Mitotic Phosphorylation of the Oct-1 Homeodomain and Regulation of Oct-1 DNA Binding Activity

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Oct-1 is a transcription factor involved in the cell cycle regulation of histone H2B gene transcription and in the transcription of other cellular housekeeping genes. Oct-1 is hyperphosphorylated as cells enter mitosis, and mitosis-specific phoshorylation is reversed as cells exit mitosis. A mitosis-specific phosphorylation site in the homeodomain of Oct-1 was phosphorylated in vitro by protein kinase A. Phosphorylation of this site correlated with inhibition of Oct-1 DNA binding activity in vivo and in vitro. The inhibition of Oct-1 DNA binding during mitosis suggests a mechanism by which the general inhibition of transcription during mitosis might occur.

OCTAMER SEQUENCE ELEMENTS have been studied in a variety of gene promoters (*1*) and are involved in transcriptional regulation of both commonly expressed genes, such as those encoding histone H2B and small nuclear RNA, as well as genes expressed in specific tissues, such as immunoglobulins. Several proteins that interact with the octamer sequence have been purified and cloned (*1*). These proteins are part of a larger family of proteins that contain POU homeodomains as their DNA binding elements (*2, 3*). Oct-1, a member of this family, has a widespread pattern of expression, consistent with a role in transcription of essential housekeeping genes (*1, 4*).

An octamer-binding site in the histone H2B gene promoter is required for the cell cycle–regulated transcription of this gene in vivo (*5*) and in vitro (*6*). Oct-1 appears to be the only octamer-binding activity in HeLa cells, which implicates Oct-1 in the cell cycle regulation of histone H2B expression. The mechanism by which it exerts this effect is unknown. Hyperphosphorylation of Oct-1 occurs as cells complete DNA synthesis and enter mitosis and is rapidly reversed as cells exit mitosis and enter the G_1 growth phase of the cell cycle (*7, 8*). Mitotic phosphorylation of Oct-1 correlates with a general inhibition of transcription that occurs during mitosis (*9–11*). Transcriptional inhibition occurs early in mitotic prophase and is reversed in telophase after the nuclear envelope reforms and the cells enter G_1. The causes of this general transcriptional inhibition during mitosis are unknown. However, the temporal correlation between mitotic transcriptional inhibition and regulated phosphorylation of Oct-1 led us to investigate the functional consequences of the mitotic phosphorylation of Oct-1.

The abundance of Oct-1 protein does not fluctuate during the HeLa cell cycle (7). To assess whether there is a significant difference in specific activity of Oct-1 DNA binding in interphase and mitotic cell extracts, we compared the amount of Oct-1 protein present in synchronized whole-cell extracts (WCE) and the Oct-1 DNA binding activity of these extracts (Fig. 1). In addition to G_1, S (DNA synthesis), and G_2 cells, we prepared mitotic cells (mitotic index >80%) by culturing elutriated S-phase cells in nocodazole (Noco) (Fig. 1A). Immunoblots revealed that mitotic extracts had two- to fourfold more Oct-1 per microgram of protein than did interphase extracts (Fig. 1B), perhaps reflecting differential extraction of Oct-1 as a consequence of changes in chromatin structure associated with mitosis. We loaded twice as much protein from mitotic cell extract in lane 5 (Fig. 1B), as compared to lane 4, to emphasize the difference between mitotic and interphase extracts. Octamer-specific DNA binding activity in each extract was measured by electrophoretic mobility-shift analysis with an oligonucleotide that contained the octamer-binding motif from the human histone H2B promoter (Fig. 1C). Less Oct-1 DNA binding activity was present in the mitotic extract than in any of the interphase extracts, although more Oct-1 protein was present. Quantitation by densitometric scanning of autoradiograms revealed a 15- to 20-fold difference in the specific activity of Oct-1 DNA binding between interphase and mitotic extracts. No consistent difference in Oct-1 DNA binding activity between G_1, S, and G_2 extracts was detected in these experiments, in agreement with other studies (*12*).

