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Follicle-stimulating Hormone Stimulates Protein Kinase A-mediated Histone H3 Phosphorylation and Acetylation Leading to Select Gene Activation in Ovarian Granulosa Cells*

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We examined the phosphorylation and acetylation of histone H3 in ovarian granulosa cells stimulated to differentiate by follicle-stimulating hormone (FSH). We found that protein kinase A (PKA) mediates H3 phosphorylation on serine 10, based on inhibition exclusively by PKA inhibitors. FSH-stimulated H3 phosphorylation in granulosa cells is not downstream of mitogen-activated protein kinase/extracellular signal-regulated kinase, ribosomal S6 kinase-2, mitogen- and stress-activated protein kinase-1, p38 MAPK, phosphatidylinositol-3 kinase, or protein kinase C. Transcriptional activation-associated H3 phosphorylation on serine 10 and acetylation of lysine 14 leads to activation of serum glucocorticoid kinase, inhibin α, and c-*fos* genes. We propose that phosphorylation of histone H3 on serine 10 by PKA in coordination with acetylation of H3 on lysine 14 results in reorganization of the promoters of select FSH responsive genes into a more accessible configuration for activation. The unique role of PKA as the physiological histone H3 kinase is consistent with the central role of PKA in initiating granulosa cell differentiation.

Maturation of ovarian follicles to a preovulatory stage requires follicle-stimulating hormone (FSH)[1] production by the pituitary gland. The FSH receptor is a member of the G protein-coupled seven-transmembrane receptor family and is coupled to adenylyl cyclase (1). It is expressed exclusively on ovarian granulosa cells in female mammals (2). Most of the actions of FSH are mediated by cAMP formation and activation of protein kinase A (PKA), based on the ability of cell-permeable cAMP analogs to mimic the known differentiation responses to FSH in granulosa cells (2) and on the ability of the PKA inhibitors H89[2] (3) and KT5720[2] to inhibit granulosa cell differentiation. The downstream consequences of FSH are well established and include, for example, the induction of receptors for luteinizing hormone (LH) and prolactin, induction of enzymes associated with the increased steroidogenic capacity of granulosa cells including P450 aromatase and cholesterol side chain cleavage, induction of proteins associated with PKA signaling including RIIβ (2, 4) and AKAP80 (5), and expression of the hormone inhibin (6). However, gene and/or protein induction for these responses to FSH is generally delayed by at least 24 h (2–4, 7). The more immediate responses to FSH, which lead to the induction of immediate early genes such as c-*fos* and serum glucocorticoid kinase (SGK) (3, 8), are less well understood. Further elucidation of the FSH signaling pathways that lead to the induction of immediate early genes would be useful to understand how FSH initiates granulosa cell differentiation. We have previously shown that FSH (via PKA) promotes activation of the p42/p44 mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (9). FSH also activates the p38 MAPK pathway (10) and downstream phosphorylation of the heat shock protein (HSP) 27, leading to granulosa cell rounding (10). We[3] and others (11) have recently identified phosphatidylinositol 3-kinase (PI-3 kinase) as a downstream target of FSH and cAMP. The transcription factor cAMP-response element-binding protein (CREB) is also phosphorylated in response to FSH in a cAMP-dependent manner (3, 12, 13).

We recently reported that histones H1 and H3 are also downstream targets of FSH and PKA in granulosa cells (14).

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[1] The abbreviations used are: FSH, follicle-stimulating hormone; TSA, trichostatin A; FITC, fluorescein isothiocyanate; PKA, protein kinase A; PKI, PKA inhibitor; HSP27, heat shock protein 27; PI-3 kinase, phosphatidylinositol-3 kinase; RSK-2, ribosomal S6 kinase-2; MSK-1, mitogen- and stress-activated protein kinase-1; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; SGK, serum glucocorticoid kinase; HAT, histone acetylase; StAR, steroidogenic acute regulatory protein; EGF, epidermal growth factor; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; CREB, cAMP-response element-binding protein; CBP, p300/CREB-binding protein; PCAF, CBP-associated factor; ChIP, chromatin immunoprecipitation assay; PMA, phorbol myristic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SF-1, steroidogenic factor-1; LH, luteinizing hormone; Ph, phosphorylated; Ac, acetylated.

[2] D. W. Carr and M. Hunzicker-Dunn, unpublished data.

[3] E. T. Maizels, L. Salvador, and M. Hunzicker-Dunn, manuscript in preparation.

Under conditions in which FSH stimulates granulosa cell differentiation and not proliferation (2, 15), histone H3 is transiently phosphorylated exclusively on serine 10 (14). The addition of factors that do not promote granulosa cell differentiation (2), such as epidermal growth factor (EGF), does not lead to histone H3 phosphorylation (14). This phosphorylation event appears to represent an early marker of differentiation and is distinct from the well established marker of chromosome condensation during mitosis (16–18) in granulosa cells as these cells undergo differentiation and not proliferation in response to FSH in serum-free cultures.

While histone H3 phosphorylation on serine 10 has traditionally been viewed as a marker for mitosis (16–18), there were also reports in the early 1980s that histone H3 was phosphorylated in response to differentiative stimuli such as nerve growth factor in PC-12 cells and cAMP/isoproterenol in C6 glioma cells (19, 20). Ten years later, the rapid phosphorylation of a small fraction of histone H3 on serine 10 was reported in quiescent C3H 10T1/2 mouse fibroblasts in response to phorbol esters, okadaic acid, EGF, and protein synthesis inhibitors coincident with the induction of immediate early genes such as c-*fos* and c-*jun* (21, 22).

Recent reports have identified two related histone H3 kinases as likely mediators in these pathways. Both ribosomal S6 kinase-2 (RSK-2), which is downstream of p42/p44 MAPK/ERK, and mitogen- and stress-activated protein kinase-1 (MSK-1), which is downstream of both MAPK/ERK and p38 MAPK (23), phosphorylate histone H3 *in vitro* (24–26). EGF-stimulated histone H3 phosphorylation was restored after the addition of the RSK-2 gene to a fibroblast cell line derived from patients with Coffin-Lowry syndrome with mutations in the RSK-2 gene (24). In quiescent C3H 10T½ mouse fibroblasts, anisomycin- and phorbol ester-stimulated histone H3 phosphorylation was inhibited by the p38 MAPK inhibitor SB203580 (27) and the MAPK/ERK kinase (MEK) inhibitor PD98059 (28, 29), respectively. Both phorbol ester- and anisomycin-stimulated histone H3 phosphorylation were inhibited by H89, based on the ability of this kinase inhibitor to inhibit MSK-1 (25) rather than its well recognized inhibition of PKA (30, 31). Similarly in JB6 epidermal C1 cells, ultraviolet B-induced histone H3 phosphorylation at serine 10 was inhibited by the MEK inhibitor PD98059 or by the p38 MAPK inhibitor SB202190 (32). These reports thus establish a prominent role for RSK-2 and MSK-1 downstream of the p38 MAPK and/or ERK/MAPK pathways in growth factor- and phorbol ester-stimulated histone H3 phosphorylation.

As FSH stimulates the MAPK/ERK pathway in granulosa cells in a PKA-dependent manner (9), we hypothesized that, as in fibroblasts and epidermal cells, histone H3 phosphorylation in granulosa cells might be mediated by either RSK-2 and/or MSK-1 activated by a MAPK pathway downstream of PKA. We additionally sought direct evidence that histone H3 phosphorylation is functionally linked to the induction of characteristic FSH-responsive immediate early genes. Finally, based on abundant evidence that histone acetylation is associated with transcriptional activation (33, 34), we sought to determine whether acetylation of histone H3 on lysine 14 and/or lysine 9 is stimulated by FSH.

EXPERIMENTAL PROCEDURES

Materials—Ovine FSH (oFSH-19) was kindly provided by Dr. A. F. Parlow of the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases (Torrence, CA). The following were purchased: H89, AG1478, GF109203X, KT5720, 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), phorbol myristic acid (PMA), and okadaic acid from LC Laboratories (San Diego, CA); PD98059, SB203580, myristoylated PKA inhibitor (PKI) 14–22 amide, and wortmannin from Calbiochem; anti-histone H3 acetylated on lysines 9 and/or 14 (anti-H3AcLys-9/Lys-14), anti-histone H3 acetylated on lysine 14 (anti-H3AcLys-14) or lysine 9 (anti-H3AcLys-9), anti-histone H3 phosphorylated on serine 10 (anti-H3PhSer-10), anti-histone H3 both phosphorylated on serine 10 and acetylated on lysine 14 (anti-H3PhSer-10/AcLys-14), anti-CREB phosphorylated on serine 133, and anti-CREB antibodies from Upstate Biotechnology (Lake Placid, NY); anti-histone H3, anti-MAPK/ERK phosphorylated on threonine 202 and tyrosine 204, anti-p38 MAPK phosphorylated on threonine 180 and tyrosine 182, anti-MSK-1 phosphorylated on serine 376, and anti-RSK phosphorylated on serine 381 (which detects RSK-1 and RSK-2) antibodies from New England Biolabs/Cell Signaling (Beverly, MA); anti-p38 MAPK antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-MAPK/ERK antibody from Zymed Laboratories Inc. (San Francisco, CA); anti-HSP27 antibody from StressGen (Victoria, British Columbia); trichostatin A (TSA) from Wako (Osaka, Japan); fluorescein isothiocyanate (FITC) from Molecular Probes, Inc. (Eugene, OR); and anti-hemagglutinin peptide antibody and H3 purified from calf thymus from Roche Molecular Biochemicals. All other chemicals were from sources described previously (14, 35, 36). Equivalent results for SDS-PAGE and acid urea gel blots were obtained with anti-H3PhSer-10 and anti-H3PhSer-10/AcLys-14 antibodies obtained from Upstate Biotechnology (and licensed from Dr. David Allis) and directly from the Allis laboratory. Recognition by anti-H3PhSer-10 antibody of H3 phosphorylated on serine 10 was not affected by acetylation of lysines 9 and/or 14 (16, 37); recognition by anti-H3PhSer-10/Lys-14 antibody was selective for H3 phosphorylated on serine 10 and acetylated on lysine 14 (37).

