Phosphorylation of *Xenopus* transcription factor IIIA by an oocyte protein kinase CK2¹

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Transcription factor IIIA (TFIIIA), isolated from the cytoplasmic 7 S ribonucleoprotein complex of *Xenopus* oocytes, is phosphorylated when incubated with [γ -³²P]ATP. This modification is due to a trace kinase activity that remains associated with the factor through several steps of purification. The kinase can use either ATP or GTP, and will phosphorylate casein and phosphovitin to the exclusion of TFIIIA. The kinase is reactive with a ten-amino-acid peptide that is a specific substrate for protein kinase CK2 (CK2; formerly casein kinase II). In addition, inhibition of phosphorylation by heparin and stimulation by spermidine indicate that the activity can be ascribed to CK2. Phospho amino acid analysis established that serine is the sole phosphoryl acceptor in TFIIIA. There are four consensus sites

for CK2 in TFIIIA; all contain serine residues at the putative site of phosphorylation. TFIIIA immunoprecipitated from oocytes, which were incubated with [³²P]orthophosphate, is also phosphorylated exclusively on serine residues. Only the cyanogen bromide fragment, which was derived from the N-terminal end of TFIIIA, is labelled *in vivo*. A recognition sequence for CK2, located at Ser¹⁶ in the β -turn of the first zinc-finger domain, is the only protein kinase consensus sequence present in this peptide. Assays *in vitro* with site-specific mutants of TFIIIA established that Ser¹⁶ is the preferred site of phosphorylation, with some secondary modification at Ser³¹⁴.

Key words: 5 S rRNA, RNA polymerase III, zinc-finger protein.

INTRODUCTION

Transcription factor IIIA (TFIIIA) has two identified functions in *Xenopus* oocytes. The factor binds to the internal promoter of the 5 S rRNA genes, as the first step in the activation of transcription by RNA polymerase III (pol III) [1]. In addition, the protein is found in the cytoplasm bound to 5 S rRNA, forming a storage particle for the nucleic acid until it is used ultimately in the assembly of ribosomes [2,3]. The rapid accumulation of 5 S rRNA during the initial stages of oogenesis is the consequence of the transcription of two 5 S rRNA multigene families [4]. The somatic genes are active during oogenesis, and then are reactivated and expressed constitutively at commencement of the mid-blastula stage of embryogenesis. The much more abundant oocyte genes are transcribed only during oogenesis.

The levels of TFIIIA parallel the expression of the 5 S rRNA genes, suggesting that the developmental silencing of the oocyte genes is due, in part, to limiting amounts of this gene-specific factor [3,5]. However, the affinities of TFIIIA for the promoters of the two types of genes are essentially the same [6], so the availability of this factor alone does not account for the differential transcription of the somatic and oocyte genes. The transcription initiation complexes that are assembled on the 5 S rRNA genes comprise TFIIIA, TFIIIB and TFIIIC [7]; the constituents of the latter two fractions have not been well characterized in *Xenopus*. There is evidence from experiments using activated egg extracts that, subsequent to meiosis, the transcription complexes that are formed on the oocytes genes are specifically destabilized relative to those assembled on the somatic genes [8]. This observation, combined with the knowledge that nucleosomes are organized differently over the two types of genes

[9,10], suggests a model in which transcription complexes on the oocyte genes are displaced during chromatin formation, resulting in the stable repression of the oocyte gene family. The transcription complexes on the somatic genes are predicted not to be affected by nucleosome structure and, thus, remain competent for transcription.

The phosphorylation of transcription factors is a common mechanism for controlling gene activity [11]. This post-translational modification can modulate the intracellular localization, stability, DNA-binding affinity or transactivation activity of a variety of these gene regulatory proteins, including some that comprise the transcription initiation complexes for pol III. In yeast, phosphorylation of the TATA-binding protein subunit of TFIIIB by protein kinase CK2 (CK2; formerly casein kinase II) is required for efficient transcription of pol III genes [12]. Conversely, in *Xenopus* eggs TFIIIB is the target of mitotic kinases that repress transcription [13]. La antigen, which binds to the 3' end of pol III primary transcripts and facilitates reinitiation by the polymerase, is down-regulated upon phosphorylation by CK2 [14]. The activity of *Xenopus* TFIIIC appears to be controlled by phosphorylation on tyrosine [15]. Of equal significance is the observation that several proteins involved in the long-term storage and processing of RNA in amphibian oocytes are phosphorylated [16–18], suggesting that these activities are also regulated by various protein kinases. Given these considerations and their relevance to the activities of TFIIIA, we undertook experiments to determine whether the factor is phosphorylated. We have identified a CK2 kinase activity that co-purifies, through several chromatographic steps, with the TFIIIA–5 S rRNA complex. Immunoprecipitates, prepared from immature oocytes incubated with [³²P]orthophosphate, establish

¹ This paper is dedicated to the memory of Dr. Veronica C. Blasquez

Abbreviations used: CK2, protein kinase CK2 (formerly casein kinase II); CNBr, cyanogen bromide; DTT, dithiothreitol; GV, germinal vesicle; pol III, RNA polymerase III; RNP, ribonucleoprotein; TFIIIA, transcription factor IIIA; S314E, Ser³¹⁴ → Glu substitution.

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that a population of TFIIIA is normally phosphorylated *in vivo* at a site within the first zinc-finger domain.

EXPERIMENTAL

Materials

Phosvitin, dephosphorylated casein, phosphorylase *b*, myelin basic protein, histone proteins, spermidine, heparin and anti-phosphoserine monoclonal antibody were obtained from Sigma. Rat CK2 was purchased from Promega. Yeast tRNA was from Life Technologies. *Escherichia coli* tRNA^{Met} and alkaline phosphatase were from Boehringer Mannheim. Plasmids that contained a copy of an oocyte (pXlo316) or a somatic (pXP-1) *Xenopus* 5 S rRNA gene were provided by the late Dr. Alan P. Wolffe (formerly at Sangamo Biosciences, Point Richmond Technology Center, Richmond, U.S.A.). The *Bam*HI fragment of pXlo316 was subcloned into pUC19 (designated pWXlo1), and the *Hind*III fragment of pXP-1 was subcloned into Bluescript II SK (designated pWXls1).

