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Histone – DNA interactions and their modulation by phosphorylation of -Ser-Pro-X-Lys/Arg- motifs

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The sea urchin sperm-specific histones H1 and H2B are multiply phosphorylated in spermatids, dephosphorylated in the final stages of spermatogenesis to give mature sperm, and rephosphorylated upon fertilization. Phosphorylation in spermatids, and probably at fertilization, occurs at repeated -Ser-Pro-X-Basic- motifs in the distinctive N-terminal basic domains of both histones and at the end of the much longer C-terminal domain of H1. Here we identify the consequences of multiple phosphorylation through comparison of some physical and biochemical properties of spermatid (phosphorylated) and sperm (dephosphorylated) chromatin and histones. Study of the DNA binding properties of the intact histones and isolated basic domains suggests that phosphorylation at three dispersed sites in the C-terminal tail of H1 has little effect on its overall DNA binding affinity, whereas, strikingly, binding of the N-terminal domains of H2B and H1 is abolished by phosphorylation at four or six tandemly repeated sites respectively. Together with the relative timing of events *in vivo*, this suggests that phosphorylation/dephosphorylation of the N-terminal (and distal end of the C-terminal) tail of H1, and/or the N-terminal tail of H2B, effectively controls intermolecular interactions between adjacent chromatin filaments, and hence chromatin packing in the sperm nucleus.

Key words: chromatin/electrospray mass spectrometry/ histones/phosphorylation/-Ser-Pro-X-Lys/Arg- motifs

Introduction

Phosphorylation/dephosphorylation is a well-documented means of modulating protein function. Histones are phosphorylated in DNA binding regions in a variety of situations, but the extent to which this has functional consequences, given the large net positive charge on the histones, has not been systematically analysed. An ideal opportunity to study the role of histone phosphorylation and dephosphorylation in modulating chromatin structure and function exists in sea urchin sperm at the end of spermatogenesis and again upon fertilization.

The highly stable, inert chromatin of mature sea urchin sperm is associated with sperm-specific histones H1 and H2B which have the same domain structure as their somatic counterparts, but differ by having extensions in their amino- (N-) terminal domains and in the case of H1, in the carboxy- (C-) terminal domain also. The extensions contain a repeated sequence motif, -Ser-Pro-X-Basic- (Poccia, 1987; Suzuki, 1989) where X is usually basic, and evidently have a specific role in sperm chromatin structure. They are multiply phosphorylated on serines in the -Ser-Pro-X-Basic- motif during spermatogenesis while the chromatin becomes condensed and inert (Hill *et al.*, 1990), dephosphorylated at the end of spermatogenesis when the chromatin is packaged most tightly, and rephosphorylated upon fertilization, probably on similar sites (Green and Poccia, 1985), as a prelude to large scale remodelling of the sperm chromatin and the reactivation of gene expression.

We have studied the structural consequences of this phosphorylation by comparison of chromatin from mature sperm and spermatids and of H1 – DNA complexes formed with unphosphorylated and phosphorylated H1. We have also measured the effect of phosphorylation on the binding to DNA of individual domains of H1 and H2B and of intact H1. The results point to the independent functional (and structural) nature of the domains of sperm H1 and H2B and suggest a role for the phosphorylatable tails in intermolecular interactions involved in close packing of the chromatin filaments in the nucleus.

Results

Sperm and spermatid chromatin

Sea urchin sperm and early spermatids (Figure 1a) differ significantly in nuclear volume [$\sim 4\ \mu m^3$ and $\sim 75\ \mu m^3$ respectively (Simpson and Poccia, 1987)]. The sperm-specific histones H1 and H2B are present in both cases but are phosphorylated in spermatids (Figure 1b and c). In the case of *Psammechinus miliaris*, but not *Echinus esculentus*, the phosphorylated and unphosphorylated forms are resolved in an SDS – 18% polyacrylamide gel (Figure 1b); a Triton/acid/urea – 14% polyacrylamide gel (Figure 1c) resolves the phosphorylated and unphosphorylated forms of both H1 and H2B from both species. *E.esculentus* and *P.miliaris* are mature at different times of the year, and both have been used in this study.

The recently developed technique of electrospray mass spectrometry gives accurate molecular masses of proteins (Fenn *et al.*, 1989). The molecular masses of sperm (unphosphorylated) and spermatid (phosphorylated) H1 from *P.miliaris* calculated from the spectra (Figure 2) show that spermatid H1, as isolated, consists of a mixed population of molecules with 7, 8 or 9 phosphates. In the most highly modified species, six of the phosphorylation sites are in -Ser-Pro-X-Basic- motifs in the N-terminal domain (Hill *et al.*, 1990), and three presumably are in the C-terminal domain. [Our previous finding, from the relative amounts of radiolabel (^{32}P) incorporated into the N- and C-terminal domains over a period of days, that only a single site was

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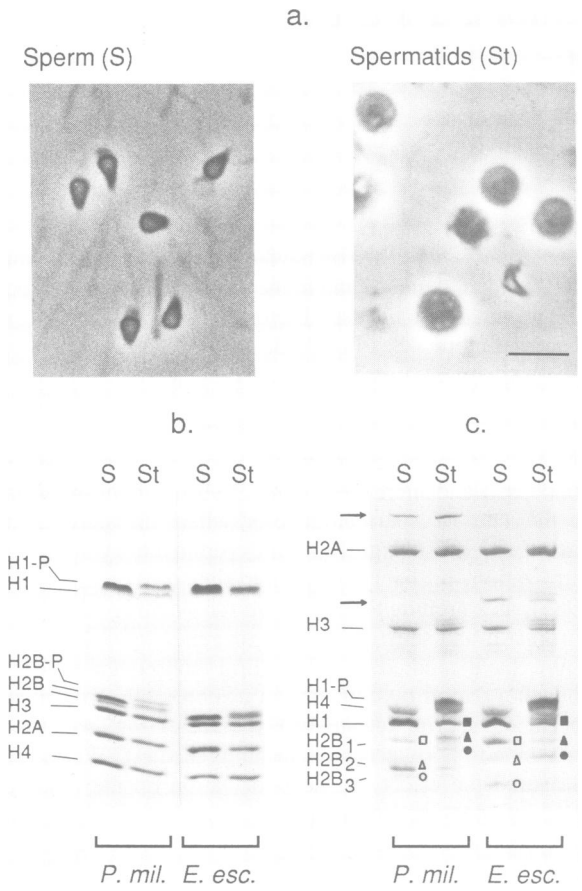


Fig. 1. Characterization of sperm and spermatid nuclei and histones. (**a**) Phase contrast microscopy of *P.miliaris* sperm and early spermatids. Scale bar represents 10 μm. (**b**) SDS−18% polyacrylamide gel electrophoresis of histones acid-extracted from *P.miliaris* and *E.esculentus* sperm (S) and spermatid (St) nuclei. H1-P and H2B-P refer to the phosphorylated forms of H1 and H2B, which are resolved from the unphosphorylated forms only in the case of *P.miliaris*. (**c**) Triton/acid/urea−14% polyacrylamide gel electrophoresis of histones from similar sperm and spermatid preparations. The three H2B subtypes are resolved; open symbols indicate unphosphorylated H2Bs; closed symbols indicate the phosphorylated H2Bs which migrate more slowly, but in the same order. The arrows indicate H3 (slower migrating) and H4 dimers formed through disulphide bonds.