Production of TAT-PKI Fusion Protein—An expression vector containing PKI fused to the cell entry leader sequence TAT was constructed by PCR. Oligonucleotides 5′-GCCGTTACCTCCCTGCTATGTGGATATTTG and 5′-CCGCTCGAGGCTTTCAGATTTTGCTGCTTCTC, which add the restriction sites KPN and XHO, respectively, were used to amplify PKI using Jurkat cell cDNA as template. The fragment was cut with the above restriction enzymes and subcloned into the pTAT vector (38) generously provided by Dr. Steven Dowdy. TAT-PKI protein was expressed in BL21 DE3 pLysS bacteria and purified by fast protein liquid chromatography. The protein was labeled with FITC and determined to enter cells using a fluorescence-activated cell sorter (Beckman Instruments). Expressed, purified protein was tested for functionality by adding it to an *in vitro* PKA assay. PKA activity in cell lysates was stimulated 3.7-fold by the addition of cAMP. This stimulation was inhibited 84% by 250 nM TAT-PKI, which is equivalent to the 83% inhibition obtained using the PKI inhibitor peptide.

Granulosa Cell Culture, Western Blotting, and Immunofluorescence—Granulosa cells were isolated from the ovaries of 26-day-old Sprague-Dawley rats that had been primed with subcutaneous injections of 1.5 mg of estradiol-17β on days 23–25 to promote the growth of preantral follicles (5, 14). Cells were either plated on 33-mm plastic dishes (Falcon) at a density of $\sim 3 \times 10^6$ cells/dish (14) or on coverslips (for immunofluorescence) and then treated with the indicated additions ~20 h after plating. For subsequent Western blotting, treatments were terminated by aspirating medium, rinsing cells once with phosphate-buffered saline, and collecting total cell extracts in 0.5 ml of SDS sample buffer (39) followed by heat denaturation. Protein concentrations were controlled by plating identical cell numbers per plate in each experiment and then loading equal volumes of total cell extract per gel lane. Ponceau S staining of the final blot was used to confirm equal protein loading. Granulosa cell proteins were separated by SDS-PAGE (10 or 12% acrylamide in running gel) (40) and transferred to Hybond-C Extra nitrocellulose (Amersham Pharmacia Biotech). Blots were incubated with primary antibody overnight at 4 °C, and antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). For acid urea gels, cells were lysed, and acid-soluble histones were extracted from nuclei (37). Acid-soluble histones and H3 standard (Roche Molecular Biochemicals) were loaded in sample buffer containing 6 M urea, 5% acetic acid, and 12.5 mg/ml protamine sulfate and then electrophoresed through a 1-cm stacking gel and 17.5-cm separating gel (15% acrylamide, 6 M urea, 5% acetic acid) for 24 h at 200 V (37). Histones were transferred to polyvinylidene difluoride (Millipore Corporation, Bedford, MA), stained with Ponceau S, and subjected to Western blotting. For immunofluorescence, cells were treated as indicated and then fixed with 3.7% formaldehyde and permeabilized with 1% Triton X-100 in phosphate-buffered saline, washed, and incubated for 2 h at 37 °C with anti-H3PhSer-10 antibody (1:50 dilution) in phosphate-buffered saline containing 5% normal goat serum. Coverslips were washed and incubated for 1 h at 37° C with FITC-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). Cells on coverslips were washed and mounted on slides in diazabicyclo[2.2.2]octane antifading medium (41).

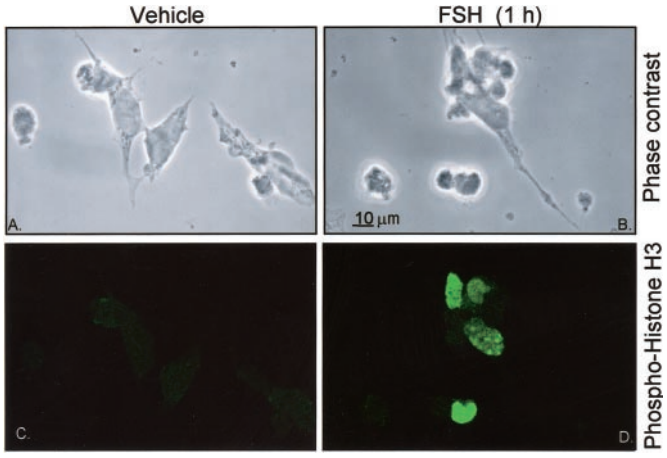

FIG. 1. **Effect of FSH on the phosphorylation of histone H3 in granulosa cells.** Granulosa cells were treated with vehicle or FSH for 1 h and then subjected to immunofluorescence using anti-phospho-histone H3 antibody. Results are representative of more than 5 separate experiments.

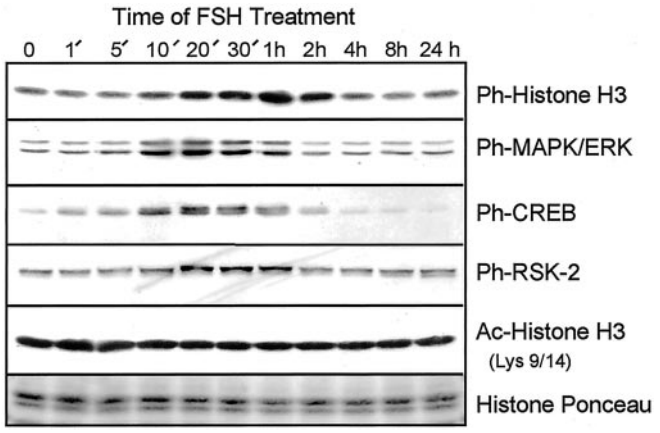

FIG. 2. **Time course of FSH-stimulated phosphorylation of histone H3, p42/44 MAPK/ERK, CREB, p90 RSK-2, and acetylated histone H3.** Granulosa cells were treated for the indicated times with 50 ng/ml FSH followed by the preparation of total cell extracts, as described under "Experimental Procedures." Following SDS-PAGE and the transfer of proteins to nitrocellulose, blots were first stained with Ponceau S to confirm equal protein loading per lane and then probed with the indicated antibodies to phosphorylated (*Ph-*) or acetylated (*Ac-*) proteins. Results are representative of 2 separate experiments.

Slides were analyzed by a Zeiss Axiovert 100M confocal microscope. For introduction of TAT-PKI, cells were plated in 1 ml of Opti-MEM I medium (Life Technologies, Inc.) on 6-well plastic dishes (Falcon). Approximately 20 h after plating, TAT-PKI was added, and cells were incubated for 3 h. Opti-MEM I medium was then aspirated off cells, serum-free Dulbecco's modified Eagle's medium/Ham's F-12 medium (5) was added, and cells were treated as indicated. Treatments were terminated as above, and total cell extracts were collected in 0.1 ml of SDS sample buffer (39).

Chromatin Immunoprecipitation (ChIP) Assay—Following indicated treatments, granulosa cells were incubated (15 min at room temperature) in 1% formaldehyde to cross-link DNA and proteins and then sonicated in cell lysis buffer as described previously (36). One-tenth of the total lysate was used for purification of total genomic DNA. The rest of the lysate was incubated with anti-H3PhSer-10, anti-H3AcLys-9/Lys-14, or anti-H3PhSer-10/AcLys-14 antibodies at 4 °C for 18 h. Following the collection of immunoprecipitates using protein A-agarose, DNA was extracted by phenol/chloroform extraction and ethanol precipitation, and PCR was performed using either total DNA or immunoprecipitated DNA in the presence of [α-^{32}P]dCTP as described previously (36). PCR products were separated on 6% nondenaturing polyacrylamide gels followed by autoradiography. Primers used for PCR correspond to sequences within the inhibin α promoter region (36) (−160 to −141) (5′-TTGGCGGGAFTGGGAGATAA-3′) and (+68 to +49) (5′-CTCTTGCCCTGACGACAGGG-3′); SGK promoter region (42) (+12 to −11) (5′-TTAGCCAACAGTGAGCTCCGGCT-3′) and (43) (−285 to −265) (5′-GCGGACGGAGGAGGCAGAGTGCAT-3′); c-*fos* promoter region (44) (−324 to −304) (5′-ACACAGGATGTCCATATTA-3′) and (−25 to −5) (5′-TGGAGTAGTAGGCGCCTCAGC-3′); and progesterone receptor promoter region (45) (−376 to −359) (5′-GTGACA-TACACTCAGAGA-3′) and (−18 to −1) (5′-GGCTCCACAGCTTTCTA-G-3′).