To test whether the increase in phosphorylation of Oct-1 as cells enter mitosis (7) might be responsible for the changes in DNA binding activity, we sought to identify the kinases responsible for phosphorylating Oct-1 in vivo. An inspection of the Oct-1 amino acid sequence for specific protein kinase recognition sequences in the PROSITE (*13*) database revealed two sites in the Oct-1 molecule that conformed to the adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) consensus recognition sequence ZZXT/SI (Z, basic amino acids; S, Ser; T, Thr; I, apolar amino acids; and X, any amino acid). These sites, Thr^{384} and Ser^{385}, are located in the homeobox domain just NH_2-terminal to the putative helix 1 of the POU homeodomain (Fig. 2A). The location of these potential Oct-1 phosphorylation site or sites suggested that phosphorylation might influence DNA binding activity.

To test whether a PKA-like enzyme is responsible for any of the phosphorylation events that occur in vivo, we labeled Oct-1 by PKA with γ-^{32}P-adenosine triphosphate

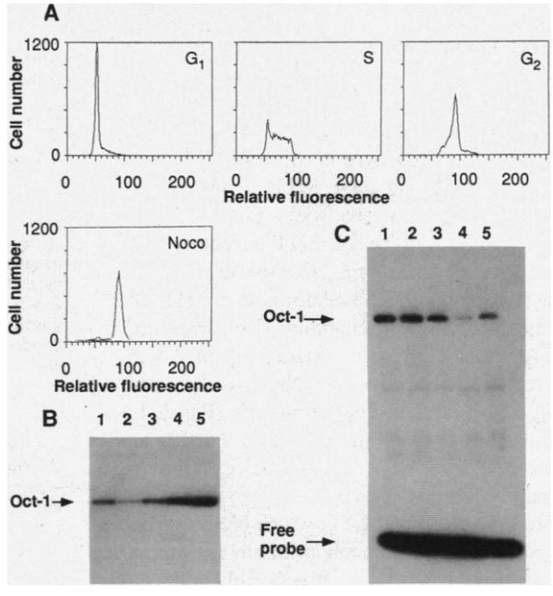

Fig. 1. Specific activity of Oct-1 DNA binding in cell cycle extracts. (**A**) WCE were prepared from HeLa cells fractionated into G_1, S, and G_2 populations by centrifugal elutriation and from cells blocked in mitosis by Noco as described (*22*). Representative graphs from FACS analysis show the relative DNA content of G_1, S, G_2, and Noco HeLa cell populations. (**B**) Immunoblot analysis of cell cycle extracts from G_1 (lane 1), S (lane 2), G_2 (lane 3), and Noco-blocked cells (lanes 4 and 5). In lane 5, 80 μg of protein was loaded; in all other lanes, 40 μg of protein was loaded. Blots were probed with antiserum to Oct-1 diluted 1:2000 (*8*) and ^{125}I-labeled protein A and were visualized by autoradiography (*23*). (**C**) Gel-shift assay of Oct-1–specific DNA binding. The same types and amounts of cell cycle extracts used in (B) were subjected to electrophoretic mobility-shift assay. Human histone H2B oligonucleotide (*24*) was used as probe.

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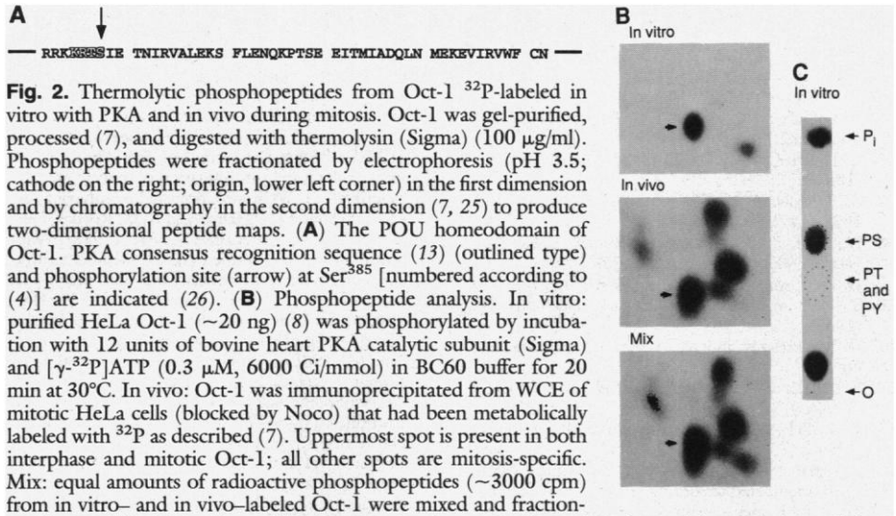

