Phosphorylation Weakens DNA Binding by Peptides Containing Multiple “SPKK” Sequences*

(Received for publication, October 15, 1992, and in revised form, February 15, 1993)

George R. Green†, Hyuck-Jin Lee, and Dominic L. Poccia

From the Department of Biology, Amherst College, Amherst, Massachusetts 01002

Sea urchin testis-specific H1 and H2B histones (Sp H1 and Sp H2B) are characterized by reversibly phosphorylated N-terminal regions consisting largely of multiple clustered “SPKK” tetrapeptides (serine-proline adjacent to two basic amino acids). This report presents data showing differences in DNA affinities between peptides containing dephosphorylated and phosphorylated N-terminal regions. Sp H1 and its phosphorylated derivative (pSp H1) were purified by hydroxylapatite chromatography. Peptides containing the N-terminal regions of Sp H1 and pSp H1 (NP and pNP, respectively) were produced by digestion with Staphylococcus aureus protease. NP and two forms of pNP differing in phosphate content were purified by DNA-cellulose chromatography. The DNA affinities of the peptides were compared using several criteria. NP was bound more tightly by DNA-cellulose than pNPs. NP precipitated DNA under a broad range of NaCl concentrations; pNPs did not. Both NP and pNPs protected DNA against thermal denaturation, but NP created a more stable DNA-peptide complex. Thirty to sixty times more pNP than NP was required to obtain equivalent inhibition of Hoechst 33258 binding to DNA. NP did not behave as a competitive inhibitor of DNA binding by Hoechst 33258 binding to DNA. We conclude that during spermatogenesis, dephosphorylation of the Sp H1 N-terminal region increases its basicity and thus its affinity for DNA.

Condensed chromatin in sea urchin sperm nuclei contains two sperm-specific histone variants, Sp H1 and Sp H2B (for review see Poccia and Green, 1992). The N-terminal regions of these histones consist largely of multiple “SPKK” tetrapeptides, which we define as a serine-proline dipeptide adjacent to two basic amino acids (R can substitute for K, T can substitute for S, and the dibasic amino acids can lie to either side of the SP dipeptide; Strickland et al., 1977, 1980; Green and Poccia, 1985; Poccia, 1987; Suzuki, 1989a). The SPKK domains bind the extraordinarily long linker DNA in sea urchin sperm chromatin to help stabilize the highly condensed DNA (Zalenskaya et al., 1981; Green and Poccia, 1988; Hill et al., 1990; Hill and Thomas, 1990; Lindsey and Thompson, 1992). Binding of SPKK tetrapeptides to DNA probably involves ionic interactions between SPKK basic residues and DNA phosphate groups, since SPKK-containing peptides can be eluted from DNA-cellulose columns with NaCl solutions (Suzuki, 1989a; Hill et al., 1991).

During spermatogenesis (Poccia et al., 1987; Hill et al., 1990, 1991) and again immediately after fertilization (Green and Poccia, 1985, 1989; Poccia et al., 1990) serine residues in the SPKK tetrapeptides appear as serine-phosphate, converting Sp H1 and Sp H2B to their phosphorylated derivatives pSp H1 and pSp H2B. Serine phosphorylation adds two negative charges to the dibasic SPKK tetrapeptide, creating a net charge of 0 at neutral pH. Since nearly all basic amino acids in the N-terminal regions of Sp H1 and Sp H2B are associated with serine phosphorylation sites, phosphorylation could effectively neutralize basicity of the entire N-terminal region and thus weaken its binding to DNA. In addition to charge neutralization, serine phosphorylation may cause disruption of SPKK secondary structure by breaking hydrogen bonds that stabilize β-turns in the SPKK domain, thereby contributing to weakened DNA binding (Green and Poccia, 1985; Poccia, 1987; Suzuki, 1989a; Erard et al., 1990; Churchill and Travers, 1991).

Naturally occurring pSp H1 and pSp H2B have been correlated with several structural differences between sea urchin spermatid chromatin (containing pSp H1 and pSp H2B) and chromatin of mature sperm (containing Sp H1 and Sp H2B). For example, spermatid chromatin is less dense, is more sensitive to micrococcal nuclease attack and thermal denaturation, and has a shorter nucleosomal repeat length than sperm chromatin (Green and Poccia, 1988). Hill et al. (1991) showed that unlike sperm chromatin fragments, spermatid chromatin fragments did not form NaCl-dependent pseudo-higher order structures and that unlike Sp H1, pSp H1 was able to exchange between chromatin fragments. They assessed relative DNA binding by Sp H1, Sp H2B, pSp H1, and pSp H2B and peptides derived from them, finding that phosphorylation within the N-terminal regions inhibited formation of H1-DNA complexes, binding to DNA-cellulose, and NaCl-dependent binding to DNA. These observations on chromatin structure and protein chemistry are consistent with the proposal that phosphorylation of SPKK tetrapeptides weakens binding by the N-terminal regions of Sp H1 and Sp H2B to linker DNA.

