Temporal Activation of the Sea Urchin Late H1 Gene Requires Stage-Specific Phosphorylation of the Embryonic Transcription Factor SSAP

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Stage-specific activator protein (SSAP) is a 41-kDa polypeptide that binds to embryonic enhancer elements of the sea urchin late H1 gene. These enhancer elements mediate the transcriptional activation of the late H1 gene in a temporally specific manner at the mid-blastula stage of embryogenesis. Although SSAP can transactivate the late H1 gene only at late stages of the development, it resides in the sea urchin nucleus and maintains DNA binding activity throughout early embryogenesis. In addition, it has been shown that SSAP undergoes a conversion from a 41-kDa monomer to a ~80- to 100-kDa dimer when the late H1 gene is activated. We have demonstrated that SSAP is differentially phosphorylated during embryogenesis. Serine 87, a cyclic AMP-dependent protein kinase consensus site located in the N-terminal DNA binding domain, is constitutively phosphorylated. At the mid-blastula stage of embryogenesis, temporally correlated with SSAP dimer formation and late H1 gene activation, a threonine residue in the C-terminal transactivation domain is phosphorylated. This phosphorylation can be catalyzed by a break-ended double-stranded DNA-activated protein kinase activity from the sea urchin nucleus in vitro. Microinjection of synthetic SSAP mRNAs encoding either serine or threonine phosphorylation mutants results in the failure to transactivate reporter genes that contain the enhancer element, suggesting that both serine and threonine phosphorylation of SSAP are required for the activation of the late H1 gene. Furthermore, SSAP can undergo blastula-stage-specific homodimerization through its GQ-rich transactivation domain. The late-specific threonine phosphorylation in this domain is essential for the dimer assembly. These observations indicate that temporally regulated SSAP activation is promoted by threonine phosphorylation on its transactivation domain, which triggers the formation of a transcriptionally active SSAP homodimer.

Normal development of a living organism requires the expression of individual genes in correct temporal and spatial patterns. In the sea urchin, there are two different families of histone genes whose expression is restricted to specific intervals during early embryogenesis (for a review, see reference 36). The early histone genes are contained in 300 to 500 tandem repeats, each of which comprises independent transcription units encoding five classes of histones. Members of the early histone gene family start to be transcribed shortly after fertilization. The messenger levels peak at about 12 h postfertilization and then abruptly decline. In contrast, the late histones are encoded by a family of low-copy-number genes. They are transcribed at low basal levels throughout the cleavage stage of embryogenesis until they are activated at the 12-h mid-blastula stage. They reach their transcriptional peak level in 24-h-old late blastula stage embryos and remain active as the somatic set of histone genes throughout the life of the organism. The accumulation of the late histone mRNAs coincides roughly with the decrease of the early histone gene transcripts (9, 22, 24, 25, 29). The dramatic switching of histone gene expression from early to late gene families provides an attractive model for studying the mechanisms governing temporal regulation of gene expression during early embryogenesis.

Previous studies of the regulation of sea urchin late H1 gene expression led to the identification of a transcription factor called stage-specific activator protein (SSAP) (12, 13, 28, 29).

SSAP is a 41-kDa polypeptide that binds specifically to an embryonic enhancer element named upstream sequence element (USE) IV, which is necessary for the transcriptional activation of the late H1 gene during the blastula stage of embryogenesis. Sequence analysis revealed that SSAP is comprised of two distinct domains. The N-terminal domain, spanning residues 1 to 180, contains two RNA recognition motifs which are commonly found in single-stranded DNA and RNA binding proteins. Functional analysis demonstrated that SSAP is capable of binding to both double- and single-stranded DNA in a sequence-specific manner through the RNA recognition motifs. The C-terminal domain of SSAP is rich in glycine and glutamine residues and thus is termed the GQ domain (12). Further investigations showed that the GQ domain can function as a potent transactivation domain which, when fused to a heterologous DNA binding activity, can stimulate the transcription of target genes in several mammalian cell lines (14). In addition, this domain interacts with various transcriptional regulators (14, 55).

There have been numerous reports of embryonic transcription factors which activate temporal specific gene expression during development (7, 26, 34, 53, 54, 56). Most of them have temporal profiles of expression that resemble those of their target genes, suggesting that genes encoding those transcription factors are themselves under the control of temporal regulatory mechanisms. Immunoblot analysis demonstrated that SSAPs are present in the sea urchin throughout embryogenesis (13). Microinjection experiments indicated that SSAP can transactivate the late H1 gene promoter through the USE IV enhancer sequence beginning at the mid-blastula stage even though its DNA binding activity is present in the embryos at

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earlier stages (12, 13). Mobility gel-shift assays have shown that during the early stages of development, when the late H1 gene is transcribed at basal levels, SSAP appears in the embryo and binds to the enhancer elements as a 41-kDa monomer. However, an increase in molecular mass of enhancer binding protein from 41 kDa to 80 to 100 kDa begins at about 12 h postfertilization (early blastula), which coincides with the late H1 gene activation (12). The higher-molecular-mass complex is believed to be a dimer containing at least one 41-kDa SSAP molecule (13). The temporal correlation between the appearance of SSAP dimer and the late H1 gene expression strongly suggests that the blastula-stage-specific transcriptional activation is governed by posttranslational modifications which mediate the formation of this dimeric species.

As one of the major classes of posttranslational modification events, the rapid, reversible, and sequence-specific phosphoryl transfer reaction is an elegant mechanism for the fine tuning of target gene expression in response to cellular signals. The addition of a negatively charged phosphate group to a specific amino acid residue can induce a conformational change and/or confer a different surface feature to the protein, thereby affecting its physiological function (for a review, see reference 20). The activity of a transcription factor can be regulated through phosphorylation by different mechanisms. First, phosphorylation can modulate the accessibility of a transcription factor to the nucleus. This mechanism might include disengagement from a cytoplasmic inhibitor, promoting nuclear entry or inhibiting nuclear import (49). Second, phosphorylation can alter sequence-specific DNA binding activity, thereby facilitating or preventing access of a transcription factor to its target gene (33, 43, 44, 51). Third, phosphorylation can modulate the interaction between a transcription factor and other proteins in the transcriptional machinery (10, 38, 45). There has been evidence in a mammalian system indicating that phosphorylation promoted by developmental cellular signals could be involved in the temporal regulation of transcription factor activity. Specifically, developmental changes in phosphorylation of the cyclic AMP (cAMP)-responsive element binding (CREB) protein correlate with the expression level of the cAMP-regulated genes in the embryonic murine palate (52). However, the mechanism by which transcription factor phosphorylation regulates temporal gene expression during development has not yet been elucidated.

