Prothymosin α in Vivo Contains Phosphorylated Glutamic Acid Residues*

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Human and monkey prothymosin α contain activated carbonyl groups on glutamic acid residues. Three lines of evidence indicate the existence of unusual phosphates. 1) Prothymosin α is phosphorylated by calf thymus protein kinases. 2) Human and monkey prothymosin α is a much less exhaustive analysis in mouse splenic lymphocytes, placed the labeled phosphate(s) on unspecified threonine residue(s) near the N terminus (22). Because prothymosin α sequences from different species are nearly identical, with −95% sequence homology over the entire protein and 100% sequence homology within the amino-terminal 30 residues (23, 24), the discrepancy was unsettling.

The specific function of prothymosin α has eluded detection. Despite a report to the contrary, prothymosin α is not present in yeast, nor are there close relatives, with the exception of the X. laevis protein, nucleoplasm. Both prothymosin α and nucleoplasm share a presumptive histone binding sequence, localize to the nucleus, and achieve high concentrations (5, 6, 14–16). However, unlike nucleoplasm, which disappears during embryogenesis (14), prothymosin α persists as an abundant protein in proliferating cells throughout the life of the organism. Other observations from which prothymosin α’s function can be intimated include 1) binding to histones in vitro (17, 18), implicating a role in chromatin remodeling; 2) interacting with the Rev protein of human immunodeficiency virus in vitro (19), suggesting involvement in RNA export from the nucleus; 3) up-regulation in the presence of Myc in specialized cells (20, 21); and 4) phosphorylation (16, 22).

There are two views of prothymosin α’s phosphates. According to Shurlati et al. (16), phosphorylation of the human and bovine protein occurs on the N-terminal acetylserylserine residue, and not on Ser at positions 8 and 9 or Thr at positions 7, 12, 13, 100, and 105 (see Fig. 1). Ser83, which is an alanine in most other mammals, and Thr85 were not rigorously excluded as possible sites in this study. The second study of phosphoproteins α, a much less exhaustive analysis in mouse splenic lymphocytes, placed the labeled phosphate(s) on unspecified threonine residue(s) near the N terminus (22). Because prothymosin α sequences from different species are nearly identical, with −95% sequence homology over the entire protein and 100% sequence homology within the amino-terminal 30 residues (23, 24), the discrepancy was unsettling.

The dynamic aspects of prothymosin α’s phosphates were equally puzzling. Prothymosin α is metabolically stable and
becomes phosphorylated equivalently at all stages of the cell cycle (16). Additionally, only 2% of the bovine protein is phosphate at steady state (16). Based on these properties, prothymosin α seemed to be involved in the continuing activities performed by the cell and not, as previously postulated (25), in the regulation of an intermittent function of the cell cycle.

Here, both the location and the stability of prothymosin α’s phosphates have been evaluated. We find that the initial sites of phosphorylation are glutamic acid residues, that the phosphates are extremely labile and readily hydrolyze during the earliest steps of the isolation procedure, and that phosphorylation on serine or threonine may occur solely in vitro when labile phosphates transfer to stable positions. Our data resolve the discrepancies between observations of Ser/Thr phosphorylation made in the human and mouse systems and explain the minute amount of phosphate found on the protein regardless of the organism studied. Based on an analysis of the peptides of human and monkey prothymosin α, the phosphorylated residues have been localized to an extremely acidic region that is homologous with the histone binding site of nucleoplasmin. Since the free energy of hydrolysis of a glutamyl phosphate is higher than that of ATP (26) and since our evidence suggests that several of prothymosin α’s glutamic acids bear phosphate simultaneously, we surmise that the protein is able to supply abundant energy for processes in the nucleus.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—** Mutant clones were obtained by making modifications in pBC12HProG, the 5-kilobase pair human prothymosin α gene cloned into pBC12B1 (5). A triple mutant in which Serα, Serα′, and Thrα′ were replaced by Ala residues was prepared using the polymerase chain reaction and appropriate oligomers to generate the desired mutations. Construction involved three steps. A single mutant in which Serα was replaced by Ala was generated first; in a second construct, Serα′ and Thrα′ were mutated; and finally, the triple mutant was obtained by shuffling fragments derived from the two mutant genes using hapaxomers (27). A map of the gene illustrating the locations of important sites and regions is shown in Fig. 1. Similar methods were used to generate clones coding for prothymosin α wild type or mutant proteins bearing six carboxyl-terminal histidine residues.

Expression of human prothymosin α in Escherichia coli was achieved by cloning the cDNA into NdeI-BamHI-cut pET3a from Stratagene. Sites were introduced into the cDNA using the polymerase chain reaction. In a related clone, the codons for six C-terminal histidine residues were introduced in the SDS/IMHI-containing part of the polymerase chain reaction-generated DNA was sequenced to select error-free molecules for further study. Recombinant genes were expressed in E. coli BL21(DE3) from Stratagene, and protein was recovered by lysing bacteria in 8 M urea made 0.1 M in sodium phosphate and 0.01 M in Tris-HCl at pH 8.0. Prothymosin α was obtained by means of a phenol extraction (6) and purified to homogeneity on a Bioselect Q2 column run with a Biologics (Bio-Rad) medium pressure liquid chromatography system.

Plasmids derived from pCH110 containing the gene for β-galactosidase were fused downstream of fragments of prothymosin α CDNA were constructed and characterized by Manrow et al. (5). Similarly, codons for KKKK were inserted into pCH110 using restriction sites and methods identical to those of Manrow et al. (5). The SV40 nuclear localization signal, VPKKKKVP, in the engineered β-galactosidase drove the protein into the nucleus as confirmed by staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (data not shown).

**Growth, Transfection, and Labeling of Cells—** HeLa cells and African green monkey kidney cells (COS-1) were grown in Dulbecco’s modified Eagle’s medium from Life Technologies, Inc. or BioFluids (Rockville, MD) containing 10% fetal bovine serum (HyClone, Logan, UT, or Life Technologies, Inc.) and 2 mM glutamine, 500 units/ml penicillin, 2.5 μg/ml streptomycin, and 5000 units/ml amphotericin from Life Technologies, Inc. in an atmosphere of 5% CO₂ at 37 °C. Cells were harvested by washing with Puck’s saline and treating them with 0.05% trypsin in Hanks’ balanced salts containing 0.5 mM EDTA. Transient transfections of COS cells were carried out in 60-mm dishes in a total volume of 0.6 ml using the DEAE-dextran method (28) with DNA that was purified by chromatography in QiaGen columns (Studio City, CA). The cells were incubated for 48–60 h and labeled either with 100 μCi/ml [³²P]orthophosphate acid for 4 h or with 200 μCi/ml of L-[³²H]glutamic acid (TRK445, 49 Ci/mmol; Amersham Corp.) for 4 h in complete medium.

