pEg2 Aurora-A Kinase, Histone H3 Phosphorylation, and Chromosome Assembly in Xenopus Egg Extract*

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In eukaryotes cell division is accompanied by phosphorylation of histone H3 at serine 10. In this work we have studied the kinase activity responsible for this histone H3 modification by using cell-free extracts prepared from Xenopus eggs. We have found that the Xenopus aurora-A kinase pEg2, immunoprecipitated from the extract, is able to phosphorylate specifically histone H3 at serine 10. The enzyme is incorporated into chromatin during in vitro chromosome assembly, and the kinetics of this incorporation parallels that of histone H3 phosphorylation. Recombinant pEg2 phosphorylates efficiently histone H3 at serine 10 in reconstituted nucleosomes and in sperm nuclei decondensed in heated extracts. These data identify pEg2 as a good candidate for mitotic histone H3 kinase. However, immunodepletion of pEg2 does not interfere with the chromosome assembly properties of the extract nor with the pattern of H3 phosphorylation, suggesting the existence of multiple kinases involved in this H3 modification in Xenopus eggs. This hypothesis is supported by in gel activity assay experiments using extracts from Saccharomyces cerevisiae.

Cell division requires accurate condensation and faithful segregation of chromosomes. Despite the great efforts invested, the mechanisms of these two processes still remain unclear. However, during the last few years an impressive progress has been made in their understanding by using two complementary approaches as follows: genetics in yeast and experiments in extracts prepared from Xenopus eggs (reviewed in Ref. 1). The biochemical manipulations of the Xenopus egg extract were extremely useful since they have led to the identification of multiprotein complexes, termed condensins, required for chromosome condensation (2). Targeting of condensins is mitotic-specific, and their phosphorylation may trigger chromosome condensation (3). Several lines of evidence indicate that the master kinase Cdc-2 might be involved in the phosphorylation and activation of condensins (3).

Chromosome assembly is also accompanied by phosphorylation of linker histone H1 (4, 5) and core histone H3 (6–9). However, the presence of linker histones is not necessary for chromosome and nucleus formation (10–14), and consequently their phosphorylation should not be required for these processes. In contrast, the phosphorylation of serine 10 in the amino-terminal domain of histone H3 is essential for cell division. Phosphorylation of histone H3 has been observed and characterized in organisms as divergent as yeast (15), Tetrahymena thermophila (16), Aspergillus nidulans (17), Caenorhabditis elegans (15, 18), plants (19), and vertebrates (20, 21). It was also described during in vitro chromosome assembly in Xenopus egg extract (22). A mutant T. thermophila strain, containing a non-phosphorylatable histone H3, exhibited perturbed chromosome condensation, abnormal segregation, and chromosome loss during mitosis and meiosis (16), demonstrating the primary significance of this histone H3 modification. This was further supported by experiments on blocking of the mitotic histone H3 kinase and, thus, histone H3 phosphorylation, which has resulted in inhibition of chromosome assembly in vitro (22) and in cells in culture (21).

Nonetheless, the mechanism of action of histone H3 phosphorylation in cell division is still poorly understood. A real progress toward elucidation of this mechanism will be the identification of the enzymes involved in the regulation of mitotic H3 phosphorylation. Recently, reports from two different groups identified two distinct kinases Never in Mitosis A (NIMA) in A. nidulans (17) and an aurora kinase Ipl1 (Increase in Ploidy) in budding yeast S. cerevisiae and air2 (aurora Ipl1-related kinase 2) in the worm C. elegans (15) as the mitotic histone H3 kinases.

In vitro NIMA phosphorylated histone H3 at serine 10, and the in vivo phosphorylation of the histone is dependent on the presence of the kinase activity (17). At the onset of mitosis, NIMA is detected on chromatin and subsequently colocalized with spindle microtubules and spindle pole bodies. The chromatin localization of this enzyme is tightly correlated with histone H3 phosphorylation (17).