In this study, we show that SSAP is a phosphoprotein throughout the early and late embryonic stages of sea urchin development. Serine 87, a cAMP-dependent protein kinase (PK) consensus site located in the N-terminal DNA binding domain, is constitutively phosphorylated. A second posttranslational modification event takes place at the mid-blastula stage, temporally correlated with SSAP dimer formation and late H1 gene activation, in which a threonine residue in the C-terminal transactivation domain is phosphorylated. In vitro studies revealed that this phosphorylation can be catalyzed by both mammalian DNA-dependent PK and a break-ended double-stranded DNA-activated PK activity in the sea urchin nucleus. In vivo transactivation assays demonstrate that phosphorylation on both serine and threonine residues is essential for SSAP to activate the late H1 gene promoter during the blastula stage. Furthermore, we have shown that SSAP can undergo blastula-stage-specific homodimerization through its GQ-rich transactivation domain and that threonine phosphorylation in this domain is required for dimerization.

MATERIALS AND METHODS

In vivo labeling of sea urchin embryos. In vivo labeling of sea urchin proteins was carried out according to the method of Childs et al. (8). Fertilized *Strongy-*

locentrotus purpuratus eggs were incubated at 17°C in phosphate-free artificial seawater (0.0846 M NaCl, 0.0103 M KCl, 0.0266 M MgCl₂, 0.0293 M MgSO₄, 0.0106 M CaCl₂ and 0.02% NaHCO₃ [pH adjusted to 8.0 by NaOH]) at a concentration of 0.5% (vol/vol). At each time point of interest, the developing embryos were pulse-labeled by incubation with 0.2 mCi of ³²P-orthophosphate/ml for 3 h. The labeling reaction was stopped by washing the embryo once with ice-cold Ca²⁺-Mg²⁺-free seawater.

Extraction and separation of ³²P-labeled histone mRNAs. The ³²P-labeled mRNA was isolated as described by Childs et al. (8) with slight modifications. The in vivo-labeled embryos were washed once with ice-cold Ca²⁺-Mg²⁺-free seawater and lysed with sodium dodecyl sulfate (SDS) buffer (2% SDS, 200 mM Tris-HCl [pH 8.0], 300 mM NaCl, 2.5 mM EDTA, and 2.5 mM EGTA [pH 8.0]). The lysate was digested with 50 µg of proteinase K/ml for 30 min at 37°C. After extraction with phenol-chloroform three times and with chloroform once, the mRNA was precipitated by the addition of 2 volumes of ethanol in the presence of ammonium acetate. The ³²P-labeled histone mRNAs were isolated by hybridization with *S. purpuratus* histone DNAs cross-linked to a nitrocellulose membrane, eluted, separated by urea-polyacrylamide gel electrophoresis (PAGE), and visualized by autoradiography.

Immunoprecipitation of ³²P-SSAP. The in vivo-labeled embryos were washed once with ice-cold STE buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA) and collected by centrifugation. The pellets were resuspended in hot SDS boiling buffer (0.5% SDS, 50 mM Tris-HCl [pH 7.4]). After the embryo suspension was boiled for 5 min, the lysate was diluted with 4 volumes of 1.25× RIPA buffer without SDS (1× RIPA is 150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40 [NP-40], 0.5% deoxycholate containing 100 µM phenylmethylsulfonyl fluoride [PMSF], 25 mM NaF, 1 mM sodium orthovanadate, 50 µM calyculin A, 1 mM EDTA, and 2 mM dithiothreitol [DTT]). Lysates were vortexed, sonicated, and cleared by centrifugation at 10,000 × g for 10 min. The cleared lysates were used for immunoprecipitation with anti-SSAP antiserum that was raised against bacterially expressed recombinant SSAP (bSSAP) (14). Typically, 10 µl of antiserum was added to the lysate for each 0.1 packed ml of embryos, and the resulting mixtures were incubated on ice for 60 min. The immunocomplex was isolated by incubation with 100 µl of 10% BCL4 protein A-Sepharose beads (Pharmacia Biotech) at 4°C for 60 min, and the immunoprecipitate was obtained by centrifugation at 500 × g for 2 min. Nonspecific competitions were performed by preincubating the lysate with preimmune serum and protein A beads on ice for 1 h followed by centrifugation at 500 × g for 2 min.

SDS-PAGE and Western blotting. Samples were subjected to SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (47). The blots were washed in TBSN (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% NP-40) following transfer. After being blocked with 2% bovine serum albumin in TBSN, the blot was washed and incubated with anti-SSAP antiserum at a 1:6,400 dilution in TBSN. To detect the bound antibody, the blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham) and developed by using the ECL kit (Amersham) under the conditions recommended by the manufacturer.

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis. Following fractionation by SDS-PAGE, ³²P-labeled proteins were transferred to a PVDF membrane and visualized by autoradiography. As described by van der Geer and Hunter (48), the portion of the membrane containing SSAP was excised, soaked in 0.5% PVP-360 in 100 mM acetic acid for 30 min at 37°C, washed with double-distilled water (ddH₂O), and then washed with freshly made 50 mM NH₄HCO₃. The protein was digested at 37°C overnight by placing the membrane in 200 µl of 50 mM NH₄HCO₃ containing 10 µg of trypsin, followed by the addition of another 10 µg of trypsin and a 2-h incubation at 37°C. Released tryptic peptides were lyophilized and oxidized with performic acid and were separated on thin-layer cellulose plates by electrophoresis at pH 1.9 (acetic acid–88% formic acid–H₂O, 156:50:1,794 [vol/vol/vol]) in the first dimension followed by chromatography in phosphochromatography buffer (*n*-butanol–pyridine–acetic acid–ddH₂O, 75:50:15:60 [vol/vol/vol/vol]) in the second dimension. Labeled phosphopeptides were detected by autoradiography.

To perform phosphoamino acid analysis, a portion of the lyophilized peptides was resuspended in boiling HCl (5.7 M) and incubated at 110°C for 1 h. The samples were then lyophilized and resolved by electrophoresis by using pH 1.9 buffer in the first dimension and pH 3.5 buffer (acetic acid–pyridine–ddH₂O, 10:1:89 [vol/vol/vol]) in the second dimension (48).

