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DNA-dependent phosphorylation of histone H2A.X during nucleosome assembly in *Xenopus laevis* oocytes: involvement of protein phosphorylation in nucleosome spacing

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ATP is required for physiological nucleosome alignment in chromatin reconstituted from high-speed nuclear supernatants of *Xenopus laevis* oocytes. Here we show that during *in vitro* nucleosome assembly the histone variant H2A.X becomes phosphorylated upon transfer onto DNA, a process which is also observed *in vivo*. Histone H2A.X phosphorylation increases in the early phase of the assembly reaction, reaching a steady state after ~16 min and is maintained with a half-life of the phosphate groups of ~2 h. After 6 h, the overall phosphorylation state of H2A.X is reduced, indicating that the phosphorylation-dephosphorylation ratio decreases considerably over time. Addition of alkaline phosphatase leads to a persistently lowered state of H2A.X phosphorylation, in contrast to other nuclear phosphoproteins which undergo rapid rephosphorylation. This suggests that H2A.X phosphorylation is a unique step in the histone-to-DNA transfer process. Selective inhibition of DNA-dependent phosphorylation of H2A.X and of other proteins causes a loss of the physiological 180 bp spacing.

Key words: chromatin assembly/nuclear proteins/nucleosome spacing/protein phosphorylation

Introduction

The basic repeating unit of chromatin structure is the nucleosome, consisting of 146 bp of DNA, wrapped around two copies each of the four histones H3, H4, H2A and H2B (for review, see van Holde, 1989). Nucleosomes are connected by discrete lengths of internucleosomal DNA which in most, though not all cases are associated with histone H1. The average length of the nucleosome repeat is a characteristic feature of the cell type and varies between 160 and 250 bp (Kornberg, 1977; van Holde, 1989). The regular arrangement of nucleosomes along DNA imposes constraints on the formation of higher order chromatin structure and in connection with sequence specific locations of nucleosomes may interfere with transcription and replication events.

Although cell-type specific spacing of chromatin has been well documented, the forces which drive nucleosomes into regular distances are poorly understood (for review see Dilworth and Dingwall, 1988). *In vitro* chromatin assembly systems using only purified histones and DNA failed to reproduce the sequence independent spacing of nucleosomes observed *in vivo* (Ruiz-Carrillo *et al.*, 1979), whereas in the case of sequence dependent positioning, regular alignment of nucleosomes was observed (Simpson *et al.*, 1985). By using rather specialized reconstitution conditions, Stein and coworkers (Stein and Künzler, 1983; Stein and Bina, 1984; Stein and Mitchell, 1988) proposed that the spacing periodicity is controlled by the value of initial average nucleosome packing density, a principle which challenges testing by the increasing number of more physiological *in vitro* assembly systems which provide regular nucleosome spacing (Laskey *et al.*, 1977; Nelson *et al.*, 1979; Glikin *et al.*, 1984; Smith and Stilman, 1989; Sessa and Ruberti, 1990; Banerjee and Cantor, 1990; Sugasawa *et al.*, 1990). Histone H1 seems to have only a modulating influence on nucleosome spacing (Rodríguez-Campos *et al.*, 1989) and its exchange with histone H5 *in vivo* does not change the nucleosome repeat length of chromatin in rat sarcoma cells (Sun *et al.*, 1990). This is in concordance with the finding of regularly spaced chromatin in the absence of histone H1 in yeast *in vivo* (Certa *et al.*, 1984) and following reconstitution from *Xenopous* oocyte extracts (Shimamura *et al.*, 1988).

Since ATP is required for reconstitution of 180 bp spaced chromatin in *Xenopus* egg or oocyte lysates (Glikin *et al.*, 1984; Ruberti and Worcel, 1986; Almouzni and Méchali, 1988), we asked whether ATP dependent modifications of proteins involved in the chromatin assembly process could play a role in the generation of physiologically spaced chromatin. Using the soluble nuclear proteins of *Xenopus laevis* for reconstitution of spaced chromatin *in vitro*, we observed a selective and DNA-dependent phosphorylation of nuclear proteins including the histone variant H2A.X during the assembly process. Reversion of these phosphorylation events during chromatin reconstitution by alkaline phosphatase treatment correlates with the loss of 180 bp nucleosome repeat length.

Results

DNA-dependent phosphorylation of histone H2A.X during nucleosome assembly in nuclear lysates of Xenopus laevis oocytes

High-speed supernatants of mechanically lysed *Xenopus* oocyte nuclei efficiently assemble DNA into nucleosomes *in vitro* as measured by the introduction of superhelical turns into relaxed circular plasmid DNA (Kleinschmidt *et al.*, 1985). The typical 180 bp spacing occurs if the assembly reaction is performed in the presence of ATP (Figure 1a; see also Sessa and Ruberti, 1990), while the 180 bp nucleosomal repeat length is reduced to 150 bp when ATP is omitted from the assembly reaction (Figure 1b).

Phosphorylation of the nuclear lysate proteins during nucleosome assembly was followed by performing the assay in the presence of [γ-^{32}P]ATP (Figure 1c). Comparison of the proteins phosphorylated during the nucleosome assembly