Table I. Distribution of phosphorylation sites in *E.esculentus* spermatid H1

		Molecular masses (daltons)	No. of phosphates*
H1	Phos.	25 897.2	9
	Unphos.	25 175.7	
N-terminal fragment H1(1−48)	Phos.	5719.1	6
	Unphos.	5239.2	
C-terminal fragment H1(112−end)	Phos.	13 481.6	3
	Unphos.	13 241.1	

* Molecular mass of phosphate = 80 daltons

phosphorylated in the C-terminal domain of *P.miliaris* H1 (Hill *et al.*, 1990), suggests that two of the sites in the C-terminal domain may be stably phosphorylated and therefore not radiolabelled.]

The 2:1 distribution of sites between the N- and C-terminal domains was confirmed by electrospray mass spectrometry of *E.esculentus* spermatid H1 (which showed predominantly a species phosphorylated at nine sites) and of proteolytic fragments containing the N- and C-terminal domains (Table I). A peptide corresponding to the N-terminal 48 residues, H1(1−48), contained six phosphates, and a peptide of about 126 residues corresponding to the C-terminal domain, H1(112−end), three phosphates. The sites in the N-terminal domain of *E.esculentus* H1 are probably the same as those mapped in *P.miliaris* H1 which has the same N-terminal sequence (Hill *et al.*, 1990). From its partial sequence (C.S.Hill *et al.*, 1989; and Figure 8b) it seems likely that the C-terminal domain of *E.esculentus* H1 contains only three -Ser-Pro-X-Basic- motifs, which are in the last 37 residues (see Materials and methods), and that these are the three sites phosphorylated. (The only other serine in the C-terminal domain occurs in a different sequence context.) The sperm-specific H1s from the two species of sea urchin thus appear to be phosphorylated on equivalent sites and to the same extent during spermatogenesis.

The long nucleosome repeat length of mature sea urchin sperm chromatin, ~240 bp for *E.esculentus* (Thomas *et al.*, 1986), is at least 25 bp longer than the longest repeat length in sea urchin embryo chromatin (Savic *et al.*, 1981) and considerably longer than the repeat in most somatic chromatin (Kornberg, 1977), and the sperm-specific H1, and possibly also H2B, play a role in this (Stein and Mitchell, 1988). Since we find that the nucleosome repeat lengths of sperm and spermatids are similar (Figure 3), at 243 ± 7 bp (however, see Green and Poccia, 1988), phosphorylation of the tail domains of H1 and H2B in spermatids evidently has no effect on the average spacing of nucleosomes, and the long repeat length appears to be established early in spermatogenesis.

Functional consequences of H1 and H2B phosphorylation

From the timing of the phosphorylation and dephosphorylation of H1 and H2B *in vivo* during spermatogenesis, Poccia *et al.* (1987) concluded that much of the chromatin condensation characteristic of inert sperm nuclei was achieved while H1 and H2B were phosphorylated, and that dephosphorylation correlated with cross-linking of close-packed chromatin fibres. In order to investigate this directly *in vitro*, we have studied the effect of phosphorylation on three previously documented properties: (i) the salt-induced H1-dependent folding of long oligonucleosomes (Thoma *et al.*, 1979; Bates *et al.*, 1981); (ii) the formation of 'pseudo-higher-order structures' which, in the case of chicken erythrocyte chromatin, appear to form by end-to-end association of short elements of higher order structure [e.g. 1−3 turns of solenoid (Finch and Klug, 1976)] and to be H5 dependent (Thomas *et al.*, 1985); and (iii) the formation and self-association of H1(H5)−DNA complexes (Clark and Thomas, 1986, 1988).

First, we compared, by electron microscopy, the effect of phosphorylation on the ability of size-fractionated sperm and spermatid chromatin to condense in a salt-dependent

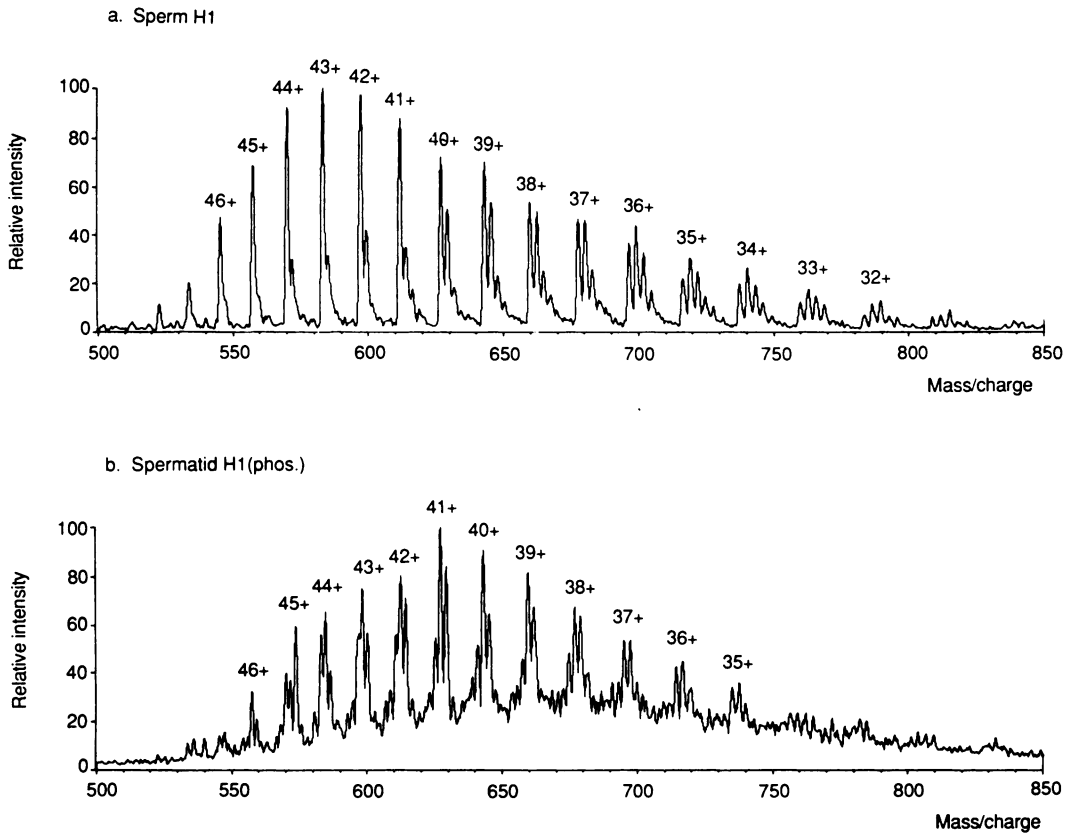


Fig. 2. Electrospray mass spectra for *P.miliaris* sperm and spermatid H1. These consist of a series of multiply charged ion peaks, arising from multiple protonation of the protein, from which the molecular mass may be computed (Fenn *et al.*, 1989). (**a**) Sperm H1. The spectrum indicates a single component of molecular mass 24 942.5 daltons; ~98 dalton adducts (Chowdhury *et al.*, 1990) cause peaks of lower m/z in particular to appear as multiplets. (**b**) Spermatid H1. The peaks are all triplets (seen most clearly for the 41+, 40+ and 39+ ions) and arise from three components of molecular mass 25 505.1, 25 582.9 and 25 665.5 daltons (all ±2.5 daltons), corresponding to H1 carrying 7, 8 and 9 phosphates respectively (the 8-phosphate species being the most abundant).