RESULTS

Immunolocalization of Phospho-histone H3 in FSH-treated Granulosa Cells—Phospho-histone H3 is undetectable in vehicle-treated granulosa cells (Fig. 1*C*). Treatment of cells with FSH for 1 h results in the detection of phospho-histone H3 in small foci in the nuclei of most but not all granulosa cells (compare Fig. 1, *B* and *D*). Overexposure results in complete nuclear staining, as seen in the cell nucleus detected in Fig. 1*D*, *lower portion*.

FSH Stimulates Histone H3, MAPK/ERK, and RSK-2 Phosphorylation in a Time-dependent Manner—Recent evidence in fibroblasts has identified both RSK-2, downstream of p42/p44 MAPK/ERK, and MSK-1, downstream of both p42/p44 MAPK/ERK and p38 MAPK, as histone H3 kinases (24, 25). We first compared the rates of phosphorylation/activation of the p42/p44 MAPK/ERK pathway, histone H3 phosphorylation on serine 10, and CREB phosphorylation on serine 133. Increased phosphorylation of CREB, an established PKA target (46), is first detected by Western blot at 1 min and increases further at 10 min after FSH addition; increased phosphorylation of histone H3 and MAPK/ERK is detected by 10 min, and increased RSK-2 phosphorylation is detected at 20 min post-FSH (Fig. 2). Histone H3 phosphorylation peaks at 1 h. By 2 h, MAPK/ERK and RSK-2 phosphorylations have returned to basal levels; CREB and histone H3 phosphorylations returned to basal levels by 4 h post-FSH. MSK-1 phosphorylation is also detected in vehicle-treated cells but is not affected by FSH (not shown). These results show that FSH promotes both MAPK/ERK and RSK-2 phosphorylation, consistent with our previous evidence that FSH stimulates RSK phosphorylation in granulosa cells incubated with $^{32}P_i$ (9).

FSH-stimulated Histone H3 Phosphorylation Is Inhibited by PKA Inhibitors but Is Not Blocked by MEK, p38 MAPK, EGF Receptor Tyrosine Kinase, PI-3 Kinase, Protein Kinase C, or RSK-2 Inhibitors—Based on evidence in fibroblasts and epidermal cells that histone H3 phosphorylation is blocked by inhibitors of the p38 MAPK and/or the MAPK/ERK pathways, we tested inhibitors of these and other pathways on FSH-stimulated histone H3 phosphorylation. Cells were pretreated with inhibitors for 30, 60, or 90 min, as indicated in the legend for Fig. 3, and then treated for 1 h with FSH, the adenylyl cyclase activator forskolin, the protein kinase C activator PMA, or the cell-permeable cAMP analog CPT-cAMP. Although a 1-h treatment with FSH is optimal for H3 phosphorylation, MAPK/ERK phosphorylation is declining or has declined to basal levels by this time (Fig. 2). Neither the MEK inhibitor PD98059 (28, 29) nor the p38 MAPK inhibitor SB203580 (27), separately or together, inhibits histone H3 phosphorylation (Fig. 3*A*). Similarly histone H3 phosphorylation is not altered by the RSK-2/protein kinase C inhibitor GF109203X (47), although this agent does inhibit the modest stimulation by PMA of histone H3 phosphorylation (Fig. 3*B*, *lanes 5* and *21*). PD98059 inhibits all detectable MAPK/ERK phosphorylation stimulated by FSH and forskolin as well as FSH- and forskolin-stimulated RSK-2 phosphorylation (Fig. 3*A*). The p38 MAPK inhibitor SB203580, which has previously been shown to inhibit FSH-stimulated p38 signaling to HSP27 (10), also abolishes RSK-2 phosphorylation (Fig. 3*A*), suggesting that in granulosa cells, as in some other cells (48–50), RSK-2 is downstream not only of MAPK/ERK but also of p38 MAPK. Histone H3 phosphorylation is also

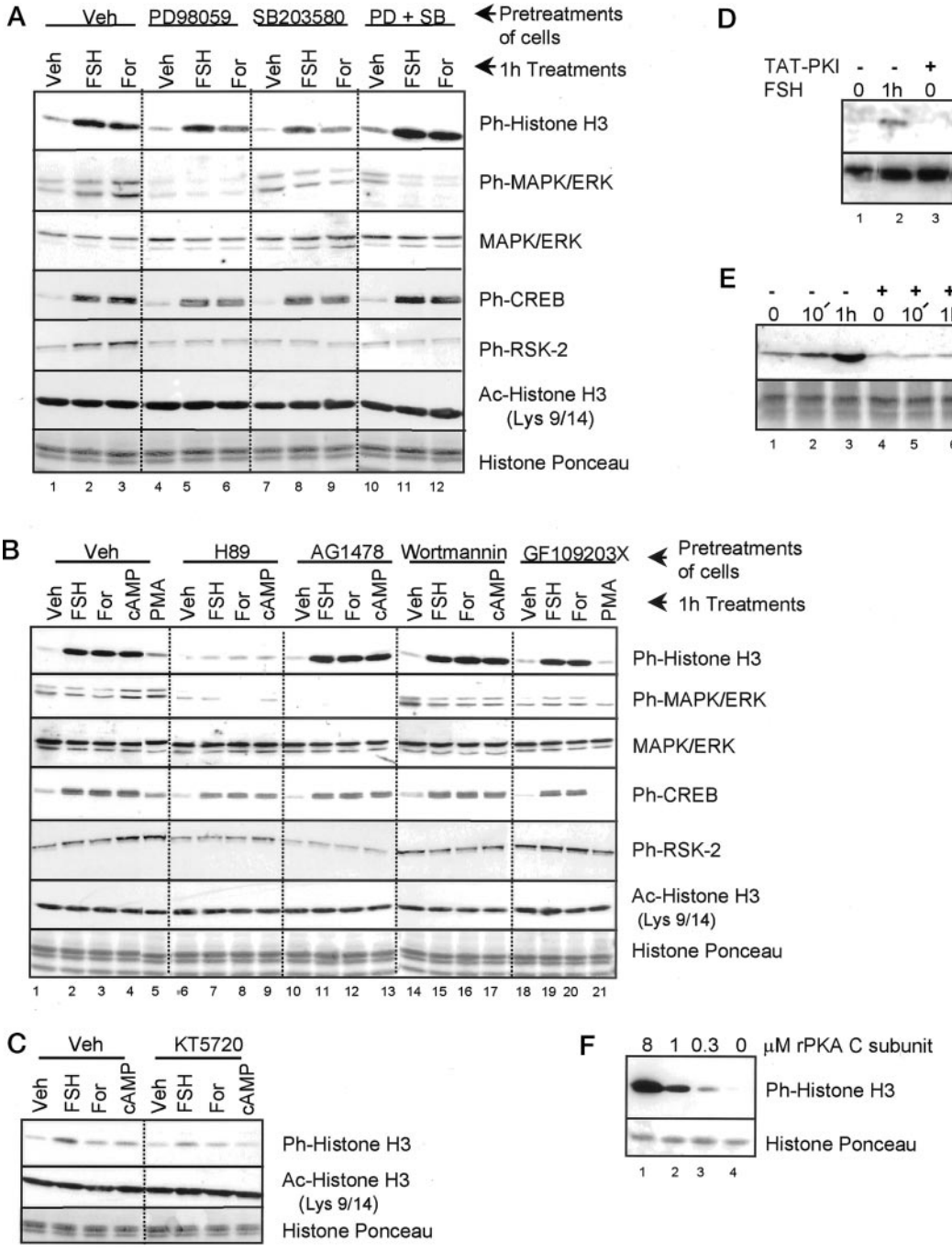

FIG. 3. **Effect of protein kinase inhibitors on FSH-stimulated histone H3 phosphorylation.** *A*, granulosa cells were pretreated with 50 μM PD98059 for 90 min, 10 μM SB203580 for 60 min, or both followed by treatment for 60 min with vehicle (*Veh*), 50 ng/ml FSH, or 10 μM forskolin (*For*), as indicated. For the rest of the details, see the legend for Fig. 2. Results are representative of 2 experiments. *B*, cells were pretreated with vehicle for 60 min, 10 μM H89 for 60 min, 250 nM AG1478 for 15 min, 100 nM wortmannin for 60 min, or 5 μM GF109203X for 30 min. Cells were then treated for 60 min with vehicle, 50 ng/ml FSH, 10 μM forskolin, 1 mM CPT-cAMP (*cAMP*), or 200 nM PMA. Results are representative of 3 experiments. *C*, cells were pretreated for 60 min with vehicle or 10 μM KT5720. For the rest of the details, see the details for *panel B*. *D*, cells were pretreated for 3 h with vehicle or 200 nM TAT-PKI and the medium was changed, followed by treatment for 1 h with vehicle or FSH, as detailed under "Experimental Procedures." Protein loading control is the HSP27 Western blot (Welsh antibody). Entry of hemagglutinin-tagged TAT-PKI is confirmed by anti-hemagglutinin antibody (not shown). Results are representative of 3 experiments. *E*, cells were pretreated for 60 min with vehicle or 50 μM Myr-PKI amide followed by treatment with vehicle or FSH for 10 min or 1 h. For the rest of the details, see the legend for Fig. 2. Results are representative of 2 separate experiments. *F*, histone H3 (5 μg, Roche Molecular Biochemicals) was incubated for 38 min at 30 °C with the indicated concentrations of recombinant (*r*) PKA catalytic subunit (*C*) in a reaction mix containing 100 μM ATP, 10 mM $MgCl_2$, and 50 mM TRIS-HCl, pH 7.5. Following SDS-PAGE of total sample and transfer to nitrocellulose, blots were processed as described in the legend for Fig. 2. Results are representative of 2 separate experiments.

unaffected by the EGF receptor tyrosine kinase inhibitor AG1478 (51), which consistently abolishes MAPK/ERK and RSK-2 phosphorylation (Fig. 3*B*). Likewise, H3 phosphorylation is unaffected by the PI-3 kinase inhibitor wortmannin (52) (Fig. 3*B*), which effectively reduces PI-3 kinase-dependent protein kinase B (AKT) phosphorylation.[3] The increased MAPK/ERK phosphorylation seen in wortmannin-treated cells could reflect inhibition by wortmannin of the inhibitory PI-3 kinase-dependent Raf phosphorylation (53).

