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M-Phase-Specific Phosphorylation of the POU Transcription Factor GHF-1 by a Cell Cycle-Regulated Protein Kinase Inhibits DNA Binding

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GHF-1 is a member of the POU family of homeodomain proteins. It is a cell-type-specific transcription factor responsible for determination and expansion of growth hormone (GH)- and prolactin-expressing cells in the anterior pituitary. It was previously suggested that cyclic AMP (cAMP)-responsive protein kinase A (PKA) phosphorylates GHF-1 at a site within the N-terminal arm of its homeodomain, thereby inhibiting its binding to the GH promoter. These results, however, are inconsistent with the physiological stimulation of GH production by the cAMP pathway. As reported here, cAMP agonists and PKA do not inhibit GHF-1 activity in living cells and although they stimulate the phosphorylation of GHF-1, the inhibitory phosphoacceptor site within the homeodomain is not affected. Instead, this site, Thr-220, is subject to M-phase-specific phosphorylation. As a result, GHF-1 DNA binding activity is transiently inhibited during the M phase. This activity is regained once cells enter G_1, a phase during which GHF-1 phosphorylation is minimal. Thr-220 of GHF-1 is the homolog of the mitotic phosphoacceptor site responsible for the M-phase-specific inhibition of Oct-1 DNA binding Ser-382. As this site is conserved in all POU proteins, it appears that all members of this group are similarly regulated. A specific kinase activity distinct in its substrate specificity and susceptibility to inhibitors from the Cdc2 mitotic kinase or PKA was identified in extracts of mitotic cells. This novel activity could be involved in regulating the DNA binding activity of all POU proteins in a cell cycle-dependent manner.

GHF-1 (Pit-1) is a cell-type-specific transcription factor originally identified by its ability to activate the growth hormone (GH) gene promoter when added to HeLa cell extracts (4). This 33-kDa protein is a member of the POU group of homeodomain proteins (3, 15) and thus contains a DNA binding domain composed of a POU-specific domain and a POU homeodomain (13). While the POU homeodomain is the minimal region required for sequence-specific DNA binding (34), the POU-specific domain contributes to high-affinity binding and participates in sequence recognition (16). A similar mode of DNA interaction is displayed by the Oct-1 POU domain protein (1, 20, 33, 38). Although it has been suggested that GHF-1 homodimerizes in solution and can also form heterodimers with Oct-1 (39, 40), most of these interactions have been found to be DNA dependent (21) and the predominant mode of DNA binding most likely involves cooperative binding of two GHF-1 molecules to each binding site composed of two half sites positioned one helical turn apart from each other, in a head-to-tail manner (reviewed in reference 36). These conclusions are consistent with the three-dimensional structure of the Oct-1 DNA binding domain-octamer site complex (19).

In addition to the GH gene, GHF-1 is responsible for activation of the prolactin (PRL) gene (6, 22, 37) and for autoregulatory activation of its own promoter (27). GHF-1 may also be involved in the activation of the gene encoding the β subunit of thyroid-stimulating hormone (22, 24). All of these genes are expressed exclusively in specific cell types of the anterior pituitary, which are also the site of GHF-1 expression (11, 32). In addition to its role in target gene activation, GHF-1 is required for expansion of committed somatotropes and lactotropes, which synthesize GH and PRL, respectively, during anterior pituitary development (6, 24). Although GHF-1 is necessary for GH and PRL expression (6, 22), it is insufficient for activation of either gene. Most somatotropes express only GH, and most lactotropes express only PRL, yet both cell types express similar amounts of functional GHF-1 (3, 18a). This suggests that additional factors, acting either positively or negatively, are responsible for selective activation of the GH and PRL genes in two related, yet distinct, cell types.

It was suggested that the target gene specificity of GHF-1 may be modified by its phosphorylation, as in vitro phosphorylation by cyclic AMP-responsive protein kinase A (PKA) inhibited the binding of GHF-1 to its high-affinity binding site within the GH promoter and had either a weak inhibitory effect, no effect, or a modest stimulatory effect on its binding to several lower-affinity sites derived from the PRL gene (17). These findings, however, are inconsistent with the known physiological regulation of GH gene expression (reviewed in reference 36). GH production is stimulated by the GH-releasing factor, a hormone which elevates the intracellular level of cyclic AMP in somatotropes (31). The GH-releasing factor stimulates GH transcription, as do cyclic AMP analogs (2), and the *cis* element which mediates this effect overlaps with the GHF-1 binding sites of the GH promoter (8, 9).

Phosphorylation was also found to inhibit the DNA binding activity of another POU protein, Oct-1, and the phosphoacceptor site that mediates this effect, Ser-385, is homologous to the phosphoacceptor site responsible for inhibition of DNA binding by GHF-1, Thr-220 (30). Although in vivo Oct-1 is likely to be phosphorylated by a mitotic protein kinase, in vitro Ser-385 is phosphorylated by PKA (30). Interestingly, this phosphoacceptor site is conserved in all POU domain proteins (17) and is located in the N-terminal arm of the POU homeo-

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domain (19). Since this residue does not appear to be directly involved in contacting the DNA (19), its function is most likely regulatory. The conservation of this residue in all POU proteins suggests that this regulatory function is of general importance. In light of the contradictory effects of PKA on GH gene transcription and on GHF-1 DNA binding activity, we reinvestigated the regulation of GHF-1 phosphorylation. We found that in vitro, the protein can indeed be phosphorylated by PKA at Thr-220 and that phosphorylation of this site inhibits its binding to DNA. However, this inhibition indiscriminately affected the binding of GHF-1 to all of the target sites that were tested. Although activation of PKA in vivo resulted in increased GHF-1 phosphorylation, the inhibitory phosphoacceptor site was hardly affected and GHF-1-dependent transactivation was not inhibited. Rather, we found that like Oct-1, GHF-1 was phosphorylated at Thr-220 only during the M phase, resulting in transient inhibition of its DNA binding activity during that phase. Extracts of mitotic cells contain an activity that phosphorylates GHF-1 at the same sites phosphorylated during the M phase in vivo, including Thr-220. This activity is different from PKA and cyclin-dependent kinases and could be responsible for the mitotic phosphorylation of all POU proteins.

MATERIALS AND METHODS

Cell culture and synchronization. GH3 cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% horse serum and 2.5% fetal calf serum. HeLa and CV1 cells were grown in DMEM plus 10% fetal calf serum. All cell cultures were incubated at 37°C with 5% CO_2.