**Characterization of Prothymosin α, Phosphorylated Prothymosin α, and Fusion Proteins—** To obtain prothymosin α, washed cells from one dish were lysed with 1 ml of the standard buffer (10 mM Tris-HCl at pH 7.5, 5 mM EDTA, 12% sucrose, 1% Triton X-100, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride-HCl (AEBSF, Sigma) at 0 °C; the nuclei that lost their prothymosin α were removed by centrifugation at 14,000 × g in a refrigerated microcentrifuge, and bovine prothymosin α and its histidine-tagged derivative were isolated by means of phenol extractions of the supernatant fluids, diluted with an equal volume of water. Protein was recovered by precipitation and, where necessary, purified further by digestion with ribonuclease in any convenient buffer. These methods were detailed by Sburlati et al. (6, 16). The wild type and tagged proteins were separated from each other and nonprotein contaminants in 18% polyacrylamide gels in the presence of SDS (Novex). ³⁴P-Protein in dried gels was imaged directly with X-AR x-ray film (Kodak). ³²H-Protein was visualized by soaking fixed gels in Enlightening (NEN Life Science Products) according to the accompanying instructions, drying the gels, and exposing them to film. In both cases, gels were stained with Comassie Brilliant Blue; quantitative evaluations of stained and autoradiographed proteins were obtained with a Molecular Dynamics Personal Laser Densitometer.

Fusion proteins containing β-galactosidase attached to all or part of prothymosin α sequences were obtained from COS-1 cells transfected with pCH110 containing a variety of inserts. In the experiments shown, cells were labeled with [³²P]orthophosphate as described above. Lysates were prepared with the standard buffer enriched with 0.1% SDS. Immunoprecipitations made use of 100 μl of Cappel rabbit anti-β-galactosidase IgG conjugated to Sepharose 4B, 1 ml of cell lysate, and the methods recommended by the manufacturer. In short, binding reactions were carried out overnight at 4 °C on a platform rocker, and the beads were collected by low speed centrifugation, washed many times with calcium- and magnesium-free phosphate-buffered saline, and treated with 1 mg/ml ribonuclease A to release bound molecules. β-Galactosidase fusion proteins were analyzed by autoradiography and quantified as noted above.

**Stability as a Function of pH—** Mock transfected or transfected COS cells were labeled with [³²P]orthophosphate, recovered from 60-mm dishes, and lysed at 4 °C in 1 ml of 5 mM EDTA, 12% sucrose, 1% Triton X-100, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride-HCl (AEBSF, Sigma) at 0 °C. The wild type and tagged proteins were separated from each other and nonprotein contaminants in 18% polyacrylamide gels in the presence of SDS (Novex). ³⁴P-Protein was isolated by dialysis into the standard buffer enriched with 0.1% SDS. Both prothymosin α and its histidine-tagged derivative were isolated by means of phenol extraction and centrifuging them to remove nuclear debris. The supernatant fluids were then brought to pH 5.1 by the addition of 3 volumes of ACE buffer (10 mM sodium acetate at pH 5.1, 50 mM NaCl, 3 mM EDTA) and made 0.5% in SDS. Prothymosin α was purified with phenol and ribonuclease-treated as described above. Control samples, in which ³⁴P-prothymosin α was studied in crude lysates, were prepared by lysing labeled cells in buffer at pH 7.5 (5 mM MES at pH 7.2, 5 mM EDTA, 1% Triton X-100, 12% sucrose, and 1 mM AEBSF), incubating them for 20 min on ice, diluting the lysates to approximately twice the volume at the stated pH, and allowing them to incubate for 30 min at the stated pH. Prothymosin α was recovered as noted. Alternatively, histidine-tagged prothymosin α was isolated from transfected, ³⁴P-orthophosphate-labeled COS cells by nickel-nitrioltriacetic acid column chromatography using 0.5 ml of matrix and instructions from Qiagen. The purified, tagged ³⁴P-prothymosin α was added to unlabeled cells that were lysed in MES. After 1 h at 0 °C at one of several pH values, prothymosin α was purified. When the fate of purified ³⁴P-prothymosin α was assessed in defined solutions, it was dissolved in MES at the stated pH, refrigerated overnight, and recovered using phenol. All samples were evaluated electrotherochemically and quantified as described above. One to two dishes of cells were used for each assay.

**Phosphorylation of Prothymosin α—** In Vitro Extracts—Extracts of 2.5 × 10⁶ COS cells, lysed in 100 μl of the standard buffer were supplemented with 30 μg of recombinant bacterial prothymosin α and 300 μCi/ml

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1 The abbreviations used are: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride-HCl, β-gal, β-galactosidase; HPLC, high pressure liquid chromatography; MES, 4-morpholinonethane sulfonic acid; PTMA, prothymosin α.
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\([\gamma^{32}P]ATP\) (Amersham, 3000 Ci/mmol) either in the presence or absence of 10 mM unlabeled ATP. The incubations were solubilized for 30 min at 37°C to allow labeling of exogenous prothymosin α to occur. Prothymosin α was recovered by means of a phenol extraction.

To test for transfer of phosphate from one prothymosin α molecule to another, 60-mm dishes of cells were mixed and lysed together. One dish contained untransfected cells labeled with 100 μCi/ml of \([\gamma^{32}P]ATP\) (Amersham, 3000 Ci/mmol) in 20 mM Tris-HCl at pH 7.5, 50 mM EDTA, and 1.0% Triton X-100 made 0.1 mM NaBH₄. Alternatively, lysis was achieved in 2 ml of Me₂SO containing 30 mM [\(^{3}H\)NaBH₄ (specific activity 1000 μCi/mmol; NEN Life Science Products) at a specific activity of 47 μCi/ml (low specific activity method) or in 1 ml of 5 mM [\(^{3}H\)NaBH₄ (222 μCi/mmol) in Me₂SO (high specific activity method). Reactions were carried out for 1–3 h. Aqueous samples were diluted with 4 volumes of 0.5% SDS, and aprotic samples composed of precipitated protein in addition to unwanted insoluble debris were either dissolved in 6 ml of ACE buffer made 0.5% in SDS or washed with Me₂SO several times and dissolved in 3 ml of the same ACE/SDS solution. Prothymosin α was recovered by means of several phenol extractions. In some experiments, cells were labeled with \([\gamma^{32}P]ATP\) for 4 h in order to incorporate radioactivity into prothymosin α before reducing with borohydride.