The budding yeast genome encodes for a single aurora kinase Ipl1 that is required for cell cycle progression, and strains bearing genetics defects in this enzyme showed abnormal chromosome segregation and suffered severe nondysjunction (23). The enzyme expression peaks at mitosis, and when a temperature-sensitive lethal Ipl1 strain was grown at permissive temp-

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1 The abbreviations used are: NIMA, Never in Mitosis A; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift analysis; bp, base pair.
perature, a markedly reduced H3 phosphorylation was detected (15). In vitro, the kinase phosphorylated both H3 and H2B in a mixture of free histones and on nucleosomes. The C. elegans genome encodes for two aurora kinases, air1 and air2, and the last one was observed on chromosomes at mitosis and meiosis (15, 18). However, only the disruption by RNA interference of air2 expression in C. elegans embryos led to unde- tectable histone H3 phosphorylation (15, 18).

The situation is different in vertebrates where three aurora kinases, recently renamed as aurora-A, -B, and -C, have been described (reviewed in Refs. 24–26) The Xenopus laevis kinase pEg2 belongs to the aurora kinase protein family, and according to the new nomenclature is the Xenopus aurora-A kinase. pEg2 was found associated with centrosomes in a cell cycle-de- pendent manner. It also binds to the spindle microtubules, and its activity is required for bipolar spindle assembly (27). In vivo pEg2 has been reported to associate with the kinesin-related protein XIEng5 and to the cytoplasmic polyadenylation element binding factor. Both proteins are phosphorylated by pEg2 in vitro in residues found phosphorylated in vivo (24, 28).

In vertebrate cells the aurora kinase(s) that phosphorylates histone H3 is not known. Moreover, none of the X. laevis H3 mitotic kinases have been identified yet. The aim of this work was to search for such enzyme(s). To this end extracts from Xenopus eggs were used, and in vitro chromosome assembly was performed. We have identified the aurora-A kinase pEg2 as a potentially good candidate for histone H3 mitotic kinase in Xenopus eggs.

**EXPERIMENTAL PROCEDURES**

**Mitic Extract Preparation and Isolation of Demembranated Sperm Nuclei**—Mitotic extracts from Xenopus eggs were prepared essentially as described (49). Dejellied eggs were crushed by centrifugation for 15 min at 15,000 rpm in an SW41 rotor (Beckman Instruments) at 16 °C in XBE2 buffer (100 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 10 mM K-HEPES, pH 7.7, 5 mM K-EGTA, 0.05 mM sucrose) supplemented with 10 μM leupeptin and aprotinin and 100 μM/ml cocktail D. The cytoplasmatic layer was collected using a 20-gauge needle via a side puncture and kept on ice. Protease inhibitors (leupeptin and aprotinin) and cocktail D (5 μM/ml) were added to the final concentration of 10 μM/ml and 120 μM/ml of 20% energy mix (20 mM phosphate buffer, 2 mM ATP, and 5 μg/ml creatinine kinase, final concentration) were added, and the extract was clarified by centrifuging at the same speed as above, but at 4 °C. The golden layer (low speed supernatant) was collected and transferred in 2 ml of polypropylene tubes for a TLS-55 rotor (Beckman Instruments) and spun at 52,000 rpm for 2 hours at 4 °C. Once the incubation completed, the tubes were washed 3 times with 10 volumes of EB. The cytosol was depleted by adding 4 volumes of extract to 1 volume of settled beads followed by incubation under rotation at 4 °C for 1 h. After centrifugation, the supernatant was treated with additional 50 μl of fresh beads under the conditions described.

Quantitative measurements showed that this protocol removed 95–98% of pEg2 present in the extract. The native pEg2 immobilized to the beads was further used in kinase assay experiments.

**Expression of pEg2 and Kinase Assay**—Recombinant pEg2 was expressed and purified essentially as described (27). Briefly, cDNA of pEg2 was prepared by differential screening of Xenopus egg cDNA library. The coding sequence of the protein was amplified by polymerase chain reaction and inserted into Xhol restriction sites of the His tag expression vector pET21 (Novagen Inc.) using primers described previously (27). The catalytically inactive form of the enzyme pEg2KR was engineered with Transformer Site-directed Mutagenesis Kit (CLON- TECH) to change the lysine 169 of pEg2 to arginine. The kinases were expressed in Escherichia coli strain BL21(DE3) and the tagged proteins purified on a nickel column (Qiagen).