Exponentially growing cultures of GH3 cells were synchronized at the beginning of the S phase by incubation with 2 mM thymidine for 14 h. Cells were released from this block by three washes with DMEM at 37°C and placed back into complete medium. When populations enriched in mitotic cells were desired, 5 h after release of the thymidine block, cell layers were extensively washed with DMEM at 37°C to remove unattached cells, nocodazole (40 ng/ml) was added, and the cells were incubated for 3 h. Mitotic cells were then collected by rocking and gentle washing of the plates with medium without detachment of the cell monolayer. A similar synchronization protocol was applied to HeLa cells, except that nocodazole was added 8 h after release of the thymidine block.

Flow cytometric analysis was performed with samples of 10^6 cells. Cells were pelleted, suspended dropwise in 300 μl of ice-cold ethanol while vortexing, washed twice with phosphate-buffered saline (PBS), suspended in 850 μl of PBS containing 5 μg of RNase A per ml, and incubated for 30 min at room temperature. Cells were stained by adding 125 μl of a propidium iodide solution (50 μg of propidium iodide per ml in 50 mM sodium citrate [pH 7.2], 0.1% Triton X-100) and analyzed on a Becton Dickinson FACScan flow cytometer by using Lysis II software.

Protein preparation and electrophoretic mobility shift assays. A *Hin*dIII-*Not*I fragment from the plasmid RSV-GHF-1 (35) was subcloned into pBluescript (SK−) to generate a pBS-GHF-1 construct. Single-stranded DNA from this construct was used as a template for oligonucleotide-directed in vitro mutagenesis with the Amersham kit. The oligonucleotides used to convert Ser-115 and Thr-220 into alanine residues were 5′-CCACCAATTTAGCTTTCCGCCTG-3′ and 5′-CGATACTGATAGCTGTCCTCCGT-3′, respectively. Point mutations were confirmed by DNA sequencing. *Bam*HI fragments from pREP9-GHF-1 derivatives, corresponding to either wild-type GHF-1 or the Ala-115, Ala-220, and Ala-115-220 mutants (see below), were inserted in frame into expression vector pRSETA (Invitrogen). These constructs were used to express and purify, through a ProBond resin (Invitrogen), recombinant polyhistidine-tagged GHF-1, as well as its mutant versions, under nondenaturing conditions by following the manufacturer's suggestions.

Whole-cell GH3 extracts were prepared from PBS-washed cell pellets suspended in NPB+PI (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.9], 140 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 1 mM dithiothreitol, 20 mM β-glycerophosphate, 100 μM sodium orthovanadate, 5 mM NaF, 2 mM sodium molybdate, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 100 μM benzamidine) (100 μl/5 × 10^6 cells). After 10 min on ice, an equal volume of 2× DC (20 mM HEPES-NaOH [pH 7.9], 50% glycerol, 840 mM NaCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM dithiothreitol, 20 mM β-glycerophosphate, 100 μM sodium orthovanadate, 5 mM NaF, 2 mM sodium molybdate, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 100 μM benzamidine) was added and the samples were rotated at 4°C for 30 min. The extracts were cleared by 10 min of centrifugation at 14,000 rpm and 4°C in a

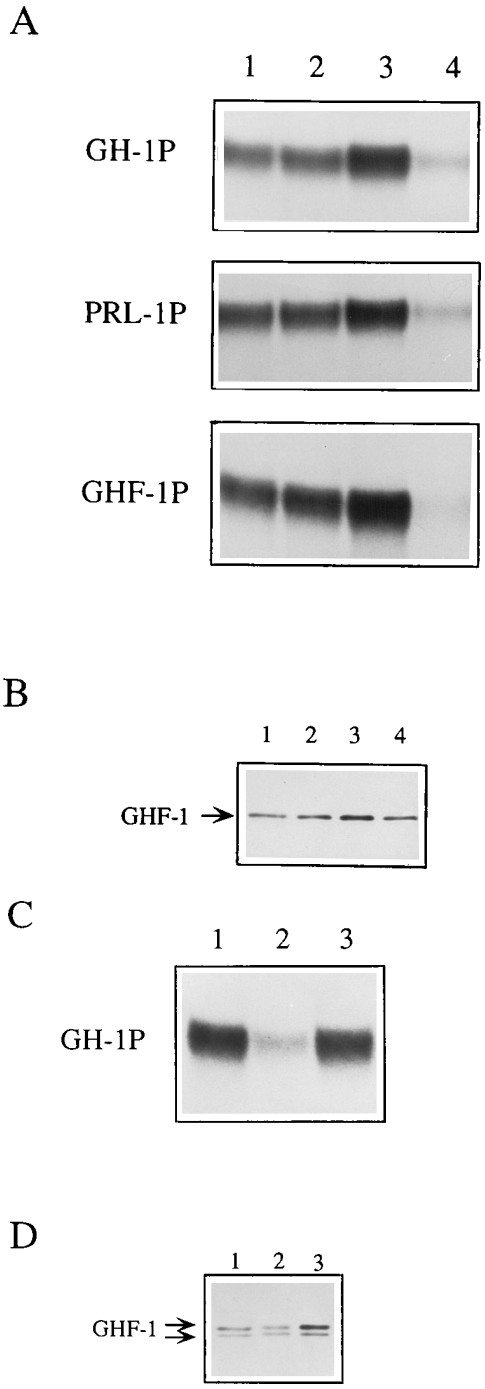

FIG. 1. The DNA binding activity of GHF-1 is cell cycle regulated. (A) Electrophoretic mobility shift assays performed with GH3 whole-cell extracts. Lanes: 1 and 2, exponentially growing cultures either untreated or treated with nocodazole for 3 h, respectively, prior to extract preparation; 3 and 4, mitotic cells isolated by the shake-off procedure (lane 4) after culture treatment with nocodazole and nonmitotic cells that remained attached to the plate (lane 3). Radiolabeled probes corresponding to high-affinity GHF-1 binding sites from the *GH* (GH-1P), *PRL* (PRL-1P), and *GHF-1* (GHF-1P) promoters were used. (B) Immunoblot analysis done by using an anti-GHF-1 antibody to quantitate the amount of GHF-1 present in the extracts tested in panel A (10 μg of total extract protein per lane). (C) Electrophoretic mobility shift assay performed with extracts prepared from either exponentially growing GH3 cells (lane 1), mitotic GH3 cells (lane 2) obtained by shaking off of nocodazole-treated cells, or mitotic GH3 cells that were replated and harvested 3 h later (lane 3). The GH-1P site was used as a probe. (D) The amount of GHF-1 present in the extracts (10 μg each) used for panel C was determined by immunoblot analysis with an anti-GHF-1 antibody.