Only the PKA inhibitors H89 (30, 31) (Fig. 3*B*) and KT5720

(54) (Fig. 3*C*) inhibit histone H3 phosphorylation stimulated by FSH, forskolin, or CPT-cAMP. H89 also inhibits MAPK/ERK and RSK-2 phosphorylation, which is consistent with evidence that FSH signals to MAPK/ERK via PKA (9). The finding that H89 only partially inhibits CREB phosphorylation (Fig. 3*B*, *lanes 6–9 versus lanes 1–4*) concurs with a recent report (11). To confirm the PKA dependence of histone H3 phosphorylation, cells were also pretreated without or with the PKA inhibitor protein PKI linked to the cell-permeabilizing TAT peptide (38, 55) and then treated for 1 h with vehicle or FSH. Results (Fig. 3*D*) show that histone H3 phosphorylation is reduced ~4-fold in cells treated with TAT-PKI. Similarly, in cells pretreated with a myristoylated PKI peptide, FSH-stimulated H3 phosphorylation is ablated (Fig. 3*E*). These results indicate that histone H3 phosphorylation in granulosa cells in response to FSH treatment is not downstream of MAPK/ERK, RSK-2, MSK-1, p38 MAPK, PI-3 kinase, or protein kinase C. Rather, histone H3 phosphorylation appears to be mediated directly by PKA. *In vitro* phosphorylation experiments confirm the ability of recombinant PKA catalytic subunit to directly phosphorylate purified histone H3 on serine 10 (Fig. 3*F*).

Effect of FSH on Histone H3 Acetylation—We next sought to determine whether the acetylation of histone H3 is linked to its phosphorylation. The results shown in Fig. 2 show strong reactivity to anti-histone H3 acetylated on either lysines 9 and/or 14 (anti-H3AcLys-9/Lys-14) antibody in granulosa cells in the absence of FSH treatment (*lane 1*). This result suggests that a portion of histone H3 is already acetylated in untreated cells. Treatment of granulosa cells with FSH over a 24-h time course does not cause a detectable change in this bulk acetylation of histone H3 (Fig. 2). Similarly, neither forskolin, PMA, CPT-cAMP, nor the addition of protein kinase inhibitors exerts a detectable modulation of histone H3 acetylated on lysines 9/14 (Fig. 3, *A–C*). To determine whether a selective deacetylation event precedes histone H3 phosphorylation on serine 10, cells were pretreated with vehicle or with the histone deacetylase inhibitor TSA. Results show that whereas pretreatment of cells for 1 h with TSA promotes the expected increase in the bulk acetylation of histone H3 (Fig. 4*A*, *lanes 6–10 versus lanes 1–5*), the FSH-stimulated phosphorylation of histone H3 on serine 10 is unaffected. Consistent with the characteristics of the phospho-H3 antibody (37), and in contrast to another phospho-H3 antibody (56), this result shows that increased acetylation of H3 does not appear to impede the ability of the anti-H3PhSer-10 antibody to detect phosphorylated H3. This result also suggests that acetylation does not predispose histone H3 to phosphorylation. We next sought to determine whether the fraction of histone H3 that becomes phosphorylated (on serine 10) also becomes acetylated (on lysine 14) in response to FSH, using an antibody reactive only with histone H3 both phosphorylated on serine 10 and acetylated on lysine 14 (anti-H3PhSer-10/AcLys-14) (37). Results show that, as is true of H3 phosphorylation, this dual modification of histone H3 is detected by 10 min (Fig. 4*B*) and increases with time after FSH treatment of cells (Fig. 4*C*). Moreover, the increase in the relative signals detected by the two antibodies over time is not significantly different at any time point (Fig. 4*C*). Similar results were obtained when granulosa cell acid-soluble histones were separated on acid urea gels (Fig. 4*D*). These results suggest either that FSH stimulates the phosphorylation of histone H3 molecules that are already acetylated on lysine 14 or that both modifications of H3 occur concurrently. That FSH most likely stimulates the apparently concurrent phosphorylation and acetylation of histone H3 is suggested by results showing that FSH also produces a modest increase by 10 min in the acetylation of histone H3 on lysine 14 (Fig. 4*E*) but not on lysine 9 (Fig. 4*F*). Consistent with the results shown in Fig. 3, the PKA inhibitor H89 abolishes the signal detected by the anti-H3PhSer-10/AcLys-14 antibody (Fig. 4*G*). Taken together, these results indicate that FSH stimulates the phosphorylation of serine 10 via PKA as well as the acetylation on lysine 14 of a small fraction of histone H3.

FSH Stimulates the Association of Phosphorylated and Acetylated Histone H3 with Promoter Sequences of Serum Glucocorticoid Kinase, Inhibin α, and c-fos, but Not with the Progesterone Receptor—Finally we sought to determine whether the phosphorylation of histone H3 in response to FSH promotes the activation of genes specific for FSH but not those responsive to LH. Granulosa cells were treated with vehicle or FSH for 1 h, chromatin was cross-linked with formaldehyde, and histone H3 that was either phosphorylated on serine 10, acetylated on lysines 9 and/or 14, or phosphorylated on serine 10 and acetylated on lysine 14 was immunoprecipitated and protein-denatured. PCR was conducted on immunoprecipitated DNA using primers specific for promoter sequences of the FSH early response genes SGK and c-*fos* as well as for the later response gene inhibin α and for the progesterone receptor, which is an LH early response gene. As a control, PCR was also conducted on total genomic DNA. SGK and inhibin α are established downstream targets in granulosa cells of FSH and PKA (3, 7), whereas c-*fos* is generally thought to be downstream primarily of MAPK/ERK and RSK-2 (57, 58). Results show that there is ~3-fold more amplified PCR product in anti-H3PhSer-10 immunoprecipitates corresponding to SGK and inhibin α promoter sequences and to a lesser extent to c-*fos* from cells treated with FSH *versus* vehicle (Fig. 5*A*, *lanes 1–4*, *lanes 9–12*, and *lanes 17–20*). The response to FSH for the SGK, inhibin α, and c-*fos* promoters was completely inhibited by pretreating cells with the PKA inhibitor H89 (compare *lanes 1* and *2* with *lanes 5* and *6*, compare *lanes 9* and *10* with *lanes 13* and *14*, and compare *lanes 17* and *18* with *lanes 21* and *22*). Promoter DNA corresponding to the progesterone receptor was not detected in the phosphorylated histone H3-chromatin pool of vehicle- or FSH-treated cells (Fig. 5*A*, *lanes 25* and *26*), although it was readily detected in total genomic DNA (*lanes 27* and *28*). This result is consistent with evidence that FSH does not promote activation of the progesterone receptor gene (4).[4]

Similar to results following immunoprecipitation with anti-H3PhSer-10 antibody, immunoprecipitation with anti-H3AcLys-9/Lys-14 antibody evokes ~2-fold increased amplification of SGK and inhibin α promoter DNA (Fig. 5*B*, *lanes 1–4* and *lanes 9–12*) but not of the progesterone receptor promoter (*lanes 13–16*). However, in contrast to phospho-histone H3-associated chromatin, the progesterone receptor promoter is readily detected in the chromatin pool associated with histone H3 acetylated on lysines 9 and/or 14 (*lanes 13–16*). Only a minor increase in c-*fos* promoter amplification is detected in response to FSH in the anti-H3AcLys-9/Lys-14 immunoprecipitates (*lanes 5–8*).

We additionally assessed whether there is a direct link between both phosphorylation on serine 10 and acetylation on lysine 14 in histone H3 and promoter activation. Immunoprecipitation with anti-H3PhSer-10/AcLys-14 antibody results in ~4-fold more amplified PCR product corresponding to SGK, inhibin α, and c-*fos* promoter sequences in FSH *versus* vehicle-treated cells (Fig. 5*C*).

These results demonstrate that although FSH treatment of granulosa cells does not promote a detectable increase by Western blotting in bulk levels of acetylated histone H3 (on lysines 9 and/or 14) (Figs. 2 and 3), in the ChIP assay, more promoter DNA characteristic of FSH response genes is associated with

[4] O. K. Parke-Sarge, personal communication.