The purified enzymes were dialyzed overnight against EB, aliquoted in 20-μl fractions, and stored at –20 °C at concentration 0.2–0.5 μg/μl. For the kinase assay 1 μl of recombinant pEg2 was added to 50 μl of EB containing 1 μg of recombinant histone 5 μg of reconstituted nucleosomes. After addition of 20 μCi of [γ-32P]ATP, the reaction was allowed to proceed for 15 min at room temperature, and then the proteins were precipitated with 20% trichloroacetic acid (final concentra-

**Immunoblotting**—The immunoblotting protocol was already described (22). The dilution used for the anti-phosphorylated histone H3 antisem was 1:3000, whereas the hybridoma supernatant was diluted 1:1000. The membranes were incubated by using the enhanced chemiluminescense system (ECL, Amersham Pharmacia Biotech) following the instruc-

**Preparation of Yeast Protein Extract**—The extract was prepared by using Saccharomyces cerevisiae strain GSA59. This yeast strain has the advantage of exhibiting low protease activity. An overnight preculture, grown in YPD medium, was diluted to Az30 = 0.2–0.3 with the same medium. The culture was grown at 30 ° C for 4 h, aliquoted in 10 μl of fresh medium, and then supplemented with nocodazole to 10 μg/ml final concentration. The control and the nocodazole-containing cultures (50 μl each) were each incubated for 5 h and harvested by centrifugation. The pellets were washed twice with ice-cold water and resuspended in 1 ml of lysis buffer (10 mM MgCl₂, 20 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsul-
fonyl fluoride, 0.05% SDS, 1 μg/ml sodium deoxycholate, 50 μg/ml Tris-HCl, pH 8.0). The suspension was then transferred in 2-ml Eppendorf tubes containing glass beads, and the cells were lysed by 5 min of vortexing. The cell debris were removed by centrifugation, and the supernatant was used in gel activity assay experiments.

**Gel Activity Assay**—The gel activity assay was performed in 10–8-μl × 1-mm 10% SDS-polyacrylamide gel containing 50 μg/ml gel-

**Immunodepletion of pEg2 Kinase from the Egg Extract**—pEg2 was depleted from the extract by the already characterized monoclonal antibody (27). The immunodepletion was carried out essentially according to a protocol described previously (37). Briefly, protein A-agarose beads (Amersham Pharmacia Biotech) were washed with EB buffer (80 mM β-glycero-phosphate, pH 7.5, 15 mM MgCl₂, 20 mM EGTA, 1 mM iodoacetamide, and blocked with 200 μl of EB containing 10 μg/ml of phytohemagglutinin at a concentration of 10 mg/ml. After three successive washings with EB, 500 μl of the hybridoma supernatant was added to 50 μl of pelleted beads, and the suspension was incubated for 1 h under rotation at 4 °C. For the mock immunodepletion, 25 μl of preimmune serum was diluted with EB to 500 μl final volume, mixed with 50 μl of settled beads and incubated as above. Once the incubation completed, the beads were washed 3 times with 10 volumes of EB. The cytosol was depleted by adding 4 volumes of extract to 1 volume of settled beads followed by incubation under rotation at 4 °C for 1 h. After centrifugation, the supernatant was treated with additional 50 μl of fresh beads under the conditions described.

Quantitative measurements showed that this protocol removed 95–98% of pEg2 present in the extract. The native pEg2 immobilized to the beads was further used in kinase assay experiments.
Assembly of Mitotic Chromosomes—Mitotic chromosomes were assembled in Xenopus egg extract at 23 °C following a standard protocol (50). 20–25 μl of extract were used for 40–80,000 demembraned sperm nuclei. Some experiments 1 μl of cyclin B390 (the nondegradable form of cyclin B) was added. To follow the kinetics of assembly, 5-μl aliquots from the mock-depleted and pEg2-depleted extract were taken at different times after initial incubation and fixed immediately with 5 μl of the fix/stain buffer (Hoechst 33258 at 1 μg/ml in 200 mM sucrose, 10 mM HEPES, pH 7.5, 7.4% formaldehyde, 0.23% 1,4-diazabicyclo[2.2.2]octane, 0.02% B NaCl, and 70% glycerol). The decondensation of sperm nuclei in heated extract was carried out as described previously (35).

Immunofluorescence—The immunofluorescence analysis was carried out as described by de la Barre et al. (50). The anti-phosphorylated histone H3 antibody was used at dilution 1:5000. Finally, the chromosomes were counterstained with 8 μl of fix/stain buffer.