Preparation of TFIIIA and 5 S rRNA

The 7 S ribonucleoprotein (RNP) particle of TFIIIA bound to 5 S rRNA was prepared from immature ovaries of *Xenopus laevis* (NASCO, Fort Atkinson, WI, U.S.A.) [19], and TFIIIA was isolated from the particle as described previously [20]. Recombinant TFIIIA was expressed in *E. coli* B strain BL21(DE3) transformed with the plasmid pTA102 [21]; a sample of transformed *E. coli* cells was given kindly by Dr. David R. Setzer (Department of Molecular Biology and Microbiology, School of Medicine, Case Western Reserve University, Cleveland, OH, U.S.A.). Cells were grown as described previously [21]; expression of TFIIIA was induced by the addition of 1 mM isopropyl β -D-thiogalactoside into the growth media. Cell lysis and the preparation of inclusion bodies were carried out according to the procedures outlined in Sambrook et al. [22]. The washed pellet, which contained inclusion bodies, was suspended in buffer containing 20 mM Hepes, pH 7.4, 250 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol (DTT), 50 μ M ZnCl₂, 10% glycerol, 1 mM PMSF and 5 M urea. The sample was mixed by inversion for 24 h at 4 °C [21]. The supernatant was taken after centrifugation at 11000 *g* for 20 min, and was dialysed at 4 °C against buffer A [50 mM Hepes (pH 7.5)/5 mM MgCl₂/1 mM DTT/10 μ M ZnCl₂/20% glycerol] containing 250 mM KCl. The sample was loaded on to a BioRex-70 column (1.25 ml bed volume) and equilibrated with buffer A containing 250 mM KCl. The procedure for chromatography was identical to that described for the purification of TFIIIA from 7 S RNP particle. Native 5 S rRNA was isolated from early stage (stages I–III) oocytes. Ovaries from immature frogs were lysed with a Dounce homogenizer and extracted with acidic guanidinium thiocyanate [23]. The total cellular RNA was resolved on a 12% polyacrylamide gel containing 7 M urea. The band corresponding to 5 S rRNA was excised from the gel, and the RNA was eluted and renatured [24].

Xenopus cell extracts

Germinal vesicle (GV) extract was prepared as described by Birkenmeier et al. [25]. Whole cell (S150) extract from immature oocytes (primarily stages I and II) was prepared by the method described by Glikin et al. [26], with the modifications specified by Wolffe [27]. OR-2 buffer [82.5 mM NaCl/2.5 mM KCl/1.0 mM CaCl₂/1.0 mM MgCl₂/1.0 mM Na₂HPO₄/5.0 mM Hepes (pH 7.8)] did not contain magnesium in the latter case.

Phosphorylation assays

Extracts or protein samples in J buffer [10 mM Hepes (pH 7.4)/70 mM NH₄Cl/7 mM MgCl₂/0.1 mM EDTA/2.5 mM DTT/10% glycerol] were incubated with 0.2–2 pmoles of [³²P]nucleoside triphosphate (4500 Ci/mM) for 30 min at 21 °C. Reactions were stopped by the addition of an equal volume of SDS-loading solution, and analysed by SDS/PAGE [28] or, in the case of the CK2 peptide substrate, on the gel system described by Schagger and von Jagow [29]. The gels were stained with Coomassie Blue dye, were dried, and then were exposed to X-ray film. The substrate peptide (RRREEETEEE), which is specific for CK2 [30], was purchased from Life Technologies. Wild-type and mutant TFIIIA proteins, which were prepared from *E. coli*, were phosphorylated *in vitro* using rat CK2. Reactions were carried out at 21 °C for the indicated time in J buffer, and contained 2 μ M TFIIIA, 50 μ Ci of [γ -³²P]ATP and 5 units of CK2.

Cyanogen bromide (CNBr) digestion

Phosphorylated TFIIIA was excised from the SDS/polyacrylamide gel using the autoradiograph as a template. The gel slice was incubated in 76% formic acid containing 50 mg/ml CNBr [31]. The sample was flushed with N₂, and the cleavage reaction was run for between 24 h to 48 h in the dark [31,32]. The digest was dried under vacuum, was taken up in SDS-loading solution, and was analysed by SDS/PAGE, followed by autoradiography.

Phospho amino acid analysis

Phosphorylated protein was purified from SDS/polyacrylamide gels. The eluted protein was dried under vacuum and then dissolved in 6 M HCl. The sample was kept for 1 h at 100 °C; the hydrolysate was dried under vacuum, resuspended in water and added to a mixture of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma). The sample was spotted on to a cellulose TLC plate, which was developed with a solvent system composed of isobutyric acid/0.5 M ammonium hydroxide (5:3, v/v) [33]. In the instances where orthophosphate was present, the amino acids were resolved further by chromatography in a second dimension using a solvent system composed of propan-2-ol/concentrated HCl/water (70:15:15, v/v/v) [34]. The dried plate was sprayed with 0.2% ninhydrin reagent to visualize the amino acid standards, followed by autoradiography to identify [³²P]phospho amino acids.

Phosphorylation of TFIIIA *in vivo*

Ovaries containing predominantly stage I–III oocytes were dissected from an immature frog, torn into small pieces and washed with J buffer containing protease inhibitors (1 mM PMSF, 1 μ g/ml each of benzamidine, pepstatin A and leupeptin, and 1 mM EDTA). A 0.5 ml volume of packed oocytes in a total volume of 0.6 ml of J buffer was incubated with 3 mCi of [³²P]orthophosphate for 4 h at 21 °C. The oocytes were washed with PBS [136 mM NaCl/2.7 mM KCl/10.1 mM Na₂HPO₄/1.8 mM KH₂PO₄ (pH 7.4)] containing protease inhibitors. The cells were lysed at 0 °C by ten strokes with a Dounce 'A' pestle. The lysate was cleared of cellular debris by centrifugation at 16000 *g* for 15 min in a microcentrifuge at 4 °C. The protein concentration of the cell extract (9.2 μ g/ μ l) was measured by the method of Bradford [35], using BSA as the standard. Oocyte extract (10 μ l) was incubated with an affinity-purified antibody specific for TFIIIA in PBS containing protease inhibitors (final volume of 50 μ l) on ice for 30 min with occasional mixing.