Purification of bSSAP. Recombinant SSAP was expressed and purified as described by Lin and Cheng (35) with slight modifications. *Escherichia coli* BL21 DE3 carrying plasmid pETSSAP (12) was grown in Luria-Bertani medium (LB) containing 150 µg of ampicillin/ml at 37°C overnight. The culture was diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 in LB-ampicillin and grown at 37°C until the OD₆₀₀ reached 0.5 when isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.4 mM. Following induction, the culture was incubated at 37°C with shaking for another 3 h. Cells were then pelleted by centrifugation. Typically, the cell pellets from a 500-ml culture were resuspended in 50 ml of buffer A (20 mM Tris-HCl, [pH 7.5], 20% sucrose, 1 mM DTT) and incubated on ice for 10 min. After pelleting by centrifugation at 4,000 × g, the cells were resuspended in 300 ml of ice-cold ddH₂O. The low-sedimentation-constant outer membrane proteins of the cell were removed by hypotonic treatment of the cells on ice for 10 min followed by centrifugation at 8,000 × g. The cell membranes were lysed by sonication of spheroplasts resuspended in 3 ml of

buffer P containing protease inhibitor ($1\times$ phosphate-buffered saline [PBS], 5 mM EDTA, 500 μ M PMSF). The cell lysate was then incubated at 37°C for 10 min in the presence of RNase A (100 μ g/ml) and DNase I (100 U/ml). To purify IPTG-induced bSSAP present in the inclusion body, the suspension was diluted by the addition of 27 ml of buffer P and subjected to centrifugation at $13,000\times g$. The crude inclusion bodies were washed three times by incubation in 30 ml of buffer W (25% sucrose in PBS, 5 mM EDTA, and 1% Triton X-100) on ice for 10 min before centrifugation at $25,000\times g$. The inclusion bodies were resuspended in 1 ml of denaturation buffer D (50 mM Tris-HCl [pH 8.0], 5 M guanidinium hydrochloride, 5 mM EDTA) and incubated on ice for an additional hour. The insoluble particles were removed by centrifugation at $12,000\times g$ for 15 min. The supernatant was dialyzed against 1 liter of renaturation buffer R (50 mM Tris-HCl [pH 8.0], 1 mM DTT, 20% glycerol, and 500 μ M PMSF) at 4°C overnight. The solution of renatured proteins was clarified by centrifugation at 13,500 cpm in a microcentrifuge at 4°C for 10 min. The supernatant contained highly purified bSSAP.

In vitro kinase assays. For the protein kinase A (PKA) assay, 1 μ g of bSSAP was incubated at 30°C with 1 U of recombinant PKA catalytic subunit (New England Biolabs) in PKA assay buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$) in the presence of 300 μ M ATP and [γ - ^{32}P]ATP. The ^{32}P -labeled bSSAP was separated from other proteins by SDS-PAGE and visualized by autoradiography.

For the DNA-dependent PK assay, 1 μ g of bSSAP was incubated at room temperature with 1 μ g of DNA-PK holoenzyme purified from HeLa cells or 5 μ g of partially purified kinase from a nuclear extract of 18-h *S. purpuratus* embryos in DNA-PK assay buffer (50 mM HEPES [pH 7.5], 100 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA) in the presence of 300 μ M ATP and [γ - ^{32}P]ATP. The ^{32}P -labeled bSSAP was separated from other proteins by SDS-PAGE and visualized by autoradiography (32).

Site-directed mutagenesis. All mutations in SSAP were made by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing using the Sequenase 2.0 kit (Amersham).

Microinjection of sea urchin one-cell zygotes. The procedure used to inject the *Lytechinus pictus* zygotes was essentially that of McMahon et al. (37) and Colin (11) and exactly as described by Lai et al. (27). Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (12).

Capped mRNA for the full-length or truncated form of SSAP for microinjection was synthesized in vitro by using a mMassenge mMachine T7 polymerase kit (Ambion). mRNA was transcribed from PCR products amplified from expression vectors carrying the coding region of the gene to be expressed (46). mRNA was diluted to an appropriate concentration in sterile deionized diethylpyrocabonate-treated water.

^{35}S labeling of exogenous proteins and coimmunoprecipitation. Capped, poly(A)⁺ mRNAs encoding the proteins to be expressed were injected into the fertilized sea urchin eggs together with 5 μ Ci of ^{35}S -methionine/ μ l. The embryos were cultured in artificial seawater at 16°C and collected at each time point of interest. For the coimmunoprecipitation experiment, 500 ^{35}S -labeled, injected embryos were resuspended in ice-cold buffer C (35 mM HEPES [pH 7.8], 40 mM KCl, 0.1 mM EDTA, 15% glycerol, 100 μ M PMSF, 1 μ M aprotinin, 1 μ M pepstatin A, 10 μ M calyculin A, 25 mM NaF, and 2 mM DTT) and lysed by sonication on ice. The lysates were centrifuged at 13,500 cpm for 10 min at 4°C to get rid of any cell debris. The supernatant was incubated on ice with 2 μ l of anti-SSAP antiserum or 4 μ l of anti-T7Tag antibody (Novagen) for 90 min followed by the addition of 50 μ l of 10% protein A-Sepharose CL4B. The samples were incubated at 4°C for another 60 min with rotating and were then centrifuged at $500\times g$ for 2 min. The pellets were washed twice with buffer C and resuspended in Laemmli buffer for SDS-PAGE.

Correction of the nucleotide sequence of SSAP. Previous studies predicted that SSAP is a 44-kDa protein encoded by a 404-amino-acid open reading frame (12). However, mass spectrometry of highly purified bSSAP revealed a protein of 41 kDa. By reanalyzing the nucleotide sequence of the SSAP cDNA, we found that a single nucleotide within the codon encoding residue 317 was omitted. The cDNA sequence after correction encodes a polypeptide of 41 kDa with a length of 382 amino acids as follows: MGEEIGKIFV GGVDNRTHAD TFRA YFEKFG KLSDIILMMD KDKPGQNKGF GFVTFADPAC VDDVTNEKNH NLEGKGLDCK RCKARGSERR MGP GDORTKK VFVGGISQQA TKEDL YELFR SHGNVEDVHI MNDTDTGKHR GFGFVTLDS EAVEKLVRMH HLELKGKSME IKKAQPKMNR GFGGPGGQGG PGGPGGFPOG GNWN QGGGQG GYGGGGSNGY GGGNQWQQM GOYGGGQGG GYQQQ RGGGQ OPGYNRQQQ PQSGYQQQG QSYGGAQSYG SYGGYG QAQQ DPTANNKQH LSSMQAKDR WVATLKEASG YGPQRGNYNQ GYSQAAPQTQ TPTPQQPQQQ SYAQVDSGQD MYGTNNYGA NMGG NQFHPY SR. The predicted amino acid sequence differs from the published sequence downstream of amino acid 317 (underlined). This error has no effect on previous published data.

Nucleotide sequence accession number. The corrected SSAP sequence has been deposited in GenBank and can be found under accession no. L15365.