manner (Thoma *et al.*, 1979) (Figure 4). At the lowest ionic strength (5 mM TEA–HCl pH 7.5, 0.2 mM Na_2EDTA), both chromatins have a similar zig-zag 'beads on a string' structure (Thoma *et al.*, 1979). As the ionic strength is increased, spermatid chromatin appears to condense less readily than sperm chromatin; for example, at ~45 mM ionic strength (40 mM NaCl, 5 mM TEA–HCl pH 7.5, 0.2 mM Na_2EDTA) the sperm chromatin consists of patches of fully condensed chromatin, linked by uncondensed regions, whereas the spermatid chromatin is still essentially uncondensed. However, at ~65 mM ionic strength the spermatid chromatin achieves substantial condensation, although possibly less than for sperm chromatin, suggesting that the effect of phosphorylation is largely offset at sufficiently high ionic strength.

Secondly, we investigated the effect of phosphorylation of H1 and H2B by comparing the ability of sperm and spermatid chromatin to form 'pseudo-higher-order structures' (Thomas *et al.*, 1985). Figure 5 shows sucrose gradient sedimentation profiles for sea urchin sperm and spermatid chromatin fragments (~12 nucleosomes) at ~55, 65 or 75 mM ionic strength. The spermatid chromatin sedimented as one peak in each case, whereas at 65 and 75 mM ionic strength (although not at 55 mM) the sperm chromatin sedimented bimodally due to self-association of oligonucleosomes found in the slower-sedimenting peak, as did oligonucleosomes of chicken erythrocyte chromatin at ~75 mM ionic strength (Thomas *et al.*, 1985). The DNA of the fast-sedimenting sperm chromatin was slightly longer, and its H1 content ~50% higher, than that of the slower-sedimenting chromatin (data not shown), but the H1 complement was nevertheless no higher than that of native chromatin [one H1 per nucleosome (Hill and Thomas, 1990)]; the slightly smaller fragments in the population probably lose their H1 more easily on handling and, being H1-depleted, then do not aggregate. Sea urchin sperm oligonucleosomes thus form aggregates at 65 mM ionic strength and above, probably resulting from the association of elements of 30 nm filaments held together through the N- (and C-) terminal domains of H1, and possibly H2B. Phosphorylation of these domains in spermatid chromatin abolishes this association.

Finally, we examined H1–DNA complexes. Sperm H1 has previously been shown to bind co-operatively to short linear DNA both at low ionic strength, giving relatively slowly sedimenting 'thin filaments' which appear from electron microscopy to consist of two molecules of DNA bridged by histone molecules, and at higher ionic strengths giving more rapidly sedimenting 'thick filaments' comprising several coiled, regular, thin filaments (Clark and Thomas, 1988). We have now compared the sedimentation behaviour of phosphorylated and unphosphorylated sperm H1 in complex formation with ~400 bp DNA. Figure 6a shows sucrose gradient profiles for H1–DNA complexes formed at low ionic strength, at an input ratio of 25% (w/w) H1:DNA. The co-existence, in both cases, of free DNA (the slower-sedimenting material) and H1–DNA complexes, presumably of the thin filament type, indicates co-operative

binding of phosphorylated H1 as well as of unphosphorylated H1 which was previously characterized (Clark and Thomas, 1988). Chemical cross-linking of the complexes with dithiobis(succinimidyl propionate) (Clark and Thomas, 1988) was also consistent with co-operative behaviour (data not shown). Thus phosphorylation of H1 appears to have no effect on its ability to bind to DNA co-operatively at low ionic strength.

At 30 mM NaCl, at an H1:DNA input ratio of 35% (w/w) (Figure 6b), the phosphorylated H1−DNA complexes still sediment as a single peak (only slightly ahead of the position of free DNA under these sedimentation conditions, which were different from those in Figure 6a), and are presumably the thin filaments also seen at low ionic strength (Clark and Thomas, 1988). In contrast, the unphosphorylated H1 gives rise to an additional fast-sedimenting peak [the thick filaments characterized for sperm H1 and other H1 variants by Clark and Thomas (1988)], together with free DNA. The N-terminal tail and/or the last 37 residues of the C-terminal tail of H1 (which contain the phosphorylation sites) thus appear to be involved in the self-association of thin filaments to form thick filaments; phosphorylation abolishes this.

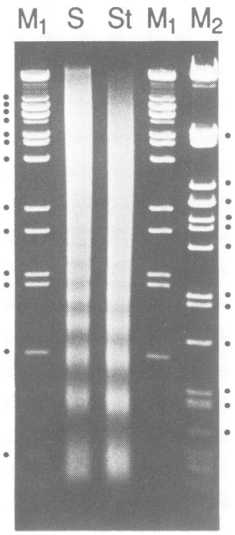


Fig. 3. The nucleosome repeat length in spermatids and sperm is the same. DNA extracted from *E.esculentus* sperm (S) and spermatid (St) nuclei that had been digested with micrococcal nuclease was analysed in a 1% agarose gel. Lane M_1: λ DNA digested with *Bst*EII; dots represent 8454, 7242, 6369, 5686, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702 and 224 bp. Lane M_2: λ DNA digested with *Pst*I; dots represent 5080/4649/4505, 2840, 2577/2454/2443, 2140, 1980, 1700, 1159, 1092, 805, 516, 448/417 and 339 bp.

Phosphorylation is required for exchange of sperm-specific H1

Exchange of H1 between chromatin fragments *in vitro* has been well characterized (Caron and Thomas, 1981; Thomas and Rees, 1983). Since the mechanism by which sperm H1 in chromatin is replaced by cleavage-stage H1 from a maternal storage pool upon fertilization (Green and Poccia, 1985) is likely to be related to exchange, we asked whether phosphorylation of sperm H1 is required for H1 exchange *in vitro*. Long sperm or spermatid chromatin fragments (>18 nucleosomes) and short rat liver chromatin fragments (~6−9 nucleosomes) were mixed and incubated for 1 h at different ionic strengths, and then separated by sedimentation through linear sucrose gradients (after dialysis at low ionic strength to break down aggregates of sperm chromatin formed at higher ionic strengths; see above).