Seasonal maturation in sea urchin populations provides an opportunity to obtain large amounts of Sp H1 and pSp H1 from gonads swollen with male germ cells (Poccia et al., 1987; Hill et al., 1990). We have purified Sp H1 and pSp H1 from this natural source by perchloric acid extraction and hydroxylapatite chromatography. A dephosphorylated N-terminal peptide (NP)

* This work was supported by the National Science Foundation Grant DMB 9004170 and by an Amherst College Faculty Research Award (to D. L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Biology Dept., Webster Center for Biological Sciences, Amherst College, Amherst, MA 01002. Tel.: 413-542-2081; Fax: 413-542-7955.

The abbreviations used are: NP, N-terminal peptide; pNP, phosphorylated N-terminal peptide; CP, C-terminal peptide; pCP, phosphorylated C-terminal peptide.
proteases were washed three times with acetone, and dried in air.

**Purification of pSp H1 and Sp H1 by Hydroxylapatite Chromatography**—The protein was dissolved in acetone (1 mg/ml), hydroxyapatite (Bio-Gel HTP, Bio-Rad) was added to the solution (1 g/10 mg of h1 histones), and the slurry was mixed by swirling for 10 min at 21°C. Hydroxylapatite was pelleted by centrifugation at 10,000 x g for 5 min, and the supernatant was removed. Hydroxylapatite was washed once with TB (10 ml/g of hydroxylapatite), and H1 histones were eluted either with gradients or by batch elutions in a centrifuge tube, first with NaCl, then with sodium phosphate. For gradient elution, hydroxylapatite was packed in a chromatography column (1 g in a 10-cm long x 1-cm diameter column; Bio-Rad) and eluted with a 30-ml gradient increasing to 1 M NaCl in TB. The column was washed twice with 30 ml of gradient and eluted with a gradient increasing to 1 M sodium phosphate, pH 8.0. The absorbance was measured at 220 nm (A220). The effluent was measured, and 2-ml fractions were collected. For batch elution, hydroxylapatite was suspended in 1 M NaCl in TB (10 ml/g of hydroxylapatite) and pelleted by centrifugation at 10,000 x g for 5 min. The supernatant (containing Sp H1) was removed, and the hydroxylapatite pellet was washed two times with 1 M NaCl in TB (5 ml/g of hydroxylapatite). The three supernatants were combined to give purified Sp H1. The hydroxylapatite was washed two times with TB (10 ml/g of hydroxylapatite) and then three times as above, except with 1 M sodium phosphate, pH 8.0. The sodium phosphate washes were combined as purified pSp H1. The purified protein solutions were dialyzed overnight against 10 mM HCl to remove NaCl or sodium phosphate and then trichloroacetic acid was added to 20% to precipitate proteins, which were washed three times with acetone and dried in air (Poccia and Green, 1986).

**Cleaveage of Sp H1 and pSp H1 with Staphylococcus aureus Protease**—Protein and peptide complexes were analyzed using a variety of methods, including protein and peptide chromatography—Testicular H1s or purified Sp H1 or pSp H1 was dissolved in TB (1 mg/ml) and digested with S. aureus protease (Sigma type XVIII; 1 mg/mg H1 histones) for 1 h at 21°C. The probable cleavage sites for the protease are aspartic acid residues located at positions 47 and 58, yielding N-terminal peptides (NP from Sp H1; pNP from pSp H1) of 47 amino acids and C-terminal peptides (CP from Sp H1 and pCP from pSp H1) of 190 amino acids. Incomplete cleavage of the C-terminal peptides, especially in some Sp H1 samples, yielded a slightly larger C-terminal peptide of 201 amino acids (see Fig. 2f).

**DNA-cellulose (Sigma; 5 mg of bound DNA/g of cellulose) was added to the digested histones, and the slurry was incubated with 1 M NaCl in TB. The A260 of the effluent was monitored, and 2-ml fractions were collected. For batch elution, the DNA-cellulose was eluted by suspension and centrifugation in sequentially increasing NaCl solutions containing TB and 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 M NaCl. The DNA-cellulose was washed twice with each solution (5 ml each wash), and the two washes were combined. Eluted peptides were precipitated by chilling the fractions to 4°C and adding 100% trichloroacetic acid to a final concentration of 20%. After 1 h on ice the precipitated peptides were collected by centrifugation at 10,000 x g for 5 min. The peptides were washed three times with acetone and dried at room temperature. Peptides (1 mg/ml) were dissolved in 1 mM HCl, and insoluble material was removed by centrifugation. The peptides were precipitated with trichloroacetic acid and washed with acetone, as above.

**Thermal Denaturation**—Salmon testis DNA (Sigma; 0.75 mg/ml in TB) was diluted with 30 volumes of 0.25 mM EDTA, pH 8.0, to yield an A260 of 0.50. Diluted DNA (10 ml in a 15-ml polypropylene centrifuge tube containing 200 mU of Salmon testis DNAase; Falcon 2002) was sheared by repeated pipetting with a plastic transfer pipette (Fisher-2171-58A) while holding the tube in an ultrasonic bath (Fisher model FS3). Three 15-cycle were performed, with 1-min delays between cycles, to avoid heating of the DNA solution. For each sample, 2.9 ml of the sheared DNA solution was placed in a Teflon-stoppered quartz cuvette, and an appropriate amount of NP or pNP-5, in volumes of less than 50 μl, was added with rapid mixing. Thermal denaturation was performed as previously described (Green et al., 1983) using a Gilford model 250 spectrophotometer fitted with a water-jacketed cuvette chamber. DNA was denatured by increasing the cuvette chamber temperature 0.5°C/min to 90°C. The A330 and was always less than 10% of the A260. No increase in the A260 was detected in any of the samples following the experiment. The Tm was defined as the temperature at which the rate of denaturation was maximal, equivalent to the peaks observed in the first derivative plots of the absorbance changes during the temperature increase.