Purification and Endoproteolytic Digestion of Prothymosin α—Prothymosin α was purified to homogeneity in a three-step process consisting of phenol extractions of postnuclear supernatants, DEAE column chromatography, and C-18 reverse phase column chromatography. For purifications involving DEAE, samples in 400 μl of deionized water were injected onto a Tosohaas TSK-gel DEAE-5PW, 7.5 mm inner diameter × 7.5 cm column and eluted at a flow rate of 1 ml/min with a gradient of 0–500 mM NaCl in 20 mM Tris-HCl at pH 7.5 using the Waters HPLC system described previously (16). Prothymosin α was recovered as a single peak at 330 mM NaCl. Further purification was carried out by C-18 reverse phase column chromatography (16). Prothymosin α eluted as a sharp peak at 30% acetonitrile; the protein was collected and dried under vacuum with a Speed Vac (Savant Instruments). When [\(^{3}H\)]samples were purified, radioactivity was determined in fractions collected in Hydrofluor (National Diagnostics) and assayed in a Packard Tri-Carb model 1500 liquid scintillation analyzer.

Peptides were generated from ~60 μg of prothymosin α with Lys-C and purified by C-4 reverse phase column chromatography essentially as detailed by Sbrurlati et al. (16). The stated digestion buffer was replaced with 0.1 mM Tris-HCl at pH 7.5 made 5% in acetonitrile. Further digestion of the peptides generated with Lys-C was performed with the proteolytic enzyme, Asp-N, in 50 mM sodium phosphate buffer at pH 8 containing 5% acetonitrile for 2 h at 37°C at an enzyme:peptide ratio of 1:100. The Asp-N peptides were purified using the same system employed for the Lys-C peptides. The enzymes were purchased from Boehringer Mannheim.

Amino Acid Analysis—Amino acid analysis was performed using the Pico-Tag methods developed by Waters. The steps include vapor phase acid hydrolysis for 22–24 h at 107°C, evaporation of the samples, redrying with twice the recommended volume of ethanol/water/triethylamine, and precolumn derivatization with phenylisothiocyanate. Injection volumes were 15 or 60 μl for unlabeled samples and up to 160 μl for samples labeled with [\(^{3}H\)NaBH₄ or for the standards used to evaluate them (see below). Fractions were collected every 23 s and counted in a Beckman 6300 amino acid analyzer equipped with an SP-4270 integrator (6).

Preparation of Standards—Homoserine and homoserine lactone, the products of borohydride reduction of aspartyl phosphate (29) were purchased from Sigma and subjected to acid hydrolysis as well as redrying and derivatization. Hydroxynorvaline, obtained similarly from glutaminolysis of [\(^{3}H\)]protein, was synthesized by deaminating proline with NaNO₂ using reactions described by Malin et al. (30). After acid hydrolysis of the treated polynorithine, the initial products are hydroxynorvaline and ornithine. However, because incubation in acid converts hydroxynorvaline to chloronorvaline (30) and because chloronorvaline cyclizes to form proline in base (31), there are at least three amino acid products, depending on the pH. Using Pico-Tag chemistry and Waters chromatography equipment as noted above, three amino acids were obtained. Ornithine was identified by hydrolyzing polynorithine in acid and finding one peak eluting near lysine on the acidic amino acid analysis column; proline was characterized both by its elution time and by mass spectrometry; the remaining peak was subjected to mass spectrometry and found to be hydroxynorvaline. With Pico-Tag chemistry, which includes the coupling of phenylisothiocyanate in base, there was no chloronorvaline.

RESULTS

Stable Phosphate in Native and Mutant Prothymosin α—The introduction of stable phosphate was investigated in COS cells using the native COS prothymosin α protein and the products of four transfected human prothymosin α genes: the wild type, a tagged variant encoding six histidine residues at the carboxyl terminus, a triple mutant in which Ser₁, Ser₈³, and Thr₈⁵ were replaced with alanine codons, and a histidine-tagged triple mutant. A gene map and amino acid sequence are presented in Fig. 1. The subtilisation of alanine residues for a subset of Ser/Thr eliminated all of the proven and suspected
sites of stable phosphate in human prothymosin α (16), whereas the histidine tag made it possible to distinguish the endogenous protein, with its greater mobility, from exogenous tagged protein, which is slightly retarded when analyzed electrophoretically in polyacrylamide gels. As shown in Fig. 2, A and B (lanes 2–5), all of the transfected genes gave rise to approximately 10-fold more prothymosin α than found in mock transfected cells (Fig. 2A, lane 1) regardless of whether the total protein (indicated by Coomassie Blue staining (Fig. 2A)) or the newly synthesized protein labeled with [3H]glutamic acid (Fig. 2B)) was examined. Furthermore, the ratio of stable phosphate to protein was the same for the endogenous and wild type transfected prothymosin α proteins (Fig. 2C, lanes 1 and 2), slightly diminished for the tagged wild type prothymosin α (lane 3), and greatly reduced for the mutant prothymosin α (lane 4) and for the tagged mutant protein (lane 5). Quantitatively, the tagged mutant prothymosin α contained 15% of the phosphate found in the tagged wild type molecule. From these observations, one can infer that the histidine tag has little effect on the amount of stable phosphate and that Ser1 is important but not the sole site of phosphate incorporation.

Among the possible explanations for the unexpected incorporation of phosphate in the absence of previously identified locations are 1) low level phosphorylation at heretofore unsuspected sites or 2) phosphorylation at primary sites common to both wild type and mutant prothymosin α proteins followed by the transfer of phosphate to a hierarchy of stable positions.