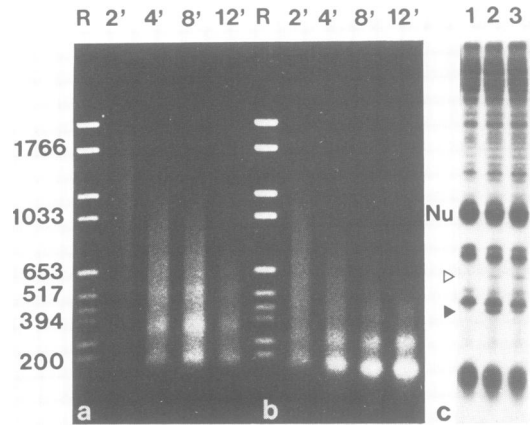

Fig. 1. Energy dependent nucleosome alignment correlates with DNA dependent phosphorylation of nuclear proteins. High-speed supernatants (100 000 *g*, 1 h) of *Xenopus laevis* oocyte nuclei assemble DNA into 180 bp spaced nucleosomes in the presence of ATP (**a**) whereas in the absence of ATP, only a limited nucleosome alignment of 150 bp repeat length is observed (**b**). Assembly products were digested with micrococcal nuclease for 2, 4, 8 and 12 min and separated on 1.5% agarose gels after digestion with proteinase K. For reference (R), DNA standards were run in parallel (pBR328, *BglI*−*HinfI*). Proteins of the nuclear extract were labeled for 20 min with [γ-^{32}P]ATP, separated by SDS−PAGE (18% acrylamide) and visualized by autoradiography (**c**). Lane 1: ^{32}P-labeled proteins in the absence of DNA, lane 2: proteins labeled in the presence of double-stranded DNA, lane 3: proteins labeled in the presence of single-stranded DNA. Positions of two polypeptides labeled in a DNA-dependent fashion (,▷) are indicated. Nucleoplasmin (Nu), which is phosphorylated independently of the presence of DNA, is marked as a reference protein.

reaction with the proteins labeled in the absence of DNA by one-dimensional gel electrophoresis (SDS−PAGE) shows some significant differences. Two polypeptides are labeled only in the presence of double-stranded or single-stranded DNA (Figure 1c, arrow heads, lanes 2 and 3), whereas the bulk of protein phosphorylation is independent of DNA addition, an example being nucleoplasmin (Figure 1c, lane 1, Nu). The lower molecular weight polypeptide (Figure 1c, filled arrow head) which is phosphorylated in a DNA-dependent manner, comigrates with histones H2A.X and H3 of *Xenopus* oocyte nuclei after separation by SDS−PAGE (for positions of *Xenopus laevis* oocyte and egg histones after gel electrophoresis see Dilworth *et al.*, 1987). The higher molecular weight polypeptide of ~23 kDa (Figure 1c, open arrow head) was labeled in the presence of DNA in most though not all experiments (cf. Figures 2, 4, 5 and 7), with this variation probably arising from the slow labeling kinetics of this protein (see below).

In order to see whether the DNA-dependently phosphorylated proteins are associated with the newly formed chromatin, we purified the assembled minichromosomes by sucrose gradient centrifugation. In these gradients the minichromosomes (Figure 2a, lanes 11 and 12) are clearly separated from the soluble proteins of the nuclear extract (Figure 2a, lanes 1−7) as shown by SDS−PAGE and staining with Coomassie blue. The corresponding autoradiograph showing the proteins phosphorylated during the first 10 min of the nucleosome assembly reaction indicates that the DNA-dependently phosphorylated polypeptide comigrating with H3 and H2A.X is associated with the purified minichromosomes (Figure 2b, arrow heads). The

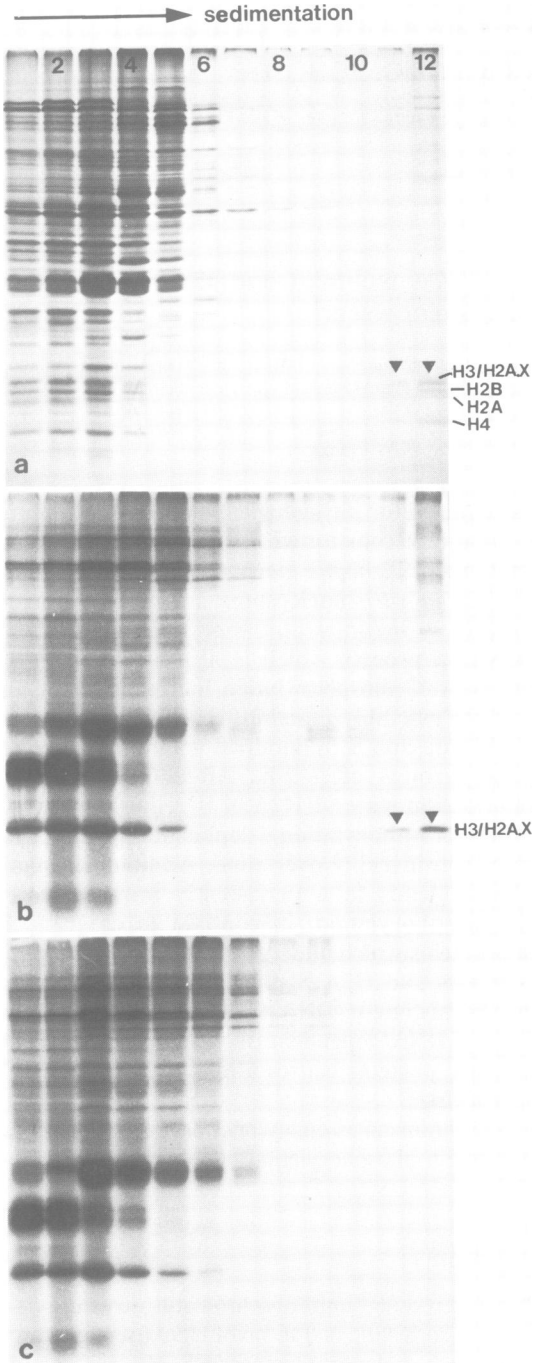

Fig. 2. A DNA-dependent phosphorylated polypeptide cosediments with the assembled chromatin. Minichromosomes assembled by incubation of circular plasmid DNA in the high-speed supernatant of *Xenopus laevis* oocyte nuclei in the presence of [γ-^{32}P]ATP (10 min pulse) were separated by sucrose gradient centrifugation (fractions 11 and 12) from the non-DNA-bound proteins (fractions 1−9), analyzed by SDS−PAGE and stained with Coomassie blue (**a**). The corresponding autoradiogram (**b**) shows the association of several phosphorylated proteins with the minichromosome. Most prominently labeled is a polypeptide at the position of histone H3 or H2A.X (arrow heads). As a control, proteins phosphorylated in the absence of DNA were fractionated in the same way (**c**, autoradiogram). Sedimentation direction is from left to right. The 23 kDa protein (open triangle in Figure 1) was not labeled during this short pulse.