Preparation of Nuclei and Nucleosomes—Hen erythrocyte nuclei were isolated as described by Mirzabekov et al. (51). Oligonucleosomes were prepared by digestion of the nuclei with micrococcal nuclease and linker histones, and non-histone proteins were removed by centrifugation of the oligonucleosomes over 5–20% sucrose, containing 0.65 M NaCl (52). After overnight dialysis against a solution of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, oligonucleosomes were aliquoted and stored frozen at −20 °C.

Nucleosome Reconstitution—In the reconstitution experiments, “bulk” nucleosomal DNA, prepared from native hen erythrocyte nuclei, was used. A strictly stoichiometric amount of the four histones (determined spectrometrically and checked on a SDS gel) in 10 mM HCl was dialyzed overnight at 4 °C against 2 mM NaCl, 50 mM Tris-HCl, pH 7.8, 1 mM EDTA. The next morning a mixture of bulk nucleosomal DNA and 32P-end-labeled 152-bp EcoRI-RsaI fragment containing a Xenopus borealis 5 S RNA gene was added to the dialysis tubing. The ratio of the added DNA to the core histone octamer was 1:9.8. Nucleosome reconstitution was performed by successively lowering the concentration of the NaCl in the dialysis buffer. Finally, the reconstituted particles were dialyzed against 10 mM Tris, pH 7.8, 10 mM NaCl, 1 mM EDTA and used for kinase assay. The extent of reconstitution and the integrity of the nucleosomes were checked by electrophoretic mobility shift analysis (EMSA) and DNase I footprinting.

EMSA and DNase I Footprinting on Reconstituted Nucleosomes—The EMSA was carried out in 2% agarose gel at room temperature in 0.5× TBE (Tris borate/EDTA) buffer. Upon completion of the electrophoresis, the gel was stained with ethidium bromide.

The footprinting of the reconstituted nucleosomes was performed by using lane 1. The digestion was carried out with 10 ng of DNase I per 10 μl of nucleosome solution (10 ng/μl) in 10 mM Tris-HCl, pH 7.6, 5 mM MgCl2 for 2 min at room temperature. The reaction was stopped by adding 100 μl of stop solution (10 mM EDTA, 0.1% SDS, 50 ng/μl proteinase K) followed by 30 min of incubation at 37 °C. Then the samples were phenol-extracted, ethanol-precipitated, and separated on 8% polyacrylamide sequencing gel containing urea. The dried gel was exposed overnight on a PhosphorImager screen.

RESULTS

Phosphorylation of Histone H3 at Serine 10 by a 40–42-kDa Polypeptide Present in Yeast Mitotic Extracts—We were interested in determining the Xenopus kinase(s) responsible for the mitotic-specific phosphorylation of histone H3 at serine 10. However, various bona fide candidates for histone H3 mitotic kinases were identified in different organisms (15, 17), which suggests that distinct kinases operate in different systems or that several kinases can act in the same organism. If the last suggestion is true, it will be important to find the relative contribution of each enzyme in the mitotic phosphorylation of histone H3. Since in vitro serine 10 of histone H3 can be phosphorylated by a multitude of kinases (protein kinase A (29), NIMA (17), Ipl1/auroA-B (15), Msk1 (30), mitogen-activated protein kinase-dependent kinases (31), and Rack-2 (32)), the determination of the active mitotic kinases could be done more easily by using yeast, which is a somewhat simpler system, instead of Xenopus. As H3 phosphorylation is highly conserved in evolution, the major mitotic kinases modifying this protein should belong to the same families in different organisms. Thus, the identification of the yeast enzymes will undoubtedly be instrumental for the analysis of the Xenopus system. Since the yeast genome is already sequenced, the kinase molecular weight determination will allow their identification. Following this rationale, we carried out a series of in gel activity assays using crude extracts isolated from nocodazole-treated or control, non-treated S. cerevisiae cells (Fig. 1). As substrates for the kinases we have incorporated into the gels either the non-mutated GST-histone H3 tail fusion (GSTH3) or the same fusion, but mutated at serine 10 to alanine (GSTH3S10), or the globular domain of histone H3 (GH3). As a control, we have used gel-incorporated GST. When the mitotic extract (isolated from the nocodazole-treated cells) was subjected to the assay, three specific bands were detected by autoradiography in the gel containing GST-H3. The strongest band migrated with molecular mass of 40–42 kDa. The incorporation of 32P in these three positions was characteristic for the yeast mitotic extract in the gel comprising GST-H3; very faint or no 32P-labeled bands with the above molecular masses were observed in all other cases (Fig. 1, compare lane 1 with lanes 2–8). These data suggest that at least three different kinases from the yeast mitotic extract phosphorylate specifically histone H3 at serine 10 when using gel activity assay. Since among them the 40–42-kDa enzyme should be the most active one, we have further concentrated on it.