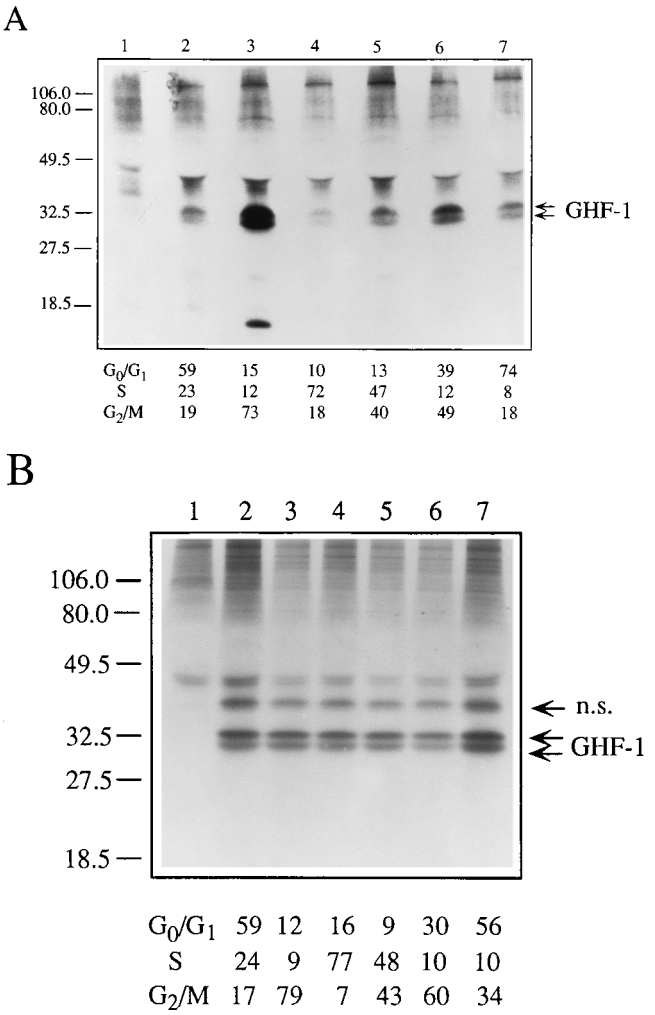

FIG. 2. Phosphorylation of GHF-1 increases in mitotic cells. (A) Immunoprecipitation of GHF-1 phosphorylated in vivo. Radioimmunoprecipitation assay lysates of GH3 cells (10^7 cells per sample) that were pulse-labeled for 1 h with $^{32}P_i$ prior to harvesting were immunoprecipitated with either preimmune serum (lane 1) or anti-GHF-1 serum (lanes 2 to 7). The cells analyzed in lanes 1 and 2 were exponentially growing. The cells in lanes 3 to 7 were first synchronized at the G_1-S interphase by a thymidine block. In lane 3, 5 h after release from the block, the cells were treated with nocodazole for 3 h prior to labeling. In lanes 4 to 7, the cells were labeled at 3, 6, 9, and 12 h after release of the thymidine block, respectively. The immunoprecipitates were separated by SDS–12% PAGE, transferred to nitrocellulose, and autoradiographed. The GHF-1-specific bands are indicated by the arrows. Unlabeled samples of cells subjected to the same treatments were analyzed on a FACScan, and the cell cycle distribution of each population (based on DNA content) is shown in percentages at the bottom. The numbers to the left are molecular sizes in kilodaltons. (B) Immunoprecipitation of ^{35}S-labeled GHF-1 from GH3 cells that were treated as described above and GH3 cells that were pulse-labeled for 1 h with Trans-^{35}S-label before harvesting. Lanes: 1, immunoprecipitation with preimmune serum; 2 to 7, immunoprecipitation with anti-GHF-1 serum. Following separation by SDS-PAGE, labeled proteins were detected by fluorography. The results of FACScan analyses performed with unlabeled samples of cultures subjected to the same treatments in parallel with the labeled cultures are indicated at the bottom. The GHF-1 bands are indicated by the arrows. n.s., nonspecific bands. The numbers to the left are molecular sizes in kilodaltons. (C) Tryptic phosphopeptide maps of GHF-1 isolated from ^{32}P-labeled GH3 cells. Exponentially growing (EG) and mitotic-cell-enriched (M) populations of GH3 cells were labeled with $^{32}P_i$ for 1 h prior to harvesting. GHF-1 was isolated as described for panel A, and its ^{32}P-labeled bands (seen in lanes 2 and 3 of panel A) above were subjected to phosphopeptide mapping. The amount of radioactivity analyzed was adjusted to reflect equal cell numbers. Repeatedly detected phosphopeptides (seen in three different experiments) are numbered. The origins of the maps are marked by arrowheads.

microcentrifuge, and the protein concentration was determined by the Bradford assay.

Electrophoretic mobility shift assays were performed with 1 to 5 ng of recombinant protein or 1 μg of total extract protein and the probes previously described (35).

Immunoblotting and antibodies. GHF-1 was detected in GH3 extracts (10 μg per lane) by immunoblotting as previously described (35). Two different rabbit anti-GHF-1 sera were used in this study. One has already been described (3). A second rabbit anti-GHF-1 serum was generated against a peptide comprising residues 274 to 285 of rat GHF-1. Similar results were obtained with both antisera.

Metabolic labeling and immunoprecipitation. GH3 cells were incubated in dialyzed fetal calf serum (5%)-supplemented, methionine-free, or phosphate-free DMEM for 30 min before labeling. Cells were labeled for 1 h with Trans-^{35}S-label (ICN) (200 μCi/ml) or $^{32}P_i$ (1 mCi/ml), respectively. After labeling, plates were placed on ice and washed once with ice-cold PBS. One milliliter of radioimmunoprecipitation assay buffer (5) was added, and the plates were incubated on ice for 30 min. Radioimmunoprecipitation assay lysates were clarified by centrifugation and immunoprecipitated with an excess of antibody as previously described (5). Protein A-Sepharose immune complexes were washed three times with radioimmunoprecipitation assay buffer and fractionated by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE). When labeling was performed with $^{32}P_i$, after electrophoresis, the proteins were electroblotted onto a nitrocellulose filter to continue with the phosphopeptide mapping (see below).