**Assay for DNA Precipitation**—Five μg of sheared salmon sperm DNA (see “Thermal Denaturation”) in 200 μl of TB containing appropriate NaCl concentrations (except the 0.25 mM EDTA sample, which did not contain TB) was placed in each well of a 96-well microplate (Corning). Appropriate amounts of NP or pNP-5 were added with mixing by drawing the sample into the pipette tip several times. After 5 min the turbidities of the samples were read as apparent A590 using a Thermomax microplate reader (Molecular Devices).

**Assay for Inhibition of Hoechst 33258 Binding to DNA**—Inhibition of fluorescence due to Hoechst 33258 binding to DNA was studied by adapting the procedure described by Reeves and Nissen (1990). DNA solutions (sheared salmon sperm DNA, 100 ng measured as phosphate) in appropriate NaCl solutions in TB (3 ml in 150 μl of degassed 10% glycerol) were mixed with TB, pNP-5, or pNP-9. The DNA-peptide mixtures were titrated with 1.25, 2.5, 5.0, 7.5, 10, 15, 20, 30, and 40 nM Hoechst 33258. Fluorescence was measured in a SPEX Fluorolog (DM1B) fluorescence spectrophotometer, with the excitation monochrometer set to 354 nm and the emission monochrometer set to 450 nm. To test for the thermal denaturation of the DNA, the concentration of the DNA was adjusted to 100 ng/ml. 10 nM NP and 100 nM DNA were centrifuged at 10,000 x g for 5 min, and the supernatant was titrated with Hoechst 33258 as described above. No differences were observed when compared with the samples that were not centrifuged. Curves were fit with Sigmaplot (Jandel) software routines to the equation F = Fmax x [Hoechst 33258]/(Ks + [Hoechst 33258]).

**Gel Electrophoresis**—Proteins and peptides were analyzed by polyacrylamide gel electrophoresis as described previously using either 12 or 20% polyacrylamide gels fitted with stacking gels (Poccia and Green, 1986). The 12% polyacrylamide gels were made with 12% acrylamide, 0.6% bisacrylamide, 0.5% acetic acid, and 5 μlurea. The 20% polyacrylamide gels were made with 20% acrylamide, 0.133% bisacrylamide, 5% acetic acid, and 5 μlurea. All gels were stained with Coomassie Blue R-250.

**Chemical Determinations**—Protein and peptide concentrations were determined using the Micro BCA procedure (Pierce). Protein and peptide-bound phosphate were determined using the method described by Buss and Stull (1983).

**RESULTS**

**Two Forms of Sp Sp H1 Are Present in Sea Urchin Testis**—The phosphorylated form of Sp H1 (pSp H1, previously referred to as protein N) usually appears as a single protein band when resolved by electrophoresis in 10–15% acetic acid-urea or acetic acid-urea-Triton X-100 polyacrylamide gels (Poccia et al., 1981, 1987; Green and Poccia, 1985, 1988, 1990; Hill et al., 1990, 1991). However, when resolved in 30-
cm acetic acid-urea polyacrylamide gels, testicular H1s (and total acid extracted testis histones) exhibited two closely migrating forms of pSp H1 (pSp H1a and pSp H1b) which were consistently present in approximately equal amounts in testicular H1s extracted from different individuals (Fig. 1a). These bands were probably not created artificially by proteolysis during protein purification, since there was no additional evidence of proteolysis in the samples, such as more rapidly migrating peptide bands. Analysis of N-terminal peptides derived from mixed pSp H1s indicated that pSp H1a and pSp H1b differed in phosphate content, with the more slowly migrating form containing more phosphate (see below).

pSp H1s Bind to Hydroxylapatite in 2 M NaCl, but Sp H1 Does Not—The large charge differential between pSp H1s and Sp H1 created by phosphorylation within the N-terminal regions suggested an obvious basis for separation using methods that depend on ionic binding to fixed matrices. However, pilot experiments employing DNA-cellulose, carboxymethyl cellulose, and phosphocellulose failed to provide adequate resolution of Sp H1 and pSp H1s, probably because other regions of the H1 molecules affected binding to these matrices.