Budding yeast has more than 100 genes coding for protein kinases (33). Twelve of these enzymes exhibited a molecular mass in the region of 40–45 kDa as described in the YPD proteome data base (www.proteom.com). Nine of them are serine/threonine protein kinases. Among them, only the Ipl1 aurora kinase was shown to participate in chromosome assembly and cell cycle progression, suggesting strongly that this is the major yeast kinase participating in phosphorylation of histone H3 at serine 10. Thus, our in gel activity assay results are in agreement with the available data identifying this enzyme as a H3 mitotic kinase in S. cerevisiae (15).

In higher eukaryotes multiple homologues to Ipl1 were described (reviewed in Ref. 24). In Xenopus, the aurora-A kinase pEg2 is active during mitosis and is shown to be involved in microtubule dynamics (27). Sequence alignment of pEg2 and
Ipl1 demonstrates a significant sequence similarity (50% identity) of the catalytic domains of both enzymes. Since Ipl1 is involved in H3 phosphorylation in *S. cerevisiae*, we hypothesized that its homologue pEg2 could play similar role in *Xenopus*.

Phosphorylation of Free Histones in Solution by Recombinant pEg2—Our first step in identifying the role of pEg2 in H3 phosphorylation was to prepare a recombinant active pEg2 (rpEg2) and to produce and purify to homogeneity the four recombinant core histones and a large number of their mutants. Next we have checked whether the enzyme can act specifically on serine 10 of histone H3. Fig. 2 and Fig. 3 show the results of the kinase assay of rpEg2 on different histone substrates. As seen, full-length histones H3 and H2B are phosphorylated (Fig. 2, lanes 2–4), whereas H2A and H4 are not substrates for the enzyme (Fig. 2, lanes 1 and 6). The radioactivity is incorporated in the tail of histone H3, since its globular domain GH3 is not labeled (Fig. 2, lane 9), whereas the GST-histone H3 tail (GST-H3) is efficiently marked (Fig. 3A, lane 4, and Fig. 3C, lane 2). The phosphorylation is specific for serine 10, because a mutation of serine 10 of H3 to alanine abolishes the incorporation of $^{32}$P in the protein (Fig. 2, compare lane 3 with lane 5, and Fig. 3C, lanes 2–4). The same type of mutation, but in the other serine of H3 tail (serine 28), has no effect on the labeling by the kinase (Fig. 2, lane 4, and Fig. 3C, lane 4). Catalytically inactive rpEg2 does not phosphorylate the tail of histone H3 (Fig. 3C, lane 6), further confirming that H3 labeling is due to the kinase itself and not to a bacterial contaminant.

rpEg2 also phosphorylates histone H2B. The site(s) of phosphorylation is located in the globular domain of the protein, since by using kinase assay GH2B is radioactively labeled (Fig. 2, lane 8), whereas the H2B tail is not (Fig. 3A, lane 3).

Specific Phosphorylation of Histone H3 at Serine 10 on Nucleosome Templates by rpEg2 Kinase—We have demonstrated that rpEg2 phosphorylates histone H3 at serine 10 and histone H2B when they are free in solution. However, in the nucleus these histones are not free but are instead organized into nucleosomes. Besides, during chromosome assembly histone H2B is not phosphorylated. Thus, if pEg2 is associated with chromosome condensation, it should be able to phosphorylate on nucleosomal templates specifically histone H3 at serine 10, but not histone H2B. To check this we have reconstituted “chimerical nucleosomes” by using bulk nucleosomal DNA and different combinations of recombinant full-length and tail-less histones. In the reconstitution reactions a trace amount of $^{32}$P-end-labeled 152-bp EcoRI-RsaI fragment containing an *X. borealis* somatic 5 S RNA gene was added. This has allowed us to follow both the efficiency of reconstitution and the integrity of the reconstituted nucleosomes (Fig. 4 and Fig. 5A). As seen, under the conditions used, complete reconstitution is achieved; the EMSA did not detect free DNA (Fig. 5A). The DNase I