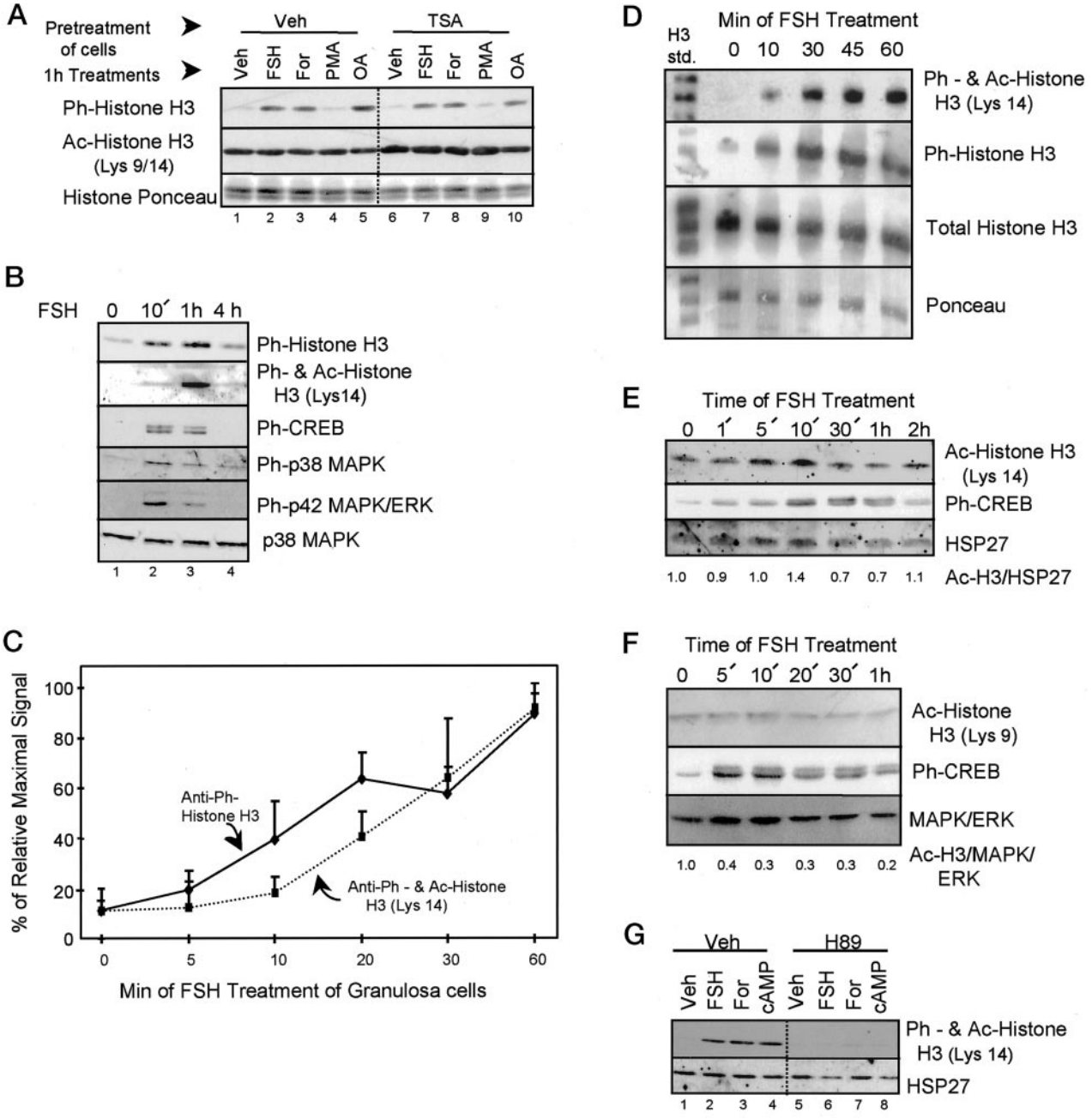

FIG. 4. **Effect of FSH on the acetylation of histone H3.** *A*, cells were pretreated without or with the histone deacetylase inhibitor TSA for 60 min. Cells were then treated for 60 min with vehicle, 50 ng/ml FSH, 10 μM forskolin, 200 nM PMA, or 1 μM okadaic acid (*OA*) followed by the preparation of total cell extracts for SDS-PAGE and Western blotting. Results are representative of 2 experiments. In *panels B, C, E and F,* cells were treated for the indicated times with FSH followed by the preparation of total cell extracts. Blots were probed with the indicated antibodies. *C*, the relative levels of phosphorylated and phosphoacetylated H3 were quantitated with the Molecular Analyst™/PC Image Analysis software program, divided by the densitometric signal for control protein load, and expressed relative to the maximal signal, and plotted as a function of min of FSH treatment. Results at each time point are means ± S.E. from 3 or 4 separate experiments and are not significantly different ($p > 0.01$) by Student's *t* test (98). The results shown in Fig. 2 and in *panel E* are from the same samples; the Ph-CREB blot in Fig. 2 is duplicated in *panel E* for reference. HSP27 antibody used in *panel E* was obtained from Dr. Michael Welsh. Acetylated H3 relative to HSP27 signal is indicated. *D*, cells were treated with FSH for the indicated times, and acid-soluble histones were extracted from nuclei and electrophoresed in acid urea gels, as detailed under "Experimental Procedures," and transferred to polyvinylidene difluoride membranes. Blots were stained with Ponceau S and then probed with the indicated antibodies. Results are representative of 4 separate experiments. The H3 standard triplet does not correspond to multiple modified forms of H3. *G*, cells were pretreated for 60 min with vehicle or 10 μM H89 and then treated with the indicated additions for 60 min followed by the preparation of total cell extracts. Following SDS-PAGE and protein transfer to nitrocellulose, blots were probed with antibody to histone H3 phosphorylated on serine 10 and acetylated on lysine 14 or to a control protein HSP27 (StressGen). Results are representative of 2 experiments.

the acetylated histone H3 after FSH treatment of granulosa cells. These results therefore support our evidence (presented in Fig. 4*E*) showing that FSH stimulated a modest increase in H3 acetylation on lysine 14, and these results are consistent with the idea that only a small fraction of total cellular histone H3 is acetylated at specific loci in response to FSH treatment of granulosa cells. In contrast to bulk histone H3 acetylation levels, increased levels of phosphorylated histone H3 as well as of histone H3 both phosphorylated on serine 10 and acetylated on lysine 14 are readily detected by Western blotting in FSH-treated granulosa cells and are associated with increased promoter amounts of FSH-responsive genes. These results thus