non-assembled portion of H3 and H2a.X proteins (Figure 2a, lanes 2−4) is not radioactively labeled since they would be visibly separated by SDS−PAGE from the slightly slower

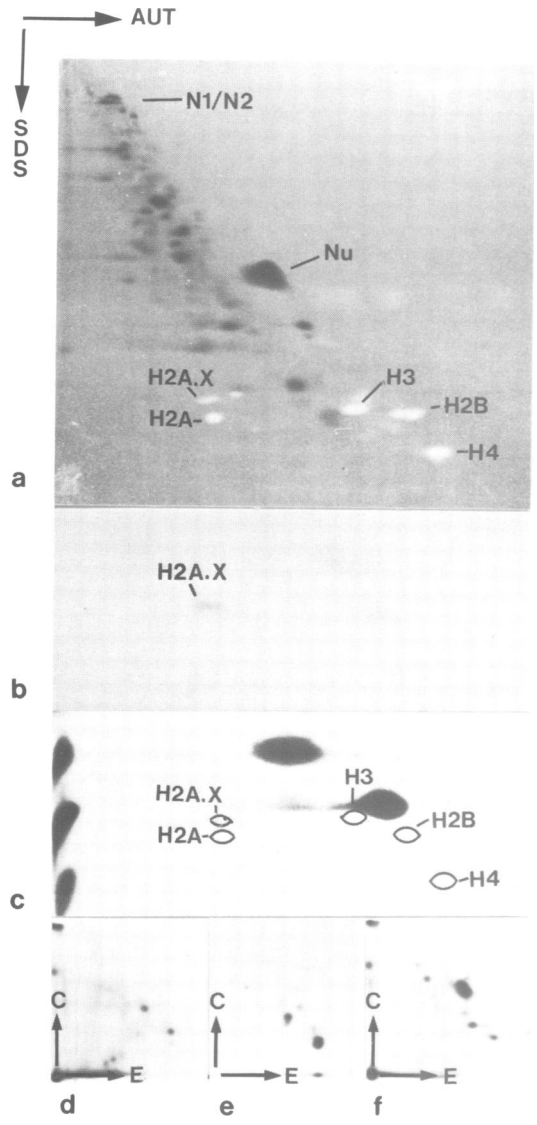

Fig. 3. The DNA-dependently phosphorylated polypeptide which associates with the minichromosomes is H2A.X. Minichromosomes assembled in the nuclear extract of *Xenopus laevis* oocytes in the presence of [γ-^{32}P]ATP were purified by gel filtration and analyzed by two-dimensional gel electrophoresis using separation in the presence of acetic acid, urea and triton (AUT) in the first dimension and sodium dodedylsulfate (SDS) in the second dimension (**a**–**c**). Identification of the histones was facilitated by addition of unlabeled nuclear extract and the histones were visualized by their enhanced light scattering effect after staining with Coomassie blue (**a**). The phosphorylated polypeptide of the purified minichromosomes comigrates with H2A.X. (**b**; autoradiogram). Two-dimensional analysis of polypeptides labeled in the absence of DNA fails to show any histone phosphorylation (**c**; autoradiogram). The identity of the light scattering spot at the position of H2A.X was further assessed by comparison of the tryptic fingerprints of H2A.X (**d**, *Xenopus*), H2A (**e**, calf thymus) and histone H3 (**f**, *Xenopus*). E, electrophoresis dimension; C, chromatography dimension. d–f show autoradiograms of iodinated peptides.

migrating radiolabeled polypeptide as shown in Figure 1 and confirmed below. Several other polypeptides bound to the minichromosomes are phosphorylated, whereas the bulk of unbound proteins shows the same labeling in the presence or absence of DNA (Figure 2b and c, fractions 1–7).

Since histones H3 and H2A.X cannot be separated sufficiently by one-dimensional SDS–PAGE, we analyzed the assembly products by two-dimensional gel electrophoresis using an acid-urea-Triton separation in the first dimension and SDS–PAGE in the second dimension. For better identification of the low amounts of histones in the assembly products, we added unlabeled total nuclear lysate proteins to the separated minichromosomes. The positions of the histones were identified by their enhanced light scattering effect in Coomassie blue stained gels (Kleinschmidt and Franke, 1982; Figure 3a). The ^{32}P-labeled polypeptide in the minichromosome fraction unequivocally comigrates with histone H2A.X (Figure 3b), while histone H3 is not labeled. The high resolution of this electrophoretic system clearly shows that no phosphate is incorporated into histones in the absence of DNA (Figure 3c). The identity of the light scattering spots with the histones was assessed by two-dimensional tryptic fingerprints of excised spots in comparison with fingerprints of calf thymus histones. *Xenopus* histone H2A.X and calf thymus H2A show a similar although not identical tryptic digestion pattern (Figure 3d and e) which is clearly different from the pattern of the *Xenopus* histone H3 (Figure 3f; very similar to calf thymus histone H3; not shown).