Fig. 2. Thermolytic phosphopeptides from Oct-1 ^{32}P-labeled in vitro with PKA and in vivo during mitosis. Oct-1 was gel-purified, processed (7), and digested with thermolysin (Sigma) (100 μg/ml). Phosphopeptides were fractionated by electrophoresis (pH 3.5; cathode on the right; origin, lower left corner) in the first dimension and by chromatography in the second dimension (*7, 25*) to produce two-dimensional peptide maps. (**A**) The POU homeodomain of Oct-1. PKA consensus recognition sequence (*13*) (outlined type) and phosphorylation site (arrow) at Ser^{385} [numbered according to (*4*)] are indicated (*26*). (**B**) Phosphopeptide analysis. In vitro: purified HeLa Oct-1 (~20 ng) (*8*) was phosphorylated by incubation with 12 units of bovine heart PKA catalytic subunit (Sigma) and [γ-^{32}P]ATP (0.3 μM, 6000 Ci/mmol) in BC60 buffer for 20 min at 30°C. In vivo: Oct-1 was immunoprecipitated from WCE of mitotic HeLa cells (blocked by Noco) that had been metabolically labeled with ^{32}P as described (7). Uppermost spot is present in both interphase and mitotic Oct-1; all other spots are mitosis-specific. Mix: equal amounts of radioactive phosphopeptides (~3000 cpm) from in vitro– and in vivo–labeled Oct-1 were mixed and fractionated in parallel with in vivo and in vitro samples. Arrows indicate phosphopeptide common to all three maps. (**C**) PAA of in vitro–labeled Oct-1. A portion of the in vitro–phosphorylated Oct-1 sample was hydrolyzed with HCl (6 N) (*25*), and the phosphoamino acids were applied to thin-layer cellulose plates and fractionated by electrophoresis (pH 1.9) as described (7). Unlabeled phosphoamino acid markers [phosphoserine, PS (20 μg); phosphothreonine, PT (20 μg); and phosphotyrosine, PY (20 μg)] were added to the sample and visualized with ninhydrin. P_i, inorganic phosphate liberated by hydrolysis; and O, loading origin. Partially hydrolyzed peptides appear just above the origin.

(ATP) in vitro and subjected it to two-dimensional phosphopeptide analysis. The resulting phosphopeptide map was compared with the phosphopeptide map of mitotic Oct-1 phosphorylated in vivo (Fig. 2B). In vitro phosphorylation of Oct-1 by PKA generated a single major phosphopeptide (Fig. 2B). This phosphopeptide comigrated with a mitosis-specific peptide present in thermolytic digests of Oct-1 immunoprecipitated from mitotically blocked cells (Fig. 2B). The minor phosphopeptide present after in vitro PKA phosphorylation (Fig. 2B) did not comigrate with any phosphopeptides labeled in vivo and may represent a partial thermolysin digestion product (Fig. 3C). Phosphoamino acid analysis (PAA) of Oct-1 phosphorylated in vitro by PKA showed that only phosphoserine is present (Fig. 2C), suggesting that Ser^{385} is the likely site of PKA phosphorylation in Oct-1.

The effect of phosphorylation by PKA on Oct-1 DNA binding activity was analyzed by electrophoretic mobility-shift analysis. Incubation of purified HeLa Oct-1 with PKA and ATP led to an almost complete inhibition of DNA binding (Fig. 3A). Control incubations of Oct-1 with PKA in the absence of ATP, with heat-inactivated PKA, with ATP alone, or with buffer alone had no effect on DNA binding activity. Thus, in vitro phosphorylation of Oct-1 by PKA mimics the inhibition of Oct-1 DNA binding observed in extracts from mitotic cells.