Figure 7a shows sucrose gradient sedimentation profiles for the separation of long sperm (S_L) or spermatid (St_L) chromatin fragments and short rat liver (R_S) fragments after incubation at ionic strengths ~25−65 mM. (Broadening of the sperm chromatin peak is presumably due to the persistence of some aggregates despite low salt dialysis.) There was no cross-contamination of chromatin fragments as shown by analysis of the DNA in the peaks (for example, see Figure 7b). Figure 7c(i) shows that unphosphorylated (sperm) H1 does not exchange with rat liver H1 at any ionic strength (it is never seen on the rat liver chromatin), although some rat liver H1 appears on the sea urchin sperm fragments. In contrast, phosphorylated (spermatid) H1 does exchange with rat liver H1, at ionic strengths of ~45 mM and above [Figure 7c(ii)]; rat liver H1 also migrates in the absence of reciprocal migration of spermatid H1 at lower ionic strengths, as also seen in Figure 7c(i). The explanation for the latter effect was found in a separate experiment (not shown) which indicated that the shorter fragments of the

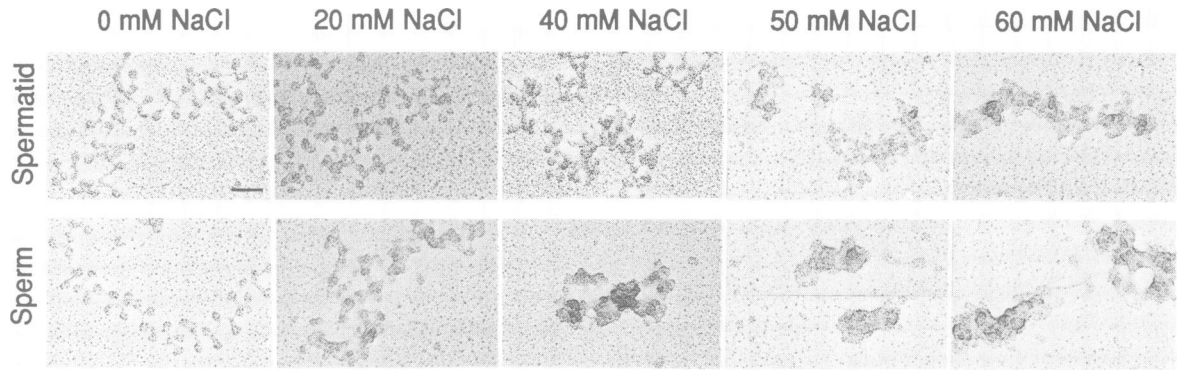


Fig. 4. NaCl-dependent folding of sperm and spermatid chromatin. Long sperm (*E.esculentus*) and spermatid (*P.miliaris*) chromatin (50−100 nucleosomes) was incubated in low salt buffer (5 mM TEA−HCl, 0.2 mM Na_2EDTA, 0.25 mM PMSF, pH 7.5) containing NaCl to give the final ionic strengths shown, and then fixed with glutaraldehyde for electron microscopy. Scale bar represents 50 nm. (Two different species of sea urchin were used because *E.esculentus* sperm are available at the same time of year as *P.miliaris* spermatids.)

sperm chromatin pool after incubation with rat liver chromatin are enriched in rat liver H1 relative to the longer fragments, implying that rat liver H1 fills up empty H1 binding sites on the shorter sperm chromatin fragments, which lose H1 more easily on handling.

These results suggest that phosphorylation of the sperm H1 tails is required for migration of the H1 on to rat liver chromatin *in vitro*, which would be consistent with the proposed role of sperm H1 phosphorylation on fertilization as a means of facilitating the replacement of sperm H1 by cleavage-stage H1 from the maternal pool (Green and Poccia, 1985).

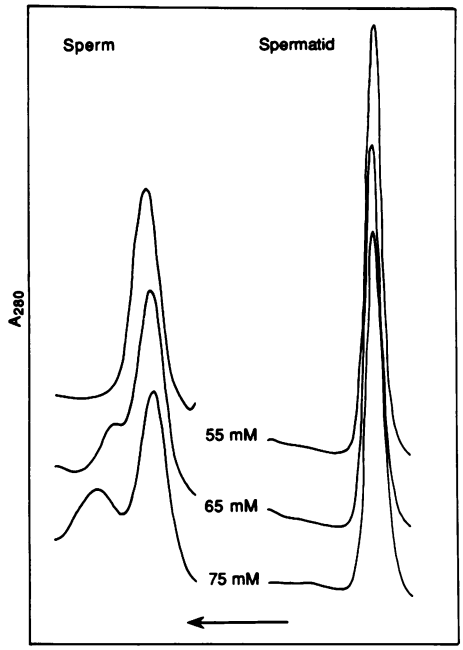


Fig. 5. Phosphorylation of H1 and H2B prevents the formation of 'pseudo-higher-order structures'. Sucrose gradient profiles of sperm (*E.esculentus*) and spermatid (*P.miliaris*) chromatin fragments (~12 nucleosomes) at various ionic strengths. Arrow shows the direction of sedimentation.

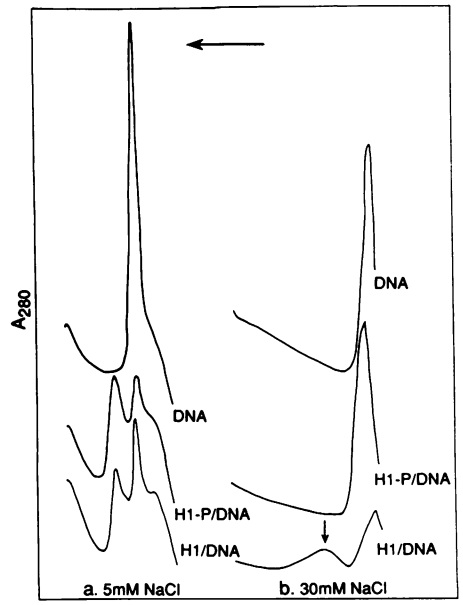


Fig. 6. Effect of phosphorylation of H1 on the formation of H1–DNA complexes at different ionic strengths. Sucrose gradient profiles of H1–DNA complexes formed from *P.miliaris* sperm H1 or spermatid H1 (H1-P) and ~400 bp DNA: (**a**) at an input ratio of 25% [histone:DNA (w/w)] in 5 mM NaCl, 1 mM Na phosphate, 0.2 mM Na_2EDTA, pH 7.4; (**b**) at an input ratio of 35% in 30 mM NaCl, 1 mM Na phosphate, 0.2 mM Na_2EDTA, pH 7.4. Note the additional peak (vertical arrow) for the unphosphorylated H1 in (b). The gradients, which were at the same ionic strengths as the samples, in (a) were 30–40% sucrose (w/v), centrifuged at 38 000 r.p.m. (SW40 rotor) for 40 h; those in (b) were 5–20% sucrose (w/v), centrifuged at 24 000 r.p.m. (SW28) for 2 h. Arrow, the direction of sedimentation.

Phosphorylation of clustered -Ser-Pro-X-Basic- motifs has a dramatic effect on the binding of the N-terminal domains of H1 and H2B to DNA

We have assessed directly the effect of phosphorylation on the DNA binding ability of the separate N- and C-terminal domains of H1 isolated after proteolytic or chemical cleavage of H1, and of the chemically synthesized N-terminal domain of H2B. *In vitro* phosphorylation of the latter using *Xenopus* 'H1 kinase' ($p34^{cdc2}$/cyclin; Gautier *et al.*, 1990), which phosphorylates serines in -Ser-Pro- motifs, has allowed us to assess the effect on DNA binding of adding an increasing number of phosphates up to a total of four. The phosphorylation levels of the intact histones and of the isolated domains of H1 were quantified by electrospray mass spectrometry, and where necessary phosphorylation sites were identified by sequence analysis.