FIG. 2. Recombinant pEg2 phosphorylates the tail of histone H3 and the “histone fold” domain of H2B. *In vitro* kinase assays were carried out by using $[\gamma-^{32}P]ATP$, recombinant pEg2, recombinant intact histones H2A, H2B, H3, and H4, their globular domains GH2A, GH2B, GH3, and GH4, and singly mutated histone H3 at serine 10 (H3S10A) and at serine 28 (H3S28A). Each reaction contained 200 ng of pEg2 and 1 µg of intact or mutated histone. After completion of the reaction, the proteins were trichloroacetic acid-precipitated and separated on 18% SDS-polyacrylamide gel. Phosphorylation was analyzed by autoradiography (A). The Coomassie-stained gel is shown in B.

FIG. 3. Specific phosphorylation of the tail of histone H3 at serine 10 by recombinant pEg2. The kinase assays were carried out with recombinant wild and mutated pEg2 by using as substrates GST fusions (GSTH2A, GSTH2B, GSTH3, and GSTH4) with the non-mutated tail of the core histones and the fusions of the tail of histone H3 but mutated at serine 10 (GSTH3S10A) and at serine 28 (GSTH3S28A). The autoradiographs and the Coomassie-stained gels are shown in A and C and B and D, respectively.

2 V. Gerson and S. Dimitrov, unpublished observations.
footprinting analysis showed the nucleosome 10-base pairs repeat with essentially no background between the different bands (Fig. 4). Thus, the particles prepared with recombinant histones exhibited overall structure closely similar to that of native nucleosomes, a result in agreement with the data in the literature (34). Once the reconstituted nucleosomes were characterized, we asked if they could be phosphorylated by rpEg2. As shown on Figs. 5 and 6, histone H3 was a good substrate for the kinase in nucleosomes comprising the four full-length histones. Moreover, this phosphorylation was specific for serine 10, since particles containing histone H3 with mutated serine 10 to alanine were not phosphorylated by the kinase (Fig. 6B, lane 3), whereas the phosphorylation of nucleosomes with histone H3 mutated at serine 28 was not affected (Fig. 6B, lane 2). Phosphorylation of histone H2B was observed neither in these particles nor on H2B in nucleosomes, reconstituted with intact H2B and the globular domains of the other three histones (Fig. 5B, lane 3). In all other cases the globular domain of histone H2B was accessible and efficiently phosphorylated by the kinase. Therefore, the tail of histone H2B impeded the phosphorylation of GH2B within nucleosomal templates.

However, in decondensed sperm nuclei the real substrates of the mitotic H3 kinase are the nucleosomal fibers. Thus, we next asked if rpEg2 could phosphorylate histone H3 at serine 10 within such complex structures. To address this problem we have prepared heat-treated Xenopus egg extract, which has allowed us to decondense sperm nuclei without histone H3 phosphorylation. Upon heating at 80 °C the kinase activities of the extract are completely eliminated (35), but its sperm decondensation ability is preserved due the thermostability of nucleoplasmin, the protein responsible for the sperm nucleus decondensation (36). As shown and in agreement with previously published data (35, 36), the incubation of sperm nuclei in the heated extract resulted in its impressive decompaction (Fig. 7, column 1). Addition of recombinant pEg2 led to the phosphorylation of histone H3 (Xenopus sperm nuclei contain an amount of H3-H4 tetramer identical to the one of somatic nuclei, for details see Ref. 37), thus demonstrating the ability of rpEg2 to penetrate decondensed sperm and act on histone H3.

The Incorporation of pEg2 in Chromatin during Chromosome Assembly Parallels the Phosphorylation of Histone H3

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**FIG. 4.** DNase I footprinting of nucleosomes reconstituted either with intact histones (ΣH), or with their globular domains (GH) only, or with intact histone H3 (H3) and the globular domains of the three remaining histones. The reconstitution was performed with nucleosomal bulk DNA, containing uniquely 32P-end-labeled 152-bp EcoRI-RsaI fragment comprising an X. borealis somatic 5 S RNA gene. After digestion with DNase I the DNA fragments were separated on 8% denaturing polyacrylamide gel and autoradiographed. In lanes 1 and 2 are shown the pattern of the guanines (G) and the DNase I digestion products of the free 152-bp DNA fragment. The arrow shows the dyad axis of the particle. The other types of reconstituted nucleosomes used in this study exhibited the same DNase I footprinting pattern, but for simplicity only the patterns of the above three ones are shown.