In order to confirm our *in vitro* observation by *in vivo* experiments, we microinjected [γ-^{32}P]ATP into the nuclei of *Xenopus* oocytes either with or without the addition of plasmid DNA. It has previously been shown that DNA microinjected into the nuclei is assembled into native chromatin (Wyllie *et al.*, 1978; Zentgraf *et al.*, 1979; Gargiulo and Worcel, 1983). Following a 10 min incubation period, the nuclei were manually isolated and proteins were analyzed by two-dimensional gel electrophoresis. Figure 4a shows that histone H2A.X becomes heavily phosphorylated *in vivo* when DNA was coinjected with ^{32}P-labeled ATP, whereas it remains unlabeled in the absence of microinjected DNA (Figure 4b). Unexpectedly, we also observed DNA dependent phosphorylation of another polypeptide (Figure 4a, arrow), in the molecular weight range of 36 kDa, the identity of which is not known.

The difference in labeling of polypeptides in the high molecular weight range is most probably caused by artificially low entry of these polypeptides in this particular gel since we never observed such a general DNA-dependent difference of phosphorylation of higher molecular weight proteins after one-dimensional gel electrophoresis.

Correlation of dephosphorylation of H2A.X with a change in nucleosome repeat length

Since we wanted to know if the DNA-dependent phosphorylation of H2A.X is one of the ATP-dependent events required for generation of physiological 180 bp spacing of nucleosomes reconstituted *in vitro*, we tried to inhibit this phosphorylation step selectively. A recently described DNA-dependent protein kinase (Lees-Miller *et al.*, 1990; Carter *et al.*, 1990) can be inhibited by inorganic phosphate (40 mM) and pyrophosphate (6 mM). In our system, they did not inhibit the phosphorylation of H2A.X (Figure 5, lanes 4 and 5, filled arrow heads) as compared with the control (Figure 5, lane 1). However, phosphorylation of the 23 kDa polypeptide was not observed in the presence of 40 mM phosphate (lane 4, open arrow head) and phosphorylation in general seems to be reduced. Inclusion of low concentrations of alkaline phosphatase from the beginning of the assembly reaction (Figure 5, lane 2) abolished labeling of H2A.X and the 23 kDa protein, as one can see after

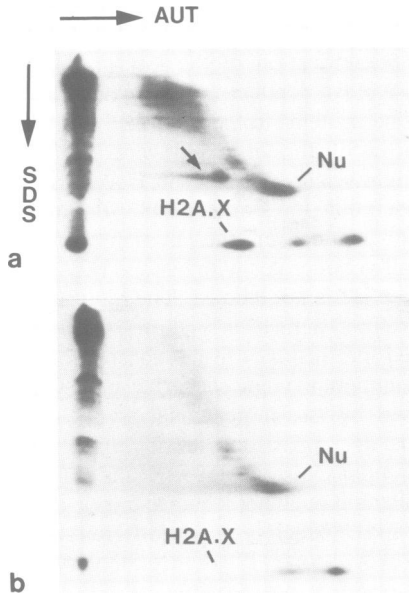

Fig. 4. DNA-dependent phosphorylation of histone H2A.X *in vivo*. [γ-^{32}p]ATP was microinjected into the germinal vesicles of *Xenopus laevis* oocytes either together with DNA (**a**) or without DNA (**b**). Manually isolated nuclei were analyzed by two-dimensional gel electrophoresis (AUT: electrophoresis in the presence of acetic acid, urea and triton; SDS: electrophoresis in the presence of SDS) after addition of unlabeled nuclear proteins for reference. Nu, nucleoplasmin; the arrow points to an unknown nuclear protein apparently labeled only in the presence of DNA.

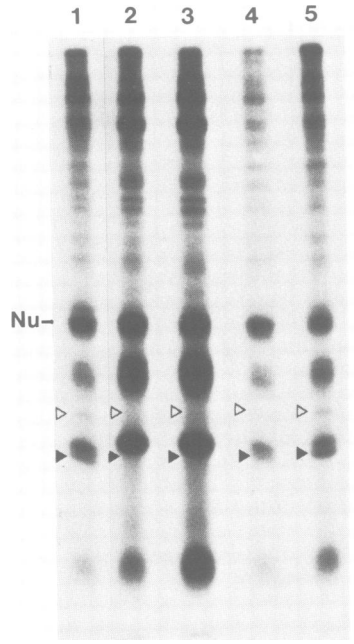

Fig. 5. Selective inhibition of DNA-dependent phosphorylation by treatment with alkaline phosphatase. Nuclear proteins of *Xenopus laevis* oocytes were labeled by incubation with [γ-^{32}P]ATP for 30 min during nucleosome assembly, analyzed by SDS–PAGE and visualized by autoradiography. Note the DNA-dependent phosphorylation of H2A.X (▶) and the 23 kDa protein (▷) (lane 1). Treatment with alkaline phosphatase at the beginning or after 12 h of nucleosome assembly sefectively abolishes phosphorylation of H2A.X and the 23 kDa protein (lanes 2 and 3). Phosphorylation in the presence of 40 mM inorganic phosphate (lane 4) reduces phosphorylation in general and seems to inhibit phosphorylation of the 23 kDa protein, but not of H2A.X. Treatment with pyrophosphate (6 mM) has no detectable effect on phosphorylation of nuclear proteins (lane 5).

a labeling period of 30 min, whereas all other polypeptides remain labeled as analyzed by one-dimensional SDS–PAGE. The same inhibition was observed when the phosphatase was added at the end of the assembly reaction (Figure 5, lane 3). Analysis of the assembly products of the same assembly reactions by treatment with micrococcal nuclease shows that inclusion of alkaline phosphatase from the beginning of the assembly reaction concomitantly reduces the 180 bp nucleosome repeat length (Figure 6a) to 160 bp (Figure 6c). This is significantly shorter than the physiological repeat length, but somewhat longer than the repeat length obtained in the absence of ATP (Figure 6b). Higher concentrations of alkaline phosphatase destroyed regular nucleosome alignment completely (data not shown). Addition of alkaline phosphatase after completion of the assembly reaction removes the phosphate of histone H2A.X without, however, influencing nucleosome spacing (Figure 6d). Treatment of the nuclear extract with inorganic phosphate or pyrophosphate does not reduce the physiological nucleosome repeat length (Figure 6e and f); this is consistent with the observation that H2A.X phosphorylation is not inhibited by these substances.