RESULTS

SSAP is differentially phosphorylated during early embryogenesis. To investigate whether SSAP is a phosphoprotein, we

FIG. 1. Time course of SSAP phosphorylation versus histone gene expression. (A) Temporal phosphorylation of SSAP. Lysates of ^{32}P -labeled sea urchin embryos 10, 18, 24, and 37.5 h postfertilization were immunoprecipitated with polyclonal antibody against SSAP. The immunoprecipitated proteins were resolved by SDS-PAGE. Positions of the molecular mass (MM) standards (Mark 12 Wide-Range Protein Standards; NOVEX) are indicated. (B) Western blot of the immunoprecipitated SSAP from the indicated stages (10, 18, 24, and 37.5 h postfertilization) of embryos. (C) Temporal expression of histone mRNAs. Total RNAs of the same ^{32}P -labeled embryos described for panel A were coincubated with a nitrocellulose membrane cross-linked to cloned DNAs containing each of the early- and late-subtype sea urchin histone genes. The hybridized histone mRNAs were then eluted and separated by urea-PAGE.

pulse-labeled staged sea urchin *S. purpuratus* embryos in artificial seawater containing ^{32}P -orthophosphate. The cell lysate of the labeled embryos was precleared with preimmune rabbit serum and then immunoprecipitated with polyclonal antibodies raised against bSSAP. As shown in Fig. 1A, a 41-kDa protein is labeled at the early blastula stage (10 h) and is more intensely labeled at later stages (18, 24, and 37.5 h). This protein is immunoreactive specifically to anti-SSAP antibody and thus is SSAP because it cannot be detected in the control immunoprecipitates by using preimmune rabbit serum (data not shown). A Western blot of immunoprecipitated SSAP (Fig. 1B) shows that there is a slight increase in SSAP levels during the blastula stage (lanes 1 and 2), but the difference in protein levels is not as dramatic as that of the increased phosphorylation. This suggests that additional phosphorylation of SSAP occurs at the mid-blastula stage of sea urchin embryogenesis. We confirmed that this phosphorylation event(s) on SSAP correlates with the activation of late H1 histone gene expression by documenting the ^{32}P -labeled histone mRNA levels

FIG. 2. SSAP is differentially phosphorylated at early and late stages. (A) Phosphoamino acid analysis of SSAP from early (10 h) and late (24 h) embryos. The results show that at the early stage, only serine residues are phosphorylated (left), while at the late stage, both serine and threonine residues are phosphorylated (right). (B) Two-dimensional tryptic phosphopeptide maps of SSAP from early and late embryos. SSAP is phosphorylated on peptides a and b (left) in early embryos and is phosphorylated on peptides a, b, c, d, and e in late samples (middle). A map of the mixture of early and late SSAP tryptic digests (right) shows that peptides a and b from early samples comigrate with those from late samples.

from embryos at the same stage as those used for immunoprecipitation of SSAP (Fig. 1C).

To analyze the phosphorylation status of SSAP during sea urchin early embryogenesis, we subjected *in vivo*- ^{32}P -labeled SSAP from early and late blastula stages to phosphoamino acid analysis and tryptic phosphopeptide mapping (48). For phosphoamino acid analysis, ^{32}P -labeled protein was hydrolyzed in 6 N HCl at 110°C, and the resulting phosphoamino acids were separated by two-dimensional electrophoresis on a thin-layer cellulose plate. As revealed in Fig. 2A, only phosphoserine is detected in SSAP from the early blastula stage, whereas both phosphoserine and phosphothreonine are detected in the late-blastula-stage sample. Phosphorylation on a threonine residue(s) is therefore a late-stage-specific modification on SSAP. Also, ^{32}P -labeled SSAP from both early (8 h) and late (21 h) embryos were subjected to tryptic digestion, and the resulting phosphopeptides were resolved on a cellulose plate by electrophoresis in the first dimension and chromatography in the second dimension. At the early blastula stage, SSAP is phosphorylated on two tryptic peptides (peptides a and b) (Fig. 2B, left). However, five tryptic peptides (peptides a to e) (Fig. 2B, middle) are phosphorylated at the late blastula stage. Comigration of tryptic phosphopeptides a and b from early blastula with those from late blastula suggests that phosphorylation on these two peptides takes place early in development, while phosphorylation on peptides c, d, and e is specific to late stages (Fig. 2B, right).

SSAP is phosphorylated on serine 87 throughout early embryogenesis. To identify the phosphorylation sites on SSAP from early and late blastula embryos, *in vitro* assays were carried out with bSSAP as a substrate for various PKs to mimic *in vivo* SSAP phosphorylation. bSSAP is phosphorylated by the

PKA catalytic subunit (PKA-c) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 3A, upper panel, lane 3). Phosphorylation of the 41-kDa protein does not occur in the absence of either PKA-c or bSSAP (Fig. 3A, upper panel, lane 1) in the labeling reaction. The 35-kDa band (Fig. 3A, upper panel, lanes 2 and 3) is believed to represent PKA-c labeled with ^{32}P via autophosphorylation (30). To address whether this phosphorylation simulates an *in vivo* event(s), tryptic fragments of ^{32}P -labeled bSSAP *in vitro* phosphorylated by PKA-c were resolved by two-dimensional phosphopeptide mapping. Two peptides are predominantly phosphorylated on bSSAP (Fig. 3A, lower panel) and comigrate with tryptic phosphopeptides a and b from authentic *in vivo*- ^{32}P -labeled SSAP (Fig. 3D, spots a and b).

We then searched for potential PKA consensus sites, (R/K)RXS/T (39), on the SSAP primary sequence and found that the amino acid sequence adjacent to serine 87 (RGS*) fell into this category. We thus considered serine 87 of SSAP a candidate acceptor of the phosphate group in the PKA-catalyzed phosphoryl transfer reaction. To verify this, we created a DNA construct encoding SSAP with serine 87 replaced by alanine. The protein, termed bSSAPS87A, was then expressed in *E. coli*, purified, and incubated with PKA-c in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. As expected, spots a and b were absent from the two-dimensional tryptic phosphopeptide map of bSSAPS87A (Fig. 4B). Therefore, SSAP is phosphorylated by PKA-c at serine 87 *in vitro*. Peptides a and b represent derivative tryptic peptides containing the same phosphoserine 87 as a result of incomplete digestion (Fig. 4A). The serine phosphorylation thus identified is also consistent with the phosphoamino acid analysis result shown in Fig. 2A. We conclude that SSAP is

FIG. 3. In vitro phosphorylation of SSAP. (A) bSSAP is phosphorylated by PKA on peptides a and b in vitro. An in vitro PKA assay was performed as described in Materials and Methods. The phosphoproteins were visualized by SDS-PAGE followed by autoradiography (upper panel). A tryptic phosphopeptide map of PKA-phosphorylated SSAP shows that SSAP was phosphorylated on peptides a and b (lower panel). (B) bSSAP can be phosphorylated by either human DNA-PK (lower panel) or a DNA-activated kinase activity from sea urchin nuclear extract (upper panel) in vitro. In vitro kinase assays were carried out as described in Materials and Methods. Bacterially expressed SSAP can be labeled by ^{32}P when coincubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of a kinase activity partially purified from 24-h sea urchin nuclear extract (upper panel, lane 3) or mammalian DNA-PK (lower panel, lane 3). The intensity of labeling was increased by the addition of DNA to both reactions (lane 4, both panels). (C) Tryptic phosphopeptide maps of the ^{32}P -labeled bSSAP as described for panel B show that bSSAP was phosphorylated on peptides c, d, and e. Upper panel: Tryptic phosphopeptide maps of SSAP phosphorylated by sea urchin DNA-activated kinase activity (left) and human DNA-PK (right). Lower panel: Phosphopeptide map of a mixture of the two tryptic samples. (D) Phosphopeptide map of a mixture of the tryptic digests of in vitro-labeled SSAP mentioned for panels A and C and in vivo-labeled SSAP from late embryos. This proves that peptides a, b, c, d, and e phosphorylated in vitro comigrate with those phosphorylated in vivo.

phosphorylated on serine 87 throughout early embryogenesis of sea urchins.