Protein A-Sepharose (Sigma) was then added and the sample was kept on ice for an additional 30 min with frequent mixing. The immunoprecipitate was collected by centrifugation, washed twice with PBS containing 0.1 M NaCl, and then washed twice with PBS. The precipitate was taken up into the loading solution, boiled and subjected to SDS/PAGE.

RESULTS

Phosphorylation of TFIIIA *in vitro*

Phosphorylation of transcription factors is a primary mechanism for the regulation of gene expression. This is true for the general factors that comprise the pre-initiation complexes of pol III. There is evidence that both TFIIIB and TFIIIC, which are required for the transcription of all the pol III genes, become phosphorylated [12,13,15,36–38]. We carried out experiments to determine whether TFIIIA, which is solely required for transcription of 5 S rRNA genes, is also phosphorylated.

Phosphorylation of TFIIIA was tested in two extracts. The S150 extract is a whole-cell extract prepared from immature oocytes (predominantly stages I and II, according to the Dumont designations [39]); both the oocyte and somatic 5 S rRNA genes are transcribed highly in these cells [4,27]. GV extract is a nuclear extract prepared from mature oocytes (predominantly stage VI) [25]. Although both types of genes are transcribed in these cells, the level of TFIIIA is more than 20-fold lower in the latter when compared with that in early stage oocytes [5]. Samples of extract were incubated with 1 μ Ci of [γ - 32 P]ATP for 30 min at 21 °C. In addition, samples of the TFIIIA–5 S rRNA particle and TFIIIA, purified from the RNP particle, were treated in the same manner. Proteins were resolved SDS/PAGE, stained with Coomassie Blue dye (Figure 1A), and subjected to autoradiography (Figure

Figure 1 Phosphorylation of TFIIIA *in vitro*

(A and B) Lanes 1, whole-cell extract prepared from immature *Xenopus* oocytes (28 μ g); lanes 2, nuclear extract prepared from mature oocytes (12 μ g); lanes 3, TFIIIA–5 S rRNA complex (6 μ g); and lanes 4, TFIIIA purified by chromatography on BioRex-70 (7 μ g), were each incubated with 1 μ Ci of [γ - 32 P]ATP and then analysed by SDS/PAGE. The gel was stained with Coomassie Blue dye (A) and then subjected to autoradiography (B). Sample amounts are given with respect to total protein [35].

Figure 2 CNBr digestion of radiolabelled TFIIIA and phospho amino acid analysis

(A) TFIIIA labelled by incubation with [γ - 32 P]ATP was purified by SDS/PAGE. A portion of the protein eluted from the gel was then reacted with CNBr. The cleavage fragments were analysed by SDS/PAGE (15% gel). Lane 1 is the gel stained with Coomassie Blue, which shows the three CNBr fragments of TFIIIA, and lane 2 is an autoradiograph of this gel. (B) A portion of the labelled protein was hydrolysed in acid, mixed with phospho amino acid standards, and then analysed by two-dimensional TLC. The standards were developed with ninhydrin reagent and [32 P]phospho amino acids identified by autoradiography. Open circles on the autoradiograph denote the positions of phosphotyrosine (Y), phosphothreonine (T) and phosphoserine (S).

1B). No incorporation of [32 P] into TFIIIA was detected either in extract or in the sample of RNP particle. Surprisingly, the purified TFIIIA was efficiently labelled by [γ - 32 P]ATP (Figure 1B, lane 4).

In order to determine whether the incorporation of the radiolabel into TFIIIA reflected the transfer of the γ -phosphoryl group of the nucleotide, we incubated the factor with other nucleoside triphosphates ([γ - 32 P]GTP, [α - 32 P]ATP, [α - 32 P]GTP and [α - 32 P]dATP). We found that [γ - 32 P]GTP also labelled the protein to nearly the same degree as [γ - 32 P]ATP; however, we observed no incorporation of [32 P] into TFIIIA with any of the other nucleoside triphosphates tested (results not shown). Subsequent treatment of the phosphorylated TFIIIA with calf intestine alkaline phosphatase removed the radiolabel, and incubation of the factor at 80 °C for 1 min prior to the addition of [γ - 32 P]ATP prevented detectable phosphorylation (results not shown). These results eliminate the possibility that there is a high affinity-binding site on TFIIIA that simply retains nucleoside triphosphate; rather, they demonstrate the direct transfer of a phosphoryl group from either ATP or GTP to the factor.

It is possible that a second protein co-migrates with TFIIIA on SDS/PAGE, and that it is the former that becomes phosphorylated in the presence of [γ - 32 P]ATP. To address this possibility and to determine the site(s) of phosphorylation on TFIIIA, we digested a sample of labelled factor with CNBr. Treatment of TFIIIA with CNBr generates three cleavage fragments of 20 kDa, 10 kDa and 8 kDa (Figure 2A, lane 1). An autoradiograph of the cleavage products (Figure 2A, lane 2) reveals radioactivity in the 10-kDa and 8-kDa peptides, establishing that the factor is multiply phosphorylated. No other radioactive fragments are visible on the autoradiograph that would indicate the presence of a second contaminating protein that co-migrates with intact TFIIIA on SDS/PAGE. The 10-kDa and 8-kDa CNBr fragments originate from the N- and C-terminal ends of TFIIIA respectively.