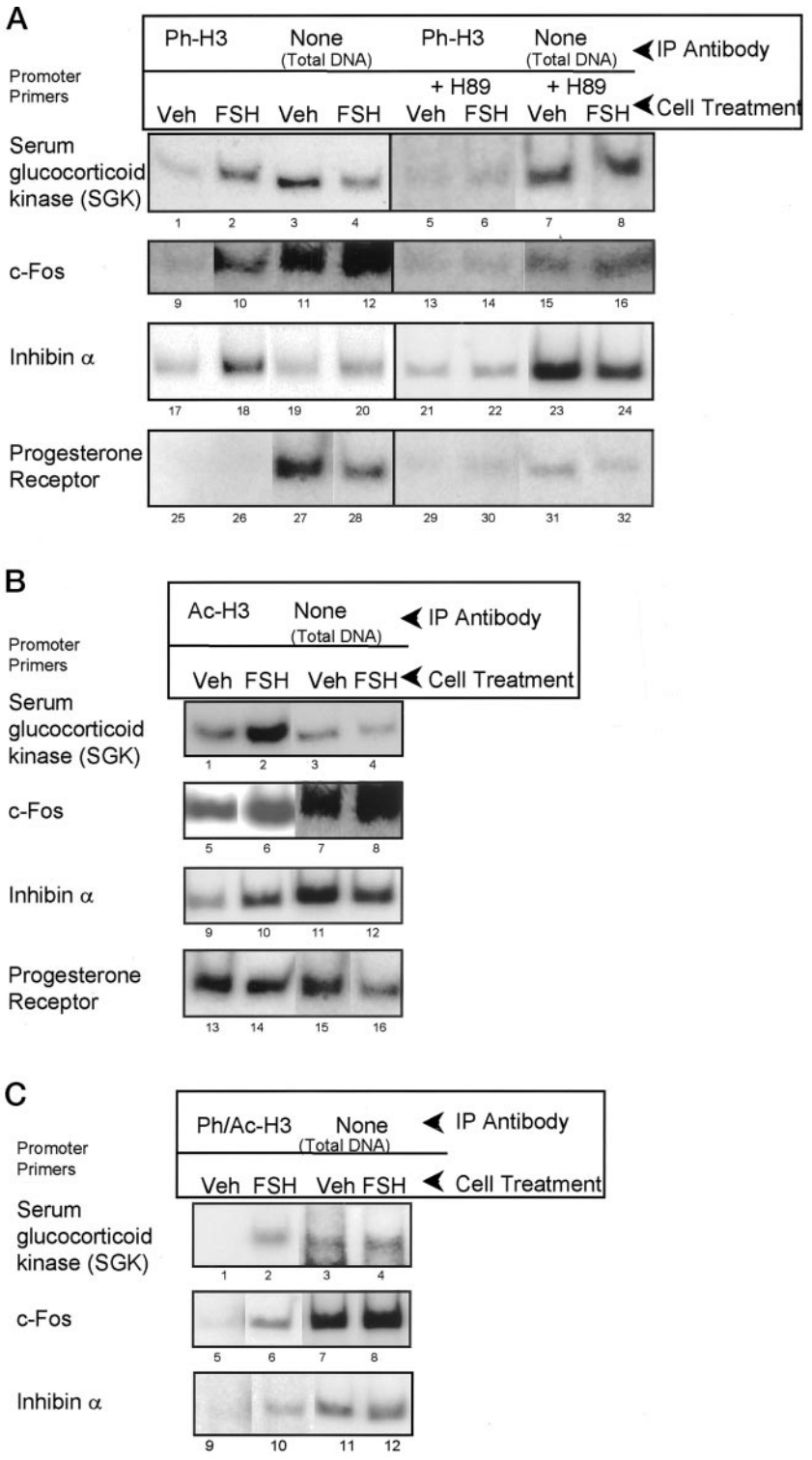

FIG. 5. **Association of histone H3 phosphorylation and acetylation with activation of FSH-dependent early response genes.** ChIP assays were performed using DNA isolated from vehicle- or FSH-treated cells. Briefly, granulosa cells were treated for 60 min with vehicle or FSH or were pretreated for 60 min with 10 μM H89 prior to cell treatment, as indicated. Following DNA and protein cross-linking with formaldehyde, an aliquot was removed for purification of total DNA, and immunoprecipitations (*IP*) were conducted using anti-histone H3 phosphorylated on serine (*A*), anti-histone H3 acetylated on lysines 9 and 14 (*B*), or anti-histone H3 phosphorylated on serine 10 and acetylated on lysine 14 (*C*). DNA was then extracted from immunoprecipitates, and PCR was conducted on total DNA and immunoprecipitated DNA with primers to the promoter regions of indicated genes, as described for the ChIP assay under "Experimental Procedures." Results are representative of at least 5 separate experiments for each panel.

provide direct evidence that both phosphorylation (on serine 10) and acetylation (on lysine 14) in histone H3 are associated with FSH-responsive promoters.

DISCUSSION

FSH stimulates the differentiation of granulosa cells to a preovulatory phenotype (2). In the intact ovary, FSH also stimulates granulosa cell proliferation (59, 60), although this response to FSH is lost in serum-free granulosa cell cultures (2, 15), presumably because of the loss of the contribution of growth factors by adjacent thecal cells (61). It is generally believed that most of the cellular pathways downstream of the FSH receptor that lead to both granulosa cell proliferation and differentiation are mediated, directly or indirectly, by PKA (2, 4, 62). It is interesting that granulosa cells appear to express the same signaling pathways that in other cells are activated by growth factors or phorbol esters and are often inhibited by PKA (63, 64), yet in granulosa cells, these pathways are activated by PKA. A prime example is the MAPK/ERK pathway, which in granulosa cells is activated downstream of PKA (9, 10, 65). Thus, granulosa cells must have evolved unique routes for PKA to modulate these conserved signaling pathways.

Consistent with the hypothesis that FSH uniquely signals predominately via PKA, we have shown in this report that

histone H3 phosphorylation in granulosa cells appears to be mediated *directly* by PKA. In other cellular models, histone H3 phosphorylation is downstream of the p38 MAPK and/or MAPK/ERK pathways and mediated by RSK-2 or MSK-1 (24, 25, 32, 66). However in granulosa cells, inhibition of these pathways (by PD98059, SB203580, GF109203X, or AG1478) does not diminish FSH-stimulated histone H3 phosphorylation. Therefore in granulosa cells, histone H3 phosphorylation is not a consequence of the activation of the MAPK pathways. Our results support the hypothesis that the catalytic subunit of PKA directly phosphorylates histone H3, based on stimulation of histone H3 phosphorylation by cell-permeable cAMP analogs as well as by the adenylyl cyclase activator forskolin and on inhibition by the ATP competitive inhibitors H89 and KT5720. Although H89 also inhibits MSK-1 (25), the absence of FSH-stimulated MSK-1 phosphorylation coupled with the lack of inhibition of FSH-stimulated histone H3 phosphorylation by inhibitors upstream of MSK-1 indicates that the effect of H89 in granulosa cells is on PKA and not on MSK-1. In support of these data, histone H3 phosphorylation was inhibited on the introduction into granulosa cells of the specific PKA inhibitor protein PKI using the TAT transducing peptide as well as inhibited by myristoylated PKI. We and others have shown that histone H3 is readily phosphorylated *in vitro* by the catalytic subunit of PKA purified from mammalian sources (14, 67, 68). We show herein that recombinant purified PKA catalytic subunit also directly phosphorylates histone H3 on serine 10, as detected with the anti-H3PhSer-10 antibody. Moreover, the catalytic subunit of PKA is known to translocate into the nucleus of granulosa cells in response to FSH treatment to phosphorylate nuclear substrates such as CREB (3, 14). Although we have been unsuccessful in our attempts to cross-link histone H3 and the catalytic subunit of PKA, the inability to demonstrate the physical association of these two proteins most likely reflects the transient nature of this catalytic reaction.

Histones are ubiquitous proteins that organize DNA into nucleosomes and chromatin. Nucleosomes consist of an inner core of two copies each of the four core histones, H2A, H2B, H3, and H4, surrounded by 146 base pairs of double-stranded DNA (69). H1 histones bind to the outer surface of the DNA that surrounds the core histones and to the stretches of linker DNA that connect histones (69). There is compelling evidence that chromatin remodeling involves the modification of core histones in nucleosomes in activated target genes (33, 70–72). These core histone modifications generally consist of the covalent addition of an acetyl or phospho group to lysines or serines, respectively, primarily in the N-terminal tails of the core histones H3 and H4. Acetylation neutralizes the positive charge of the histone, and phosphorylation adds a negative charge to histone, thereby decreasing the affinity of histone for DNA (33). The predicted result of these histone modifications is the destabilization of nucleosomes and chromatin structure, resulting in the access of select promoter regions to transcription factors and co-activators (72, 73). In support of these suppositions, mutational analysis of yeast Gcn5, one of the well known nuclear histone acetyltransferases (HATs), indicates a direct role for histone acetylation in the transcriptional activation of target genes *in vivo* (74, 75). It has been similarly shown that the substitution in Gcn5 of arginine 164 for alanine, a residue close to serine 10 in the structure of the ternary Gcn5-CoA-histone H3 complex, reduces promoter activity of a set of Gcn5-dependent genes; mutation of serine 10 to alanine in histone H3 impairs transcription of the same set of genes (76).

Recent evidence from yeast and EGF-treated C3H 10T½ fibroblasts indicates that acetylation on lysine 14 rapidly follows phosphorylation on serine 10 in histone H3 (37, 76). Consistent with these results, *in vitro* studies show that several HATs, including p300 and p300/CREB-binding protein (CBP)-associated factor (PCAF) and Gcn5, display increased HAT activity toward a histone H3 peptide phosphorylated on serine 10 *versus* the unphosphorylated or mutated peptide (37, 76). However, these authors did not detect global modifications in histone H3 acetylation (by Western blotting with anti-H3AcLys-9/Lys-14 antibody) (37), suggesting that these modifications are restricted to a small portion of growth factor-responsive genes in the genome. While our data show that FSH stimulates both the phosphorylation (on serine 10) and acetylation (on lysine 14) of histone H3 in primary rat granulosa cells and that both modifications occur on at least a pool of the same H3 molecules, phosphorylation and acetylation occurred in a statistically simultaneous manner, although in some experiments, phosphorylation appeared to precede acetylation (Fig. 4*C*). Additional studies are needed to resolve the precise timing of H3 phosphorylation *versus* acetylation in response to FSH in granulosa cells. It has been proposed that transcriptional activation requires both the serine 10 phosphorylation and acetylation on lysine 14 of histone H3, whereas mitotic activity requires only histone H3 phosphorylation (73). As granulosa cells exhibit differentiation and proliferation responses to FSH in the absence and presence, respectively (2), of growth factors, this cell offers an ideal model to explore this hypothesis.

We detected increased representation of c-*fos* and SGK promoter DNA in the phosphorylated, acetylated, and dual-phosphorylated and acetylated histone H3-chromatin pools in response to FSH treatment of granulosa cells in ChIP assays. Similarly in C3H 10T1/2 fibroblasts, EGF-stimulated phosphoacetylation of H3 is associated with c-*fos* activation (37, 56). However, the activation of c-*fos* in granulosa cells most likely reflects signaling to the c-*fos* gene via PKA and CREB rather than the more commonly used MAPK/ERK pathway (57, 58) as the c-*fos* promoter contains a functional cAMP response element (77) that can bind phospho-CREB, leading to the recruitment of CBP and perhaps other co-activators to activate the c-*fos* gene.

Transcriptional activation of the SGK gene in response to FSH/PKA requires an Sp1/Sp3 binding site (78). Sp1 sites are also required both for FSH/PKA induction of the LH receptor gene (79) and the cholesterol side chain cleavage CYP11A gene (80) and for LH/PKA induction of the progesterone receptor gene (81), but the mechanism by which PKA modulates Sp1-directed gene activation is not known. Because we have shown that both phosphorylation and acetylation of histone H3 are linked to the rapid activation of the SGK gene, based on our evidence that FSH increases the amount of SGK promoter DNA in immunoprecipitates of phosphorylated, acetylated, and dual-phosphorylated and acetylated histone H3, we anticipate that co-activators with HAT activity, such as CBP or PCAF, may complex with Sp1 at the SGK promoter. These findings also raise the possibility that some of the transcriptional effects of PKA may reflect H3 phosphorylation and chromatin reorganization rather than, or in addition to, direct phosphorylation of transcription factors.