**FIG. 5.** Recombinant pEg2 phosphorylates only histone H3 in nucleosomes reconstituted with intact histones. Nucleosomes were reconstituted by using bulk nucleosomal DNA and different combinations of recombinant histones; ΣH, particles containing intact histones; ΣGH, nucleosomes reconstituted with the globular domains of the histones; H2A, “chimerical” particles containing intact histone H2A only and the globular domains GH2B, GH3, and GH4 of the other histones; H2B, H3, and H4, “chimerical” nucleosomes, comprising either intact H2B or H3 or H4 and the globular domains of the three other histones, respectively. The kinase reaction using the different nucleosomal samples was carried out as described in Fig. 2. A, EMSA of the reconstituted nucleosomes in 2% agarose gel. The staining was performed with ethidium bromide; B and C represents the autoradiograph and the Coomassie-stained gel of the proteins from the respective kinase assays.
above data show that histone H3 is a good substrate for pEg2. The next question addressed was whether the native enzyme, present in the extract, is also able to phosphorylate efficiently H3 at serine 10. To this end we have immunoprecipitated pEg2 with a monoclonal antibody from the extract and carried out a kinase assay by using as substrates H3, H3S10A, and H3S28A. As seen on Fig. 8A, the radioactive label is incorporated only in H3 and H3S28A. The mutation of serine 10 completely abolished the labeling. This fact clearly shows that native pEg2 specifically modifies histone H3 at serine 10.

In addition, incubation of sperm nuclei in Xenopus mitotic extract resulted in the uptake of the kinase from the extract and in its association with the remodeled nuclei structures (Fig. 8B). In other words during chromosome assembly native pEg2 binds to chromatin. Importantly, the kinetics of histone H3 phosphorylation strictly parallels the kinetics of uptake of pEg2 from the extract; the more pEg2 is found bound to chromatin, the more histone H3 phosphorylation is observed. These results suggest that pEg2 participates in the phosphorylation of histone H3. However, quantitative estimation showed that the chromatin-bound enzyme represented no more than 1–2% of the enzyme present in the extract (data not shown). This low amount of the chromatin-associated enzyme could explain the failure of previous attempts to localize pEg2 on chromosomes by using indirect immunofluorescence (27).
Depletion of pEg2 and Histone H3 Phosphorylation—We have found that in vitro pEg2 selectively and efficiently phosphorylated serine 10 of histone H3 when using as substrates reconstituted nucleosomes or decondensed sperm in heated extracts. Furthermore, during chromosome assembly in Xenopus egg mitotic extracts the kinase associates with chromatin, and the time dependence of the amount of bound kinase is very similar to the kinetics reflecting the increase of phosphorylation of histone H3 at serine 10. Taken together, these data strongly suggest that pEg2 could be involved in mitotic-specific phosphorylation of histone H3. We have addressed this hypothesis by immunodepleting pEg2 from the extract followed by chromosome assembly. The structural transitions of the sperm nuclei and the presence and distribution of phosphorylated histone H3 at serine 10 were visualized by using indirect immunofluorescence.

pEg2 was very efficiently removed from the extract at the conditions used for immunodepletion (Fig. 9A). Quantitative measurements show that more than 95–98% of pEg2 present in the extract was depleted by the monoclonal antibody (data not shown). Histone H3 is efficiently phosphorylated in the sperm nucleus structures formed in both control and pEg2-depleted extracts (Fig. 9B). Incubation of sperm nuclei in the different extracts (Fig. 9C) results in the already described sperm nucleus rearrangements (22) and culminates in the formation of condensed chromosomes. The time course of H3 phosphorylation pattern as well as the specific structures of the chromosome intermediates assembled in the control and pEg2-depleted extracts were very similar (Fig. 3C). In some experiments a delay in the kinetics of chromosome assembly was observed, but the final chromosome structures as well as the histone H3 distribution were essentially the same (data not shown).