Dynamics of protein phosphorylation during nucleosome assembly in vitro

Since phosphorylation events obviously play a role in the assembly of physiologically spaced chromatin, we followed the kinetics of phosphate addition and turnover on nuclear proteins during the nucleosome assembly reaction. Most proteins which are phosphorylated independently of the nucleosome assembly process—for example nucleoplasmin (Nu)—show a rather steady increase in phosphate labeling during the first 60 min (Figure 7a–c; for quantification see Figure 8, squares).

Histone H2A.X phosphorylation, however, rapidly increases in the first 4 min of the assembly reaction and reaches nearly a steady state after ~16 min (Figure 7a; Figure 8, filled arrow head). The 23 kDa polypeptide is labeled in a slower, DNA-dependent reaction after 8–16 min (Figure 7a, open arrow head). Two polypeptides comigrating with N1/N2 are labeled more rapidly than H2A.X and reach a maximum after 2 min (Figure 7a; Figure 8, dots); thereafter they appear to be slowly dephosphorylated. Prolonged labeling for 6 h results in a decreased phosphorylation of H2A.X and the 23 kDa protein, whereas most other proteins remain unchanged (Figure 7a). H2A.X and the 23 kDa protein do not become labeled in the absence of DNA, whereas the polypeptides comigrating with N1/N2 are phosphorylated even more rapidly while their dephosphorylation seems to be slowed (Figure 7b, arrow heads and dot). Treatment with alkaline phosphatase leads to an accelerated dephosphorylation of the polypeptides at the N1/N2 position (Figure 7c, dot) and prevents detectable labeling of the 23 kDa protein (Figure 7c, open arrow head). Under these conditions, H2A.X is transiently phosphorylated, the DNA-dependent phosphorylation being lost after 1 h (Figure 7c, filled arrow head). The overall phosphorylation pattern under these conditions remains constant as measured at 3 and 6 h (data not shown).

In order to estimate the phosphate turnover of H2A.X and

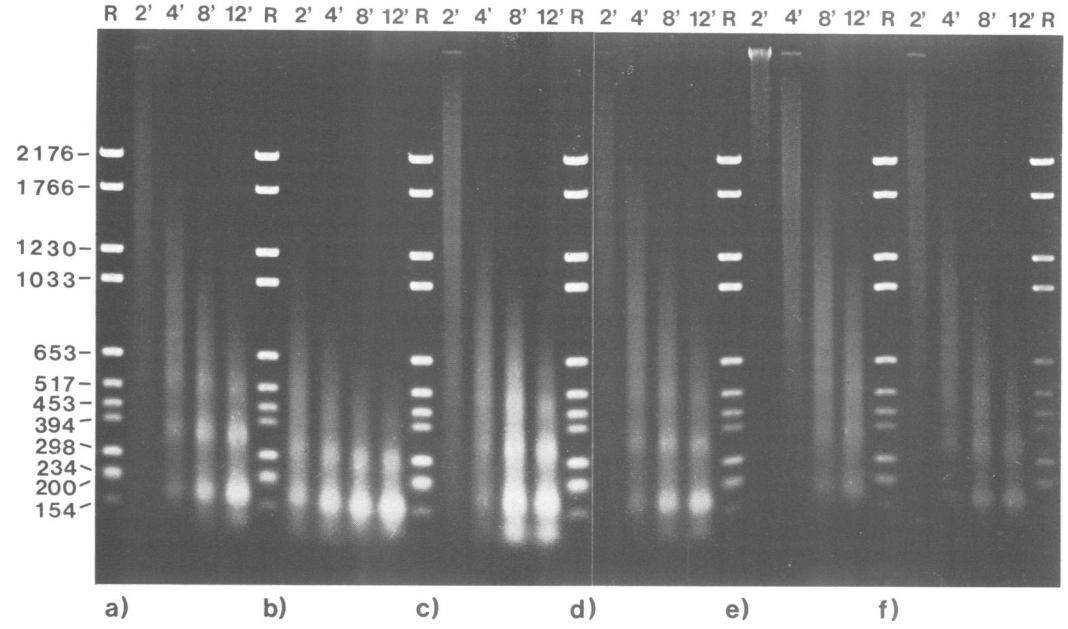

Fig. 6. Effect of alkaline phosphatase on nucleosome alignment. Nucleosome assembly products were treated for 2, 4, 8 and 12 min with micrococcal nuclease, deproteinated, analyzed by 1.5% agarose gel electrophoresis and stained with ethidum bromide. The physiological 180 bp spacing observed in the presence of ATP (**a**), is reduced to 150 bp if ATP is omitted (**b**). When alkaline phosphatase is included throughout the whole assembly process, nucleosome spacing is reduced to 160 bp (**c**), whereas addition of alkaline phosphatase at the end of the assembly process has no effect on nucleosome alignment (**d**). Incubation with inorganic phosphate (40 mM) or pyrophosphate (6 mM) does not significantly change the nucleosome repeat length (**e**,**f**).