The identity of the amino acids that become phosphorylated was determined by subjecting a sample of the labelled protein to acid hydrolysis, and separating the phospho amino acids by two-

Figure 3 Specificity of the TFIIIA-associated kinase activity

TFIIIA (1 μ g) and [γ - 32 P]ATP (1 μ Ci) were incubated with representative proteins of known substrate activity (5 μ g each except for the histone sample which is 16 μ g). The samples were then analysed by SDS/PAGE. The gel was stained with Coomassie Blue dye (A) and then subjected to autoradiography (B). Lane 1 contains only TFIIIA and isotope. Note that the sample of phosphatidylcholine (lane 2) does not become stained with the dye.

dimensional cellulose TLC (Figure 2B). Serine is the only amino acid residue that becomes labelled with [32 P] in this experiment.

A CK2 activity phosphorylates TFIIIA

Substrate specificity can be used to distinguish various kinase activities [40]. We tested several proteins for phosphorylation by the kinase associated with TFIIIA (Figure 3). In this experiment the individual proteins were incubated with 1 μ g of TFIIIA and 1 μ Ci of [γ - 32 P]ATP. The proteins were analysed by SDS/PAGE (15% gel). The gel was stained for protein with Coomassie Blue dye (Figure 3A), and then was subjected to autoradiography (Figure 3B). In this experiment, casein and phosphatidylcholine become

phosphorylated to the exclusion of TFIIIA. The other proteins tested are not phosphorylated and do not interfere with the phosphorylation of TFIIIA. These results indicate that a CK2 activity is responsible for the phosphorylation of TFIIIA. On the basis of these results, we tested the phosphorylation of a synthetic ten-amino-acid peptide [30], which is a specific substrate for CK2. Phosphorylation of this peptide confirmed that the activity can be classified as a CK2 protein kinase (see below). Moreover, this classification is in accordance with the determination that serine residues of TFIIIA become phosphorylated and that either ATP or GTP can act as phosphoryl donors [40]. Preliminary experiments indicate that TFIIIA is recovered with CK2 upon immunoprecipitation of the latter from oocyte extract; this suggests a possible interaction between the two proteins (M. Q. Malik and P. W. Huber, unpublished work).

Two well-established diagnostic effectors of CK2 activity are heparin (inhibitor) and spermidine (activator) [40]. We examined the effect of both on the TFIIIA-associated kinase activity. The phosphorylation of the CK2 substrate peptide (Figure 4A) and TFIIIA (Figure 4B) are markedly inhibited by nanomolar concentrations of the polyanion. The concentration of heparin resulting in 50% inhibition of TFIIIA phosphorylation (Figure 4B) is 1 nM, which is in agreement with the cited literature value of 1.4 nM [40,41]. The phosphorylation of the peptide substrate is slightly less sensitive to heparin. Using TFIIIA as the substrate, we measure a 2.5-fold stimulation of phosphorylation in the presence of spermidine (Figure 4C). CK2 activities, purified from *Xenopus* oocytes, exhibit comparable stimulation by spermidine [41,42], as do CK2 activities that specifically phosphorylate RNA-binding proteins [42,43].

The minimal recognition motif for CK2 is S/T-X-X-E/D, where the initial residue of the sequence is the site of phosphorylation [40]. Nearly all identified physiological targets have a serine residue in this position, and glutamic acid is the favoured acidic residue. Additional acidic residues to the C-terminal side of the serine residue or a β -turn secondary structure are also positive determinants for recognition. There are four canonical CK2 sequences in TFIIIA (at amino acid residues 16, 244, 314 and 328); all contain a serine residue at the putative phosphorylation site, which is in agreement with our determination that TFIIIA is phosphorylated on serine after incubation with [γ - 32 P]ATP (Figure 5). On the basis of the results in Figure 2(A), it appears that at least two sites are phosphorylated *in vitro*. The 20-kDa CNBr fragment is absent in the autoradiograph, establishing that Ser²⁴⁴ is not modified.

Figure 4 Effects of heparin and spermidine on the kinase activity associated with TFIIIA

(A) TFIIIA (1.8 μ g), the CK2 substrate peptide (2 μ g) and [γ - 32 P]ATP (10 μ Ci) were incubated in the presence of 0, 0.06, 0.13, 0.25, 0.50 and 1.0 μ M heparin (lanes 1–6 respectively). The samples were analysed on polyacrylamide gels prepared according to the procedure of Schägger and von Jagow [29]. (B) TFIIIA (1 μ g) and [γ - 32 P]ATP (1 μ Ci) were incubated in the presence of 0, 1, 10, 100 and 1000 nM heparin (lanes 1–5 respectively). (C) TFIIIA (2 μ g) and [γ - 32 P]ATP (0.5 μ Ci) were incubated in the presence of 0, 0.5, 1, 5, 10 and 20 mM spermidine (lanes 1–6 respectively). The reactions in (B) and (C) were analysed on SDS/PAGE.

Figure 5 Location of consensus sequences for CK2 in TFIIIA

The numbered boxes indicate the positions of the nine zinc-finger domains in TFIIIA. The molecular masses identify the origin of the three CNBr fragments that result from cleavage at residues 90 and 265. The four CK2 sequences begin with the putative phosphorylation site.

Figure 6 Inhibition of the phosphorylation of TFIIIA by nucleic acids

TFIIIA (1.7 μ M) and [γ - 32 P]ATP (1 μ Ci) were incubated with (A) 0, 0.35, 1.7 and 8.7 μ M tRNA^{Met} (lanes 1–4), *Xenopus* oocyte 5 S rRNA (lanes 5–8) or unfractionated yeast tRNA (lanes 9–12); (B) 0, 13, 67 and 330 nM *Xenopus* oocyte 5 S rRNA gene as the plasmid pWXlo1 (lanes 1–4), *Xenopus* somatic 5 S rRNA gene as the plasmid pWXIs1 (lanes 5–8), or pUC19 (lanes 9–12). Samples were analysed by SDS/PAGE (12.5% gel) followed by autoradiography.

