division.

It may be premature to speculate about the function of prothymosin α and the effect of phosphorylation on its activity. The situation appears particularly murky because only 2% of

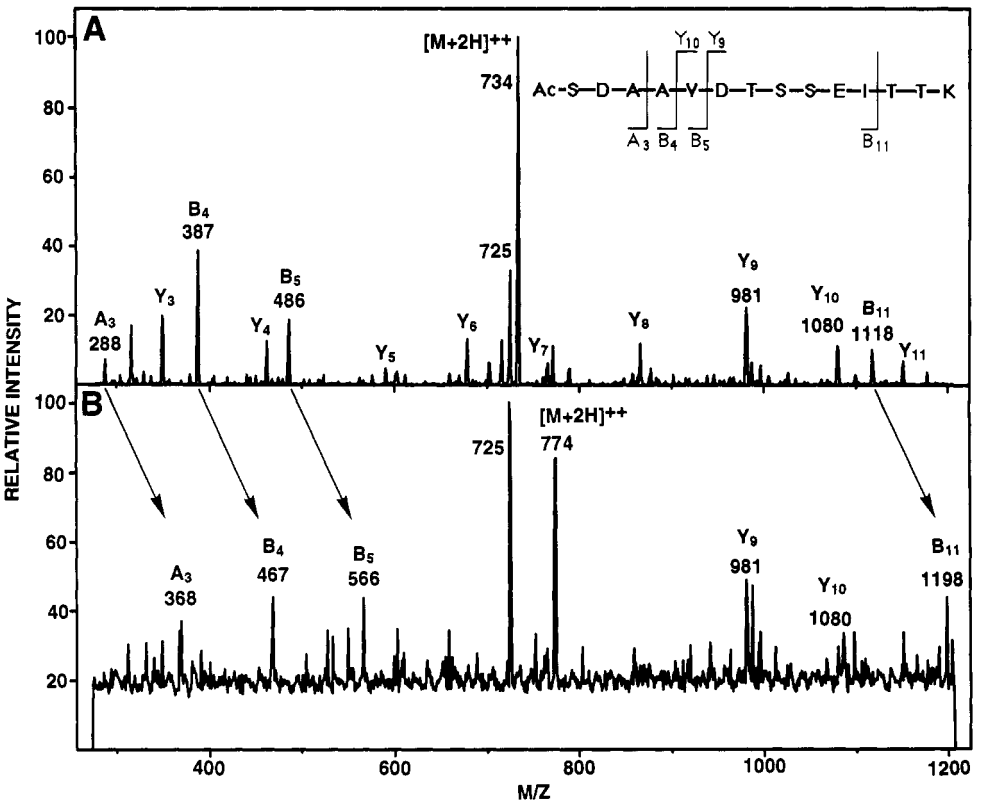

FIGURE 7: Mass spectra of daughter ions produced upon fragmentation of the doubly charged 14-mer and the doubly charged phospho-14-mer generated by Lys-C digestion of prothymosin α from bovine thymus. The unphosphorylated 14-mer (A) and the phosphorylated 14-mer (B) were isolated from a thymus different from those used for the data in Figure 4 and were fractured in the mass spectrometer. Relative intensities in panels A and B are unrelated. Ions are defined at the upper right corner of panel A as follows: A ions, fragments from the N-terminus which fracture on the C-terminal side of the α carbon to produce aldimines; B ions, fragments from the N-terminus which fracture through the peptide bond resulting in acylium ions; and Y ions, C-terminal fragments with a structure much like enzymatic hydrolysis products. In this case, for a 14-mer, a B-ion tetramer includes amino acids 1–4, whereas a Y-ion 10-mer contains amino acids 5–14 (amino acids 1–10 counting from the carboxyl terminus). Arrows indicate ions that have shifted as a consequence of phosphorylation. More detailed descriptions and structures of fragment ions can be found in Carr et al. (1991). All ions bear one positive charge with the exception of the doubly charged parent ions denoted $[M + 2H]^{2+}$ and the doubly charged ion at m/z 725.

prothymosin α molecules are phosphorylated at steady state. However, prothymosin α is plentiful during rapid growth, representing 0.3 pg/cell, or about 0.02% of total protein. Thus, the number of phosphorylated prothymosin α molecules, 340 000 per cell, is significant, and the fraction phosphorylated is comparable to that of inhibitor-1, a thermostable phosphatase inhibitor [reviewed in Cohen (1989)]. Inhibitor-1, which must be activated by a kinase, is <10% phosphorylated in perfused rat hemicorpus. We suggest that the low level of phosphorylation we observed be viewed as a clue, which together with the labeling kinetics is consistent with phosphate turnover on preexisting molecules. Although there are many roles prothymosin α might fulfill, we favor a testable hypothesis in which phosphorylation is dynamic and occurs during each cycle of activity performed by the protein.