The effect of phosphorylation on the salt-induced dissociation from DNA–cellulose of the N- and/or C-terminal domains of H1 and H2B and of intact H1 is shown in Figure 8a(i–iv). In the case of the N-terminal domains this is striking. Increasing phosphorylation (from one to four phosphates) steadily lowers the affinity for DNA of the N-terminal domain of H2B [peptide H2B(1–24); Figure 8b], the ionic strength at the midpoint of the dissociation curve being reduced by ~50 mM for the addition of each phosphate [Figure 8a(i)]. At ~150 mM ('physiological') ionic strength the unphosphorylated peptide is essentially completely bound, whereas the fully phosphorylated form is not bound at all. Similarly, phosphorylation at six sites in the N-terminal domain of H1 [peptide H1(1–48); Figure 8b] dramatically reduces its affinity for DNA [Figure 8a(ii)]; the ionic strengths at the midpoints of the dissociation curves are at ~90 mM and ~330 mM for the phosphorylated and unphosphorylated peptides respectively.

In contrast, phosphorylation at three sites in the isolated C-terminal domain of H1 [peptide H1(112–end); Figure 8b] has virtually no effect on its DNA binding [Figure 8a(iii)]. In addition, phosphorylation of the whole H1 molecule at nine sites, six in the N-terminal domain and three at the distal end of the C-terminal domain, has only a small effect on its affinity for DNA [Figure 8a(iv)]; the midpoints of the dissociation curves are at ~630 mM and ~680 mM for the phosphorylated and unphosphorylated forms respectively. The C-terminal domain dominates the DNA binding of H1 (Thoma *et al.*, 1983), and indeed, the C-terminal peptide and the intact H1 dissociate at fairly similar salt concentrations (~580 mM and ~680 mM respectively). [A recent report (Suzuki *et al.*, 1990) that phosphorylation *in vitro* by a cAMP-dependent protein kinase from sea urchin eggs has a large effect on the DNA binding affinity of sperm H1 from another species of sea urchin is hard to evaluate since the phosphorylation level and sites were not

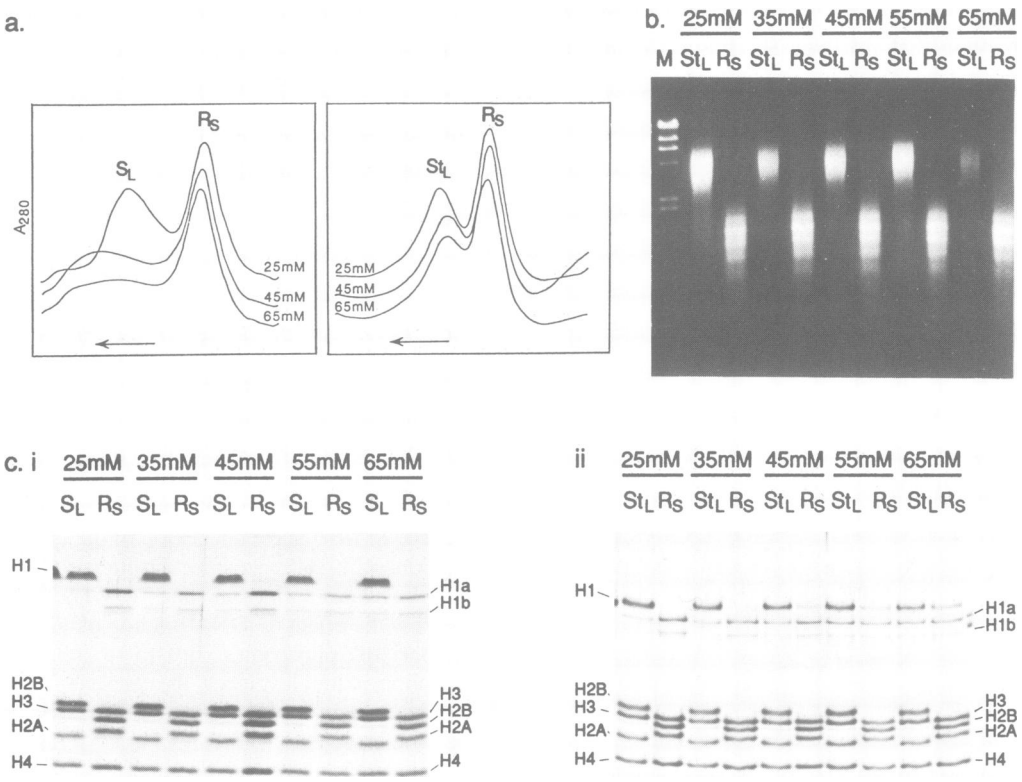


Fig. 7. Phosphorylation is required for exchange of sperm-specific H1. (**a**) Selected sucrose gradient profiles of fragments of long sperm chromatin (S_L) (*E.esculentus*) and short rat liver chromatin (R_S) (left-hand panel); and long spermatid (St_L) (*E.esculentus*) and short rat liver chromatin (R_S) (right-hand panel), separated at low ionic strength after mixing at the ionic strengths shown. Arrows show the direction of sedimentation. (**b**) 1% agarose gel analysis of DNA extracted from the fragments of spermatid (St_L) and rat liver chromatin (R_S) separated after mixing at the ionic strengths shown. M is λ DNA digested with *Hin*dIII; the fragments are 23 190, 9416, 6557, 4361, 2322 and 2027 bp. (**c**) SDS−18% polyacrylamide gel electrophoresis of histones extracted from (i) fragments of long sperm (S_L) and short rat liver chromatin (R_S); and (ii) long spermatid (St_L) and short rat liver chromatin (R_S), separated after mixing at the ionic strengths shown.

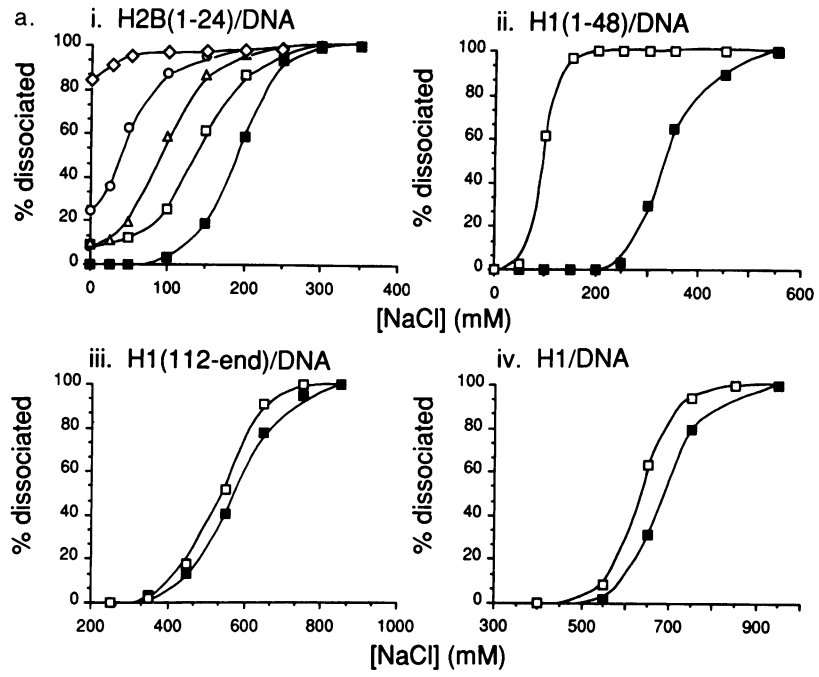
determined, and the relevance to the *in vivo* phosphorylation which we have studied is not clear.]