Inhibition of phosphorylation by nucleic acids

We detected no phosphorylation of TFIIIA in either the S150 or GV extracts; likewise, the 7 S RNP particle of TFIIIA bound to 5 S rRNA is not phosphorylated. This suggests that either the sites of phosphorylation on TFIIIA are masked when bound to a nucleic acid or that the kinase activity itself is sensitive to nucleic acids. We measured the phosphorylation of TFIIIA in the presence of increasing concentrations of various nucleic acids (Figure 6). The specified nucleic acids were added to a constant concentration of TFIIIA, followed by the addition of [γ - 32 P]ATP. The 5 S rRNA and plasmids, carrying either the oocyte or somatic form of the 5 S rRNA genes, inhibited phosphorylation of TFIIIA. However, noncognate nucleic acids, including tRNA and the plasmid pUC19, inhibited phosphorylation equally well, suggesting that nucleic acids, in general, inhibit this kinase activity. We observed an identical pattern of inhibition by these nucleic acids on the phosphorylation of casein (results not shown), which confirms that they must be affecting the enzyme directly. These results are similar to those of Gatica et al. [42], who reported that both RNA and DNA inhibit the nuclear CK2 activity of *Xenopus* oocytes. Furthermore, the binding of the α subunit of *Xenopus* CK2 to DNA inhibits its catalytic activity [44]. These experiments do not eliminate the possibility that sites of phosphorylation on TFIIIA are masked by the binding of one or both cognate nucleic acids; nonetheless, the results do establish that the kinase activity is sensitive to their presence and explain the absence of detectable phosphorylation in S150 and GV extracts. Additionally, it is important to point out that an inhibitor of CK2 is present in *Xenopus* oocytes, which masks the activity of the enzyme in unfractionated extract [41].

Phosphorylation of TFIIIA *in vivo*

The experiments presented above demonstrate that TFIIIA can be phosphorylated by a CK2 activity that co-purifies with the factor and that may be associated with the 7 S RNP storage particle. These results compelled us to determine whether phosphorylation of TFIIIA occurs in oocytes, since this modification could play a pivotal role in controlling the activity of the

Figure 7 Phosphorylation of TFIIIA *in vivo*

Oocytes, predominantly stages I to III, were incubated with 3 mCi [32 P]orthophosphate for 4 h at 21 $^{\circ}$ C. Cell lysate was prepared as described in the Experimental section and 10 μ l aliquots (9.2 μ g protein/ μ l) were incubated for 30 min with the antibody against TFIIIA. Antibody complexes were retrieved from the mixture with Protein A–Sepharose. Samples were analysed by SDS/PAGE, and the gel was stained with Coomassie Blue dye (A) and then subjected to autoradiography (B). Lanes 1–4, oocyte lysate incubated with 0, 2, 5 and 10 μ l of antibody (Ab) respectively; lane 5, antibody alone; lane 6, 1 μ l of oocyte lysate loaded directly on to the gel; lane 7, molecular-mass markers of BSA (66 kDa), TFIIIA (38 kDa) and carbonic anhydrase (29 kDa).

Figure 8 CNBr digestion of TFIIIA labelled *in vivo* and phospho amino acid analysis

(A) TFIIIA from oocytes incubated with [32 P]orthophosphate was isolated from an SDS/polyacrylamide gel and was reacted with CNBr. The cleavage fragments were analysed by electrophoresis. Lane 1 is the gel stained with silver, and lane 2 is the corresponding autoradiograph of the sample. (B) A portion of the radiolabelled TFIIIA was hydrolysed in acid, mixed with phospho amino acid standards, and analysed by one-dimensional TLC. The standards, phosphotyrosine (Y), phosphothreonine (T) and phosphoserine (S), were detected with ninhydrin reagent, and the [32 P]phospho amino acids identified by autoradiography.

factor and, by extension, the synthesis of 5 S rRNA. Oocytes (predominantly stage I and II) were incubated for 4 h with [32 P]orthophosphate. Cell extract was prepared and was incubated with affinity-purified antibody against TFIIIA. The antigen-antibody complexes were recovered specifically from

the extract by incubation with Protein A-Sepharose, and were analysed by SDS/PAGE (Figure 7). The autoradiograph shows that TFIIIA immunoprecipitated from the oocyte extract is indeed phosphorylated. Because of the high concentration of TFIIIA in immature oocytes, it is also possible to observe the factor directly in a sample of whole cell lysate (Figure 7A, lane 6); a prominent phosphoprotein at the position corresponding to TFIIIA is also detected in the autoradiograph of this sample (Figure 7B, lane 6). Oocyte TFIIIA also reacts with an anti-phosphoserine monoclonal antibody in Western-blot assays (results not shown).

As an initial step towards identifying the sites on TFIIIA that are phosphorylated *in vivo*, we isolated the factor from oocytes incubated with [32 P]orthophosphate and digested the protein with CNBr. In this case, we detected labelling of only the 10-kDa fragment derived from the N-terminus of the factor (Figure 8A). A sample of the labelled protein was hydrolysed with acid and the phospho amino acids identified by one-dimensional TLC (Figure 8B). Once again, serine is the sole [32 P]phospho amino acid detected. There are four serine residues in the 10-kDa CNBr fragment. Computer-assisted analysis of this fragment using PC/GENE (IntelliGenetics) identified the CK2 site at Ser¹⁶ as the only consensus phosphorylation site for serine. Secondary digestion of the 10-kDa CNBr fragment with *Staphylococcus aureus* V8 protease generated a labelled peptide; however, attempts to sequence this fragment were unsuccessful, suggesting that it is derived from the blocked N-terminal end of TFIIIA [45]. These results, nonetheless, are consistent with Ser¹⁶ being the site of phosphorylation *in vivo*.