Tryptic phosphopeptide mapping. In vivo- and in vitro-phosphorylated GHF-1 was fractionated by SDS–12% PAGE, transferred to nitrocellulose by electroblotting, and autoradiographically detected. Radiolabeled GHF-1 bands were excised and digested with trypsin as previously described (25). The resulting phosphopeptides were collected and resolved by electrophoresis with pH 1.9 buffer, followed by chromatography in phosphochromatography buffer (5).

Transfections. *Hin*dIII-*Not*I fragments from pBS-GHF-1 and its Ala-115, Ala-220, and Ala-115-220 mutant derivatives were inserted into the pREP9 mammalian expression vector (Invitrogen) to generate pREP9-GHF-1(WT) and its mutant derivatives. CV-1 cells on 35-mm-diameter plates were cotransfected by using Lipofectamine (Gibco/BRL) with 100 ng of a −422PRL-Luc (15) or −312GH-Luc (35) reporter, along with 100 ng of pREP9 or the respective pREP9-GHF-1 expression vector. Cells were incubated with 10^{-5} M forskolin or with ethanol (vehicle control) 33 h after transfection and harvested 3 h later to determine luciferase activity.

In vitro kinase assays. Extracts (50 μg per reaction) of whole HeLa cells prepared as described above were used to phosphorylate 200 μg of either wild-type or Ala-115 or Ala-220 mutant recombinant polyhistidine–GHF-1. The reactions were performed in a 100-μl volume containing 20 mM HEPES-NaOH (pH 7.6), 15 mM $MgCl_2$, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM EDTA, 60 mM β-glycerophosphate, 5 mM dithiothreitol, 100 μM benzamidine, 20 μM ATP, and 5 μCi of [γ-^{32}P]ATP and incubated for 30 min at 30°C. The recombinant polyhistidine proteins were isolated on Probond resin and, after elution with SDS-PAGE sample buffer, fractionated by SDS-PAGE and autoradiographed. PKA phosphorylation reactions were performed in the same buffer as above with 100 ng of substrate. Lactoglobulin (1 μg per reaction) was added as a carrier. Once the reaction was concluded, the proteins were ethanol precipitated in the presence of 5 μg of bovine serum albumin and analyzed by SDS-PAGE and autoradiography as described above. Alternatively, after phosphorylation, the DNA binding activity of recombinant GHF-1 proteins was analyzed by electrophoretic mobility shift assay.

RESULTS

The DNA binding activity of GHF-1 is cell cycle regulated. Previous findings suggest that GHF-1 is required for prolifer-

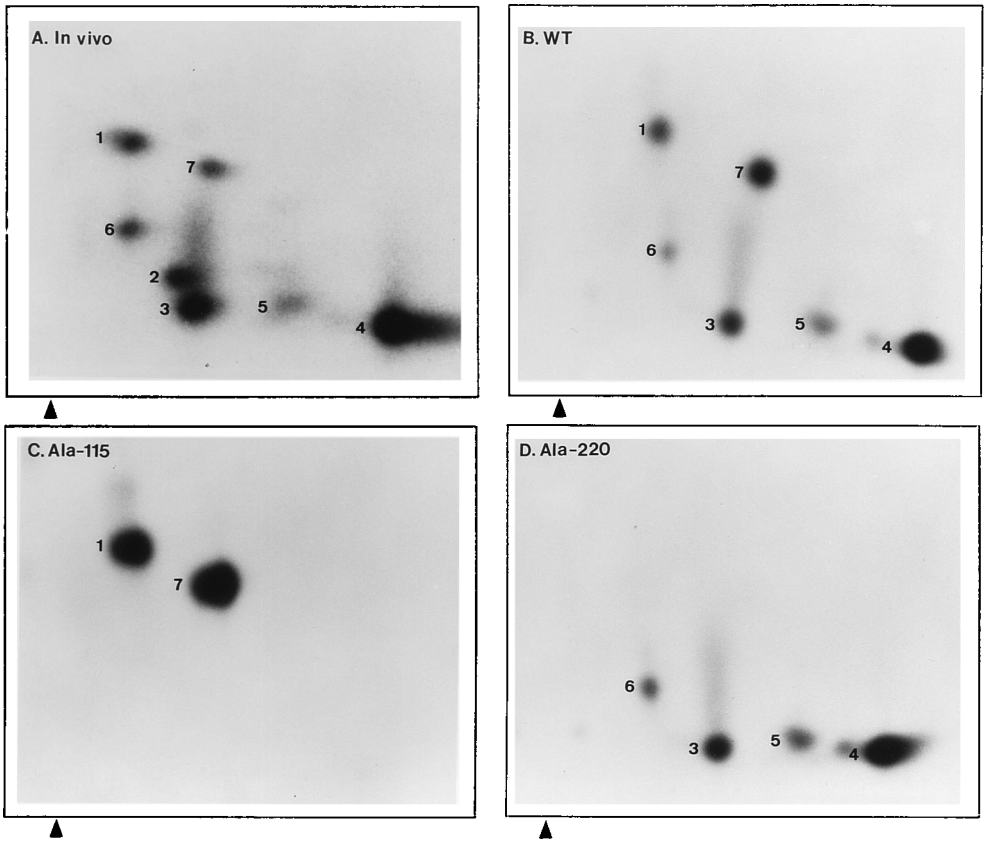

FIG. 3. GHF-1 is phosphorylated at Ser-115 and Thr-220 in mitotic cells. Tryptic phosphopeptide maps of in vivo-phosphorylated GHF-1 isolated from GH3 cultures that were enriched in mitotic cells (A) and PKA-phosphorylated recombinant wild-type (WT) GHF-1 (B), GHF-1(Ala-115) (C), and GHF-1(Ala-220) (D). Samples of tryptic digests of all four proteins were analyzed in parallel. Equivalent phosphopeptides are numbered as in Fig. 2C. The origin of each map is marked by an arrowhead.