In contrast, hydroxylapatite was found to separate pSp H1s from Sp H1 completely (Fig. 1, b–d). Sp H1 eluted from hydroxylapatite in 2 M NaCl (Fig. 1, b and d), leaving pSp H1s bound. pSp H1s subsequently eluted in 1 M sodium phosphate (Fig. 1, c and d). Sp H1 and pSp H1s obtained from hydroxylapatite were very pure, judged by heavily loaded polyacrylamide gels (Fig. 1d). It may be possible to differentially elute pSp H1a and pSp H1b from hydroxylapatite using a shallow sodium phosphate gradient, but this was not attempted in the present work. Hydroxylapatite has been used previously to separate egg yolk proteins differing in phosphate content (Bernardi and Cook, 1960). Its utility for purification of pSp H1 shown here suggests that hydroxylapatite may be of more general application for purifying phosphorylated protein derivatives. We do not know the basis for the extreme affinity of pSp H1s for hydroxylapatite, but we speculate that multiple phosphate groups clustered in the pSp H1 N-terminal region may interact with calcium ions fixed in the hydroxylapatite matrix. In the present application, hydroxylapatite provided a simple, very efficient means for purification of large amounts of pSp H1.

Cleavage of Testicular H1s with S. aureus Protease Yields N-terminal Peptides Containing Five SPKK Tetrapeptides—H1 histones, including Sp H1 and pSp H1s, contain four distinct structural regions defined by sensitivity to trypsin proteolysis. These include a variable N-terminal trypsin-sensitive region (which is unusually large in Sp H1 because of the presence of multiple SPKK tetrapeptides); a conserved globular trypsin-resistant region; an α-helical region, rich in lysine and alanine (and trypsin-resistant in sea urchin sperm chromatin); and a long C-terminal region, rich in basic amino acids, proline, and serine (Fig. 2a; Allan et al., 1980; Strickland et al., 1980; Hill et al., 1989; Vodicka et al., 1990).

Aspartic acid residues at positions 47 and 58 of the Sp H1 amino acid sequence (Parechinus angulosus; Strickland et al., 1980) provided sites for selective cleavage by S. aureus protease (Fig. 2, a and b). The resultant 47-amino acid N-terminal peptide has nine potential phosphorylation sites, defined as the hydroxyamino acids serine and threonine (the tyrosine residues in Sp H1 are not actively phosphorylated in pronuclear or spermatic chromatin; Green and Poccia, 1985; Poccia et al., 1987). NP contains five SPKK tetrapeptides plus a degenerate SPKK tetrapeptide (residues 3–7), all of which are actively phosphorylated in vivo during spermatogenesis (Hill et al., 1990). Additional potential phosphorylation sites include serine 27 and a serine-threonine dipeptide (residues 40 and 41) at the border of the N-terminal and globular domains. Serines 27 and 40 and threonine 41 apparently do not undergo phosphorylation at rates comparable to serines contained in the SPKK tetrapeptides (Hill et al., 1990). Serine 62, the site phosphorylated by CAMP-regulated protein kinase in many H1 histones (Porter et al., 1988), is not present in NP. The larger C-terminal peptide contains 190 amino acids C-terminal to aspartic acid 58. Cleavage of pSp H1 with S. aureus protease yielded two N-terminal peptides, pNP-5 and pNP-9, named to reflect their phosphate contents (see below), and one phosphorylated C-terminal peptide, pCP (Fig. 2b). NPs and CP migrated more rapidly in acid-urea gels than the larger and less basic pNPs and pCPs.

pNPs Bind Less Tightly to DNA-Cellulose Than NP—DNA-cellulose was used in this investigation both as a method to assess DNA affinity and for purification of NP and pNPs derived by digestion of Sp H1 and pSp H1 with S. aureus

![Fig. 1. Purification of pSp H1. Samples of testicular H1 histones (T H1s) obtained from several sea urchins were resolved by electrophoresis in 30-cm 12% acetic acid-urea polyacrylamide gels (panel a). The high resolution gels show that two forms of pSp H1 were present, designated pSp H1a and pSp H1b. To separate Sp H1 and pSp H1s, 2 mg of testicular H1s (1 mg/ml in TB) were mixed with 100 mg of hydroxylapatite. Bound protein was eluted from the hydroxylapatite with two gradients, the first increasing to 1 M NaCl (panel b), the second to 1 M sodium phosphate (panel c). The A220 of the eluted protein was monitored continuously. The purified fractions were analyzed by electrophoresis in a 15-cm 20% acetic acid-urea polyacrylamide gel (panel d).](image-url)
**Phosphorylation Weakens DNA Binding by SPKK**

**FIG. 2.** DNA-cellulose chromatography of *S. aureus* protease digested Sp H1 and pSp H1. Amino acid sequence data for *Parachinus angulosus* Sp H1 (Strickland et al., 1980) was used to construct a linear map emphasizing four structural regions (N-terminal, globular, α-helical, and C-terminal) which have been identified in Sp H1 (panel a). The locations of SPKK tetrapeptides (S-P adjacent to E-E), additional potential phosphorylation sites (S or T), and *S. aureus* protease cleavage sites (E) are indicated, as are the relative sizes of peptides generated by cleavage with *S. aureus* protease (NP and CP). The peptides produced by digestion of these proteins with *S. aureus* protease were resolved by electrophoresis in a 15-cm 20% acetic acid-urea polyacrylamide gel (panel b). The peptides derived by digestion of mixed (panel c) or hydroxylapatite purified (panel d) testicular H1s were resolved by DNA-cellulose chromatography, using either a continuous NaCl gradient (panel c) or washes of increasing NaCl concentrations (panel d). Peptide concentrations of the eluted fractions were determined by *A*$_{220}$ (panel c) or by the BCA assay (panel d). Peptides eluting in the fractions were analyzed by electrophoresis in 15-cm 20% acetic acid-urea polyacrylamide gels (panels e and f).
protease (Fig. 2). Either testicular H1s (containing a mixture of Sp H1 and pSp H1; Fig. 2c) or hydroxylapatite-purified Sp H1 and pSp H1 (Fig. 2d) were mixed with DNA-cellulose. In the first experiment, mixed Sp H1 and pSp H1 digestion products were resolved by elution of a DNA-cellulose chromatography column with a continuous NaCl gradient increasing to 1.0 M, so that the relative NaCl concentrations required for elution of the various DNA-binding peptides could be determined. In the second experiment, designed to obtain purified NP and pNPs, digestion products from purified Sp H1 and pSp H1 were eluted from DNA-cellulose with solutions increasing to 1 M NaCl, in steps of 0.1 M.