The consensus sequence for CK2 in the 10-kDa fragment is particularly interesting, because it is located in the β -turn of the first zinc-finger domain. Structural elements that maintain the

Figure 9 Phosphorylation of TFIIIA mutants

The serine residues at putative CK2 phosphorylation sites in TFIIIA were changed individually to glutamic acid. (A) Wild-type or mutant TFIIIA expressed in *E. coli* was incubated for the indicated times with rat CK2 and [γ - 32 P]ATP. Samples were analysed by SDS/PAGE, followed by autoradiography. (B) The kinase activity associated with the 7 S RNP particle was isolated by ion-exchange chromatography on BioRex-70, and incubated with either wild-type TFIIIA or the S16E mutant and [γ - 32 P]ATP for the indicated times. Kinase incubated with [γ - 32 P]ATP only for 5 h is shown in the right-hand lane.

phospho-acceptor segment in a β -turn conformation markedly increase the efficiency of phosphorylation by CK2, particularly at sites that lack a cluster of acidic residues [40]. The turn connecting the β -sheets of the zinc-finger domain is expected to be quite stable due to metal co-ordination; consequently, both sequence and structural elements in combination should promote phosphorylation at this position by CK2.

Phosphorylation of TFIIIA Mutants

The 10-kDa and 8-kDa CNBr fragments of TFIIIA became phosphorylated *in vitro* by the CK2 activity associated with the factor; however, we detected phosphorylation of only the 10-kDa fragment in oocytes. In order to characterize the three putative CK2 sites, the individual serine residues at positions 16, 314 or 328 were changed to glutamic acid. Phosphorylation of the wild-type and mutant proteins was measured using commercial CK2 (Figure 9A). Substitution of Ser¹⁶ with glutamic acid abolished phosphorylation of TFIIIA by CK2. There is a modest reduction of phosphorylation for the S314E (Ser³¹⁴ → Glu substitution) mutant and no effect for the S328E mutant. Identical results were observed with mutants in which the serine residues were changed to alanine (results not shown). The CK2 site at Ser¹⁶ is the kinetically preferred site of phosphorylation, which is in accordance with the assays *in vivo* that detected phosphorylation of the CNBr fragment derived from the N-terminal end of TFIIIA. Secondary phosphorylation is possible at Ser³¹⁴, which explains the results of the assays *in vitro* with TFIIIA purified from the 7 S RNP particle that showed phosphorylation at sites in both the 10-kDa and 8-kDa CNBr fragments.

Xenopus TFIIIA is purified from the 7 S RNP particle by chromatography on BioRex-70 ion-exchange resin. We found that the associated kinase activity elutes from this column in fractions immediately preceding and overlapping those containing the transcription factor. Using an early fraction from the column that contains the kinase, but no TFIIIA, we showed that this activity can phosphorylate TFIIIA expressed in *E. coli* (Figure 9B). However, there was no detectable phosphorylation of the S16E mutant (Figure 9B), providing additional evidence that the associated kinase activity is CK2 and that Ser¹⁶ is the preferred site of phosphorylation. Incubation of the kinase fraction from the BioRex-70 column with [γ -³²P]ATP in the absence of exogenous TFIIIA does not produce a radioactive band at 38 kDa (Figure 9B).

DISCUSSION

We have detected a CK2 activity that co-purifies with the TFIIIA–5 S rRNA complex isolated from *Xenopus* oocytes. CK2 is particularly abundant in *Xenopus* oocytes [46]. The levels of mRNA encoding the α and β subunits of the enzyme increase approximately 3-fold during oogenesis, resulting in a 12- to 15-fold increase in CK2 activity by stage VI of oogenesis. In previous studies to characterize the physical properties of TFIIIA, Bieker and Roeder [47] noted a contaminating kinase activity in preparations of the factor that could be removed by analytical gel filtration chromatography. This kinase activity was detected using casein as the substrate in the assay; however, the phosphorylation of TFIIIA was not tested. The experiments in the present study, which show that a casein kinase co-purifies with the 7 S RNP particle, parallels these previous observations. Additionally, our results potentially account for an ATPase activity attributed to TFIIIA [48].

There is substantial evidence that components of the pol III transcriptional machinery are phosphorylated in response to a

variety of different physiological conditions, and also with respect to the position within the cell cycle (reviewed in [49–51]). However, to our knowledge, this is the first reported observation of phosphorylation of TFIIIA. Two other pol III-associated factors are also phosphorylated by CK2: the TATA-binding protein subunit of TFIIIB [12] and the La antigen [14]. The consequences of phosphorylation by CK2 in these two cases are quite distinct. In *Saccharomyces cerevisiae*, phosphorylation of TFIIIB by CK2 is required for efficient transcription by pol III [52]. Assays in extract prepared from a temperature-sensitive mutant of CK2 showed greatly reduced levels of pol III transcription that can be rescued by the addition of the kinase or TFIIIB [50]. Whereas phosphorylation of TFIIIB by CK2 has a positive effect in yeast, phosphorylation of the human La antigen by CK2 appears to inhibit recycling of pol III transcription complexes, resulting in greatly reduced levels of transcription [14]. It is not clear at this time how to reconcile these apparently opposite effects of CK2 on pol III transcription.

The two activities of TFIIIA are compartmentalized. In the nucleus, the protein is a positive activator of 5 S rRNA gene transcription and in the cytoplasm it forms a storage RNP particle for 5 S rRNA. Since CK2 is found in both the cytoplasm and nucleus of *Xenopus* oocytes, it is not possible to speculate on the possible functional consequences of the phosphorylation of TFIIIA. It is equally likely that phosphorylation could influence the cytoplasmic, rather than the nuclear, activity of the factor. Kinase activities, including CK2, are found associated with RNP particles in amphibian oocytes [17,18,53]. Indeed, the experiments presented here indicate that CK2 is associated with the TFIIIA–5 S rRNA complex. The phosphorylation of proteins within these RNP structures appears to have a role in the storage and expression of the constituent RNAs. Beginning at stage III of oogenesis, ribosomal protein L5 displaces TFIIIA from much of the stored cytoplasmic pool of 5 S rRNA. The L5–5 S rRNA complex then travels to the nucleolus for assembly into nascent ribosomes. Interestingly, L5 is also associated with [54] and phosphorylated by CK2 [55]. Moreover, it appears that the phosphorylated protein binds to 5 S rRNA with 5-fold less affinity. Preliminary experiments indicate that TFIIIA phosphorylated *in vitro* with CK2 or serine-to-glutamate mutants of TFIIIA exhibit no significant change in binding affinity for 5 S rRNA (R. Ghose and P. W. Huber, unpublished work). Given the dual roles of TFIIIA, it is not surprising that the protein is phosphorylated in *Xenopus* oocytes. The imperative now is to determine whether this modification controls one of these essential activities.