To positively identify the site or sites in Oct-1 that are phosphorylated by PKA, we subcloned the cDNA sequence encoding the POU homeodomain of Oct-1 into the pGEX bacterial expression vector (*14*). This wild-type (WT) fusion protein bound an octamer-containing oligonucleotide (Fig. 3B), and, like native Oct-1, incubation of WT fusion protein with PKA and ATP led to complete inhibition of DNA binding (Fig. 3B). We constructed a second fusion protein that contained a point mutation (PM) that changed the predicted PKA phosphorylation site Ser^{385} to Ala^{385} (*14*). The PM fusion protein also bound to octamer-containing DNA, but it was no longer inhibited by incubation with PKA and ATP (Fig. 3B). Two-dimensional peptide mapping of affinity-purified, PKA-phosphorylated WT fusion protein (Fig. 3C) showed that the site of PKA phosphorylation was the same as that observed in native Oct-1 and confirmed that phosphorylation occurred in the homeodomain. Incubation of the PM fusion protein with PKA and [γ-^{32}P]ATP under the same reaction conditions as those used for the WT fusion protein resulted in severely reduced incorporation of ^{32}P. Overexposure of autoradiograms of two-dimensional phosphopeptide maps of WT and PM fusion proteins allowed visualization of background spots (Fig. 3C, arrows) and demonstrated that neither the major nor the minor Oct-1 phosphopeptides are present in the PM peptide map. This demonstrates that Ser^{385} is the in vitro site of PKA phosphorylation on Oct-1 and that the minor phosphopeptide is likely to result from partial thermolysin cleavage of the phosphopeptide represented by the major spot.

Both histone and nonhistone nuclear proteins are phosphorylated during mitosis (*15*). One possible explanation for the mi-