ation of somatotropic and lactotropic cells (6, 22). This possible involvement in cell proliferation suggested that the DNA binding activity of GHF-1 is cell cycle regulated, as previously shown for Oct-1 (29, 30). We therefore used different protocols to synchronize GH-synthesizing GH3 cells at different phases of the cell cycle. Whole-cell extracts were prepared, and GHF-1 DNA binding activity was measured by an electrophoretic mobility shift assay. These experiments revealed that GHF-1 DNA binding activity was dramatically inhibited during the M phase (Fig. 1A, lane 4). The degree of inhibition was not influenced by the nature of the target site used to measure GHF-1 binding. As enrichment for mitotic cells involved treatment with the antimicrotubule drug nocodazole, we examined the effect of the drug itself on GHF-1 DNA binding. A 3-h incubation of exponentially growing cells with nocodazole did not affect GHF-1 binding activity (lane 2), and even prolonged incubation with the drug did not inhibit GHF-1 binding activity in those cells that remained attached to the plate (lane 3). A similar inhibition of GHF-1 DNA binding was observed in mitotic cells selected by the shake-off procedure (37) without drug treatment (data not shown). Immunoblotting of cell extracts revealed that the inhibition of GHF-1 DNA binding activity was not due to degradation of the protein (Fig. 1B). The inhibition of DNA binding activity was reversible, and 3 h after replating of mitotic cells, GHF-1 binding activity was restored nearly to the basal level (Fig. 1C and D). Similar results were obtained with other GHF-1-expressing cells, including GC, 235, and GHFT-1 cells, regardless of whether they express GH, PRL, or neither (data not shown). We also observed inhibition of Oct-1 and Oct-2 DNA binding activities in mitotically enriched populations of B-JAB cells (unpublished results).

Phosphorylation of GHF-1 increases in the M phase. The inhibition of GHF-1 DNA binding during mitosis resembles the mitotic inhibition of Oct-1 activity (29). As the mitotic inhibition of Oct-1 activity is due to its phosphorylation (30), we examined the cell cycle dependence of GHF-1 phosphorylation. GH3 cells that were synchronized at the G_1-S-phase boundary by a thymidine block were allowed to progress through the cell cycle after removal of the drug. To enrich for M-phase cells, 5 h after thymidine removal the cells were treated with nocodazole for 3 h. A portion of the cells were removed for flow-cytometric analysis of their DNA content, and the rest were labeled with $^{32}P_i$. As shown in Fig. 2A, the level of GHF-1 phosphorylation was lowest at the beginning of the S phase (lane 4) and then began to increase as cells progressed through the cycle and reached the M phase (lanes 5 and 6). After the majority of the cells had traversed the M phase, the level of GHF-1 phosphorylation declined (lane 7). In the population highly enriched in mitotic cells (lane 3), the level of GHF-1 phosphorylation was at least 10-fold higher than in nonsynchronized cells (lane 2). A similar analysis, in which [^{35}S]methionine, instead of $^{32}P_i$, was used to label GHF-1, indicated that its rate of synthesis was fairly uniform throughout the cell cycle (Fig. 2B).

Two-dimensional tryptic phosphopeptide mapping indicated

that not only was the level of GHF-1 phosphorylation higher in mitotic cells than in nonsynchronized cells, but also its pattern was different (Fig. 2C). While four tryptic phosphopeptides, labeled 1 to 4, were detected in GHF-1 isolated from nonsynchronized cells, the same four and three additional tryptic phosphopeptides were detected in M-phase cells. While the intensity of phosphopeptide 2 was relatively invariant, the intensities of the signals generated by phosphopeptides 1, 3, and especially 4 were higher in mitotic cells. Tryptic phosphopeptide mapping of GHF-1 isolated from a population enriched in early S-phase cells indicated that phosphopeptide 2 was the predominant species (data not shown). As the cells progressed through the cycle and the population became enriched in mitotic cells, the levels of the other phosphopeptides gradually increased (data not shown). Similar results were obtained when two different anti-GHF-1 antibodies were used and when the level of each phosphopeptide was normalized to the amount of protein analyzed instead of the cell number (data not shown). Phosphoamino acid analysis of GHF-1 isolated from exponentially growing or mitotic cells revealed the presence of only phosphoserine and phosphothreonine (data not shown).

GHF-1 is phosphorylated at Ser-115 and Thr-220 during mitosis. Both GHF-1 and Oct-1 can be phosphorylated in vitro by PKA on sites that inhibit their binding to DNA (17, 30). In Oct-1, the PKA phosphorylation sites are identical to the mitotic phosphorylation sites (30). Since our phosphopeptide maps of GHF-1, isolated from either exponentially growing or mitotic cells, seemed different from those of Kapiloff et al. (17), we used our conditions to map GHF-1 phosphorylated in vitro by PKA. We found that every single phosphopeptide present in GHF-1 phosphorylated in mitotic cells, with the exception of phosphopeptide 2, was also present in GHF-1 phosphorylated by PKA (Fig. 3A and B). Even the relative intensities of the different phosphopeptides were, by and large, similar in the two preparations of phospho-GHF-1.

PKA has been reported to phosphorylate GHF-1 on Ser-115 and Thr-220 (17). To confirm these results, we expressed and purified two variant GHF-1 proteins, GHF-1(Ala-115) and GHF-1(Ala-220). Both proteins were phosphorylated by PKA and subjected to phosphopeptide mapping. While GHF-1(Ala-115) lacked phosphopeptides 3, 4, 5, and 6, GHF-1(Ala-220) contained these phosphopeptides but lacked phosphopeptides 1 and 7 (Fig. 3C and D). These results were also supported by phosphoamino acid analysis, which revealed the presence of phosphothreonine in spots 1 and 7 and phosphoserine in spots 3, 4, 5, and 6 (data not shown). Collectively, these results indicate that PKA phosphorylated GHF-1 at Ser-115 and Thr-220. These are also the sites subject to phosphorylation during the M phase. The presence of multiple phosphopeptides corresponding to each of these phosphorylation sites is due to partial tryptic digestion caused by the several adjacent positively charged residues that flank these sites.

Next we compared the effect of PKA on the DNA binding activity of wild-type GHF-1 to its effect on GHF-1(Ala-115) and GHF-1(Ala-220). While the binding of wild-type GHF-1 and GHF-1(Ala-115) to three different target sites derived from either the *GH*, *PRL*, or *GHF-1* gene was inhibited by PKA-mediated phosphorylation, the DNA binding activity of GHF-1(Ala-220) was resistant to inhibition (Fig. 4). Therefore, as previously concluded by Kapiloff et al. (17), phosphorylation at Thr-220 inhibits the binding of GHF-1 to DNA. However, unlike those authors, we found that PKA phosphorylation inhibited the binding of GHF-1 to both *GH*- and *PRL*-derived binding sites indiscriminately.