The pH Stability of Prothymosin α's Phosphates—At the moment of cell lysis, prothymosin α should contain only those phosphates acquired in vivo. If phosphate attaches initially at Ser and Thr residues and persists, the phosphate should be resistant to changes in pH under gentle conditions regardless of their locations. Alternatively, if phosphorylation in vivo occurs on any other type of amino acid found in prothymosin α, it might be possible to infer the nature of the phosphoamino acid bond from its stability as a function of pH. When prothymosin α in transfected COS cells was exposed to a range of lysates buffers at different pH values and subsequently purified using the standard methods, the specific activity of the [32P]ectopic wild type protein or the [32P]-tagged triple mutant exhibited bell-shaped pH stability curves (Fig. 3A). Endogenous prothymosin α in untransfected cells and the tagged wild type protein behaved similarly (data not shown). Such behavior is typical of
an acyl phosphate such as acetyl phosphate (32), aspartyl phosphate (33), or the phosphorylated residue of acetate kinase (see Ref. 34 and references therein). It is not characteristic of phosphoramidates (35). These data show that 1) the amount of stable phosphate obtained depends profoundly on the conditions of lysis; 2) at least 90% of the phosphate is labile as indicated by its susceptibility to hydrolysis at the extremes of pH; and 3) the shape of the pH stability curve does not substantively change in the presence or absence of introduced mutations that replace serine and threonine residues. Taken together, the data argue against Ser or Thr as the primary site of phosphorylation but, instead, support the idea of unstable phosphates whose transient survival influences the production of phosphoserine or phosphothreonine as a secondary event. Clearly, labile phosphate will either hydrolyze and remain undetected or transfer to more stable positions, where it can be analyzed.

Controls that support these conclusions make use of the data in Fig. 3B. Here, [32P]prothymosin α, which was purified to homogeneity and incubated in buffers from pH 3 to 11, was evaluated and found to be completely resistant to strong acid and base (solid line). Furthermore, purified [32P]histidine-tagged prothymosin α, when added to cells before lysis, incubated briefly in cell extracts prepared as in Fig. 3A, and subsequently purified by nickel chelate chromatography, also remained stable (dotted line). These controls show that at mild temperatures the stable phosphate of prothymosin α is unaffected by defined solutions at different pH values and by any of the components of complete cell extracts prepared over a broad range of pH. In the third curve (dashed line in Fig. 3B), the fate of [32P]prothymosin α, which was labeled in vivo, was determined after a brief delay at pH 7; lysates at neutrality were incubated 20 min on ice, diluted with each of a range of buffers to generate extracts comparable with those in Fig. 3A, and used for analysis of phosphoprophymosin α. Again, the specific activity of prothymosin α was independent of pH. Labile phosphate in vivo, after a brief sojourn in extracts at pH 7, gave rise to phosphate that was stable in vitro throughout the pH range studied. These results emphasize the fact that phosphate on freshly isolated prothymosin α undergoes change and that the static conditions encountered when phosphoprophymosin α is purified to homogeneity do not reflect the properties immediately apparent upon cell disruption. It is worth reiterating that even at pH 7, where phosphate recovered on prothymosin α is maximal, only 2% of the total purified prothymosin α contains phosphate (16) and the amount of phosphate initially incorporated and then lost is unknown.

Phosphatas as the cause of the disappearance of prothymosin α’s phosphates were also investigated. As illustrated in Table I, the specific activity of [32P]prothymosin α labeled in vivo and purified remained unchanged regardless of whether a phosphatase inhibitor such as sodium fluoride, okadaic acid, or calyculin A was included in the lysis buffer. From these data, one suspects that the loss of labile phosphate occurs independent of phosphatases and, therefore, stems from an inherent property of the phosphorylated residue.

Phosphorylation at Serine 1 in Vitro—To tally prothymosin α’s phosphates, one must consider phosphorylation in vivo as well as the acquisition or loss of phosphate occurring as artifacts during the purification procedure. Phosphorylation of prothymosin α was examined during isolation in the standard EDTA-containing buffer at pH 7. Extracts of COS cells supplemented with [γ-32P]ATP either in the presence or absence of unlabeled ATP were unable to label prothymosin α (data not shown). EDTA appears to sequester the Mg2+ necessary for kinases utilizing ATP. In a slightly different format, 32P-labeled endogenous prothymosin α labeled in vivo with [32P]orthophosphate was tested as a phosphate donor; when the labeled COS cells were mixed and lysed together with unlabeled COS cells, which were transfected with the tagged prothymosin α gene, 32P-labeled molecules including labeled prothymosin α were unable to transfer phosphate to an excess of tagged prothymosin α molecules (data not shown). Furthermore, in the accompanying paper (36), we searched for and failed to find kinases capable of phosphorylating serine 1 in cell extracts composed of whole cells or the isolated cytosol under a wide variety of conditions. These data are consistent with the idea that the phosphate on Ser1 of prothymosin α is not acquired intermolecurally from cell extracts, that cells lack the capability of direct phosphorylation of serine 1, and that phosphate on serine or threonine is captured as a result of an intramolecular transfer from unstable to stable positions.

Identification of the Region of Prothymosin α Responsible for the Transfer of Phosphate—If labile phosphate is indeed transferred from a region of prothymosin α bearing acyl phosphates to its N-terminal portion, it should be possible to identify the acidic region and use it to distribute phosphate to stable positions in an irrelevant recipient. The experiment was performed by creating proteins composed of β-galactosidase with all or part of the prothymosin α coding sequence (minus its own initiator codon) fused in frame upstream of the β-gal gene but downstream of the AUG codon in pCH110 (5). A construct in which the SV40 nuclear localization sequence was inserted into the same position in the β-galactosidase gene in the same vector was also made. When these genes were transfected into COS cells, it was evident that β-galactosidase targeted to the nucleus by means of the SV40 nuclear targeting signal and β-galactosidase bearing 51 amino acids from prothymosin α’s amino terminus were poorly phosphorylated or unphosphorylated on stable positions (Table II). In contrast, the addition of either prothymosin α or the C-terminal portion of prothymosin α to the bacterial protein resulted in a substantial increase in stable phosphate. It is important to note that the C-terminal fragment of prothymosin α, PTMA-(30–109), unlike the protein in its entirety, does not include the sites of stable phosphorylation found within the first 14 amino acids of prothymosin α but does include the nuclear localization signal. These data suggest that the acyl phosphates are located between amino acid 30 and the C terminus of prothymosin α (or, more restrictively, downstream of residue 51) and that the presence of this region is capable of conferring stable phosphate on an irrelevant protein in very close proximity.