SSAP is phosphorylated in the transactivation domain by a DNA-PK-like activity at the late blastula stage. To characterize the late-stage-specific phosphorylation events, we carried out column fractionation of nuclear extracts from 24-h-old sea urchin embryos (unpublished data) to identify kinases that potentially phosphorylate SSAP. A kinase activity was obtained that can phosphorylate bSSAP in vitro (Fig. 3B, upper panel, lane 3). We compared the tryptic phosphopeptide map of bSSAP in vitro phosphorylated by this kinase activity (Fig.

3C, upper panel, right) with that of ^{32}P -labeled SSAP from late blastula embryos (Fig. 2B, right) and found that the three tryptic peptides phosphorylated in vitro comigrated with the late-stage-specific phosphopeptides from authentic SSAP (Fig. 3D, peptides c, d, and e). This indicates that this activity might represent the late-stage-specific kinase activity. Interestingly, this kinase activity can be significantly stimulated by the addition of break-ended double-stranded DNA (Fig. 3B, upper panel, lane 4). The best characterized protein kinase which can be activated in the presence of break-ended double-stranded DNA fragments is the so-called DNA-dependent PK (DNA-

FIG. 4. SSAP is phosphorylated by PKA at serine 87 in vitro. (A) Amino acid sequences of tryptic peptides from wild-type (WT) and mutant bSSAP (S87A). As described in Materials and Methods, an amino acid substitution was introduced by site-directed mutagenesis followed by expression of the protein by using a prokaryotic system. (B) Tryptic phosphopeptide maps of wild-type bSSAP (left panel), mutant (S87A) bSSAP (middle panel) after incubation with PKA in the presence of [γ - 32 P]ATP, and a mixture of the two samples (right panel).

PK) present in the mammalian and amphibian systems (for a review, see reference 31). Since SSAP is phosphorylated by a sea urchin DNA-activated kinase activity, we next asked whether this activity resembles mammalian DNA-PK. To answer this question, we performed an in vitro DNA-PK assay using bSSAP as the substrate. As shown in the lower panel of Fig. 3B, highly purified human DNA-PK catalyzes bSSAP phosphorylation which is significantly activated by DNA fragments (lanes 3 and 4). The phosphorylated bSSAP was then subjected to tryptic phosphopeptide mapping (Fig. 3C, upper panel, left). The major tryptic phosphopeptides resolved on the map comigrate with those from bSSAP phosphorylated by the sea urchin kinase (Fig. 3C, lower panel). These experiments suggest, therefore, that the sea urchin kinase contains a DNA-PK-like activity which phosphorylates SSAP at the mid-blastula stage.

Phosphoamino acid analysis of SSAP phosphorylated by the sea urchin DNA-PK-like activity determined the modification event to be threonine phosphorylation (Fig. 5A). A number of DNA-PK substrates contain glutamine residues immediately preceding or succeeding the serine/threonine phosphorylation sites; however, this QS/T or S/TQ motif is neither sufficient nor necessary to define a DNA-PK consensus site (32). Inspection of the amino acid sequence of SSAP identified a cluster of three threonines (threonines 339, 341, and 343) in the GQ domain. Of these, threonines 339 and 341 are positioned next to glutamine residues. A surface probability analysis (data not shown) revealed that they are among a cluster of residues which have dramatically higher surface probabilities than the flanking amino acids, suggesting that they are at the surface of the protein and are accessible to modification enzymes. bSSAP in vitro phosphorylated by the sea urchin kinase was subjected to chymotryptic digestion as well as combined tryptic-chymotryptic digestions. The two-dimensional phosphopeptide of the chymotryptic digestion was identical to that obtained from the combined chymotryptic-tryptic digestions (data not shown), suggesting that the phosphothreonine was contained in a chymotryptic peptide(s) devoid of arginine and lysine residues. We deduced from the primary sequence of SSAP that amino

acids 339, 341, and 343 are located on a chymotryptic peptide (amino acids 333 to 352) devoid of tryptic cleavage sites. To determine whether threonines 339, 341, and 343 are candidate targeting sites for sea urchin DNA-PK-like kinase, we then changed these threonine residues to alanine by generating site-directed mutations on a prokaryotic expression vector carrying SSAP cDNA. The recombinant SSAPs containing the appropriate amino acid substitutions (Fig. 5B) were expressed in *E. coli* and subjected to in vitro kinase assays. As shown in Fig. 5C, SSAP phosphorylation catalyzed by sea urchin DNA-PK-like kinase was abolished in the mutant SSAP protein in which threonines 339, 341, and 343 were converted to alanines (lane 5). However, none of the single-amino-acid substitutions on SSAP attenuates its phosphorylation level (Fig. 5C, lanes 2, 3, and 4) or alters its tryptic 32 P-phosphopeptide map pattern (Fig. 5D) significantly. The three phosphopeptides on the two-dimensional map comigrate during electrophoresis (Fig. 5E, lane 4), indicating that they do not represent tryptic peptides with differing extents of phosphorylation (5). In addition, tryptic phosphopeptides derived from SSAP containing either single or double mutations on residues 339, 341, and 343 retain the same mobility as those from the wild-type protein during electrophoresis (Fig. 5E). If these peptides were phosphorylated on three threonines, any of the single-residue conversions would have caused them to migrate faster during electrophoresis due to the reduction of the negative charge. If two residues were phosphorylated, any of the double residue conversions would have either abolished the labeling on at least one peptide or conferred higher mobility upon it during electrophoresis by eliminating at least one phosphorylation (5). We therefore ruled out the possibility of multiple phosphorylation on these tryptic peptides. We believe that each of the three peptides contains only one phosphothreonine, either threonine 339, 341, or 343. Since the phosphopeptides were resolved by chromatography, they obviously differ in their relative hydrophobicities (5). A similar phenomenon was observed by Alvarez et al. (1) when studying the epidermal growth factor threonine PK phosphorylation of Jun and is believed to be the result of peptide degradations during sample preparation or