A mitotic kinase phosphorylates GHF-1 in vitro. The results

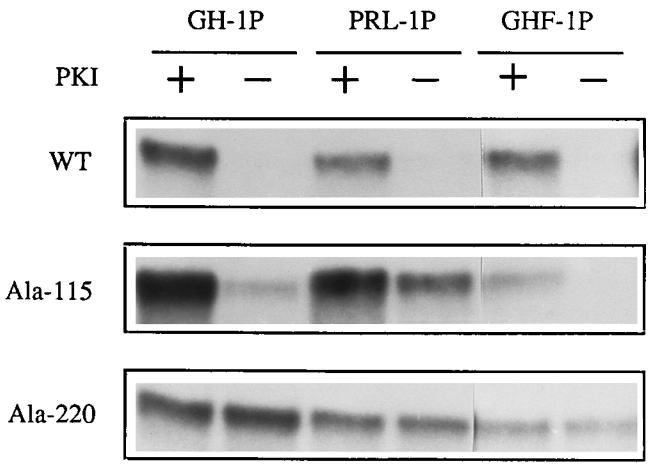

FIG. 4. Phosphorylation of Thr-220 inhibits the binding of GHF-1 to its target sites. Electrophoretic mobility shift assays were performed with recombinant wild-type (WT) GHF-1 and the GHF-1(Ala-115) and GHF-1(Ala-220) variants. The proteins were subjected to an in vitro phosphorylation by PKA in the presence or absence of PKI, as indicated, prior to analysis of DNA binding. In the reactions carried out in the absence of PKI, this peptide was added once the phosphorylation reaction was completed. Oligonucleotides containing the high-affinity GHF-1 binding sites of the *GH* (GH-1p), *PRL* (PRL-1p), and *GHF-1* (GHF-1P) promoters were used as probes.

described so far indicate that in vitro GHF-1 is phosphorylated by PKA on the same sites that are phosphorylated in vivo during the M phase, with a sole exception (the site that accounts for phosphopeptide 2). To investigate the relationship between the mitotic GHF-1 kinase and PKA, we prepared extracts of either nonsynchronized, exponentially growing or M-phase-enriched whole HeLa cells. HeLa cells are a legitimate source of this activity, as exactly the same mode of regulation observed for GHF-1 is exhibited by Oct-1 in these cells (29, 30). Either wild-type GHF-1 or GHF-1(Ala-115-220) was used as the substrate. As shown in Fig. 5A, neither protein was efficiently phosphorylated by the nonsynchronized cell extract, whereas wild-type GHF-1, but not the variant GHF-1(Ala-115-220), was efficiently phosphorylated by the mitotic extract. Immunoblot analysis demonstrated that the lack of GHF-1 phosphorylation by the nonsynchronized cell extract is not due to its degradation by this extract (Fig. 5A, bottom). Tryptic phosphopeptide mapping of GHF-1 phosphorylated by the mitotic cell extract indicated that the pattern of phosphorylation was identical to that obtained with PKA (Fig. 5B). Therefore, the mitotic kinase phosphorylates GHF-1 in vitro on the same sites that are phosphorylated during the M phase in vivo and has substrate specificity similar to that of PKA.

Next we examined whether the GHF-1 kinase activity present in the mitotic extract is sensitive to inhibition by protein kinase inhibitor (PKI), a specific inhibitor of PKA (41). While addition of PKI to the phosphorylation reaction inhibited the phosphorylation of GHF-1 by either PKA alone (90% inhibition) or by PKA mixed with the mitotic extract (80% inhibition), it had very little effect, if any, on the phosphorylation of GHF-1 by the kinase activity present in the mitotic extract (Fig. 5C, compare lanes 1 and 2 to lanes 5 and 6).

PKA does not inhibit GHF-1 activity in vivo. To examine whether activation of PKA in vivo has any effect on GHF-1 activity, we cotransfected CV-1 cells with a GHF-1 expression vector and two GHF-1-dependent reporters, −312GH-Luc and −422PRL-Luc. After transfection, the cells were incubated in the absence or presence of forskolin, an activator of