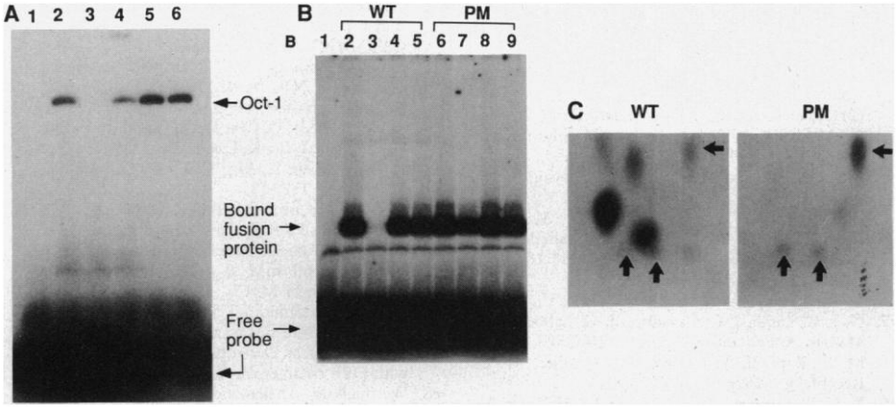

Fig. 3. Effect of PKA phosphorylation on Oct-1 DNA binding and identification of the site of PKA phosphorylation in vitro. (**A**) Electrophoretic mobility-shift analysis (*24*) of Oct-1 (~25 ng) purified from HeLa nuclear extract (*8*) and incubated as described (*27*). Lane 1, probe alone; lane 2, kinase reaction with PKA alone; lane 3, PKA and ATP; lane 4, heat-inactivated PKA (boiled 5 min) and ATP; lane 5, ATP alone; and lane 6, buffer alone. (**B**) Gel-shift analysis (*24*) of WT and PM Oct-1 homeodomain fusion proteins (*14*), as indicated, phosphorylated by incubation with PKA and ATP. Equal amounts of bacterial extract containing the induced fusion proteins were incubated as in (A), except that the kinase reaction buffer was BC60 and 10 units of bovine heart PKA catalytic subunit (Promega) were used per well. Lane 1, probe; lane 2, buffer; lane 3, PKA and ATP; lane 4, PKA; lane 5, ATP; lane 6, buffer; lane 7, PKA and ATP; lane 8, PKA; and lane 9, ATP. (**C**) Phosphopeptide analysis of WT and PM Oct-1 homeodomain fusion proteins phosphorylated by PKA. Bacterially expressed fusion proteins were affinity purified (*21*) and incubated with 10 units of bovine heart PKA catalytic subunit (Promega) and [γ-^{32}P]ATP (0.3 μM, 6000 Ci/mmol) in BC60 buffer for 20 min at 30°C. Thermolytic phosphopeptide maps were prepared exactly as in Fig. 2B. Equal amounts of radioactive phosphopeptides (3000 cpm) were loaded on WT and PM chromatograms. Autoradiographs of chromatograms were overexposed in order to visualize the background spots (arrows), which were needed to align the maps.

totic inhibition of transcription is that it reflects a need to remove these and other proteins from the chromosomes before condensation. If, as has been hypothesized (*16*), phosphorylation plays a role in this process, then phosphorylation of Oct-1 by PKA or a PKA-like mitotic kinase might represent a specific example of this phenomenon. Alternatively, specific modification of transcription machinery at mitosis might be important for the regulation of specific transcriptional events during the subsequent cell cycles.

Mitosis-specific phosphorylation of Oct-1 by a PKA-like enzyme suggests that PKA activity is regulated in a cell cycle–dependent fashion. Inhibition of PKA activity is necessary for nuclear envelope breakdown (NEBD) to occur (*17*), supporting the idea that PKA is active early in mitosis. This correlates with the onset of transcription inhibition, which begins early in prophase before nucleolar dissolution or NEBD (*10*). Thus, by the time NEBD has occurred, PKA may have already served a role in inhibiting transcription. In this context, Oct-1 may provide an experimental substrate for analyzing the mechanisms governing the mitotic regulation of PKA activity.

A site analogous to the Ser^{385} of Oct-1 in another POU homeodomain protein Pit-1 has been shown to be phosphorylated by PKA in vitro and in response to cAMP in vivo (*18*). Like Oct-1, in vitro phosphorylation of this analogous site in Pit-1 inhibits DNA binding. Conservation of this site within the POU homeodomain family suggests that phosphorylation could modulate the DNA binding activity of all these proteins. Together with our results, these observations suggest a mechanism, other than through the well-characterized cAMP response element–binding protein CREB (*19*), for mediating the transcriptional response to cAMP. The important role of cAMP in the regulation of cell growth and differentiation (*20*) suggests that regulation of the POU homeodomain family of proteins may be fundamental to many transcriptional responses observed during these processes.