A comparison of prothymosin α with other acidic proteins of the nucleus was undertaken as a further aid to deducing function. In the search for parallel relationships, the circumstances of its phosphorylation and its location, structure, abundance, and net charge had to be considered. Polypeptides with acidic stretches have been implicated in the following nuclear processes: DNA repair [the RAD6 gene product; reviewed in Prakash (1989)]; DNA replication, recombination, and bending [HMG proteins; reviewed in Lilley (1992)]; structure of the centromere (CENP-B; Earnshaw et al., 1987); transcriptional activation (GAL4, GNC4, and acid blobs; Ma & Ptashne, 1987; Hope & Struhl, 1986; Sigler, 1988); and assembly of nucleosomes (nucleoplasmin; Laskey et al., 1978) and ribonucleoprotein particles (nucleophosmin; Schmidt-Zachmann, 1987). With the exception of the assembly operations, all of these functions require stable association with DNA or chromatin. As noted above, prothymosin α does not remain bound to insoluble nuclear components (Sburlati et al., 1990; Manrow et al., 1991) and, given the prevalence of acidic residues throughout the molecule, exhibits no affinity for DNA or RNA (R. E. Manrow and S. L. Berger, unpublished results). In contrast, prothymosin α bears a marked resemblance to the assembly factors. These proteins are characterized by low isoelectric points, the ability to undergo phosphorylation and oligomerization, and competence as molecular chaperones [reviewed in Dingwall and Laskey (1990)]. Since prothymosin α is excluded from the nucleolus (Manrow et al., 1991), the site of nucleophosmin function, nucleoplasmin is the more apt model.

Nucleoplasmin is the most abundant protein in *Xenopus laevis* germinal vesicles (Mills et al., 1980; Krohne & Franke, 1980). It consists of heat-resistant pentamers, exhibits varying levels of phosphorylation, does not constitute an integral part of chromatin, readily leaks out of the nucleus, and combines reversibly with histones [Laskey et al., 1978; Earnshaw et al., 1980; Krohne & Franke, 1980; Mills et al., 1980; Cotten et al., 1986; reviewed in Dingwall and Laskey (1990)]. It has been shown to facilitate assembly of nucleosomes and has been found associated with active chromatin and with the products of transcription (Laskey et al., 1978; Earnshaw et al., 1980; Moreau et al., 1986). It should be pointed out that this protein has no direct analog in frog somatic cells or in other organisms (Burglin et al., 1987), although nucleosome

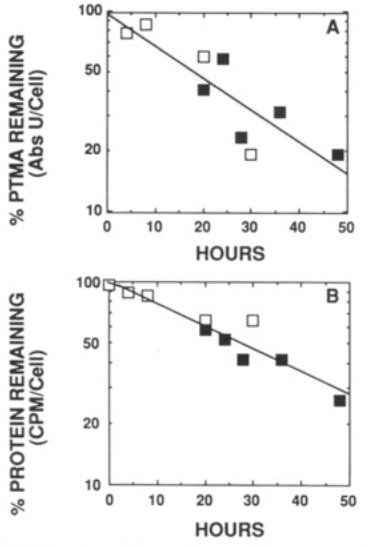

FIGURE 8: Half-life of prothymosin α. Human myeloma cells were pulse-labeled with [^{3}H]glutamic acid and chased for the stated period. (A) [^{3}H]Prothymosin α (PTMA) was quantified by means of a scanning densitometer after extraction with phenol, electrophoretic purification in an SDS–polyacrylamide gel, and fluorography of the gel. Points represent normalized values of absorbance (arbitrary units) due to prothymosin α/cell. The number of cells varied from 1×10^7 to 20×10^7, depending on the length of the chase period. Normalization of data from different experiments (each with its own symbol) was carried out as follows: (1) the intercept on the ordinate [representing log (prothymosin α)] was evaluated by extrapolating a fitted line in each experiment to 0 time on the abscissa; (2) the 0-time value in arbitrary absorbance units/cell was set equal to 100% remaining; (3) all other data in the same experiment were corrected accordingly. (B) Turnover of bulk ^{3}H-labeled protein, obtained by determining trichloroacetic acid precipitable radioactivity per cell in an aliquot of the same samples used for assay of prothymosin α, was normalized similarly. Best-fit lines were computer generated with the aid of Cricket Graph.