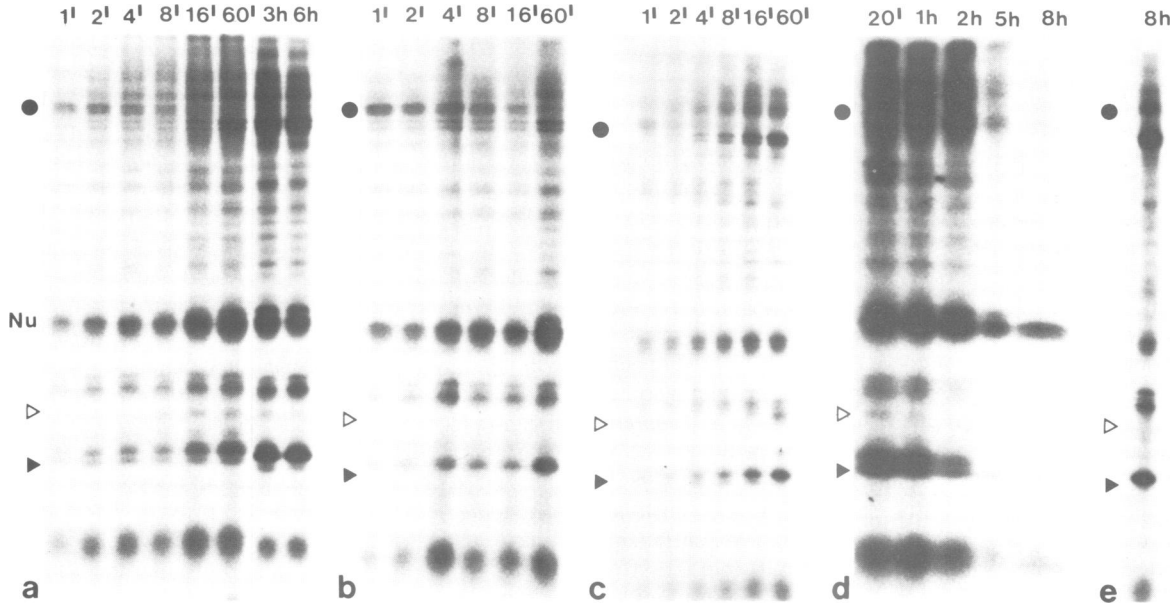

Fig. 7. Dynamics of protein phosphorylation during nucleosome assembly in nuclear extracts of *Xenopus laevis* oocytes. Soluble nuclear proteins of *Xenopus laevis* oocytes were labeled with [γ-^{32}P]ATP in the presence (**a**) or absence (**b**) of DNA or in the presence of DNA and alkaline phosphatase (**c**). Protein phosphorylation was stopped at the time points indicated at the top margin by addition of 9 vol of acetone. Precipitated proteins were analyzed by SDS–PAGE and ^{32}P-labeling was visualized by autoradiography. In (**d**), nuclear proteins were labeled with [γ-^{32}P]ATP for 20 min in the presence of DNA and then chased by addition of an excess of unlabeled ATP and dialyzed against assembly buffer containing 3 mM ATP. At the time points indicated, samples were taken and analyzed by SDS–PAGE and autoradiography. Phosphorylation of nuclear proteins after the completion of nucleosome assembly (after 8 h) is shown in (**e**).

the 23 kDa protein, we labeled nuclear proteins with [γ-^{32}P]ATP in the presence of DNA for 20 min and then chased the ^{32}P label by the addition of an excess of unlabeled ATP and dialyzed against assembly buffer containing unlabeled ATP (Figure 7d). One can see that H2A.X labeling is relatively stable and disappears after ~5 h, a time frame which is required to achieve proper nucleosome spacing *in vitro* (Ruberti and Worcel, 1986). Labeling of the 23 kDa polypeptide has already disappeared between 1 and 2 h. Since we wanted to know if H2A.X

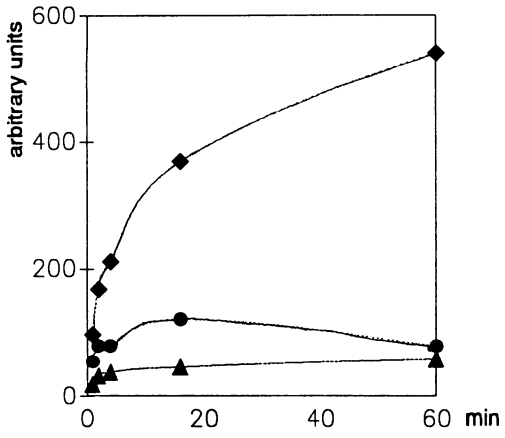

Fig. 8. Quantification of polypeptides phosphorylated during nucleosome assembly *in vitro*. ^{32}P-labeling of H2A.X (▲) and the polypeptides comigrating with an N1/N2 (●) were scanned and compared with the labeling of nucleoplasmin (◆), the phosphorylation of which is not influenced by the addition of DNA.

becomes rephosphorylated after completion of the nucleosome assembly process, we performed a 20 min labeling 8 h after the onset of the nucleosome assembly process (Figure 7e). We were unable to observe H2A.X phosphorylation under these conditions (Figure 7e, filled arrow head), in contrast to other proteins which are labeled independently of the assembly process. Phosphorylation of the 23 kDa protein is not clearly detected (Figure 7e, open arrow head).

Discussion

High-speed supernatants of oocytes and eggs of *Xenopus laevis* have most successfully been used to reconstitute *in vitro* sequence-independent, physiologically spaced nucleosomes (Laskey *et al.*, 1977; Glikin *et al.*, 1984; Ruberti and Worcel, 1986; Shimamura *et al.*, 1988; Almouzni and Méchali, 1988; Rodríguez-Campos *et al.*, 1989; Sapp and Worcel, 1990). The mechanism responsible for the regular alignment of nucleosomes in these extracts has not yet been elucidated. Nucleosome assembly and spacing can also be demonstrated in high-speed supernatants of isolated *Xenopus* oocyte nuclei, which are highly enriched in the proteins required for chromatin formation (Kleinschmidt *et al.*, 1985; Sessa and Ruberti, 1990; this study). The two histone complexes recently purified from these nuclei are sufficient for nucleosome core formation (Kleinschmidt *et al.*, 1990), but not for nucleosome spacing (J.A.Kleinschmidt, unpublished results). The ATP requirement of the spacing reaction pointed to a separate, energy dependent activity which has been lost during purification of the core assembly activity. Among the different possibilities of using ATP for a nucleosome alignment process, we have investigated the involvement of protein modification in the reaction.