Reductive Cleavage of Covalently Bound Phosphate on Prothymosin α by Sodium Borohydride—Aspartyl phosphate and glutamyl phosphate, but not phosphoserine, phosphothreonine, or phosphotyrosine, react with sodium borohydride to give altered amino acids that no longer contain phosphate (29). Such reactions can be carried out in an aqueous medium or in aprotic solvents such as Me2SO. Since the phosphate on [32P]prothymosin α rapidly disappears upon rupturing cells, it was not

**Table I**

| Buffer          | [32P]Prothymosin α/Prothymosin α |
|-----------------|----------------------------------|
| Control         | Okadaic acid                     |
|                 | Calyculin A                      | NaF               |
| 9.4             | 9.9                              | 10.5              | 8.7               |
| 10.5            | 8.4                              | 9.2               | 8.4               |

**Experimental Procedure:**

1. Cells were labeled with [32P]orthophosphate in vivo.
2. Cells were lysed and extracts prepared.
3. Extracts were analyzed for [32P]phosphate content.
4. Extracts were treated with phosphatase inhibitors.
5. Extracts were analyzed for [32P]phosphate content.

**Results:**

- In the presence of phosphatase inhibitors, [32P]phosphate content remained unchanged.
- In the absence of phosphatase inhibitors, [32P]phosphate content decreased.

**Conclusion:**

Phosphatases are responsible for the disappearance of [32P]phosphate content in prothymosin α.
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TABLE II
Phosphorylation of β-galactosidase fusion proteins

| β-Gal fusion partner | Specific activity ([32P]-labeled fusion protein) | Exp. I | Exp. II |
|----------------------|-----------------------------------------------|-------|--------|
| SV40 NLS             |                                               | 0     | <8.6   |
| PTMA residues 1–51   |                                               | 0     | <5.2   |
| PTMA residues 30–109 |                                               | 7     | 22     |
| PTMA residues 1–109  |                                               | 20    | 13.1   |

* Values recorded are overestimates because the optical density on the film was diffuse, unlike the sharp discrete bands exhibited by all fusion proteins on the stained gel and on the autoradiograms for β-gal-PTMA-(1–109) and β-gal-PTMA-(30–109).

possible to purify a suitably phosphorylated protein before initiating treatment with sodium borohydride. Accordingly, [32P]prothymosin α phosphorylated in vivo was studied in cells lysed either in aqueous solution or in Me2SO, both with and without NaBH4. Fig. 4 shows an electropherogram of Coomassie Blue-stained prothymosin α and the accompanying autoradiogram obtained under aqueous conditions. The presence of the borohydride had little effect on the recovery of prothymosin α, compared with a sample isolated in the absence of the reagent (Fig. 4, left), but had a major effect on the retention of phosphate on prothymosin α (Fig. 4, right). In quantitative terms, the specific activity ([32P]-prothymosin α/total prothymosin α) was ~5-fold higher in the absence of borohydride than in its presence. Similar results were obtained in Me2SO except that the untreated [32P]prothymosin α had a specific activity ~5-fold higher than the borohydride-reacted sample (data not shown). The results are consistent with the presence of acyl phosphates in prothymosin α, which are displaced by borohydride.

**Estimation of the Number of Phosphorylated Acidic Residues in Prothymosin α**—The reaction of prothymosin α with borohydride yields informative products. 1) reductive cleavage of acyl phosphates by borohydride treatment generates the corresponding alcohols; 2) the alcohols and their acid hydrolysis products allow identification of the type of amino acid bearing the activated carboxyl group; and 3) tritium donated by [3H]NaBH4 becomes incorporated into the protein at the location of the acyl groups (29). Cells containing unlabelled prothymosin α were lysed in the presence or absence of very high concentrations of sodium borohydride in aqueous solutions or necessarily lower concentrations in Me2SO, and the products were purified. After reduction with borohydride, prothymosin α was purified to homogeneity using phenyl-Superose to remove other proteins, and two successive column chromatography steps (see “Experimental Procedures”) to remove nucleic acids and other contaminants. The purified protein was hydrolyzed and subjected to amino acid analysis. Table III displays the results from different approaches. Using the Pico-Tag method to visualize amino acids, the number of acidic residues found in the absence of borohydride was always greater than that in its presence. There were 4–8 missing acidic residues in borohydride-reduced samples. The large number of acidic amino acids in prothymosin α and the proximity of the peaks for derivatized glutamate and aspartate made it difficult to determine which type(s) was missing. It was clear from many analyses that the Pico-Tag methods almost always underestimated the number of acidic residues in prothymosin α but that borohydride treatment always caused a reduction in acidic residues. The correct number of residues was obtained for all other amino acids, in particular lysine and arginine.

As a control, purified prothymosin α was also treated with borohydride. Prothymosin α was first purified to homogeneity (a process during which the labile phosphates disappear), reacted with NaBH4, and repurified to homogeneity using the methods noted above. Amino acid analysis of these samples revealed no deficit in acidic residues relative to controls (data not shown). Hence the methods themselves are not responsible for the results. To corroborate these findings, we repeated the analyses of borohydride-treated and untreated aqueous prothymosin α samples using the ninhydrin method to visualize amino acids. Here too, reduced prothymosin α was missing an average of four acidic residues in a reaction that did not underestimate the acidic residues in untreated protein. Taken together, the data suggest that there are a few to many phosphorylated acidic residues/molecule of prothymosin α and that other phosphorylated amino acids are not present at detectable levels.
levels. Because the borohydride must compete with water in the reaction, our methods can only underestimate the number of mixed anhydrides.