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14. A partial clone of human Oct-1 [nucleotides 455 to 1330 [numbered according to (*4*)]] that contains the POU homeodomain region was cloned from a λZAP II cDNA expression library (Stratagene) made from HeLa cell mRNA prepared by R. Pine. This clone was used in a polymerase chain reaction (PCR) (Perkin-Elmer Cetus Instruments) with an oligonucleotide that contained nucleotides 1076 to 1098 of Oct-1 and an oligonucleotide that hybridized to the T7 promoter sequence in the vector. The PCR product thus contains flanking Eco RI restriction sites that were used to subclone into vector pGEX-3X (*21*) to produce the WT fusion protein construct (nucleotides 1104 to 1330 of the Oct-1 cDNA). The 5′ Eco RI site is located in the Oct-1 cDNA sequence at nucleotide position 1100 and places the fragment in an open-reading frame for the vector. We made the PM fusion protein using the same strategy except that an oligonucleotide that spanned nucleotide number 1104 to 1164 was used as the 5′ primer. Nucleotides corresponding to numbers 1153, 1154, and 1155 were changed from the A, G, and C, respectively in the WT, to G, C, and A in the mutant clone. This introduced a serine to alanine amino acid substitution in the PM fusion protein at Oct-1 amino acid position 385. We verified the sequence of both the WT and PM clones through the region containing the putative phosphorylation site (Ser^{385}) and found it to be identical to the published Oct-1 sequence (*4*). WT and PM fusion proteins were produced and purified as in (*21*), except that BC60 buffer [50 mM tris (pH 7.9), 1 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM $MgCl_2$, and 60 mM KCl] was used during extraction.
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22. HeLa D cells in suspension (3×10^9) were fractionated by counter-flow centrifugal elutriation (Beckman JE5.0 rotor) at 20°C [F. LaBella, P. Gallinari, J. D. McKinney, N. Heintz, *Genes Dev.* **3**, 1982 (1989)]. We used Joklik's modified Eagle's medium to collect and wash the cells. Mitotic cells were produced by inoculating an S-phase population of elutriated cells into culture with Noco (5 ng/ml); after 12 hours the mitotic index was >80%. Cells were prepared for fluorescence-activated cell sorter (FACS) (Becton Dickinson FACScan Flow Cytometer) analysis and determination of mitotic index as described (7). WCE were prepared essentially as for nuclear extracts [N. Heintz and R. G. Roeder, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2713 (1983)], with modifications as described (*12*). Protein concentrations of extracts were typically 10 to 20 mg/ml.
23. Immunoblots were prepared as described [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)]. Aliquots of WCE (40 or 80 μg of protein) were diluted in SDS–polyacrylamide gel electrophoresis sample buffer, boiled 5 min, and fractionated on a 7.5% polyacrylamide gel. The gel was transferred to nitrocellulose at 4°C. Antiserum to Oct-1 was diluted 1:2000 and incubations were for 1 hour at room temperature. Bound antibody was detected with ^{125}I-labeled protein A (Du Pont Biotechnology Systems) and autoradiography.
24. Electrophoretic mobility-shift assays were performed as described [M. Fried and D. M. Carothers, *Nucleic Acids Res.* **9**, 6505 (1981); M. M. Garner and A. Revzin, *ibid.*, p. 3047], with the following modifications: Reactions (20 μl) were done in binding buffer [20 mM Hepes (pH 7.9), 4% w/v Ficoll, 40 mM KCl, and 2.5 mM $MgCl_2$] with labeled oligonucleotide (0.5 ng), poly(dI-dC) (40 μg/ml) and the indicated amount of protein for 20 min at room temperature. Binding reactions were analyzed by electrophoresis in a 6% polyacrylamide gel in 0.25× TBE [0.025 M tris-borate (pH 8.0), 0.5 mM EDTA, and 0.02% NP-40] for 90 min at 120 V. Gels were dried and subjected to autoradiography. Oligonucleotides were synthesized by the Rockefeller University Protein Sequencing Laboratory on a DNA synthesizer (Applied Biosystems). Double-stranded oligonucleotides were end-labeled with [α-^{32}P]ATP and DNA polymerase I Klenow fragment (New England Biolabs). A double-stranded oligonucleotide from the human histone H2B gene, which contained the octamer-binding element (underlined) was used in the electrophoretic mobility-shift assay: dTTCAACTCTTCACCTTATTTGCATAAGCGATTCTACTGCTCGAT.
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26. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
27. Oct-1 was incubated with and without 12 units of bovine heart PKA catalytic subunit (Sigma) and 30 μM ATP in kinase buffer (15 μl) [25 mM MOPS (pH 7.2), 60 mM β-glycerophosphate, 5 mM EGTA, 15 mM $MgCl_2$, 5 mM dithiothreitol, and 100 μM benzamidine] for 20 min at 30°C. Each reaction mixture was assayed (2 μl) for DNA binding activity in an electrophoretic mobility-shift assay with H2B octamer-containing probe (*24*).

28. We thank M. Thelen and A. Aderem for help with the phosphopeptide analysis; J. Philips for isolation of Oct-1 cDNA clones; J. McKinney, L. Dailey, R. Martinelli, and J. C. Courvalin for advice and discussion of the manuscript. Supported by NIH grants GM 13752 (to N.S.) and GM 32544 (to N.H.) and the Howard Hughes Medical Institute.

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