We also detected increased representation of inhibin α promoter DNA in the phosphorylated, acetylated, and dual-phosphorylated and acetylated histone H3-chromatin pools in response to FSH treatment of granulosa cells. Inhibin α is generally considered to be a relatively late FSH responsive gene (82) as maximal mRNA levels are not reached until 12–24 h after FSH addition. However, inhibin α mRNA is detectable as early as 4 h after FSH (83). Interestingly in our ChIP assays, histone H3, which contains the dual modifications of phosphorylation and acetylation, is already associated with the inhibin α promoter by a 1-h post-FSH treatment. Perhaps the ChIP

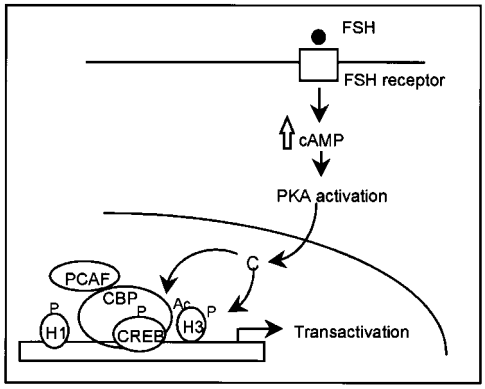

FIG. 6. **Proposed model for FSH signaling to activate histone H3.** FSH via the catalytic (*C*) subunit of PKA catalyzes both histone H3 and CREB phosphorylation as well as histone H1 phosphorylation. Phosphorylated CREB, possibly in conjunction with SF-1 and Sp1, recruits CBP and possibly other HATs such as PCAF, inducing histone H3 acetylation and gene transactivation.

assay detects a reorganization of the promoter into a relatively more accessible configuration for the entry of various co-activators prior to the activation of transcription. Activation of the inhibin α gene is regulated by the nuclear transcription factor steroidogenic factor-1 (SF-1) (36). Recent studies have shown that SF-1, in conjunction with PKA, CREB, and CBP, synergize to strongly activate the inhibin α promoter (36). SF-1 and CREB constitutively bind to adjacent sites on the inhibin α promoter and physically interact (36). CBP is known to be recruited by and to bind not only to phospho-CREB (84, 85) but also to SF-1 (86, 87) and to enhance transcription of SF-1-regulated genes (87). Using the ChIP assay, it was shown that PKA and SF-1 increased the histone H4 acetylation associated with the inhibin α promoter (36). The increased HAT activity is believed to derive either from the recruitment of CBP (88, 89) or the recruitment by CBP of PCAF (90, 91). The role of PKA in these events is believed to be primarily via its phosphorylation of CREB (46), thereby leading to CBP recruitment (36, 85). Perhaps in response to the translocation of the catalytic subunit of PKA to the nucleus of granulosa cells in response to FSH, both CREB and histone H3 are phosphorylated, leading to the formation of a complex between phospho-CREB and adjacent SF-1, recruitment of CBP and possibly PCAF, acetylation of histones H3 and H4, engagement of basal transcription machinery, and initiation of transcription. It is interesting that PCAF has been shown to preferentially acetylate histone H3 (92). Since SF-1 also regulates the transcription of aromatase (13, 93, 94), CYP11A (86), high density lipoprotein receptor (95), and steroidogenic acute regulatory protein (StAR) (96) genes, histone H3 may also be involved, along with CREB and PKA, in the induction of these FSH-responsive genes.

PKA-dependent activation of both CYP11A and StAR genes requires not only SF-1 but also Sp1 (80, 97). SF-1 and Sp1 have been shown to bind to nearby sites on the CYP11A promoter (80) and to physically interact, as demonstrated both in yeast two-hybrid and gel mobility shift assays (80, 97). As SF-1 can recruit and interact with CBP (86, 87) and CBP can enhance transcription of CYP11A reporter constructs (87) and recruit PCAF (89), the role of PKA in the activation of the CYP11A promoter and possibly the StAR promoter, like that of inhibin α, may also involve phosphorylation of histone H3 followed by its acetylation catalyzed by CBP or PCAF.

We hypothesize that the phosphorylation on serine 10 and rapid acetylation on lysine 14 of histone H3 constitutes a necessary step in the transcriptional activation of FSH responsive genes leading to granulosa cell differentiation and that the kinase responsible for this phosphorylation is PKA, as depicted in the model shown in Fig. 6. Perhaps it is histone H3, in its phosphorylated and acetylated conformation, that functions as a scaffold to mediate the assembly of the multiprotein complex that leads to transcription (25, 73). Future studies are planned to evaluate the components of the multimeric complex associated with phospho-histone H3 at the promoters of the FSH responsive genes in granulosa cells.

It is also entirely possible that PKA functions to promote histone H3 phosphorylation, acetylation, and resulting gene activation in the many other physiological models in which hormone actions are mediated by PKA. Thus, the actions of PKA in granulosa cells to modulate chromatin structure may be duplicated in target cells for such hormones as thyroid-stimulating hormone, LH, epinephrine, and vasopressin, for instance. PKA-mediated histone H3 phosphorylation could prove to be a universal mechanism to regulate gene activation.

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REFERENCES

1. Sprengel, R., Braun, T., Nikolics, K., Segaloff, D. L., and Seeburg, P. H. (2000) *Mol. Endocrinol.* **4,** 525–530
2. Hsueh, A. J. W., Adashi, E. Y., Jones, P. B. C., and Welsh, T. H., Jr. (1984) *Endocr. Rev.* **5,** 76–110
3. Gonzalez-Robayna, I., Alliston, T. N., Buse, P., Firestone, G. L., and Richards, J. S. (1999) *Mol. Endocrinol.* **13,** 1318–1337
4. Richards, J. S. (1994) *Endocr. Rev.* **15,** 725–751
5. Carr, D. W., DeManno, D. A., Atwood, A., Hunzicker-Dunn, M., and Scott, J. D. (1993) *J. Biol. Chem.* **268,** 20729–20732
6. Woodruff, T. K., Meunier, H., Jones, P. B., Hsueh, A. J., and Mayo, K. E. (1987) *Mol. Endocrinol.* **1,** 561–568
7. Pei, L., Dodson, R., Schoderbek, W. E., Maurer, R. A., and Mayo, K. E. (1991) *Mol. Endocrinol.* **5,** 521–534
8. Delidow, B. C., Lynch, J. P., White, B. A., and Peluso, J. J. (1992) *Biol. Reprod.* **47,** 428–435
9. Das, S., Maizels, E. T., DeManno, D., St. Clair, E., Adam, S. A., and Hunzicker-Dunn, M. (1996) *Endocrinology* **137,** 967–974
10. Maizels, E. T., Cottom, J., Jones, J. R., and Hunzicker-Dunn, M. (1998) *Endocrinology* **139,** 3353–3356
11. Gonzalez-Robayna, I. J., Falender, A. E., Ochsner, S., Firestone, G. L., and Richards, J. S. (2000) *Mol. Endocrinol.* **14,** 1283–1300
12. Mukherjee, A., Park-Sarge, O. K., and Mayo, K. E. (1996) *Endocrinology* **137,** 3234–3245
13. Carlone, D. L., and Richards, J. S. (1997) *Mol. Endocrinol.* **11,** 292–304
14. DeManno, D. A., Cottom, J. E., Kline, M. P., Peters, C. A., Maizels, E. T., and Hunzicker-Dunn, M. (1999) *Mol. Endocrinol.* **13,** 91–105
15. Dorrington, J., Chuma, A. V., and Bendell, J. J. (1988) *Endocrinology* **123,** 353–359
16. Hendzel, M. J., Wei, Y., Mancini, M. A., VanHooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P., and Allis, C. D. (1997) *Chromosoma* (*Berl.*) **106,** 348–360
17. Bradbury, E. M. (1992) *Bioessays* **14,** 9–15
18. Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L., and Tobey, R. A. (1978) *Eur. J. Biochem.* **84,** 1–15
19. Halegoua, S., and Patrick, J. (1980) *Cell* **22,** 571–581
20. Harrison, J. J., Suter, P., Suter, S., and Jungmann, R. A. (1980) *Biochem. Biophys. Res. Commun.* **96,** 1253–1260
21. Mahadevan, L. C., Willis, A. C., and Barratt, M. J. (1991) *Cell* **65,** 775–783
22. Barratt, M. J., Hazzalin, C. A., Cano, E., and Mahedevan, L. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4781–4785
23. Frodin, M., and Gammeltoft, S. (1999) *Mol. Cell. Endocrinol.* **151,** 65–77
24. Sassone-Corsi, P., Mizzen, C. A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A., and Allis, C. D. (1999) *Science* **295,** 886–891
25. Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J., and Mahadevan, L. C. (1999) *EMBO J.* **18,** 4779–4793
26. Frodin, M., Jensen, C. J., Merienne, K., and Gammeltoft, S. (2000) *EMBO J.* **19,** 2924–2934
27. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) *FEBS Lett.* **364,** 229–233
28. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7686–7689
29. Alessi, D., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. A. (2000) *J. Biol. Chem.* **270,** 27489–27494
30. Hidaka, H., Watanabe, M., and Kobayashi, R. (1991) *Methods Enzymol.* **201,** 328–339
31. Engh, R. A., Girod, A., Kinzel, V., Huber, R., and Bossemeyer, D. (1996) *J. Biol. Chem.* **271,** 26157–26164