**Treatment of Prothymosin α with [3H]NaBH₄**—The reaction of prothymosin α in crude cell lysates was repeated using [3H]NaBH₄ in Me₂SO. When labeled borohydride was included in the cell lysis solution, tritium became incorporated into prothymosin α as well as into most other macromolecules. Tritium may be incorporated as a consequence of reduction, but in the vast majority of cases, radioactive protons generated during the reaction merely exchange with their nonradioactive counterparts. Thus, purification of prothymosin α was required not only to obtain pure protein but also to eliminate large amounts of exchangeable tritium from the products. With this goal, we designed the procedure shown in Fig. 5. Here phenol-extracted prothymosin α was partially purified on a DEAE-methacrylate copolymer column and visualized at 214 nm (Fig. 5A). Although the pattern appears to be complex, prothymosin α was easily recognized as the peak at 214 nm that did not have absorbance at either 260 (data not shown) or 280 nm due to the absence of aromatic residues. The chromatogram displays entities not usually seen in gels (see Fig. 2, A and B), because RNA, as well as salts and solvents, absorbs at 214 nm and acquires exchangeable tritium but does not stain with Coomasie Blue or label with [3H]glutamic acid. Prothymosin α, indicated by the arrow, was purified to homogeneity using a C-18 reverse phase HPLC. The arrow marks prothymosin α.

**FIG. 5. Purification of [3H]NaBH₄-treated prothymosin α by sequential DEAE and C18 column chromatography using HPLC.** A, prothymosin α, obtained by means of a phenol extraction, was used to charge a DEAE column. The eluent was monitored at 214 (thick line), 230 (data not shown), 260 (data not shown), and 280 (thin line) nm. Fractions were collected each minute and counted. The inset shows an expanded version of the chromatogram from 30 to 40 min. With the exception of the peaks between 30 and 38 min, all of the peaks at 280 nm exhibited substantially greater absorbance at 260 nm, suggesting that they contain RNA. The arrow denotes the position of prothymosin α. B, the peak eluting at 35–36 min on DEAE was purified using C-18 reverse phase HPLC. The arrow marks prothymosin α.

**FIG. 6. Amino acid analyses of [3H]NaBH₄-treated prothymosin α and standards using Pico-Tag technology.** A, three chromatograms have been superimposed: that of homoserine, which also gives rise to homoserine lactone (dashed line); that of deaminated polyornithine, which gives rise to ornithine, hydroxynorvaline, and proline (thin solid line); and that of acid-hydrolyzed prothymosin α isolated from cells lysed in the presence of [3H]NaBH₄ in Me₂SO using the high specific activity method (thick solid line). The eluate was collected in fractions. To confirm the precise location of the peaks representing homoserine (Hse), hydroxynorvaline (Hnv), and proline (Pro), the absorbance in the separated fractions of the standard sample was measured. B, two chromatograms of acid-hydrolyzed prothymosin α have been superimposed: that of prothymosin α isolated from pelleted cells lysed in the presence of [3H]NaBH₄ in Me₂SO using the low specific activity method (■) and that of prothymosin α purified by means of a phenol extraction and resuspended in a mixture of water and Me₂SO containing [3H]NaBH₄ to mimic the mixed Me₂SO/aqueous solvent used for the fresh protein (●). The results of three chromatograms have been averaged.
Prothymosin α was treated with \[^3H\]borohydride in dimethylsulfoxide at the moment of cell lysis. Peptides were purified and analyzed by acid hydrolysis and amino acid analysis. All radioactivity incorporated was found in the peptide Lys-C-(21–87) shown below. Lys-C peptides in which no radioactivity was found are included only to emphasize the absence of label. Lys-C-(21–87) was further digested with Asp-N. Major peptides were purified to homogeneity and analyzed. Values represent the dpm in tritium, with background subtracted, found in proline in the stated peptide upon amino acid analysis. Sp. Act. refers to the specific activity/mole of peptide. Carrier prothymosin α present only in experiments I and II was not included in specific activity calculations. BKG, background; NA, not applicable. —, the peptide was produced in the experiment but not analyzed.

### Table IV: Location of glutamyl phosphates

| Peptide          | Exp. I | Exp. II | Exp. III | Exp. I | Exp. II | Exp. III |
|------------------|--------|---------|----------|--------|---------|----------|
| Lys-C-(21–87)    | 310    | 1120    | 50       |        |         |          |
| Asp-N-(31–87)    | 330    | 300     | 160      | 50     |         |          |
| Asp-N-(31–30)    | 20     |         |          |        |         |          |
| Asp-N-(31–47)    | BKG    |         | NA       |        |         |          |
| Asp-N-(48–87)    | 170b   | 560     | 120b     | 80     |         |          |
| Lys-C-(1–14)b    | BKG    |         | NA       |        |         |          |
| Lys-C-(88–101)   | BKG    |         | NA       |        |         |          |

^a Corrected value based on analysis of approximately one-third of the total quantity of peptide.

^b Value corrected for contamination by Lys-C-(21–87) as indicated by the presence of substoichiometric amounts of arginine in the hydrolysate.

Fig. 6A also displays the behavior of the radioactive products obtained from prothymosin α that was reduced with \[^3H\]NaBH₄ in Me₂SO immediately as cell lysis occurred, purified to homogeneity, and hydrolyzed (\textit{thick solid line}). There are two major peaks of radioactivity: a peak eluting at 8 min, which is composed in part of Tris and NH₃ but does not contain any known amino acid or any of the derivatives, and a smaller peak of radioactivity, which coelutes with proline. The presence of labeled proline following the reductive cleavage of prothymosin α with tritiated sodium borohydride strongly suggests that prothymosin α initially contained γ-glutamyl phosphate.

To demonstrate that the radioactivity associated with proline was incorporated specifically, rather than by nonspecific exchange of \[^3H\]H₂O with ionizable hydrogen ions in the protein, a sample of purified prothymosin α was used as a control. Purified prothymosin α does not contain acyl phosphates, based on the pH stability studies shown in Fig. 3, and should not generate \[^3H\]proline upon treatment with \[^3H\]borohydride. Cells were lysed in Me₂SO/\[^3H\]borohydride, and in the same experiment the homogeneous protein was suspended in a Me₂SO/H₂O/\[^3H\]borohydride mix to duplicate the conditions encountered with crude samples. The amino acid analysis of the reduced, purified peptide (Fig. 6B, \textit{dashed line}) gave rise to a pattern almost identical to that of the crude prothymosin α (\textit{solid line}) with one exception; radioactivity in proline was absent from hydrolyzed prothymosin α that had been purified to homogeneity first and only later treated with the reducing agent. Hence, radioactivity associated with proline was specifically incorporated into prothymosin α when the reaction with borohydride occurred immediately upon cell lysis. Our data suggest that γ-glutamyl phosphate is present on prothymosin α in vivo but disappears during the subsequent scheme of purification.