The phosphorylated N-terminal peptides pNP-5 and pNP-9 were bound less tightly to DNA-cellulose than NP. Nearly all pNP-9 eluted from DNA-cellulose in 0.1 M NaCl, whereas nearly all pNP-5 eluted in 0.2 M NaCl (Fig. 2, c-f), demonstrating that these two peptides differed in their DNA affinities. The purity of the pNP-9 (0.1 M NaCl) fraction was about 90%, judged from the polyacrylamide gel, with the major contaminant being pNP-5. The pNP-5 (0.2 and 0.3 M NaCl) fraction was about 85% pure, with the major contaminants being pNP-9 and a peptide of slightly greater mobility than pNP-5. A similar value (0.09 M) for elution of a phosphorylated N-terminal Sp H1 peptide from DNA-cellulose was obtained by Hill et al. (1991). Unphosphorylated NP eluted at 0.5 M NaCl, a higher NaCl concentration than those observed previously (Suzuki, 1989a; Hill et al., 1991). This difference, and those noted above for pNPs, may be due to peptide denaturation caused by harsher conditions used to prepare Sp H1 N-terminal peptides (48 h in 70% acetic acid plus an unreported amount of cyanogen bromide by Suzuki, 1989a; 24 h in 0.1 M HCl, 2 mg/ml cyanogen bromide by Hill et al., 1991). Sp H1 and pSp H1 C-terminal peptides eluted last, with pCP eluting at a slightly lower NaCl concentration (0.7 M) than CP (0.8 M). Thus increased phosphate content within both the N-terminal and C-terminal Sp H1 peptides was associated with weaker binding to DNA-cellulose.

pNP-5, pNP-5, and pNP-9 Differ in Phosphate Content—Separation of NP, pNP-5, and pNP-9 into highly pure fractions by DNA-cellulose chromatography permitted measurement of the phosphate contents of these peptides, using the phosphomolybdate-acetate green method to determine total phosphate (Buss and Stull, 1983) and the BCA assay to quantify protein. The phosphate content of NP was found from four determinations to be 0.01 ± 0.01 mol of phosphate/mole of peptide, essentially devoid of phosphate (net charge +1.5). Five phosphate determinations of pNP-5 yielded an average value of 5.2 ± 0.7 mol of phosphate/mole of peptide. The peptide was therefore designated "pNP-5" (net charge = +4.6 ± 1.4). Four phosphate measurements of pNP-9 yielded an average value of 9.3 ± 2.9 mol of phosphate/mole of peptide, suggesting that all 9 serines and threonines are occupied by phosphate (net charge = −3.6 ± 5.8). We conclude that NP, pNP-5, and pNP-9 had identical amino acid compositions but varied in phosphate content, causing the peptides to be highly basic (NP), moderately basic (pNP-5), or essentially neutral (pNP-9).

Phosphate determinations of Cp and pCP yielded values of 0.12 ± 0.07 mol of phosphate/mole of peptide (six determinations) and 0.38 ± 0.21 mol of phosphate/mole of peptide (10 determinations), respectively. We conclude that CP was essentially devoid of phosphate and that on average pCP contained one phosphate per peptide; both peptides would carry strong net positive charge. These data are similar to those of Hill et al. (1991) who described a peptide equivalent to a mixture of pNP-5 and pNP-9 as containing six phosphates and a peptide equivalent to pCP as containing three phosphates (NP and CP were not analyzed in their report). The higher phosphate content obtained for pCP by Hill et al. (1991) may indicate that pSp H1 contains more phosphate in its C-terminal region earlier in spermatogenesis, since these workers used relatively immature animals for their studies.

NP Precipitates DNA, but pNPs Do Not.—The addition of NP to DNA solutions (25 μg/ml DNA) in a wide range of NaCl concentrations led to precipitation of the peptide-DNA complex as the peptide mass (3–6 μg) approached the mass of the DNA (5 μg; Fig. 3a). Precipitation apparently does not occur in extremely dilute DNA solutions used for Hoechst 33258 binding studies (33 ng/ml; see “Assay for Inhibition of Hoechst 33258 Binding to DNA” under “Experimental Procedures”). Precipitation was not observed when up to twice the equivalent mass of pNP-5 was added to DNA in either 0.25 mM EDTA (Fig. 3b) or in 50 mM NaCl (Fig. 3c), showing that even partial phosphorylation of the Sp H1 N-terminal peptide abolished its ability to precipitate DNA under these conditions. These data extend the observations of Lindsey and Thompson (1992) who attributed NaCl-dependent precipitation of H1-depleted sea urchin sperm chromatin to binding of linker DNA by the Sp H2B N-terminal region (containing three to four SPKK tetrapeptides) and of Hill et al. (1991) who showed that intact Sp H1 formed pseudo-higher order structures, whereas pSp H1 did not. Here we show that the Sp H1 N-terminal region alone is sufficient to precipitate DNA and that partial phosphorylation abolishes this ability.