FIG. 5. The sea urchin DNA-PK-like activity-phosphorylated SSAP at threonine 339, 341, or 343 in vitro. (A) Phosphoamino acid analysis of bSSAP phosphorylated by sea urchin DNA-PK-like activity. (B) Amino acid sequences of tryptic peptides from wild-type (WT) and the indicated mutant SSAPs. As described in Materials and Methods, an amino acid substitution was introduced by site-directed mutagenesis followed by expression of the protein by using a prokaryotic expression system. (C) In vitro kinase assay using wild-type and the indicated mutant bSSAPs as substrates of sea urchin DNA-PK-like activity. (D) Tryptic phosphopeptide maps of bSSAP from the in vitro kinase assay shown in panel C. (E) One-dimensional tryptic phosphopeptide electrophoresis of mutated bSSAP bearing single or double threonine-to-alanine conversions phosphorylated by sea urchin DNA-PK-like activity. The tryptic peptide samples used are indicated above each lane. Lanes 5 and 10 show results obtained with mixtures of the peptides used in lanes 1 through 4 and lanes 7 through 9, respectively.

possibly deamination of the glutamine residues by extraction and lyophilization of the peptide in the presence of formic acid (1).

We conclude from in vitro kinase assays (Fig. 3B) followed by comparative ^{32}P -phosphopeptide mapping (Fig. 3C and D) and phosphoamino acid analysis (Fig. 5A), in combination with amino acid substitution studies (Fig. 5C and D), that SSAP is alternatively phosphorylated in its GQ-rich transactivation domain on threonine 339, 341, or 343 by a DNA-PK-like kinase at late stages of sea urchin embryogenesis.

Phosphorylation on serine 87 and threonine 339, 341, or 343 is essential for temporal activation of the late H1 promoter. We tested the physiological significance of the phosphorylation events on SSAP by comparing the abilities of wild-type and phosphorylation-defective mutant SSAP to transactivate re-

porter gene constructs when overexpressed in the sea urchin embryos. The system we applied involves microinjection of SSAP mRNA with a reporter gene construct into fertilized sea urchin eggs and has been successfully used to express exogenous DNAs and mRNAs in the developing sea urchin embryos (11, 37). We made use of a reporter gene construct, pGC364 (12) (Fig. 6A), in which the bacterial CAT gene is under the control of upstream nucleotide sequence corresponding to the embryonic enhancer element, USE IV, together with the basal H1 gene promoter (−106 to +8). The three tandem copies of an oligonucleotide containing the USE IV sequence subcloned 3' to the simian virus 40 splicing poly(A) immediately downstream of the CAT gene has been proven to be a late H1 promoter-specific enhancer (12). Therefore the enzymatic activity of CAT represents the late H1 promoter-specific en-

FIG. 6. Both serine and threonine phosphorylation of SSAP are essential for the activation of late H1 gene. (A) Schematic representation of linearized reporter gene DNA. pGC364 is a construct in which a 145-bp partial enhancer containing sequences from -227 to -372 was placed upstream of the basal promoter (this fragment has two SSAP-binding sites), and three tandem copies of the 27-bp USE IV oligonucleotide were cloned in the *Bam*HI site downstream of the CAT gene. (B) In vivo transactivation activity of wild-type and mutant SSAPs. Fertilized eggs were injected with pGC364 (0.2 pg) alone or coinjected with capped synthetic mRNAs (0.2 pg) encoding wild-type or mutated SSAP. Injected embryos were collected at 12 h postfertilization, and CAT assays were performed as described in the text. Bar graphs show means $\pm$ standard deviations of fold activation levels from triplicate experiments relative to CAT activity in embryos injected with pGC364 alone. The autoradiographic image of CAT assays is a representative of three independent experiments. In S87A, serine is replaced by alanine at amino acid 87; in T-A, threonines 339, 341, and 343 are replaced by alanine residues. (C) Expression levels of exogenous proteins. Equal amounts of mRNAs encoding wild-type or mutant SSAP proteins were introduced into fertilized eggs. Exogenous proteins were metabolically labeled by including 35 S-methionine in the microinjection buffer. Anti-SSAP antibody was used to immunoprecipitate the embryo lysate. The immunocomplexes were separated by SDS-PAGE, and the proteins isolated were visualized by autoradiography. The gel shows the exogenous SSAP expressed in the embryos injected with buffer only (lane 1), wild-type SSAP mRNA (lane 2), SSAP (S87A) mRNA (lane 3), or SSAP (T-A) mRNA (lane 4).

hancer activity of the USE IV elements. Previous work has shown that pGC364 is temporally expressed with a pattern identical to that of the late H1 histone gene in the sea urchin embryos during development and that it is further transactivated with the same temporal pattern when overexpressing wild-type SSAP (12).

To test the effect of SSAP phosphorylation on the activation level of the late H1-specific enhancer, capped mRNAs of wild-type or mutant SSAP were coinjected with pGC364, and the injected embryos were collected at the mid-blastula stage and assayed for CAT enzyme activity. We compared these activities to those assayed from embryos injected with pGC364 alone. As shown in Fig. 6B, there was about a two- to threefold increase of CAT activity when pGC364 was coinjected with wild-type SSAP mRNA (rows 1 and 2). How-

ever, when identical amounts of mRNA encoding mutated SSAP with amino acid substitutions at either serine 87 or threonines 339, 341, and 343 were coinjected with the reporter construct, CAT activities were only about 74 and 88%, respectively, of that in embryos injected with pGC364 alone (Fig. 6B, rows 3 and 4). We confirmed by immunoprecipitating the metabolic 35 S-labeled exogenous SSAP molecules in the sea urchin embryos that the mutant proteins can be overexpressed to levels similar to that of wild-type SSAP (Fig. 6). Therefore, the reporter gene transactivation assay suggests that SSAP fails to transactivate the USE IV containing the late H1 promoter when either of the phosphorylation sites is absent. We thus conclude that both the serine and threonine phosphorylations on SSAP are essential for activation of the late H1 gene.

Threonine phosphorylation in the GQ domain mediates homodimerization of SSAP at mid-blastula stage of embryogenesis. Gel mobility-shift assays and Superose 12 gel filtration chromatography showed that SSAP is a 41-kDa species early in embryogenesis and changes to an 80- to ~100-kDa species coincident with the activation of late H1 gene transcription (12, 13). However, no additional proteins were copurified with SSAP through protein-protein interaction, as determined by either affinity chromatography or immunoprecipitation under nondenaturing conditions (12, 14a). These observations suggest that SSAP might exist in late blastula embryos as homodimers. This hypothesis is supported by studies using the yeast two-hybrid system and far-Western binding assays using bacterially expressed proteins (14a, 55a).