32. Zhong, S.-P., Ma, W.-Y., and Dong, Z. (2000) *J. Biol. Chem.* **275,** 20980–20984
33. Struhl, K. (1998) *Genes Dev.* **12,** 599–606
34. Walia, H., Chen, H. Y., Sun, J.-M., Holth, L. T., and Davie, J. R. (1998) *J. Biol. Chem.* **23,** 14516–14522
35. Mukherjee, S., Gurevich, V. V., Jones, J. C. R., Casanova, J. E., Frank, S. R., Maizels, E. T., Bader, M.-F., Kahn, R. A., Palczewski, K., Aktories, K., and Hunzicker-Dunn, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5901–5906
36. Ito, M., Park, Y., Weck, J., Mayo, K. E., and Jameson, L. J. (2000) *Mol. Endocrinol.* **14,** 66–81
37. Cheung, P., Tanner, K. G., Chueng, W. L., Sassone-Corsi, P., Denu, J. M., and Allis, C. D. (2000) *Mol. Cell* **5,** 905–915
38. Nagahara, H., Vocero-Akbani, A. M., Snyder, E. L., Ho, A., Latham, D. G., Lissy, N. A., Becker-Hapak, M., and Dowdy, S. F. (1998) *Nat. Med.* **12,** 1449–1452
39. Hunzicker-Dunn, M. (1981) *J. Biol. Chem.* **256,** 12185–12193
40. Hunzicker-Dunn, M., Cutler, R. E., Jr., Maizels, E. T., DeManno, D. A., Lamm, M. L. G., Erlichman, J., Sanwal, B. D., and LaBarbera, A. R. (1991) *J. Biol. Chem.* **266,** 7166–7175
41. Goldfinger, L. E., Stack, M. S., and Jones, J. C. R. (1998) *J. Cell Biol.* **141,** 255–265
42. Webster, M. K., Goya, L., Ge, Y., Maiyar, A. C., and Firestone, G. L. (1993) *Mol. Cell. Biol.* **13,** 2031–2040
43. Maiyar, A. C., Huang, A. J., Phu, P. T., Cha, H. H., and Firestone, G. L. (1996) *J. Biol. Chem.* **271,** 12414–12422
44. Treisman, R. (1985) *Cell* **42,** 889–902
45. Park-Sarge, O.-K., and Mayo, K. E. (1994) *Endocrinology* **134,** 709–718
46. Montminy, M. (2000) *Annu. Rev. Biochem.* **66,** 807–822
47. Alessi, D. (1997) *FEBS Lett.* **402,** 121–123
48. Lian, J. P., Huang, A. J., and Badwey, J. A. (1999) *J. Immunol.* **163,** 4527–4536
49. Merienne, K., Jacquot, S., Zeniou, M., Pannetier, S., Sassone-Corsi, P., and Hanauer, A. (2000) *Oncogene* **37,** 4221–4229
50. Horstmann, S., Kahle, P. J., and Borasio. G. D (1998) *J. Neurosci. Res.* **15,** 483–490
51. Levitzki, A., and Gazit, A. (1995) *Science* **267,** 1782–1788
52. Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., and Ui, M. (1994) *J. Biol. Chem.* **269,** 3563–3567
53. Zimmermann, S., and Moelling, K. (1999) *Science* **286,** 1741–1744
54. Hidaka, H., and Kobayashi, R. (1992) *Annu. Rev. Pharmacol. Toxicol.* **32,** 377–397
55. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) *Science* **285,** 1569–1572
56. Clayton, A. L., Rose, S., Barratt, M. J., and Mahadevan, L. C. (2000) *EMBO J.* **19,** 3714–3726
57. DeCesare, D., Jacquot, S., Hanauer, A., and Sassone-Corsi, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12202–12207
58. Karin, M., Liu, Z.-G., and Zandi, E. (1997) *Curr. Opin. Cell Biol.* **9,** 240–246
59. Rao, M. C., Midgley, J. S., Jr., and Richards, J. S. (1978) *Cell* **14,** 71–76
60. Kumar, T. R., Wang, Y., Lu, N., and Matzuk, M. M. (1997) *Nat. Genet.* **15,** 201–204
61. Bendell, J. J., and Dorrington, J. (1988) *Endocrinology* **123,** 941–948
62. Robker, R. L., and Richards, J. S. (1998) *Mol. Endocrinol.* **12,** 924–940
63. Cook, S. J., and McCormick, F. (1993) *Science* **262,** 1069–1072
64. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1993) *Science* **262,** 1065–1069
65. Cameron, M. R., Foster, J. S., Bukovsky, A., and Wimalasena, J. (1996) *Biol. Reprod.* **55,** 111–119
66. Chadee, D. N., Hendzel, M. J., Tylipski, C. P., Allis, C. D., Bazett-Jones, D. P., Wright, J. A., and Davie, J. R. (1999) *J. Biol. Chem.* **274,** 24914–24920
67. Taylor, S. S. (1982) *J. Biol. Chem.* **257,** 6056–6063
68. Shibata, K., Inagaki, M., and Ajiro, K. (1990) *Eur. J. Biochem.* **192,** 87–93
69. Wolffe, A. P. (1992) *FASEB J.* **6,** 3354–3361
70. Wu, C. (1997) *J. Biol. Chem.* **272,** 28171–28174
71. Gibori, G., Kalison, B., Vasuray, R., Rao, M. C., and Hunzicker-Dunn, M. (1984) *Endocrinology* **115,** 1157–1163
72. Wolffe, A. P., and Pruss, D. (1996) *Curr. Biol.* **6,** 234–237
73. Strahl, B. D., and Allis, D. (2000) *Nature* **403,** 41–45
74. Kuo, M. H., Zhou, J., Jambeck, P., Churchill, M. E., and Allis, C. D. (1998) *Genes Dev.* **12,** 627–639
75. Wang, L., Liu, L., and Berger, S. L. (1998) *Genes Dev.* **12,** 640–653
76. Lo, W.-S., Trievel, R. C., Rojas, J. R., Duggan, L., Hsu, J.-Y., Allis, C. D., Marmorstein, R., and Berger, S. L. (2000) *Mol. Cell* **5,** 917–926
77. Sassone-Corsi, P., Visvader, J., Ferland, L., Mennon, P. L., and Verma, I. M. (1988) *Genes Dev.* **2,** 1529–1538
78. Alliston, T. N., Maiyar, A. C., Buse, P., Firestone, G. L., and Richards, J. S. (1997) *Mol. Endocrinol.* **11,** 1934–1949
79. Hu, Y. L., Lei, Z. M., and Rao, C. V. (1996) *Endocrinology* **137,** 3897–3905
80. Liu, Z., and Simpson, E. R. (1997) *Mol. Endocrinol.* **11,** 127–137
81. Park-Sarge, O.-K., and Sarge, K. D. (1995) *Endocrinology* **136,** 5430–5437
82. Woodruff, T. K., and Mayo, K. E. (1989) *Annu. Rev. Physiol.* **52,** 807–821
83. Mukherjee, A., Urban, J., Sassone-Corsi, P., and Mayo, K. E. (1998) *Mol. Endocrinol.* **12,** 785–800
84. Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature* **365,** 85585.9
85. Kwok, R. P. S., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachiger, H. P., Brennan, R. G., Roberts, S. G. E., Green, M. R., and Goodman, R. H. (1994) *Nature* **370,** 223–225
86. Clemins, J. W., Lala, D. S., Parker, K. L., and Richards, J. S. (1994) *Endocrinology* **134,** 1499–1508
87. Monte, D., DeWitte, F., and Hum, D. W. (1998) *J. Biol. Chem.* **273,** 4585–4591
88. V. W.V. V.Ogryzko, V. V., Schiltz, R. L., Russanov, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87,** 953–959
89. Bannister, A. J., and Kouzarides, T. (1996) *Nature* **384,** 641–643
90. Yang, X. J., V. V.Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) *Nature* **382,** 319–324
91. Blanco, J. C., Minucci, S., Lu, J., Yang, X. J., Walker, K. K., Chen. H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) *Genes Dev.* **12,** 1638–1651
92. Herrera, J. E., and Schiltz, R. L. B. M. (2000) *J. Biol. Chem.* **275,** 12994–12999
93. Fitzpatrick, S. L., and Richards, J. S. (1993) *Mol. Endocrinol.* **7,** 341–354
94. Lynch, J. P., Lala, D. S., Peluso, J. J., Luo, W., Parker, K. L., and White, B. A. (1993) *Mol. Endocrinol.* **7,** 776–786
95. Lopez, D., Sandoff, T. W., and McLean, M. P. (1999) *Endocrinology* **140,** 3034–3044
96. Sugawara, T., Holt, J. A., Kiriakidou, M., and Strauss, J. F., Jr. (1996) *Biochemistry* **35,** 9052–9059
97. Sugawara, T., Saito, M., and Fujimoto, S. (2000) *Endocrinology* **141,** 2895–2903
98. Bender, F. E., Douglass, L. W., and Kramer, A. (1982) in *Statistical Methods for Food and Agriculture*, AVI Publishing Co., Inc., Westport, CT