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REFERENCES

- Engelke, D. R., Ng, S.-Y., Shastry, B. S. and Roeder, R. G. (1980) Specific interaction of a purified transcription factor with an internal control region of 5 S RNA genes. *Cell* (Cambridge, Mass.) **19**, 717–728.
- Pelham, H. R. B. and Brown, D. D. (1980) A specific transcription factor that can bind either the 5 S RNA gene or 5 S RNA. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4170–4174.
- Honda, B. M. and Roeder, R. G. (1980) Association of a 5 S gene transcription factor with 5 S RNA and altered levels of the factor during cell differentiation. *Cell* (Cambridge, Mass.) **22**, 119–126.
- Wolffe, A. P. and Brown, D. D. (1988) Developmental regulation of two 5 S ribosomal RNA genes. *Science* (Washington, D.C.) **241**, 1626–1632.

- 5 Shastry, B. S., Honda, B. M. and Roeder, R. G. (1984) Altered levels of a 5 S gene-specific transcription factor (TFIIIA) during oogenesis and embryonic development of *Xenopus laevis*. *J. Biol. Chem.* **259**, 11373–11382
- 6 McConkey, G. A. and Bogenhagen, D. F. (1988) TFIIIA binds with equal affinity to somatic and major oocyte 5 S RNA genes. *Genes Dev.* **2**, 205–214
- 7 Lassar, A. B., Martin, P. L. and Roeder, R. G. (1983) Transcription of class III genes: formation of preinitiation complexes. *Science (Washington, D.C.)* **222**, 740–748
- 8 Wolffe, A. P. and Brown, D. D. (1987) Differential 5 S RNA gene expression *in vitro*. *Cell (Cambridge, Mass.)* **51**, 733–740
- 9 Chipev, C. C. and Wolffe, A. P. (1992) Chromosomal organization of *Xenopus laevis* oocyte and somatic 5 S rRNA genes *in vivo*. *Mol. Cell. Biol.* **12**, 45–55
- 10 Engelke, D. R. and Gottesfeld, J. M. (1990) Chromosomal footprinting of transcriptionally active and inactive oocyte-type 5 S RNA genes of *Xenopus laevis*. *Nucleic Acids Res.* **18**, 6031–6037
- 11 Whitmarsh, A. J. and Davis, R. J. (2000) Regulation of transcription factor function by phosphorylation. *Cell. Mol. Life Sci.* **57**, 1172–1183
- 12 Ghavidel, A. and Schultz, M. C. (1997) Casein kinase II regulation of yeast TFIIIB is mediated by the TATA-binding protein. *Genes Dev.* **11**, 2780–2789
- 13 Gottesfeld, J. M., Wolf, V. J., Dang, T., Forbes, D. J. and Hartl, P. (1994) Mitotic repression of RNA polymerase III transcription *in vitro* mediated by phosphorylation of a TFIIIB component. *Science (Washington, D.C.)* **263**, 81–84
- 14 Fan, H., Sakulich, A. L., Goodier, J. L., Zhang, X., Qin, J. and Maraia, R. J. (1997) Phosphorylation of the human La antigen on serine 366 can regulate recycling of RNA polymerase III transcription complexes. *Cell (Cambridge, Mass.)* **88**, 707–715
- 15 Reynolds, W. F. (1993) The tyrosine phosphatase cdc25 selectively inhibits transcription of the *Xenopus* oocyte-type tRNA^{tyr} gene. *Nucleic Acids Res.* **21**, 4372–4377
- 16 Deschamps, S., Jacquemin-Sablon, H., Triqueneaux, G., Mulner-Lorillon, O., Potier, M., Le Caer, J. P., Dautry, F. and le Maire, M. (1997) mRNP3 and mRNP4 are phosphorylatable by casein kinase II in *Xenopus* oocytes, but phosphorylation does not modify RNA-binding affinity. *FEBS Lett.* **412**, 495–500
- 17 Cummings, A. and Somerville, J. (1988) Protein kinase activity associated with stored messenger ribonucleoprotein particles of *Xenopus* oocytes. *J. Cell Biol.* **107**, 45–56
- 18 Kandror, K. V. and Stepanov, A. S. (1984) RNA-binding protein kinases from amphibian oocytes is a casein kinase II. *FEBS Lett.* **170**, 33–37
- 19 Hanas, J. S., Bogenhagen, D. F. and Wu, C.-W. (1983) Cooperative model for the binding of *Xenopus* transcription factor A to the 5 S RNA gene. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2142–2145
- 20 Rawlings, S. L., Matt, G. D. and Huber, P. W. (1996) Analysis of the binding of *Xenopus* transcription factor IIIA to oocyte 5 S rRNA and to the 5 S rRNA gene. *J. Biol. Chem.* **271**, 869–877
- 21 Del Rio, S. and Setzer, D. R. (1991) High yield purification of active transcription factor IIIA expressed in *E. coli*. *Nucleic Acids Res.* **19**, 6197–6203
- 22 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Expression of cloned genes in *Escherichia coli*. In *Molecular Cloning: A Laboratory Manual*, pp. 36–39, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- 23 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162**, 156–159
- 24 Huber, P. W. and Wool, I. G. (1984) Nuclease protection analysis of ribonucleoprotein complexes: Use of the cytotoxic ribonuclease α -sarcin to determine the binding sites for *Escherichia coli* ribosomal proteins L5, L18, and L25 on 5 S rRNA. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 322–326
- 25 Birkenmeier, E. H., Brown, D. D. and Jordan, E. (1978) A nuclear extract of *Xenopus laevis* oocytes that accurately transcribes 5 S RNA genes. *Cell (Cambridge, Mass.)* **15**, 1077–1086