**DISCUSSION**

During cell division in all eukaryotic organisms studied chromosome assembly is accompanied by a phosphorylation of the flexible tail of histone H3 at serine 10 (38). The evolutionary conserved character of this modification suggests that it should play an important role in both mitosis and meiosis. Since the discovery of histone H3 phosphorylation in the late seventies (6) numerous studies have addressed this problem, but it still remains an enigma (for recent reviews see Refs. 38 and 39). The identification of the mitotic kinase(s) responsible for the phosphorylation of histone H3 at serine 10 will undoubtedly be of great help in the understanding of the function of this modification. It is quite possible that these enzymes exist in the cell as high molecular mass protein complexes. The kinase partners in the complexes should exhibit the property to target the kinases to chromatin, to help them in a local remodeling of chromatin structure, and to make available histone H3 tails for efficient phosphorylation. Thus, the understanding of the mechanism of histone H3 kinase action during cell division is inherently related to the isolation and characterization of such complexes. The extracts isolated from Xenopus eggs represent the perfect reagent for these types of experiments, since they contain very strong kinase activities that phosphorylate serine 10 of histone H3 during in vitro chromosome assembly (22).

**pEg2, a Good Candidate for Histone H3 Mitotic Kinase—**Our long term goal is to characterize the H3 mitotic kinase complex(es) and to understand the relevance of histone H3 phosphorylation for cell division. As a first step toward this goal, we have tried to identify the kinase phosphorylating histone H3 at serine 10 in Xenopus egg extract. Since kinases from divergent families were found to exhibit mitotic histone H3 phosphorylation activity (15, 17), initially we have concentrated on a much simpler system, the yeast _S. cerevisiae_. By using in gel activity assay, we have confirmed that in _S. cerevisiae_ the p41 aurora kinase is likely to be the major H3 mitotic kinase. We further hypothesized that in Xenopus, enzymes from the same family should be able to phosphorylate histone H3 and to play a similar role in mitosis. We have focused on pEg2, the major _Xenopus_ mitotic aurora-A kinase, and have studied _in vitro_ its histone phosphorylation capability. We have shown that recombinant pEg2 phosphorylates both H3 and H2B when free in solution, but in nucleosomes only histone H3 phosphorylation at serine 10 was detected. Moreover, decondensation of sperm nuclei in heated extract made histone H3 accessible to rpEg2 and phosphorylatable by the enzyme. Native pEg2, isolated by immunoprecipitation from the Xenopus egg extract, was also able to phosphorylate specifically histone H3 at serine 10. In addition, _in vitro_ chromosome assembly pEg2 was re-recruited from the extract and associated with the remodeled sperm nuclei. Importantly, the kinetics of histone H3 phosphorylation parallels closely that of pEg2 association with chromatin. All these data designate pEg2 as a good candidate for an H3 mitotic kinase in Xenopus eggs.

**Multiple Kinases Might Be Involved in the Mitotic Phosphorylation of Histone H3—**Mitotic chromosome assembly is a very
complex process, and undoubtedly it should require the active participation of numerous protein factors. Two well defined chromosome assembly factors are topoisomerase II and the condensin complex (1). A good candidate for a third one is the kinase phosphorylating histone H3 in mitosis (22, 38, 39). The data presented in this study strongly suggest that pEg2 is a histone H3 mitotic kinase in Xenopus eggs. However, immunodepletion of pEg2 from the extract has no considerable effect on both chromosome formation and histone H3 phosphorylation. Thus, if pEg2 is a bona fide histone H3 mitotic kinase, how could this result be explained? A plausible explanation is the existence of several and redundant in function kinases involved in the mitotic phosphorylation of H3. This hypothesis is supported by our in gel activity assay data that suggest the existence in S. cerevisiae of three H3 mitotic kinases with different molecular masses. After chromatographic fractionation of Xenopus extract, several distinct activities able to phosphorylate nodepletion of pEg2 from the extract has no considerable effect in escence in 3 L. Scrittori, F. Hans, M. Charra, and S. Dimitrov, unpublished observations.

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pEg2 Kinase and H3 Phosphorylation 30009
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pEg2 Aurora-A Kinase, Histone H3 Phosphorylation, and Chromosome Assembly in *Xenopus* Egg Extract

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