Precipitation probably resulted from neutralization of DNA phosphate negative charges by lysine and arginine positive charges in NP, as has been shown for NaCl-dependent chromatin condensation by H1 histone (Clark and Kimura, 1990). The nearly equal masses of DNA and peptide which produced precipitation in our assays contained nearly equal numbers of positive and negative charges (3 nmol of phosphate negative charges/μg of DNA versus 3.2 nmol of peptide positive charges/μg of peptide, assuming molecular weights of 660 g/mol for DNA phosphate and 5,090 g/mol for NP).

Both NP and pNP Protect DNA against Thermal Denaturation, but NP Forms a More Stable DNA-Peptide Complex—The hyperchromic shift accompanying conversion of double-stranded DNA to single-stranded DNA is a measure of the total interaction energy of packing the DNA by histones (Kaplan et al., 1984). DNA strand separation is favored by electrostatic repulsion of the negatively charged phosphate groups in the DNA backbone, which can be partially neutralized by positively charged counterions. Histones, as polycations, are more effective at charge neutralization than monovalent ions because fewer are required to give the same degree of neutralization in this entropically unfavorable interaction (Manning, 1978; Clark and Kimura, 1989). Thermal stabilization of DNA by nuclear proteins has been documented for various particles derived by micrococcal nuclease digestion of different chromatins (Simpson, 1978; van Holde, 1989) and has been shown to be especially large for DNA in sea urchin sperm chromatin, presumably because of tight binding by the variant N-terminal regions of Sp H1 and Sp H2B (Paolotti and Huang, 1969; Ozaki, 1971; Green and Poccia, 1988). However, the complex structures of nucleosomes prohibit attribution of observed differences in chromatin thermal stabilities to specific histone regions.

To assess directly differences in thermal stability imparted by NP and its phosphorylated derivative pNP-5, reconstituted peptide-DNA complexes were examined. Sheared salmon sperm DNA denatured at a maximal rate at 41 °C, indicated as the peak of the first derivative plot of the absorbance change (Fig. 4). The addition of either pNP-5 or NP caused stabilization of the DNA, producing maximal denaturation rates of 85 and 88 °C, respectively. The data provide evidence
Phosphorylation Weakens DNA Binding by SPKK

That at low ionic strength both pNP-5 and NP bind to DNA, with pNP-5 creating a less stable peptide-DNA complex. pNP-5 may bind to DNA via its residual positive charges. By reducing the net charge on the histone through phosphorylation, more molecules would need to bind to contribute the same degree of charge neutralization. At the equivalent protein/DNA ratios used in our experiments the lower $T_m$ for the pNP-5-DNA complex can be attributed to less complete charge shielding by NP-5. In addition to charge shielding, other features of the DNA-histone interaction, which may, for example, enhance DNA base stacking, could stabilize the DNA duplex. The difference in thermal stabilization by NP and pNP shown here (3 °C) is greater than the 1 °C difference reported for chromatin reconstituted with either unphosphorylated or in vitro phosphorylated H1 histones (Kaplan et al., 1984). It is less than the difference measured for natural chromatin of spermatic and spermatozoa (Green and Poccia, 1988). However, other histone and nonhistone proteins may have contributed to the relative stabilities of these chromatin.

**NP Inhibits DNA Binding by Hoechst 33258 More Effectively Than pNPs**—Hoechst 33258 forms a fluorescent complex with DNA. At low Hoechst 33258 concentrations the dye binds predominantly to the DNA minor groove, with preference for AT-rich regions (Loontiens et al., 1990; Fjura et al., 1987; Teng et al., 1988; Carrondo et al., 1989). Fluorescence inhibition by DNA-binding proteins has been used previously to estimate binding constants for a peptide similar to NP (S6; Suzuki, 1989a) and for regions of the high mobility group protein I molecule containing the tetrapeptide TPRK (Reeves and Nissen, 1990). We therefore used this method to assess the relative DNA binding affinities for NP, pNP-5, and pNP-9.