We observed a clear-cut correlation of histone H2A.X phosphorylation with the establishment of 180 bp spaced chromatin *in vitro*, which corresponds to the nucleosome repeat length of chromatin formed *in vivo* in the *Xenopus* oocyte system (Gargiulo and Worcel, 1983). The phosphorylation reaction is strictly DNA-dependent and also occurs *in vivo* as demonstrated by microinjection of plasmid DNA into *Xenopus* oocyte nuclei. Shimamura and Worcel (1989) observed a correlation of histone H4 deacetylation with the assembly of regularly spaced nucleosomes. However, inhibition of deacetylation by butyrate did not influence the formation of spaced nucleosomes. In contrast to this finding, we show here that inhibition of H2A.X phosphorylation by treatment with alkaline phosphatase abolished proper nucleosome alignment. This suggests a physiological role of H2A.X phosphorylation during chromatin assembly especially in the nucleosome spacing process. Nevertheless, we cannot exclude the possibility that the increased phosphate turnover on other phosphorylated proteins observed during phosphatase treatment may also influence the nucleosome spacing reactions. In addition, several other polypeptides may be dephosphorylated by alkaline phosphatase if their rephosphorylation is a slow reaction; for example, the 23 kDa polypeptide is also phosphorylated in a DNA-dependent way. Inhibition of phosphorylation of the 23 kDa polypeptide by incubation of the nuclear proteins with 40 mM phosphate did not influence the spacing reaction, suggesting that this protein does not play a major role in nucleosome spacing. H2A.X, on the other hand, seems to be a prime candidate for playing a role in phosphorylation dependent nucleosome alignment, since it is part of the nucleosome structure which itself has to be aligned. Exchange for example of the oocyte specific H2B–H2A histone fraction containing the H2A.X variant against H2B and H2A from *Xenopus* erythrocytes causes a change in nucleosome repeat length from 180 bp to 160 bp (Sapp and Worcel, 1990). This observation becomes interpretable considering that *Xenopus* erythrocyte chromatin contains histone H5, whereas the oocyte and egg seem to be devoid of a histone H1 type molecule (see below). Thus, the phosphorylatable H2A variant might functionally replace the missing H1 to establish physiologically spaced chromatin.

The presence of histone H1 in *Xenopus* oocyte and egg extracts remains controversial. While van Dongen *et al.* (1983) reported the accumulation of a pool of 8–10 ng of histone H1A during oogenesis based on immunological criteria, Dilworth *et al.* (1987) and Shimamura *et al.* (1988) could not detect any histone H1 in purified chromatin assembly products. Likewise, minichromosomes assembled in the lysate of oocyte nuclei also do not contain stoichiometric amounts of polypeptides in the histone H1 region (J.A.Kleinschmidt, unpublished data). Whether the histone H1-like protein reported by Smith *et al.* (1988) is bound to chromatin assembled in oocyte or egg extracts of *Xenopus* laevis has still to be proven. The fact that yeast have spaced chromatin without detectable histone H1 (Certa *et al.*, 1984) raises the possibility that the 180 bp spacing of nucleosomes is achieved in the *Xenopus* oocyte and egg system without the aid of histone H1. Clearly, histone H1 can have a modulating influence on nucleosome spacing under certain conditions (Stein and Mitchell, 1988; Rodríguez-Campos *et al.*, 1989). It seems, however, not to play the dominant role in determining tissue specific nucleosome repeat length *in vivo* as recently shown by Sun *et al.* (1990).

The kinetics of H2A.X phosphorylation show a rapid DNA-dependent phosphorylation of the protein during the initial phase of histone deposition and only a slow turnover of the phosphate in a time course similar to the maturation of native chromatin *in vitro* and *in vivo* (Ruberti and Worcel, 1986; Shimamura *et al.* 1988; Levy and Jakob, 1978; Worcel *et al.*, 1978; Annunziato and Seale, 1982). The

failure to rephosphorylate H2A.X after this stage of chromatin assembly together with a decrease in H2A.X phosphorylation after 6 h indicates a change in the phosphorylation rate of H2A.X. The fact that dephosphorylation of H2A.X by alkaline phosphatase after this maturation time has no influence on nucleosome repeat length suggests that phosphorylation is required for the formation of correct spacing rather than for the maintenance of the spaced structure. Since removal of the phosphate from H2A.X parallels the gradual maturation of a spaced chromatin structure, one could speculate that this phosphate cycling reaction accompanies conformational changes associated with nucleosome sliding during the process of nucleosome alignment. This interpretation fits well with the finding of Rodríguez-Campos *et al.* (1989) that an excess of exogenously added histone H1 can change nucleosome repeat length when added at the beginning, but not when added at the end of the assembly reaction. An attractive alternative, although not mutually exclusive, explanation is that phosphorylation of H2A.X directly influences the initial nucleosome packing density by steric hindrance or charge reduction, a principal proposed by Stein and coworkers (Stein and Bina, 1984; Stein and Mitchell, 1988). In support of this interpretation are the observations of Shimamura *et al.* (1988) and Rodríguez-Campos *et al.* (1989) who obtained a higher number of regularly spaced nucleosomes with a shorter repeat length (18 nucleosomes with a 160 bp interval) assembled on a 2.9 kb circular plasmid at 37°C as compared with only 16 nucleosomes formed with a repeat length of 180 bp at 27°C in the *Xenopus* oocyte system. It would be interesting to see if the observed influences of temperature (and pH) on nucleosome spacing are due to a change in the phosphorylation–dephosphorylation kinetics of H2A.X or if it just reflects the higher thermal motion of the molecules, allowing more nucleosome sliding and the higher packing densities.