To investigate SSAP homodimerization during sea urchin embryogenesis, we coinjected mRNAs of full-length SSAP and a T7Ag-tagged truncated form of SSAP containing the C-terminal GQ transactivation domain (amino acids 181 to 382) (Fig. 7A) into fertilized eggs. The embryos are metabolically labeled by including ^{35}S -methionine in the microinjection buffer. The injected embryos were collected at 7.5 h (morula stage) and 13.5 h (blastula stage) postfertilization. The ^{35}S -labeled proteins were immunoprecipitated by using the polyclonal antibody recognizing the C-terminal GQ domain of SSAP. As shown in Fig. 7B, both full-length SSAP (~41 kDa) and T7TAG-GQ (~23 kDa) are immunoprecipitated at the morula stage (lane 1) and blastula stage (lane 5). To test for interaction between the two proteins in the injected embryos, a monoclonal antibody immunoreactive specifically to the T7TAG epitope was used. At the morula stage, 7.5 h postfertilization, when the late H1 gene enhancer is inactive, very low levels of full-length SSAP were coimmunoprecipitated with the T7TAG-GQ (Fig. 7B, lane 2). However, at later stages, when late H1 gene is actively transcribed, quantitatively comparable amounts of full-length SSAP molecules were coprecipitated with the T7TAG-GQ (Fig. 7B, lane 6). Therefore, SSAP dimerizes through its C-terminal GQ transactivation domain when the late H1 gene enhancer is activated.

We next asked whether temporal phosphorylation on the GQ domain is a mechanism that is essential for dimerization. To investigate this, we coinjected mRNAs encoding wild-type full-length SSAP and mutant T7TAG-GQ fusion protein in which threonines 339, 341, and 343 are replaced by alanines. Immunoprecipitation of the injected embryo lysates using anti-T7TAG monoclonal antibody revealed that full-length SSAP was not quantitatively coprecipitated with T7TAG-GQ with threonine to alanine conversions at either early (Fig. 7B, lane 4) or late (Fig. 7B, lane 8) stages. The mRNAs of wild-type SSAP were efficiently translated as demonstrated by the control immunoprecipitation experiment using anti-SSAP antibody (Fig. 7B, lanes 3 and 7). The inability of the phosphorylation-deficient GQ domain to coimmunoprecipitate with the full-length protein at the late stages demonstrates that dimerization of SSAP depends on temporally regulated phosphorylation.

DISCUSSION

To elucidate the mechanisms governing the temporal regulation of gene expression during development, we characterized the posttranslational modification events of the sea urchin embryonic transcription factor SSAP. We found that SSAP is phosphorylated on serine residues early in development, whereas it is phosphorylated on both serine and threonine residues at later stages (Fig. 2A). The stage-specific threonine

phosphorylation of SSAP occurred within the time interval in which late H1 gene transcription is activated.

To obtain enough material to investigate the phosphorylation of this low-abundance embryonic transcription factor, we expressed recombinant SSAP (bSSAP) in bacteria and performed *in vitro* kinase assays. Tryptic phosphopeptide maps of *in vitro*-phosphorylated bSSAP were compared with those of authentic SSAP.

We found that the PKA catalytic subunit can phosphorylate SSAP *in vitro* on the peptides (a and b) that were phosphorylated in both early and late embryos (Fig. 3A and C). Substitution of the potential PKA target serine residue at position 87 by alanine abolished the phosphorylation on both tryptic peptides a and b by PKA *in vitro* (Fig. 4). Thus, phosphorylation on serine 87 in the N-terminal DNA-binding domain constitutes the serine phosphorylation on SSAP throughout sea urchin embryogenesis. *In vivo* transactivation experiments showed that serine 87 is required for the transactivation of the late H1 promoter (Fig. 6). The physiological significance of this phosphorylated residue is still not understood. An attractive model is that phosphorylation in the DNA-binding domain can regulate the sequence-specific interaction with DNA by SSAP. Previous work suggests that native SSAP exhibits higher DNA binding affinity and a more distinct coding to noncoding strand preference than bSSAP (12). However, we failed to detect significant differences in DNA binding properties between PKA-phosphorylated and nonphosphorylated bSSAP (data not shown). Although the catalytic subunit of PKA can phosphorylate SSAP on serine 87 *in vitro*, we cannot conclude that it is an SSAP targeting kinase *in vivo*. Application of a potent, cell-membrane-permeable PKA inhibitor, H-89, to developing sea urchin embryos did not block the accumulation of late H1 transcripts in the blastula stage (data not shown). Therefore, it is possible that sea urchins contain another kinase which recognizes and phosphorylates SSAP on serine 87.

bSSAP is also a target of mammalian DNA-dependent PK *in vitro* (Fig. 3B). The mammalian DNA-PK is a nuclear serine/threonine PK which as a holoenzyme contains a 460-kDa catalytic subunit and one of two Ku regulation subunits of 70 and 80 kDa (18). Binding of break-ended double-stranded DNA to Ku70 results in the release of the catalytic subunit from inhibition. A number of transcription factors, such as p53, SP1, c-Myc and c-Jun, are known to be substrates of this kinase (3, 15, 21, 23, 32). Significantly, a sea urchin kinase activity that can phosphorylate SSAP was found in nuclear extracts of late blastula embryos (Fig. 3B). This kinase activity resembles DNA-PK in several aspects. First, two-dimensional phosphopeptide mapping showed that this kinase phosphorylates SSAP on the same peptides that are phosphorylated by human DNA-PK (Fig. 3C). In addition, this kinase can also be activated by the addition of break-ended double-stranded DNA (Fig. 3B). Fractionation of the partially purified kinase with a Superose 12 fast protein liquid chromatography gel filtration column resulted in the elution of this kinase activity in an early fraction (data not shown), demonstrating that it is a component of a large protein complex with a molecular mass of several hundred kilodaltons. Since the kinase applied to the Superose 12 column survived several high-salt washes during purification procedures, we believe that the early fraction contains the holoenzyme of the kinase itself. These observations support the argument that the sea urchin kinase is a functional homologue of mammalian DNA-PK. Both phosphopeptide mapping and phosphoamino acid analysis indicate that this sea urchin DNA-PK phosphorylates SSAP *in vitro* on the same threonine residue that is phosphorylated *in vivo* (Fig. 3D).