Phosphorylation has a marked effect on the binding of H1 in chromatin in contrast to the results just described for H1 binding to DNA. When samples of size-fractionated sperm and spermatid chromatin were sedimented through linear sucrose gradients containing 300−700 mM NaCl, chromatin that contained H1 was recovered in the pellet and fully H1-depleted chromatin in the gradient; appearance in the gradient occurred at 400 mM NaCl for spermatid chromatin and 600 mM NaCl for sperm chromatin (data not shown). The proportionally greater effect of phosphorylation on binding of H1 to chromatin than to DNA might be because chromatin is its natural substrate, and is probably reflected in the ability of phosphorylated, but not unphosphorylated sperm H1 to exchange with other H1s (Figure 7) [although of course exchange occurs at a much lower salt concentration than does dissociation (Caron and Thomas, 1981; Thomas and Rees, 1983)].

Discussion

The results described here suggest that the phosphorylatable regions of H1 and H2B have a predominantly cross-linking role in sperm chromatin structure, rather than a role in the formation and stabilization of condensed chromatin filaments. This supports previous suggestions [based on the timing of sperm histone dephosphorylation relative to the decrease in nuclear volume during spermatogenesis (Poccia *et al.*, 1987)], that the bulk of chromatin condensation occurs while H1 and H2B are phosphorylated, and that dephosphorylation provides DNA binding 'arms' that interact with exposed (linker?) DNA in adjacent filaments (Poccia *et al.*, 1987; Hill and Thomas, 1990; Hill *et al.*, 1990). We have also shown that phosphorylation of sperm H1 is a prerequisite for its exchange with other H1 variants *in vitro*, providing evidence in support of the mechanism proposed for replacement of sperm H1 with cleavage-stage H1 on fertilization (Green and Poccia, 1985). Although in that case only the phosphorylated sperm H1 is chromatin-bound, whereas the cleavage-stage H1 exists in a maternal storage pool, the mechanism must be related to exchange as it occurs before the onset of DNA replication (Green and Poccia, 1985).

The DNA binding experiments reported here suggest strongly that the separate domains of H1 (and H2B) act independently and presumably have distinct functional roles. Clustering of -Ser-Pro-X-Basic- motifs in the N-terminal domains of H1 and H2B means that when the serine residues are phosphorylated, these otherwise DNA binding domains



b. **H2B(1-24)** *E.e* PKSPSKSSPRKGSPRKASPKRGGK

H1(1-48) *P.a.* PGSPQKRAASPRKSPRKSPKKSPRKASASPRRKAKRARASTHPPVLEM
E.e PGSPQKRAASPRKSPRKSPKKSPRKASASPRR.....AATHPPVIDM

H1(112-end) *P.a.* RVGAVAKPKKAKKTSAAAKAKKAKAAAAKKARRAKAAAKRKAALAKKKAAAAKRKAAAKAKKAKKPKKK-
E.e RVGAVAKPKKAKKTSAAAKAKKEKARARAAAKKAKAAAKRKAALAKKKAAAAKRKAATKAKKAKKPKKK-

AA-KKAKKPA-KKSPKKAKKPAKKSPKKKKAKRSPKKAKKAAGKRKPAAKKARRSPRKAGKRRSPKKARK
TAAKKAKKPAAKKAKKPAKK----------SPKKAKKAAGKR.....

Fig. 8. Effect of phosphorylation on the binding of H1 and H2B and isolated domains to DNA. (**a**) i–iv, salt-dependent dissociation of phosphorylated and unphosphorylated peptides and H1 from DNA cellulose. (i) H2B(1–24): ■, unphosphorylated; □, +1 phosphate; △, +2 phosphates; ○, +3 phosphates; ◇, +4 phosphates. (ii) H1(1–48): ■, unphosphorylated; □, +6 phosphates. (iii) H1(112–end): ■, unphosphorylated; □, +3 phosphates. (iv) H1: ■, unphosphorylated; □, +9 phosphates. The amount of peptide eluted at each ionic strength was quantified by the appropriate method (Materials and methods). (**b**) Amino acid sequences of the peptides in (a). H2B(1–24) is from *E.esculentus* (*E.e*) sperm $H2B_2$ (Hill and Thomas, 1990). H1(1–48) from *E.esculentus* (C.S.Hill *et al.*, 1989), is shown aligned with the N-terminal region of *P.angulosus* (*P.a*) sperm H1, for which the complete sequence is known (Strickland *et al.*, 1980). H1(112–end) from *E.esculentus* (C.S.Hill *et al.*, 1989) is shown aligned with residues 112–248 of *P.angulosus* H1 (Strickland *et al.*, 1980). *, known phosphorylation sites (Hill *et al.*, 1990), o, possible phosphorylation sites. Note that *P.angulosus* H1 has five -Ser-Pro-Basic-Basic- motifs in its C-terminal domain, whilst *E.esculentus* H1 probably only has three. -----, alignment gaps, ..., residues not sequenced.

are released at physiological ionic strength (~150 mM), although the DNA binding affinity of the rest of the molecule is essentially unaffected. Phosphorylation of three residues at the distal (C-terminal) end of the C-terminal domain of H1 has very little effect on the binding of this domain as a whole to DNA (although there may be a more pronounced effect at its distal end which we would not have detected). This is not altogether surprising in view of the large net positive charge (~56+) on the C-terminal domain even after phosphorylation at three sites, in contrast to the effect of phosphorylation at six clustered sites in the much shorter N-terminal domain which leads to a substantial reduction in net positive charge (from 16+ to 7+ if each phosphate contributes approximately −1.5 at neutral pH).

The tails of H1 are thought to interact with the linker DNA to achieve chromatin condensation (Allan *et al.*, 1986), whereas the central globular domain of H1 interacts with the nucleosome at the dyad (Allan *et al.*, 1980; Staynov and Crane-Robinson, 1988). Since dephosphorylation seems to occur after the filaments are fully condensed, the N-terminal domains of H1 and H2B and the distal end of the C-terminal domain of H1 are probably exposed on the outside of the 30 nm filament, making them available to a phosphatase at the end of spermatogenesis and to a kinase at fertilization, and allowing them to interact with adjacent chromatin filaments on dephosphorylation. If the tails are indeed associated with linker DNA, this implies that part at least of the linker DNA may also be exposed in the 30 nm filament (see also R.J.Hill *et al.*, 1989); in the supercoiled linker model for the 30 nm filament (McGhee *et al.*, 1983) some of the linker DNA in sea urchin sperm chromatin is indeed exposed. We also note that if the arrangement of H1 molecules along the nucleosome filament is polar, i.e. head-to-tail (Lennard and Thomas, 1985), then the N-terminal

domain of one H1 molecule would be close to the distal end of the C-terminal domain of the next, and linker interactions disrupted by phosphorylation would be highly localized.