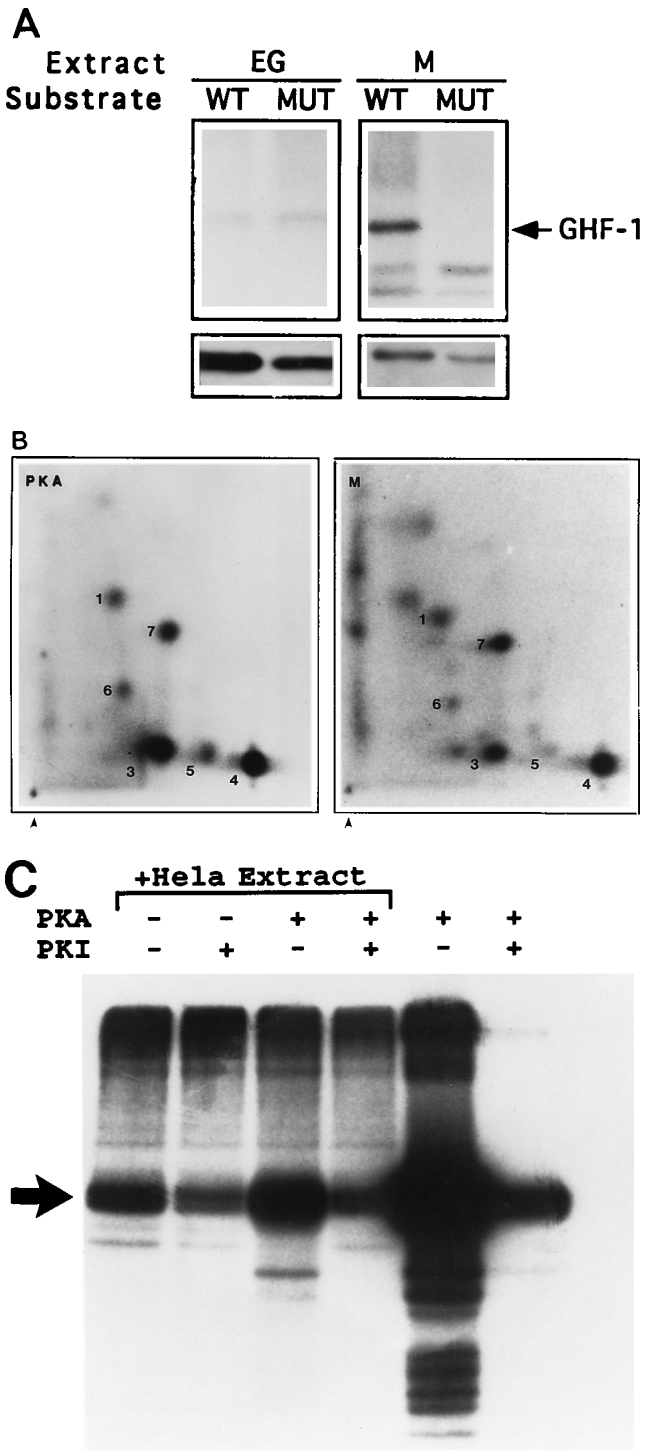

FIG. 5. GHF-1 kinase activity is enriched in mitotic cell extracts and is not inhibited by PKI. (A) In vitro phosphorylation of wild-type GHF-1 (WT) and GHF-1(Ala-115-220) (MUT) using extracts of either exponentially growing (EG) or mitotic-cell-enriched (M) populations of HeLa cells. After the phosphorylation reactions were terminated, recombinant GHF-1 proteins were isolated on Probond beads, fractionated by SDS-PAGE, and electroblotted to a nitrocellulose membrane. Protein phosphorylation was detected by autoradiography (top), and the total amount of recovered GHF-1 protein was determined by immunoblotting. (B) Tryptic phosphopeptide maps of recombinant wild-type GHF-1 phosphorylated by either PKA or the mitotic HeLa cell extract (M). The origin of each map is indicated by the arrowhead. (C) Recombinant wild-type GHF-1 was phosphorylated in vitro by either a mitotic HeLa cell extract (lanes 1 and 2), a mitotic-cell extract supplemented with PKA (lanes 3 and 4), or PKA alone (lanes 5 and 6) in the absence or presence of PKI, as indicated. After isolation of recombinant GHF-1 with Probond beads, its phosphorylation was analyzed by SDS-PAGE and autoradiography. The GHF-1 band is indicated by the arrow.

adenylyl cyclase that is known to stimulate GH promoter activity in pituitary cells (9). Instead of inhibiting GHF-1 activity, forskolin stimulated the activation of both the *GH* and *PRL* promoters (Fig. 6). This stimulation, however, did not involve phosphorylation of GHF-1, as the transcriptional activity of the variant GHF-1(Ala-115-220) was also stimulated.

To examine whether activation of PKA stimulates the phosphorylation of GHF-1 in GH3 cells, we incubated ^{32}P-loaded cells with forskolin, nocodazole, or ethanol (vehicle control) and isolated GHF-1 by immunoprecipitation. SDS-PAGE analysis and autoradiography indicated that forskolin treatment stimulated GHF-1 phosphorylation almost as efficiently as incubation of cells with nocodazole (data not shown). However, when equal amounts of ^{32}P-labeled GHF-1 isolated from either forskolin- or nocodazole-treated GH3 cells were digested with trypsin and subjected to two-dimensional phosphopeptide analysis, it was clear that forskolin did not stimulate the phosphorylation of Thr-220 (Fig. 7). While digestion of ^{32}P-labeled GHF-1 isolated from nocodazole-treated cells gave rise to all of the tryptic phosphopeptides seen before, including phosphopeptides 1 and 7, which denote phosphorylation of Thr-220, ^{32}P-labeled GHF-1 recovered from forskolin-treated cells was deficient in phosphopeptides 1 and 7. In fact, the phosphopeptide map of GHF-1 from forskolin-stimulated cells was very similar to that of GHF-1(Ala-220) phosphorylated in vitro by PKA (Fig. 3). Thus, although forskolin treatment stimulates the phosphorylation of GHF-1, Thr-220 is not one of the affected sites.

DISCUSSION

As shown in this report, phosphorylation of GHF-1 at Thr-220 inhibits its binding to DNA. However, by contrast to a previous report (17) but consistent with the physiological control of GH expression, activation of PKA does not cause the phosphorylation of GHF-1 at Thr-220. Instead, this site, responsible for inhibition of DNA binding, is phosphorylated by a mitosis-specific kinase. Interestingly, Thr-220 is highly conserved among members of the POU group of homeodomain proteins, which contain either Ser or Thr at this position. Indeed, phosphorylation of Oct-1, another POU protein, at Ser-385, the counterpart of Thr-220 of GHF-1, also inhibits DNA binding (30). This residue is located in the N-terminal arm of the POU homeodomain (Ser-7 in the Oct-1 DNA binding domain, whose structure was solved by Klemm et al. [19]). Although Ser-385 is not in direct contact with the DNA, residues located next to it contact the phosphates on the DNA or make hydrogen bonds to phosphodiester oxygens (19). Therefore, phosphorylation of this residue is likely to interfere with DNA binding through electrostatic repulsions, a mechanism similar to that proposed for inhibition of c-Jun binding to DNA through phosphorylation of residues located next to its basic region (5, 14, 23). Alternatively, phosphorylation of this critical Ser or Thr residue may alter the conformation of the N-terminal arm and disrupt its interaction with the minor groove of the recognition site. By contrast to Kapiloff et al. (17), we found that phosphorylation of GHF-1 at Thr-220 indiscriminately inhibited its binding to all of the target sites tested, including sites such as PRL-1P and GHF1-1P (referred to as PB-1 in reference 17), with which those authors observed no effect of phosphorylation on binding activity. The indiscrim-

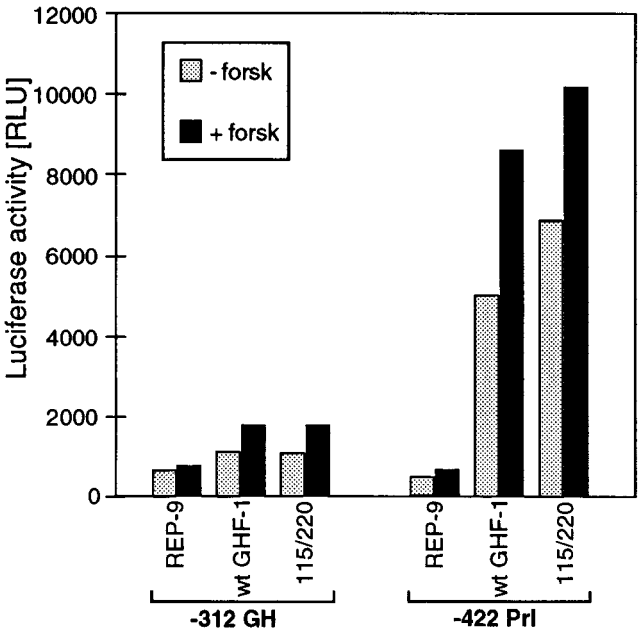

FIG. 6. PKA activation does not inhibit GHF-1 transcriptional activity. CV-1 cells were cotransfected with either the −312GH-Luc or the −422PRL-Luc reporter and either an empty expression vector (pREP-9) or an expression vector encoding wild-type (wt) GHF-1 or a mutant lacking PKA phosphorylation sites, GHF-1(Ala-115-220). After 33 h, the cells were incubated with either ethanol (vehicle control) or 10 μM forskolin (forsk) in ethanol. Cell extracts were prepared 3 h later, and luciferase activity was determined. RLU, relative light units.