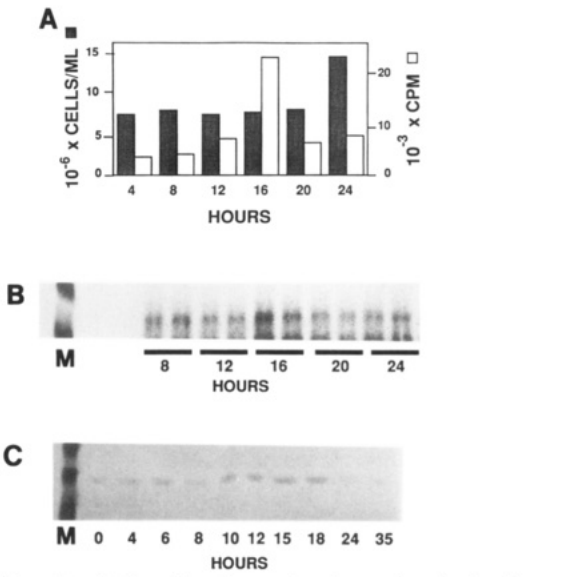

FIGURE 9: Phosphorylation of prothymosin α in synchronized cells. Cells in stationary phase were transferred to fresh medium and distributed into 16 dishes, each containing 10 mL of medium. Cells were counted and pulse-labeled in duplicate for 4 h by the addition of both 0.06 μCi/mL [^{3}H]thymidine and 10 μCi/mL [^{32}P]orthophosphoric acid as detailed in Materials and Methods. (A) At the stated time, an aliquot of 50 μL from each dish was used to count cells (filled bars). The remainder of the cells were lysed and fractionated. The amount of [^{3}H]thymidine incorporated into DNA during a pulse terminated at the stated time was ascertained by phenol-extracting the nuclei (open bars). Values reported are averages of duplicates. (B) The amount of ^{32}P incorporated into prothymosin α during a pulse terminated at the stated time was determined by phenol-extracting cytoplasmic supernatants of the same cells. Duplicate samples were treated with RNase A and analyzed in an 18% polyacrylamide gel. The dried gel was exposed to film for 5 days. The lane designated M displays bovine trypsin inhibitor and lysozyme as markers. (C) Cells in stationary phase were transferred to fresh medium and resuspended at 10^6/mL. The 15% polyacrylamide gel displays Coomassie blue-stained prothymosin α obtained from 5 mL of culture at 0, 4, 6, 8, 10, 12, 15, 18, 24, and 35 h after growth stimulation. The lane designated M displays the two markers in lane M of panel B as well as β lactoglobulin.

assembly and transcription are functions shared by almost all tissues. Similarly, prothymosin α is rich in negative charge, plentiful, probably unfolded (Watts et al., 1989, 1990), and stable. It has been reported to undergo oligomerization (Haritos et al., 1984a, 1987; Palvimo & Linnala-Kankkunen, 1990), becomes phosphorylated, does not associate stably with nuclear components (Sburlati et al., 1990), and promptly diffuses out of the nucleus during preparative procedures (Manrow et al., 1991). More importantly, it possesses an acidic motif virtually identical to the presumed histone binding region of nucleoplasmin (Burglin et al., 1987; Dingwall et al., 1987; R. E. Manrow et al., unpublished results).

Chromatin is a dynamic structure which not only packages and preserves DNA for mitosis and further generations but also molds it for transcription. van Holde et al. (1992) have incorporated the kinetic aspects of chromatin into a novel view of transcription: they postulate that partial unfolding of nucleosomes accompanies the passage of RNA polymerase along DNA. Implicit in the theory is a physiological means for loosening or displacing histone dimers in a reversible manner. Such nucleosome displacement during transcription has now been demonstrated (Clark & Felsenfeld, 1992). Although there is no experimental evidence to implicate prothymosin α in the process, it should be noted that this protein apparently functions throughout the cell cycle, that it is soluble in nucleoplasm, that it is depressed in transcriptionally inactive, quiescent cells, and that, under rigorously controlled conditions, cells treated with antisense oligonucleotides to prothymosin α mRNA synthesized reduced amounts of total mRNA and its precursors (A. R. Sburlati, unpublished results).

Concluding Remarks. The overall data tend toward a link between prothymosin α and chromatin. Since neither the amount of prothymosin α nor the interval during which it is phosphorylated appears to vary with the cell cycle, how can a possibly subtle effect on chromatin be reconciled with a function essential for cell division? Prothymosin α might influence the onset of mitosis by controlling the accumulation of products needed to traverse each phase of the cell cycle: a deficit would lead to a cessation of growth by causing the cell to default on successive cell cycle commitments.

NOTE ADDED IN PROOF

Since submitting our manuscript, two papers on the phosphorylation of prothymosin α have appeared [Barcia, M. G., Castro, J. M., Jullien, C. D., González, C. G., & Freire, M. (1992) *FEBS Lett. 312*, 152–156; Barcia, M. G., Castro, J. M., Jullien, C. D., & Freire, M. (1993) *J. Biol. Chem. 268*, 4704–4708]. Both papers claim that prothymosin α, when phosphorylated in vivo, contains phosphothreonine within the amino terminal tryptic peptide. As documented above, our findings differ, and we believe that we understand the nature of the discrepancy: they did not rule out the possibility that significant phosphorylation had occurred as an artifact during the isolation procedure, whereas we did; they assumed that a phosphorylated peptide would comigrate with its unphosphorylated counterpart, whereas we showed that the two

resolve using an almost identical peptide purification scheme; and they did not determine the precise location of the phosphorylated residue, whereas we used mass spectrometry to find it. Not only did we identify N-terminal acetylserine as the source of the phosphate, but also we excluded the threonine residues near the N-terminus. Their data suggest to us that the label and the peptide may have resided in unrelated molecules.

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