### Purification of a Peptide Homologous to an Acidic Region of Nucleoplasmin

Peptides derived from \[^3H\]borohydride-reduced fresh prothymosin α were prepared by treating the isolated protein with endopeptidases, purifying the products, and subjecting the peptides to amino acid analysis. As Table IV illustrates, all of the radioactivity was found in a single Lys-C peptide composed of residues 21–87; other Lys-C peptides were not radioactive. However, of the 34 glutamic acid residues in prothymosin α, Glu18 and Glu107 were not included in peptides of sufficient size to be retained by the HPLC column and were not pursued. Upon further digestion of Lys-C-(21–87) with Asp-N, four major products, Asp-N-(31–87), Asp-N-(21–30), Asp-N-(31–47), and Asp-N-(48–87), were obtained, purified, and identified by amino acid analysis (Table IV). Further characterization of these peptides indicated that residues 21–30 contained an occasional glutamyl phosphate, which, because of poor labeling, was difficult to quantify; residues 31–47 did not include a phosphorylation site; and residues 48–87 harbored virtually all of the radioactivity incorporated specifically into prothymosin α (Table IV). This region conforms precisely to that tentatively identified by the experiments with β-galactosidase fusion proteins in Table II, \textit{i.e.} it is an acidic region located downstream of amino acid 51 of prothymosin α.

The chromatogram of the amino acid analyses of four representative peptides is illustrated in Fig. 7. Tritiated Lys-C-(21–87), Asp-N-(31–87), and Asp-N-(48–87) each contain \[^3H\]proline as the sole radioactive amino acid, whereas the fourth peptide in Fig. 7, Lys-C-(88–101), was devoid of \[^3H\]proline. As the peptides underwent purification, radioactivity in the unidentified peaks at the beginning and end of the chromatogram became minor, and the peak at 8 min, attributed primarily to contaminating Tris, also became less pronounced relative to proline. (Compare the amino acid analysis of the intact protein in Fig. 6A with the patterns in Fig. 7.) It is worth reiterating that this peak does not overlap with any known amino acid or derivative and cannot be assigned to spurious products generated from putative phosphoramidates, because arginine and lysine are not found in two of the peptides analyzed in Fig. 7. Because experiment III (Table IV) did not include carrier, it was used to compare the specific activities of radioactivity incorporated per mole of peptide; the specific activity of the large Lys-C peptide from 21 to 87 was nearly identical to that of the smaller Asp-N-(48–87) peptide. Therefore, it is unlikely that significant radioactivity occurred elsewhere in the protein. The presence of glutamyl phosphate in this highly acidic region of prothymosin α is notable because the presumed histone binding region of nucleoplasmin, a chromatin remodeling pro-
point to the presence of activated carbonyl groups bearing phosphate as an integral part of prothymosin $\alpha$. 5) When $[\text{H}]\text{NaBH}_4$ was the reducing agent, tritium was incorporated into prothymosin $\alpha$ specifically and localized to a peptide composed of residues 48–87. Upon amino acid analysis, the label was found in proline, which arises only when a hydroxynorvaline residue, the initial product obtained from borohydride treatment of a protein-bound glutamic acid anhydride (29, 30), is converted first to chloronorvaline during acid hydrolysis and later to proline during derivitization in base (31). Although borohydride reduction of proteins produces few artifacts beyond a low level of $\beta$-amino alcohols, the reaction is clearly not stoichiometric even in well defined solutions (29). In our experiments, where reduction was carried out in the presence of the contents of the entire cell, under conditions in which proteins were largely insoluble, the proline generated did not begin to equal the glutamic acid residues lost. Indeed, radioactivity was essential for discovering reduced products. The low yield is probably caused by competing reactions in very crude solutions which do not favor hydrolytic regeneration of the acid.

These data strongly support the idea that prothymosin $\alpha$ contains glutamyl phosphates which are synthesized in vivo. Our experiments with sodium borohydride in aprotic solvents show clearly that some glutamic acid residues in a restricted region of prothymosin $\alpha$ from position 48 to 87 have activated carbonyl groups (i.e. they give rise to proline) and that $^{32}\text{P}$-labeled groups are lost. One can postulate structures in which glutamic acid residues are covalently connected via anhydride linkages to more complex $^{32}\text{P}$-labeled entities, but such fabrications appear to be inconsistent with the pH stability curves of $^{32}\text{P}$prothymosin $\alpha$, which match those of acyl phosphates. Furthermore, measurements of the molecular weight of purified prothymosin $\alpha$ by mass spectrometry indicate the absence of unsuspected adducts (data not shown). We therefore conclude that prothymosin $\alpha$ contains phosphoglutamate. This study is the first to demonstrate phosphoglutamate in a mammalian protein and the first to use borohydride reduction in very crude solutions as an element of proof.

The acquisition of phosphate on glutamic acid residues as a posttranslational modification raises questions about the relationship, if any, between these labile phosphates and other phosphorylated residues on prothymosin $\alpha$, such as the phosphate found on Ser$^3$ in 2% of bovine prothymosin $\alpha$ molecules (16) and the $^{32}\text{P}$-labeled phosphothreonine residues found in much lower amounts in mouse prothymosin $\alpha$ (22). Several lines of evidence suggest that the stable phosphates of prothymosin $\alpha$ are artifacts that arise upon transfer of labile glutamyl phosphates, intramolecularly, to any convenient hydroxyamino acid. 1) The amount of stable phosphate found on prothymosin $\alpha$ varies with the solutions used to rupture cells; buffers that support the transient survival of acyl phosphates enhance the recovery of stable phosphate. Since 90% of the stable phosphate found at pH 7 disappears at the extremes of pH, our data suggest that 10% of 2% or, at most, 0.2% of prothymosin $\alpha$ molecules might possess a stable phosphate obtained in vivo. Such low, erratic levels of phosphorylation should always be scrutinized. 2) The recovery of stable phosphate does not improve in the presence of phosphatase inhibitors. Thus, there is no reason to believe that stable phosphate is attacked during protein purification and that the amounts obtained should be greater than those noted above. Instead, the amount of phosphate could be abnormally high, due to kinase activity in vitro. 3) Prothymosin $\alpha$ does not acquire phosphate directly on serine or threonine during and after cell lysis. Using buffers with EDTA, it proved impossible to label prothymosin $\alpha$ in vitro with $^{32}\text{P}$ATP in the presence or absence of added nonradioactive