The abilities of NP, pNP-5 and pNP-9 to inhibit Hoechst 33258 binding to DNA were examined over a range of Hoechst 33258 concentrations from 1 to 40 nM and peptide concentrations of 0.5–64 nM at a fixed concentration of 100 nM DNA. Fluorescence inhibition curves were constructed for various concentrations of NP (Fig. 5, a–d) in solutions of four different ionic strengths (0.01, 0.05, 0.15, and 0.30 mM NaCl) and in the presence of increasing amounts of NP. The data were fit to a curve of the form $F_l = (F_{l_{max}} × [Hoechst 33258])/(K_{Hoechst 33258} + [Hoechst 33258])$, from which apparent fluorescence maxima ($F_{l_{max}}$) and binding constants for Hoechst 33258 ($K_{Hoechst 33258}$) were estimated. In the absence of NP, $K_{Hoechst 33258}$ estimates were $1.9 ± 0.04$, $5.1 ± 0.4$, $8.4 ± 0.7$, and $8.4 ± 1.3$ nM in solutions of 0.01, 0.05, 0.15, and 0.30 mM NaCl, respectively, in agreement with published values (Loontiens et al., 1990; Nissen et al., 1991). Similar values were obtained when the data were analyzed graphically using a double-reciprocal plot (Fig. 5e). At high salt concentrations (0.30 mM), NP was less effective in inhibiting fluorescence, suggesting that NP binding to DNA involves ionic interactions between lysine residues in the SPKK tetrapeptides and phosphate groups in the DNA backbone.

Apparent fluorescence maxima were decreased by the pres-
Phosphorylation Weakens DNA Binding by SPKK

**Fig. 5. Inhibition of Hoechst 33258-DNA binding by NP.** The abilities of NP to inhibit DNA binding by Hoechst 33258 were examined in 0.01 (panel a), 0.05 (panel b), 0.15 (panel c), and 0.30 M NaCl (panel d). DNA concentrations were fixed at 100 nM phosphate, and appropriate amounts of NP were added with rapid mixing. The DNA-peptide complexes were titrated with Hoechst 33258, and the fluorescence (F1) of each sample was measured following each Hoechst 33258 addition. Data from (panel b) were replotted in double-reciprocal form (panel e).

The fluorescence inhibition by NP was not competitive, since the curves do not intersect at the same point on the y axis (Fig. 5e). Nor do the curves intersect at the same point on the x axis, as would be expected of a noncompetitive interaction. Two clusters of curves in the double-reciprocal plot were formed by the lower and higher NP concentrations, showing that the mode of inhibition of Hoechst 33258 binding to DNA changed to reflect weaker NP binding as the concentration of NP increased. The NP concentration at which the change in binding affinity changed (8 nM) corresponds with the calculated point of DNA phosphate charge neutralization by NP positive charges, suggesting that ionic interactions between DNA negative charges and NP positive charges contribute to stability of the native DNA-peptide complex. We conclude that fluorescence inhibition by NP is due neither to competitive nor to noncompetitive interactions with Hoechst 33258, but is rather a complex phenomenon reflecting various interactive and noninteractive modes of DNA binding by the two ligands.

Double-reciprocal plots of data from the experiment in 0.05 M NaCl confirm that fluorescence inhibition by NP was not competitive, since the curves do not intersect at the same point on the y axis (Fig. 5e). Nor do the curves intersect at the same point on the x axis, as would be expected of a noncompetitive interaction. Two clusters of curves in the double-reciprocal plot were formed by the lower and higher NP concentrations, showing that the mode of inhibition of Hoechst 33258 binding to DNA changed to reflect weaker NP binding as the concentration of NP increased. The NP concentration at which the change in binding affinity changed (8 nM) corresponds with the calculated point of DNA phosphate charge neutralization by NP positive charges, suggesting that ionic interactions between DNA negative charges and NP positive charges contribute to stability of the native DNA-peptide complex. We conclude that inhibition of fluorescence by NP is due neither to competitive nor to noncompetitive interactions with Hoechst 33258, but is rather a complex phenomenon reflecting various interactive and noninteractive modes of DNA binding by the two ligands.

Binding constants could not be assigned to the complex...
Phosphorylation Weakens DNA Binding by SPKK

interactions described in Fig. 5, prohibiting a simple comparison of the DNA affinities of NP, pNP-5, and pNP-9. However, it is clear from the fluorescence assay that both pNP-5 and pNP-9 bind to DNA much more weakly than NP, since in 0.05 M NaCl 64 nM pNP-5 and 64 nM pNP-9 were required to inhibit fluorescence to levels attained with only 2.0 and 1.0 nM NP, respectively (Fig. 6). pNP-9 at 64 nM produced slightly less fluorescence inhibition than pNP-5 at 32 nM, indicating that pNP-9 binds DNA less tightly than pNP-5, consistent with our other experiments showing that DNA affinity of the Sp H1 N-terminal region was reduced by increased phosphate content.

DISCUSSION

Three peptides of identical amino acid sequence but differing phosphate contents were analyzed for their abilities to bind DNA. Thirty to sixty times more pNP-5 or pNP-9, respectively, than NP were required to produce equivalent inhibition of Hoechst 33258 binding to DNA, indicating much weaker binding by the phosphorylated peptides. NP was bound to DNA-cellulose relatively tightly, eluting in 0.5 M NaCl, well above physiological ionic conditions (equivalent to about 0.15 M NaCl). Therefore the N-terminal region of Sp H1 must be tightly bound to DNA in sperm chromatin. pNPs were less tightly bound to DNA-cellulose, eluting in 0.1 M (pNP-9) and 0.2 M (pNP-5) NaCl. In vivo, the N-terminal region of pSp H1a (represented by pNP-9) may not be bound to DNA, and the N-terminal region of pSp H1b (represented by pNP-5) may be weakly bound.