inate inhibition of DNA binding by phosphorylation at Thr-220 or its equivalent is entirely consistent with the important role of the N-terminal arm in DNA binding (19) and the similar effect of Ser-385 phosphorylation on binding of Oct-1 to its target sites (30).

Another similarity between GHF-1 phosphorylation and Oct-1 phosphorylation is their cell cycle dependence. Phosphorylation of Oct-1 at Ser-385 occurs as cells enter the M phase and is reversed as cells reenter the G_1 phase (29, 30). Likewise, phosphorylation of GHF-1 at Thr-220 occurs upon entry into the M phase, while reentry into G_1 seems to be associated with dephosphorylation of the site (Fig. 2). The protein kinase that is responsible for Oct-1 phosphorylation was not characterized. However, its mitotic phosphorylation site is phosphorylated by PKA in vitro and therefore the mitotic kinase was proposed to be a PKA-like enzyme (30). While the mitotic kinase that phosphorylates GHF-1 and Oct-1 recognizes sites that are also recognized by PKA in vitro (i.e., a Ser or Thr residue preceded by several positively charged residues; 18), we provide evidence suggesting that this protein kinase is distinct from PKA. While PKA activity is inhibited by the specific peptide inhibitor PKI (41), the mitotic kinase that phosphorylates GHF-1 (and presumably also Oct-1) in vitro is refractory to this inhibitor (Fig. 5). Since the pattern of regulation and the substrate specificity of this in vitro activity are identical to those of the mitotic kinase acting on GHF-1 and Oct-1 in vivo, we presume that the two activities are the same. We also found that activation of PKA in living cells does not result in increased phosphorylation of Thr-220, although phosphorylation of other sites on GHF-1 is stimulated. It has not been reported that PKA can phosphorylate Oct-1 in vivo either or inhibit the activation of an Oct-1-dependent reporter gene.

Is the mitotic protein kinase activity that phosphorylates the POU homeodomain related to previously identified mitotic kinases? The best characterized mitotic protein kinase is Cdc2 (reviewed in references 12 and 28), which, like other members of its family, recognizes a Ser or Thr residue followed by a Pro at the P+1 position while the P+2 position shows preference for a positively charged residue. The Pro at the P+1 position is strictly required for phosphorylation by Cdc2 or other cyclin-dependent kinases (12, 18). However, the mitotic phosphorylation sites within the POU domains of GHF-1 and Oct-1 do not contain Pro at P+1. Thus, it is very unlikely that Cdc2 or a related activity is responsible for phosphorylation of these proteins. In agreement with these conclusions, GHF-1 was not phosphorylated by Cdc2 in vitro (5a). Three sites that are phosphorylated late in the M phase were detected on glial fibrillary acidic protein, a component of intermediate filaments. These sites are phosphorylated in vitro by PKA but not by protein kinase C (26). Thus the glial fibrillary acidic protein may be phosphorylated by the same mitotic kinase that phosphorylates the POU proteins.

The strict conservation of the mitotic phosphorylation site in the POU protein suggests that it is of structural or regulatory importance. Since the phosphoaccepting Ser residue is not directly involved in contacting the octamer site (19), it is more likely to have a regulatory role. A possible function for the mitotic phosphorylation of POU proteins is their removal from the DNA (chromatin) as cells enter mitosis. Indeed, analysis of GH3 cells arrested early in mitosis by indirect immunofluorescence indicates that most of GHF-1 has been displaced from the condensing chromatin (18a). As suggested by Segil et al. (30), such displacement is likely to be involved in the general transcriptional inhibition that occurs during the M phase (10). In addition to their well-established role in transcription, POU proteins may also be involved in cellular DNA replication. At least one protein, Oct-1, plays a critical role in assembly of the initiation complex at the origin of adenovirus replication (7). Interference with GHF-1 expression inhibits the proliferation of pituitary cells in vitro (6), and its inactivation through mutations prevents the expansion of somatotropic-lactotropic progenitors in vivo (6, 24), suggesting some involvement in cell proliferation and, possibly, DNA replication. In that case, the mitotic phosphorylation of such proteins may be responsible for disassembly of origin recognition complexes. Reentry into the G_1 phase is associated with rapid dephosphorylation of the mitotic phosphorylation sites, thus allowing POU proteins to resume their transcriptional activation role, as well as their putative replicative functions.

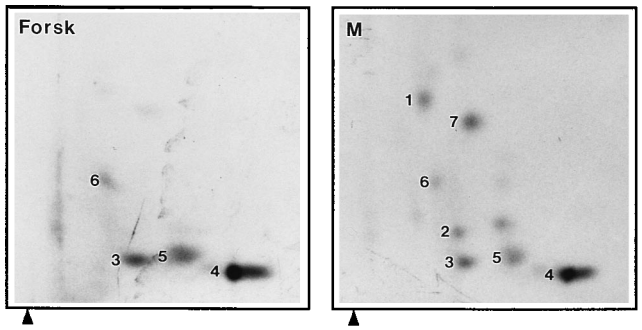

FIG. 7. PKA activation does not result in phosphorylation of Thr-220. ^{32}P-labeled GHF-1 was isolated from $^{32}P_i$-labeled GH3 cells treated with either forskolin (Forsk; 10 μM for 30 min) or a thymidine block followed by nocodazole (40 ng/ml for 3 h) prior to harvesting. Equal amounts of ^{32}P-labeled protein were digested with trypsin and subjected to two-dimensional analysis as described in the legend to Fig. 3. An arrowhead indicates the origin of each map.

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