FIG. 7. Amino acid analyses of peptides of $[\text{H}]\text{NaBH}_4$-treated prothymosin $\alpha$ using Pico-Tag technology. Prothymosin $\alpha$ was isolated from cells lysed in the presence of $[\text{H}]\text{NaBH}_4$ using the high specific activity method and digested with Lys-C. Then an aliquot of the peptide, Lys-C-(21–87) was further digested with Asp-N. All peptides were purified, hydrolyzed, and subjected to amino acid analysis. Four chromatograms have been superimposed: that of Lys-C-(88–103) (thin line, no symbols), Lys-C-(21–87) (●), Asp-N-(31–87) (●), and Asp-N-(48–87) (●).
ATP or to label histidine-tagged prothymosin α in nonradioactive, transfected cells with molecules from 32P-labeled untransformed cells when both populations were mixed and lysed together. Hence, prothymosin α neither acquires new label nor loses label to phosphatases as a consequence of the disruption of cells; label has already been incorporated. 4) A short incubation in the cell lysate converts pH-sensitive phosphate to pH-resistant phosphate. These data strongly suggest that prothymosin α's phosphates are in flux, with a hydroxylaminio acid (or water) as the ultimate destination. 5) The polyglutamyl stretch, which we have shown contains glutamyl phosphate in vivo, mediates the transfer of phosphate to stable positions on an irrelevant molecule, β-galactosidase. In contrast, the N terminus of prothymosin α, which contains the phosphorylated serine and threonine residues, does not cause β-galactosidase to accumulate phosphate. Hence, we have been able to stably phosphorylate a non-phosphate-bearing bacterial protein expressed in mammalian cells by inserting the prothymosin α sequence that bears the glutamyl phosphates. We conclude that the phosphate flux is a transferable property mediated by the acidic region of prothymosin α. 6) Cells cannot phosphorylate Ser1 of prothymosin α (36). When specific peptides bearing N-terminal acetylserine or the amino-terminal sequence of prothymosin α were added to the lysis buffer under a variety of conditions including those most conducive to phosphorylation by a traditional kinase (γ32PATP and Mg2+), or incubated with extracts of whole cells labeled with [32P]inorganic phosphate in vivo, no phosphorylated specific peptides were recovered (36). These experiments argue against a cellular activity capable of phosphorylating prothymosin α directly on Ser1 in vivo. We have failed to find the tools for direct stable phosphorylation of prothymosin α and, therefore, propose that the mechanism must be indirect. By eliminating the traditional kinase pathway and discovering the ability of specific prothymosin α sequences to direct phosphorylation of a heterologous substrate, our experiments lead to a model in which labile phosphate obtained in vivo migrates during cell lysis to any conformationally available stable position at sufficient concentration, e.g., those on prothymosin α, those remaining on mutant prothymosin α, or those on fused β-galactosidase.

Glutamyl phosphates are largely invisible. Rapid hydrolysis in vitro is the hallmark of an acyl phosphate; prothymosin α loses its glutamyl phosphates, which were acquired in vivo, almost instantaneously upon cell lysis, and the aspartyl phosphates of Che Y (39) and NtrC (40, 41), which were labeled in vitro under carefully defined conditions, disappear with half-lives of ~6 s and 3.5 min, respectively. Che Y and NtrC, each with one aspartyl phosphate, are examples of bacterial response regulator proteins, which comprise the second member of two-component systems (reviewed in Refs. 42–44). These phosphorylated proteins, as well as examples drawn from the ATPases (reviewed in Ref. 45), have an acyl phosphate as the sole element of homology with prothymosin α. Very few bona fide examples of glutamyl phosphates in eukaryotes have been described. The best documented case is that of the α2 chains of type I chicken bone collagen (46), where 4–5 atoms of organic phosphorus/mol of collagen were found in the absence of phosphorylated hydroxylaminio acids, phosphoamidated amino acids, or phosphorylated sugars (47). These glutamyl phosphate groups, which survived in part during the several hours needed for purification of the protein, were later localized to a specific peptide (48). The enrichment in glutamyl phosphate found in collagen from mineralized tissues compared with that from unmineralized tissues prompted speculation that these groups play a role in mineralization (48). Such γ-glutamyl phosphates on a structural protein appear to be functionally unrelated to those of prothymosin α.

Two other examples of glutamyl phosphates have been cited: guinea pig brain (Na+, K+) ATPase (49) and ATP-citrate lyase from rat liver (50). However, the hydroxamate derivatives used in the identification in both cases have been questioned. The acyl phosphate of the ATPase is now thought to be an aspartyl phosphate, as in the kidney enzyme (51), whereas the unusual phosphate in the lyase has been assigned to a histidine residue (52, 53). The only other known example is that of frog nucleoplasmin.

Homologies between prothymosin α, whose specific function is unknown, and nucleoplasmin, a protein involved in remodeling chromatin, have been noted. Both proteins are abundant, acidic, and located exclusively in the nucleus (germinal vesicle in Xenopus) (Ref. 5 and 6; reviewed in Refs. 54–56). Both bind histones (17, 18, 57–59), presumably by means of an acidic sequence in common (15) that contains prothymosin α's phosphoglutamate. Both also leak out of the nucleus during preparative procedures (16, 55), a fact that suggests that neither protein remains tightly bound to a positively charged partner to perform its specified role. Furthermore, our laboratory has shown that nucleoplasmin, also, has glutamyl phosphate. With so many features in common, it is tempting to speculate that prothymosin α, like nucleoplasmin, interacts with chromatin. Further evidence for a role involving chromatin comes from studies of the half-life of prothymosin α's glutamyl phosphates in vivo (36). Our laboratory will show that these phosphates undergo a pronounced increase in stability upon the cessation of transcription, suggesting the nucleosome as a likely target for the functional activity of prothymosin α. The mechanism by which a nucleosome makes way for the RNA polymerase complex is a hotly debated issue that may depend on investigations such as these for resolution. Although the ideas are still in their formative stages, it should now be clear that studies of purified prothymosin α, performed in vitro, are suspect and that the prothymosin α's high energy γ-glutamyl phosphate may be the key to understanding its function.

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