The DNA-dependent phosphorylation of histone H2A.X and of further nuclear polypeptides adds evidence to the general role of DNA-dependent phosphorylation events observed so far (Walker *et al.*, 1985; Lees-Miller and Anderson, 1989; Jackson *et al.*, 1990). Recently a DNA-activated protein kinase has been purified from HeLA cells (Lees-Miller *et al.*, 1990; Carter *et al.*, 1990) and it is conceivable that a similar enzyme is present in *Xenopus* oocyte nuclei. Two characteristics of the DNA-dependent phosphorylation of H2A.X are somewhat divergent from the behavior of this described protein kinase: H2A.X phosphorylation seems also to be activated by single-stranded DNA and it seems not to be significantly inhibited by phosphate or pyrophosphate (Lees-Miller *et al.*, 1990). Further analysis will determine if a single kinase or a family of such enzymes is involved in the regulation of protein–DNA interactions.

Materials and methods

Isolation of oocyte nuclei and nucleosome assembly reactions

Germinal vesicles of stage IV–VI oocyte (Dumont, 1972) of *Xenopus laevis* were isolated by the large scale isolation procedure of Scalenghe *et al.* (1978) as modified by Kleinschmidt and Franke (1982). Nuclei were collected in buffer A (83 mM KCl, 17 mM NaCl, 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 2.5 mM D-dithiothreitol (DTT) and lysed by low speed centrifugation at 3600 *g* for 20 min at 4°C. The supernatant was again centrifuged at 100 000 *g* for 1 h at 4°C and concentrated 10-fold by vacuum dialysis against buffer A without DTT. The 10-fold concentrated nuclear lysate had a protein concentration of ~10 mg/ml and was stored at −70°C.

Different extracts were titrated for nucleosome assembly activity with the supercoil assay, using 0.5–5 μl of 10× nuclear supernatant, 300 ng of relaxed circular plasmid DNA, 2 mM ATP in a final volume of 15 μl in buffer A without DTT at 26°C for 2 h. Assembly products were extracted with phenol and analyzed on 1% agarose gels. The lowest extract concentration allowing full supercoil induction was used for micrococcal nuclease digestion experiments, in which the assembly reaction was scaled up to 3 μg of DNA in a volume of 150 μl and incubated overnight at 26°C. For digestion with micrococcal nuclease the assembly reaction was adjusted to 3 mM $CaCl_2$ and digested with 30 U of micrococcal nuclease (Boehringer, Mannhiem, FRG) for 2, 4, 8 and 12 min. Digestion was stopped by addition of 30 μl reaction aliquots (~600 ng DNA) to 30 μl of stop mix (50 mM EDTA, 0.4% SDS). DNA was deproteinated by treatment with 6 μl proteinase K (10 mg/ml) for 4 h at 37°C, precipitated with ethanol and analyzed as outlined by Shimamura *et al.* (1988). In order to interfere with protein phosphorylation, we added 5 U of calf intestinal alkaline phosphatase (Boehringer, Mannheim, FRG) per 3 μg DNA at the beginning or one hour before completion of the assembly reaction. Alternatively, the assembly reaction was performed in the presence of 40 mM phosphate or 6 mM pyrophosphate.

For ^{32}P-labeling of proteins 0.5 μl [γ-^{32}P]ATP (10 mCi/ml, Amersham, UK) was included in a 15 μl assembly reaction. The concentration of cold ATP was reduced to 0.75 mM, except for the experiment shown in Figure 5 where 0.5 μl [γ-^{32}p]ATP (10 mCi/ml) was added to 30 μl of the assembly reaction performed for micrococcal nuclease digestion analysis shown in Figure 6. The reaction was stopped by addition of 9 vol of acetone at the time points indicated. Precipitated proteins were sedimented, dried, and solubilized in sample buffer for electrophoretic analysis. In the pulse–chase experiments, unlabeled ATP was added to a final concentration of 3 mM after a 20 min pulse with [γ-^{32}P]ATP (10 mCi/ml) and the assembly reaction was dialyzed against 0.5 μl of buffer A (without DTT) containing 3 mM ATP. Samples were taken at the time points indicated and analyzed as described above.

Electrophoretic methods and peptide mapping

One-dimensional SDS–PAGE was performed according to Thomas and Kornberg (1975) using 18% acrylamide gels. The first dimension of acid-urea-Triton gel electrophoresis was performed essentially as described by Zweidler (1978). The second dimensions were run with the conditions described above for one-dimensional gels. Individual spots containing polypeptides separated by two-dimensional gel electrophoresis were excised, radioiodinated and subjected to analysis of tryptic peptides essentially as described by Elder *et al.* (1977).

Purification of assembled minichromosomes

Preparative nucleosome assembly was performed with 10 μg DNA (pBR322) in a volume of 200 μl. Assembly products were separated from the nuclear proteins by a 10–30% sucrose gradient centrifugation for 90 min at 55 000 *g* and 4°C in a SW60 Ti rotor (Beckman Instruments, USA). Alternatively they were chromatographed on a Superose 6 column (ϕ1 cm; 9.5 cm long; Pharmacia/Uppsala, Sweden) with a flow rate of 0.3 ml/min at 4°C. Fractions were taken according to peaks detected by absorption at 280 nm, proteins were precipitated with trichloracetic acid (final concentration 15%) and analyzed by gel electrophoresis.

Microinjection

The nuclei of stage V oocytes were microinjected according to Colman (1984) either with 20 nl of [γ-^{32}P]ATP in injection buffer (10 mCi/ml) per nucleus or with 10 ng DNA in addition to [γ-^{32}P]ATP. After 10 min incubation at 21°C, oocytes were fixed with 10% trichloracetic acid, nuclei were manually isolated and solubilized in sample buffer for acid-urea-Triton gel electrophoresis.

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