Our experimental observations indicate that ionic bonds between NP lysine and arginine residues and DNA phosphate groups are essential for the stability of the NP-DNA complex. Disruption of NP-DNA bonds and subsequent elution of NP from DNA-cellulose occurred in 0.5 M NaCl, suggesting that the NP-DNA bond is ionic in nature (see also Suzuki, 1989a; Hill et al., 1991). DNA precipitated from its normally highly soluble form when positive charges from added NP approximated the number of negative charges present as DNA phosphate. Inhibition of Hoechst 33258 binding to DNA by NP occurred in at least two modes, which showed differences in peptide affinity for DNA above and below the calculated point of DNA phosphate charge neutralization by NP. Thus once all DNA phosphates are bound, additional NP must bind to the DNA-peptide complex by a different mechanism. Ionic binding between DNA and Sp H1 and Sp H2B N-terminal regions is also suggested by the close correlation between the number of negative charges in the long linker DNA of sperm chromatin and the number of basic amino acids located in the N-terminal regions of these histones (Green and Poccia, 1985).

In addition to charge neutralization, phosphorylation-induced alteration of SPKK secondary structure may contribute to weakening of DNA binding, assuming that β-turns in the SPKK domains are important in DNA binding (Green and Poccia, 1985; Poccia, 1987; Suzuki, 1989a). Since a predicted β-turn can be stabilized by two H-bonds between the i+2 serine residue and the NHs in the i+2 and i+3 positions of the tetrapeptide (Suzuki, 1989a), phosphorylation is likely to disrupt the β-turn. Details of the relative importance of charge neutralization and disruption of β-turn secondary structure in weakening DNA binding by the SPKK domain must await determination of the structure of the SPKK tetrapeptide-DNA complex.

It has been suggested that SPKK tetrapeptides prefer to bind the minor groove of AT-rich regions of DNA, by analogy with certain Hoechst 33258-DNA complexes (Suzuki, 1989a; Churchill and Suzuki, 1989). However, the SPKK tetrapep-

![Fig. 6. Inhibition of Hoechst 33258-DNA binding by pNP-5 and pNP-9](image-url)
tide is both more bulky and more basic than Hoechst 33258 and generates hydroxyl-radical cleavage patterns that differ from those produced by Hoechst 33258 (Churchill and Suzuki, 1989a). We did not observe competitive behavior by the very similar SPKK tetrapeptide-DNA interaction, which remains unknown.

Evidence is presented in this paper for two forms of pSp H1 and H2B in sea urchin testis which differ in phosphate content within the N-terminal regions of the molecules. First noticed as two pNP tetrapeptides differentially eluting from DNA-cellulose and later confirmed for intact pSp H1s on long acetic acid urea polyacrylamide electrophoresis gels, these data suggest that sperm maturation is attained in part by progressive dephosphorylation of maximally phosphorylated H1 histones, with one major intermediate (pSp H1b). According to this scheme, pSp H1a (containing pNP-9) first loses four phosphates to become pSp H1b (containing pNP-5). Then Sp H1b loses its remaining phosphates to become Sp H1 (containing NP), the latter transition taking place in late spermatids, during sperm maturation (Poccia et al., 1987). Progressive dephosphorylation would produce progressive strengthening of the ionic interaction between the H1 N-terminal region and DNA. Alternatively, the two forms could coexist in all spermatogenic cells and become dephosphorylated in parallel in late spermatids.

Dephosphorylated Sp H1 and Sp H2B only exist in the spermatozoa, suggesting that binding of SPKK domains to linker DNA is required for packaging the highly condensed sperm chromatin. An electrostatic mechanism for chromatin folding predicts that binding of dephosphorylated SPKK domains to linker DNA will reduce the residual electrostatic free energy of the DNA, which opposes close approximation of linker segments (Clark and Kimura, 1990). In the case of the dense packing of chromatin fibers in the sea urchin sperm nucleus (Green and Poccia, 1985; Poccia, 1987), a premium is placed on electrostatic shielding by histone. A second role may be played by the Sp H1 and Sp H2B N-terminal regions if such binding generates cross-links between adjacent chromatin fibers (Green and Poccia, 1986; Poccia, 1987), but evidence for chromatin cross-linking in vivo has not been presented.

Unlike protamine-type molecules, Sp H1 and Sp H2B must be able to function during spermatogenesis and immediately after fertilization in those cells that undergo such activities as replication, transcription, and mitosis (Poccia and Green, 1992). Phosphorylation of the Sp H1 and Sp H2B N-terminal regions during these times might prevent the N-terminal regions from interacting with chromosomal DNA, allowing the remainders of each molecule, which resemble their somatic counterparts, to function normally. Since nonclustered SPKK sequences exist in many somatic H1 histones (Poccia, 1987), and in several nonhistone DNA-binding proteins (Poccia and Green, 1992) modulation of DNA binding by protein phosphorylation may be of more general occurrence in the control of access to DNA. An especially important function for phosphorylation within the DNA binding regions of somatic histones may be control of access to DNA during transcription (Suzuki, 1989b; Hunter and Karin, 1992).

REFERENCES