- 26 Glikin, G. C., Ruberti, I. and Worcel, A. (1984) Chromatin assembly in *Xenopus* oocytes: *in vitro* studies. *Cell (Cambridge, Mass.)* **37**, 33–41
- 27 Wolffe, A. P. (1988) Transcription fraction TFIIIC can regulate differential *Xenopus* 5 S RNA gene transcription *in vitro*. *EMBO J.* **7**, 1071–1079
- 28 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 29 Schagger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379
- 30 Kuenzel, E. A. and Krebs, E. G. (1985) A synthetic peptide substrate specific for casein kinase II. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 737–741
- 31 McDonald, O. B., Merrill, B. M., Bland, M. M., Taylor, L. C. E. and Naji, S. (1993) Site and consequences of the autophosphorylation of Ca²⁺/calmodulin-dependent protein kinase type 'Gr'. *J. Biol. Chem.* **268**, 10054–10059
- 32 Smith, D. R., Jackson, I. J. and Brown, D. D. (1984) Domains of the positive transcription factor specific for the *Xenopus* 5 S RNA gene. *Cell (Cambridge, Mass.)* **37**, 645–652
- 33 Cooper, J. A., Sefton, B. M. and Hunter, T. (1983) Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol.* **99**, 387–402
- 34 Kuchino, Y., Hanyu, N. and Nishimura, S. (1987) Analysis of modified nucleosides and nucleotide sequence of tRNA. *Methods Enzymol.* **155**, 379–396
- 35 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 36 Conesa, C., Swanson, R. N., Schultz, P., Oudet, P. and Sentenac, A. (1993) On the subunit composition, stoichiometry, and phosphorylation of the yeast transcription factor TFIIIC/ τ . *J. Biol. Chem.* **268**, 18047–18052
- 37 Hoeffler, W. K., Kovelman, R. and Roeder, R. G. (1988) Activation of transcription factor IIIC by the adenovirus E1A protein. *Cell* **53**, 907–920
- 38 White, R. J., Stott, D. and Rigby, P. W. (1990) Regulation of RNA polymerase III transcription in response to Simian virus 40 transformation. *EMBO J.* **9**, 3713–3721
- 39 Dumont, J. N. (1972) Oogenesis in *Xenopus laevis* (Daudin) I. Stages of oocyte development in laboratory maintained animals. *J. Morph.* **136**, 153–180
- 40 Pinna, L. A. (1990) Casein kinase 2: an 'eminence grise' in cellular regulation? *Biochim. Biophys. Acta* **254**, 267–284
- 41 Mulner-Lorillon, O., Marot, J., Cayla, X., Pouhle, R. and Belle, R. (1988) Purification and characterization of a casein-kinase-II-type enzyme from *Xenopus laevis* ovary. Biological effects on the meiotic cell division of full-grown oocyte. *Eur. J. Biochem.* **171**, 107–117
- 42 Gatica, M., Allende, C. C. and Allende, J. E. (1989) Nucleic acids can regulate the activity of casein kinase II. *FEBS Lett.* **255**, 414–418
- 43 Holcomb, E. R. and Friedman, D. L. (1984) Phosphorylation of the C-proteins of HeLa cell hnRNP particles. *J. Biol. Chem.* **259**, 31–40
- 44 Allende, J. E. and Allende, C. C. (1995) Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *FASEB J.* **9**, 313–323
- 45 Taylor, W., Jackson, I. J., Siegel, N., Kumar, A. and Brown, D. D. (1986) The developmental expression of the gene for TFIIIA in *Xenopus laevis*. *Nucleic Acids Res.* **14**, 6185–6195
- 46 Wilhelm, V., Rojas, P., Gatica, M., Allende, C. C. and Allende, J. E. (1995) Expression of the subunits of protein kinase CK2 during oogenesis in *Xenopus laevis*. *Eur. J. Biochem.* **232**, 671–676
- 47 Bieker, J. J. and Roeder, R. G. (1984) Physical properties and DNA-binding stoichiometry of a 5 S gene-specific transcription factor. *J. Biol. Chem.* **259**, 6158–6164
- 48 Hazuda, D. J. and Wu, C.-W. (1986) DNA-activated ATPase activity associated with *Xenopus* transcription factor A. *J. Biol. Chem.* **261**, 12202–12208
- 49 Brown, T. R., Scott, P. H., Stein, T., Winter, A. G. and White, R. J. (2000) RNA polymerase III transcription: its control by tumor suppressors and its deregulation by transforming agents. *Gene Expression* **9**, 15–28
- 50 Ghavidel, A., Hockman, D. J. and Schultz, M. C. (1999) A review of progress towards elucidating the role of protein kinase CK2 in polymerase III transcription: regulation of the TATA-binding protein. *Mol. Cell. Biochem.* **191**, 143–148
- 51 Gottesfeld, J. M. and Forbes, D. J. (1997) Mitotic repression of the transcriptional machinery. *Trends Biochem. Sci.* **22**, 197–202
- 52 Hockman, D. J. and Schultz, M. C. (1996) Casein kinase II is required for efficient transcription by RNA polymerase III. *Mol. Cell. Biol.* **16**, 892–898
- 53 Stepanov, A. S., Kandror, K. V. and Elizarov, S. M. (1982) Protein kinase activity in RNA-binding proteins of *Amphibia* oocytes. *FEBS Lett.* **141**, 157–160
- 54 Kim, J. M., Cha, J. Y., Marshak, D. R. and Bae, Y. S. (1996) Interaction of the β subunit of casein kinase II with the ribosomal protein L5. *Biochem. Biophys. Res. Commun.* **226**, 180–186
- 55 Park, J.-W. and Bae, Y.-S. (1999) Phosphorylation of ribosomal protein L5 by protein kinase CKII decreases its 5 S rRNA binding activity. *Biochem. Biophys. Res. Commun.* **263**, 475–481

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