The structure of the phosphorylated motif in sperm histones, -Ser-Pro-X-Basic-, is not known, although it is predicted to form a β-turn (Poccia, 1987; Suzuki, 1989). DNA-footprinting experiments suggest that it binds preferentially in the narrow minor groove of (A+T)-rich sequences and might adopt a structure that specifically recognizes this (Churchill and Suzuki, 1989). We have been unable to find any evidence by high resolution ^{1}H- nuclear magnetic resonance spectroscopy for the formation of β-turns in two model peptides containing the motif (a pentapeptide, Gly-Ser-Pro-Arg-Lys, and the 24mer whose sequence is given in Figure 8b) in a variety of solution conditions. In particular we do not see any proton−proton nuclear Overhauser enhancements (NOEs) characteristic of β-turns (Wüthrich *et al.*, 1984) or indeed any folded structure (C.S.Hill, E.D.Laue, J.O.Thomas, unpublished). This does not rule out stabilization of structure on binding to DNA, but we have been unable to detect any structure in the 24 mer even when bound to the self-complementary oligonucleotide CGCGAATTCGCG.

Histone phosphorylation in other systems

The events of spermatogenesis have certain parallels in other systems where reversible phosphorylation and modulation of DNA binding occur, in particular H5 during erythropoiesis and H1 at metaphase. Chicken erythrocyte H5 is phosphorylated on four major sites when it is newly synthesized in polychromatic erythrocytes (Sung, 1977; Sung and Freedlender, 1978). Two sites (not precisely mapped) are on serines in -Ser-Pro-X-Basic- motifs in the highly basic C-terminal domain, but by analogy with sea urchin sperm H1, phosphorylation of these would not be expected to have a large effect on its DNA binding affinity. The other two sites are close together in the N-terminal domain, at serines 3 and 7. On dephosphorylation the extreme N-terminal region becomes less (although is still) negatively charged, perhaps allowing an adjacent positively charged region which abuts the globular domain to bind DNA more strongly, probably contributing to the high degree of chromatin condensation seen in chicken erythrocytes.

Our findings are also relevant to H1 phosphorylation during mitosis. H1 is phosphorylated during S-phase at two sites on average, but during mitosis most subtypes are phosphorylated at five or six sites (reviewed by Lennox and Cohen, 1988), mostly in the C-terminal domain and probably on -Ser/Thr-Pro-X-Basic- motifs distributed throughout it (Langan, 1976), maximal phosphorylation correlating with chromosome condensation. It is not obvious how H1 phosphorylation could promote chromatin condensation, unless the phosphates form strategic salt links with basic side-chains, although it has been proposed (Gurley *et al.*, 1978; Marion *et al.*, 1985; Lennox and Cohen, 1988) that phosphorylation relieves constraints through loosening of protein−DNA interactions, allowing some other mechanism to condense the chromatin, maybe at the level of the nuclear scaffold. Only relatively subtle changes in H1−DNA interactions may be necessary since, by analogy with sea urchin sperm H1 described here, phosphorylation at dispersed sites in the C-terminal domain at mitosis will probably have only a small effect on DNA binding.

Materials and methods

Preparation of nuclei, chromatin and H1

Sperm nuclei from *P.miliaris* or *E.esculentus* (Thomas *et al.*, 1986) and rat liver nuclei (Hewish and Burgoyne, 1973) were prepared exactly as described. Spermatid nuclei were isolated from immature *P.miliaris* or *E.esculentus* male gonads by a modification of the procedure described previously (Hill *et al.*, 1990). After centrifuging the homogenate to remove contaminating sperm, the supernatant was treated with 0.25% (v/v) Triton X-100 at 0°C for 3 min to remove residual membranes surrounding the nuclear membrane. The spermatid nuclei were collected by centrifugation for 10 min at 10 000 r.p.m. (Sorvall SS-34 rotor), and washed several times with 0.34 M sucrose−buffer A (Hewish and Burgoyne, 1973).

Chromatin was prepared from sperm, spermatid and rat liver nuclei by digestion in this buffer with micrococcal nuclease (Noll *et al.*, 1975), typically 15 U/ml for 6 min (long sea urchin sperm chromatin), 15 U/ml for 4 min (long spermatid chromatin) and 30 U/ml for 5 min (short rat liver chromatin), and lysing into 0.2 mM Na_2EDTA, pH 7 for 1 h at 4°C. The soluble chromatin was size-fractionated by centrifugation through 5−30% (w/v) linear sucrose gradients containing 10 mM Tris−HCl, pH 7, 1 mM Na_2EDTA, 0.25 mM phenylmethylsulphonyl fluoride (PMSF) as described previously (Butler and Thomas, 1980).

H1 in its unphosphorylated and phosphorylated forms was purified from sperm and spermatid nuclei, respectively, exactly as described for sperm H1 (Clark *et al.*, 1988).

Determination of nucleosome repeat length

The repeat lengths of *E.esculentus* sperm and spermatid chromatin were determined graphically by measurement of DNA lengths for micrococcal nuclease-digested nuclei as described (Thomas and Thompson, 1977; Pearson *et al.*, 1983).

Redistribution (exchange) of H1 and the formation of 'pseudo-higher-order structures'

The redistribution of H1 between sperm or spermatid chromatin and rat liver chromatin was studied as described previously (Thomas and Rees, 1983). Briefly, equal amounts (2.5 A_{260} units) of long size-fractionated sperm or spermatid chromatin fragments (>18 nucleosomes) and short rat liver chromatin fragments (6−9 nucleosomes) were mixed in a total vol of 3 ml of low salt buffer [5 mM triethanolamine (TEA)−HCl pH 7.5, 0.2 mM Na_2EDTA, 0.25 mM PMSF], and the ionic strength was adjusted with 1 M NaCl to a final NaCl concentration of 20, 30, 40, 50 or 60 mM. After 1 h at 0°C, the samples were dialysed against low salt buffer for 5 h at 4°C, and then the fragments were separated by centrifugation in a Beckman SW28 rotor at 19 000 r.p.m. for 12 h. The gradients were fractionated and monitored at 280 nm, and fractions were pooled and analysed for histone and DNA content.

In experiments to investigate the formation of 'pseudo-higher-order-structures' (Thomas *et al.*, 1985), size-fractionated sperm or spermatid fragments were dialysed into low salt buffer (see above). Aliquots of 2.5 A_{260} units were adjusted to a volume of 1.5 ml, and 1 M NaCl was added to give the required salt concentration. After 1 h at 0°C, the samples were loaded on to 17 ml linear sucrose gradients (5−30%, w/v) containing low salt buffer and the appropriate concentration of NaCl, and the gradients were centrifuged in a Beckman SW28 rotor typically at 13 000 r.p.m. for 12 h.

Formation and fractionation of H1−DNA complexes

H1−DNA complexes were formed from unphosphorylated or phosphorylated sperm H1 and ~400 bp mixed sequence DNA at various ionic strengths, exactly as described (Clark and Thomas, 1988).