Numerous studies have attempted to determine the consen-

FIG. 7. Threonine phosphorylation in the GQ domain is essential for the temporally regulated dimerization of SSAP. (A) Schematic representation of open reading frame structures of microinjected synthetic capped SSAP mRNAs. Open boxes represent RNP repeats in the DNA-binding domain, black boxes represent GQ domains, and grey boxes represent T7TAg sequences. (B) Immunoprecipitation of exogenous proteins. Full-length SSAP mRNAs (0.4 pg) were coinjected into fertilized eggs with mRNAs encoding either wild-type (WT) or mutated (MUT) T7TAg-GQ (0.4 pg). 35 S-methionine were included in the injection solution to metabolically label the proteins translated. Embryos were collected at the indicated developmental stages, and coimmunoprecipitation was performed as described in the text. The 35 S-labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. Early (lanes 1 to 4), proteins from embryos collected at 7.5 h postfertilization. Late (lanes 5 to 8), proteins from embryos collected at 13.5 h postfertilization. Lanes: 1, 3, 5, and 7, proteins immunoprecipitated with anti-SSAP polyclonal antibody; 2, 4, 6, and 8, proteins immunoprecipitated with anti-T7TAg monoclonal antibody; 1, 2, 5, and 6, proteins from embryos injected with mRNA encoding full-length SSAP and that of T7TAg-wild-type GQ domain; 2, 4, 7, and 8, proteins from embryos injected with mRNA encoding full-length SSAP and that of T7TAg mutated GQ domain in which threonines 339, 341, and 343 are replaced by alanine residues. Positions of the molecular mass standards (BenchMark Prestained Protein Ladder; NOVEX) are indicated.

sus sequences flanking the DNA-PK phosphorylation site (for a review, see reference 31). A number of DNA-PK substrates are phosphorylated on serine or threonine residues with adjacent glutamines (QS/SQ or QT/TQ), which can be used to evaluate whether a peptide sequence is a candidate DNA-PK target (32). However, many QS/T- and S/TQ-containing se-

quences cannot be phosphorylated by DNA-PK in vitro. In addition, there are reports that several DNA-PK substrates contain no such sequence. Particularly, serine 288 of c-Fos in the sequence RSVP is phosphorylated by DNA-PK in vitro (2). In another report, DNA-PK was shown to phosphorylate the seventh serine in the heptapeptide repeat (YSPTSPS) of the

C-terminal domain of RNA polymerase II (40). Our studies reveal three threonines as phosphorylation sites of a sea urchin DNA-PK-like PK (Fig. 5). Of the three, two of them (threonines 339 and 341) harbor Q/T or T/Q motifs. The third one was not adjacent to glutamine residues; rather, it was flanked by the sequence PT*PQPPQ. This finding contributes a new member to the non-QS/T or -S/TQ class of DNA-PK targeting sites. Although all of them are potential DNA-PK sites, only one threonine is modified on each SSAP molecule. Phosphorylation on one threonine residue probably plays a negative role in recognition of the other two sites by DNA-PK. Charge effect itself does not seem to be a mechanism because previous studies have shown that basic residues in the flanking region negatively regulate DNA-PK site recognition while acidic residues positively regulate it (32). It is possible that the addition of the phosphate group on one threonine induces a conformational change in the surrounding sequence which results in a poor substrate for the kinase. Further study of the mechanism of this effect might shed light on the determination of the site-targeting regulation element on the substrate of DNA-PK.

Our results strongly suggest that late-stage-specific threonine phosphorylation in the GQ domain of SSAP is catalyzed by a DNA-PK-like kinase in sea urchin embryos. Unlike DNA-PK-dependent phosphorylation of the glucocorticoid receptor and Oct-1 (17), which negatively regulates transcriptional activity, phosphorylation of SSAP correlates with activation of the late H1 gene enhancer (Fig. 1B). SSAP is the only sequence-specific late histone H1 enhancer DNA-binding activity identified in the sea urchin embryo nucleus (13). In vivo transactivation experiments demonstrated that wild-type but not mutant SSAP is able to activate an embryonic enhancer composed of multimers of the USE IV binding site in a stage-specific pattern (Fig. 6). Interestingly, threonine phosphorylation in the GQ-rich transactivation domain also coincides with the formation of the higher-molecular-mass USE IV-binding SSAP complex (12, 13). The functional connection between phosphorylation and transcription factor multimer formation has been well established in a number of systems. Both homodimerization (STAT-1) and heterooligomerization (VP16-Oct1-HCF complex, ISGF-1 and CREB-CBP complex) could result from transcription factor phosphorylation (10, 38, 45, 50). In each of the cases listed above, protein-protein interaction between transcription factors is involved in the formation of a transcriptional preinitiation complex, thereby triggering the activation of the target gene. Coimmunoprecipitation experiments demonstrate that full-length wild-type SSAP can form stable complexes with a truncated protein containing the C-terminal GQ domain in late blastula sea urchin embryos but not in early blastula embryos (Fig. 7B, lanes 2 and 6), suggesting that at the late stages, the GQ domain of one SSAP molecule possesses a higher affinity for another molecule. This observation supports the idea that a homodimer is the transcriptionally active form of SSAP. Phosphorylation on the DNA-PK-like kinase targeting sites is critical for interaction between the full-length and truncated SSAP because abolishing these sites on T7Tag-GQ would disrupt dimer formation in late blastula embryos (Fig. 7B, lane 8). We thus conclude that temporal phosphorylation of the threonine residue in the transactivation domain of SSAP promotes the stage-specific homodimerization of this protein.

Neither threonine phosphorylation nor dimerization seems to be a prerequisite for the sequence-specific DNA binding activity of SSAP, since SSAP can bind USE IV as a monomer early in development (12). Our microinjection experiments show that an SSAP mutant lacking the target threonine residues lost its capability to activate the late H1 promoter (Fig.

6B), strongly suggesting that this modification is critical for the intrinsic transactivation activity of the C-terminal GQ domain. One possibility is that the introduction of negative charge to the C-terminal region by adding a phosphate group strengthens the protein-protein interaction between monomers, thereby stabilizing the transcriptionally active dimeric species. This hypothesis is supported by the observation that a single conversion of basic residue K369 or R382 to an acidic amino acid (E) or neutral amino acid (L), respectively, can produce transactivational gain-of-function mutants in a yeast one-hybrid system (4a). Previous studies showed that oligomers of GQ domains possess a high transactivation potential. The Gal4-GQ fusion protein exhibits a dramatic transactivation potential in HeLa cells (14, 55). In this case, GQ dimerization is stabilized by dimerization of Gal4 DNA binding motifs.

There are numerous observations that somatic histone gene expression is coupled with DNA synthesis (4, 19, 41, 42). It is believed that the transcription of histone genes is upregulated in the S phase of the cell cycle (42). Early in development, the sea urchin embryo undergoes a period of very rapid cell cleavage. The requirement of rapid histone biosynthesis for efficient packaging of newly synthesized DNA is apparently fulfilled by the sharp increase of early histone mRNAs transcribed from high-copy-number templates in the genome (36). The sea urchin embryo builds up its normal cell cycle after it enters the blastula stage of development, when late histone gene transcription is activated (36). There has been evidence that DNA-PK activity can be stimulated by the formation of DNA replication intermediates containing single- and double-stranded regions, suggesting that DNA-PK is activated during S phase of the cell cycle (6). Therefore, late H1 gene activation through phosphorylation of SSAP by the DNA-PK-like kinase might reflect a mechanism to modulate the developmental variation of the S-phase index of cell cycles during sea urchin embryogenesis.

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