Preparation of phosphorylated and unphosphorylated histones and peptides

H2B(1−24). A peptide containing the N-terminal 24 residues of *E.esculentus* $H2B_2$ (Hill and Thomas, 1990) was synthesized and purified at the AFRC Microchemical Facility, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge. The peptide, H2B(1−24), was phosphorylated at one to four sites on serines in the -Ser-Pro-X-Basic- motif by treatment with 'H1 kinase' ($p34^{cdc2}$/cyclin; Gautier *et al.*, 1990) which had been adsorbed from *Xenopus* egg extract (Murray and Kirschner, 1989) on to $p13^{suc1}$−Sepharose beads (Meijer *et al.*, 1989). The peptide (200 μg) was phosphorylated at one or two sites by treatment with 40 μl of packed beads containing the immobilized kinase in 400 μl assay buffer (50 mM β-glycerophosphate, pH 7.5, 7 mM NaF, 0.3 mM Na_2EDTA, 15 mM $MgCl_2$, 2 mM dithiothreitol) containing 1 mM ATP and 20 μCi [γ-^{32}P]ATP, for 3 h at 23°C. To phosphorylate the peptide at three or four

sites, 25 μg peptide was treated with 40 μl beads in 200 μl assay buffer. The supernatants after incubation were fractionated by reversed phase HPLC using a C18 Spherisorb 3 ODS II column (HPLC Technology, Macclesfield, UK) and a gradient of acetonitrile (8–25% in 60 min) in 0.3% (v/v) trifluoroacetic acid (TFA). The different phosphorylated forms separated under these conditions.

The phosphorylated peptides were characterized by electrospray mass spectrometry (see below) and peptide mapping after tryptic digestion (Thomas, 1989). Mass spectrometry gave molecular masses of 2505.4, 2585.4, 2665.4 and 2745.4 daltons for the unphosphorylated, mono-, di- and tri-phosphorylated peptides respectively; for reasons that are not clear the tetra-phosphorylated peptide (see below) did not give a spectrum. The autoradiograms of the tryptic peptide maps of the di-, tri- and tetra-phosphorylated species were identical, indicating that the occupancy of three of the phosphorylation sites was random; the map of the singly phosphorylated peptide did not contain a radiolabelled N-terminal peptide (Pro-Lys-Ser-Pro-Ser-Lys), indicating that phosphorylation did not occur first on Ser3 or Ser5. The sites of phosphorylation in the tetra-phosphorylated peptide were determined by amino acid sequence analysis using an Applied Biosystems Model 477A pulsed liquid sequencer, by coupling the peptide via its C-terminus to Sequelon-AA membrane (MilliGen) according to the manufacturer's instructions, and counting the eluent from each round of sequencing for ^{32}P. This analysis indicated phosphorylation of serines 3, 8, 13 and 18 (all in -Ser-Pro-X-Basic- motifs) but not serines 5 and 7.

H1(1–48). A peptide containing the N-terminal 48 residues of *E.esculentus* H1, H1(1–48), in its phosphorylated and unphosphorylated forms was prepared by cleavage of spermatid or sperm H1 (0.2 mg/ml in 0.1 M HCl) with cyanogen bromide [10:1 (w/w), CNBr:protein] for 24 h at 23°C. After lyophilization, the peptides were dissolved in 0.3% TFA (v/v) and fractionated by reversed phase HPLC as described above using a gradient of acetonitrile (20–40% in 60 min) in 0.3% (v/v) TFA, and characterized by SDS–18% polyacrylamide gel electrophoresis (Thomas and Kornberg, 1978) and electrospray mass spectrometry (Table I).

H1(112–end). A peptide resulting from cleavage after Phe111 of *E.esculentus* H1, H1(112–end), in its phosphorylated and unphosphorylated forms was generated by digesting sperm or spermatid H1 (100 μg) with chymotrypsin [1:50 (w/w), enzyme to protein] in the presence of an equal weight (to enzyme) of Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) for 10 min at 24°C (Clark *et al.*, 1988). The reaction was stopped with 0.5 mM PMSF (from a 50 mM stock in propan-2-ol), TFA was added to 0.3% (v/v) and the peptides were fractionated by reversed phase HPLC as above, using a gradient of acetonitrile (17–45% in 60 min) in 0.3% TFA, and characterized by electrospray mass spectrometry (Table I). The *E.esculentus* peptide (which has not been fully sequenced) is evidently shorter than the equivalent peptide from *P.angulosus* (Strickland *et al.*, 1980) which has a molecular mass of 14 550 daltons, and is (probably) 126 residues instead of 137 residues (see Figure 8b).

Binding of phosphorylated and unphosphorylated histones and peptides to DNA

The effect of phosphorylation on the binding of the peptides H1(1–48), H1(112–end), H2B(1–24) and of H1 to DNA was investigated using step-wise salt elution from DNA–cellulose (Sigma: 4 mg double-stranded DNA/g cellulose). Aliquots of the DNA–cellulose (6.4 mg) were washed several times with 10 mM Tris–HCl, pH 7, incubated with 2.5 μg peptides or 20 μg H1 in 100 μl of the same buffer for 30 min at 4°C, and then washed four times with 100 μl of 10 mM Tris–HCl, pH 7, with centrifugation for 2 min at high speed in a microfuge between the washes. The DNA–cellulose was then washed successively with 100 μl portions of the same buffer containing increasing concentrations of NaCl (25–950 mM). The supernatants were assayed either by Cerenkov counting [for radiolabelled H2B(1–24) peptide]; or [for unphosphorylated H2B(1–24) peptide] by measuring peak heights after reversed phase HPLC of each fraction; or [for H1, H1(112–end) and H1(1–48)] by precipitation with trichloroacetic acid, followed by analysis of the proteins by SDS–18% polyacrylamide gel electrophoresis (Thomas and Kornberg, 1978) and quantitation by densitometry.

Electron microscopy

Samples of size-fractionated chromatin in 5 mM TEA–HCl, pH 7.5, 0.2 mM Na_2EDTA, 0.25 mM PMSF containing 0, 20, 40, 50 or 60 mM NaCl were incubated for 1 h at 0°C and then fixed with glutaraldehyde [0.1% (v/v) final concentration]) for 15 h at 4°C. The samples were applied to grids, shadowed with platinum and examined by electron microscopy as described previously (Thoma *et al.*, 1979).

Electrospray mass spectrometry

Mass spectrometry was performed on a VG Bio-Q instrument with an electrospray ion source operating at atmospheric pressure coupled to a quadruple mass analyser with a mass range for singly charged ions of 3000 daltons. Samples of sperm and spermatid H1 (~1 nmol) were dissolved in ~80 μl 50% aqueous methanol containing 1% acetic acid and 10 μl aliquots were introduced into the source at 5 μl/min. The range m/z 500–1000 daltons was scanned at 11 s/scan cycle and several scans were summed to obtain the final spectrum in each case. One and two 10 μl aliquots of solution, respectively, were introduced, and data were acquired over 3 and 10 min for sperm and spermatid H1 respectively. Mass scale calibration was based on the singly charged ions from a separate introduction of polyethylene glycol 600. Spectra for the peptides H1(1–48), H1(112–end) and H2B(1–24) and their phosphorylated derivatives were acquired similarly with appropriate adjustment of the m/z range scanned and the number of scans.

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