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### **PAGE 2588:**

In the left column, the last sentence of the first paragraph should read as follows: "A 5'-end sense primer (5'-GAG ATG GCC TGA GTG AGT CAC-3') and a 3'-end antisense primer (5'-TGG GTA GAG TGA CGT GCA TTC-3') were used for amplifying a 318-bp-long sequence in promoter II/I.3 regulatory region for 35 cycles."

### **PAGE 2585:**

Also, in the print version, some of the author names (Jianfeng Zhou, Ayse Gonca Imir, Mehmet Bertan Yilmaz, Zhihong Lin) were transposed. They are shown correctly above.

We suggest that subscribers photocopy these corrections and insert the photocopies in the original publication at the location of the original article. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

# A Novel Role of Sodium Butyrate in the Regulation of Cancer-associated Aromatase Promoters I.3 and II by Disrupting a Transcriptional Complex in Breast Adipose Fibroblasts\*

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The aromatase gene encodes the key enzyme for estrogen formation. Aromatase enzyme inhibitors eliminate total body estrogen production and are highly effective therapeutics for postmenopausal breast cancer. A distal promoter (I.4) regulates low levels of aromatase expression in tumor-free breast adipose tissue. Two proximal promoters (I.3/II) strikingly induce *in vivo* aromatase expression in breast fibroblasts surrounding malignant cells. Treatment of breast fibroblasts with medium conditioned with malignant breast epithelial cells (MCM) or a surrogate hormonal mixture (dibutyryl (Bt<sub>2</sub>)cAMP plus phorbol diacetate (PDA)) induces promoters I.3/II. The mechanism of promoter-selective expression, however, is not clear. Here we reported that sodium butyrate profoundly decreased MCM- or Bt<sub>2</sub>cAMP + PDA-induced promoter I.3/II-specific aromatase mRNA. MCM, Bt<sub>2</sub>cAMP + PDA, or sodium butyrate regulated aromatase mRNA or activity only via promoters I.3/II but not promoters I.1 or I.4 in breast, ovarian, placental, and hepatic cells. Mechanistically, recruitment of phosphorylated ATF-2 by a CRE (–211/–199, promoter I.3/II) conferred inductions by MCM or Bt<sub>2</sub>cAMP + PDA. Chromatin immunoprecipitation-PCR and immunoprecipitation-immunoblotting assays indicated that MCM or Bt<sub>2</sub>cAMP + PDA stabilized a complex composed of phosphorylated ATF-2, C/EBPβ, and cAMP-response element-binding protein (CREB)-binding protein in the common regulatory region of promoters I.3/II. Overall, histone acetylation patterns of promoters I.3/II did not correlate with sodium butyrate-dependent silencing of promoters I.3/II. Sodium butyrate, however, consistently disrupted the activating complex composed of phosphorylated ATF-2, C/EBPβ, and CREB-binding protein. This was mediated, in part, by decreased ATF-2 phosphorylation. Together, these findings represent a novel mechanism of sodium butyrate action and provide evidence that aromatase activity can be ablated in a signaling pathway- and cell-specific fashion.

Inhibitors of the aromatase enzyme are currently the most effective and most commonly used noncytotoxic therapeutic compounds in estrogen receptor-positive postmenopausal breast cancer (1–5). These

compounds decrease aromatase activity indiscriminately at all body sites and thus cause severe estrogen deprivation. Recent evidence is indicative that local aromatase enzyme within the breast tumor tissue is the most critical source of estrogen for cancer cells (6–8). Because the aromatase P450 (P450arom)<sup>4</sup> gene is regulated by alternatively used distinct promoters in a signaling pathway-specific manner in multiple tissues, including the brain, bone, skin, and adipose tissue, it is possible to target the signaling pathway that regulates the P450arom gene primarily in breast cancer but to permit P450arom expression at other sites (9). This approach would potentially obviate total estrogen deprivation and may still be as effective as the currently used aromatase inhibitors.

Aromatase P450 catalyzes the conversion of C<sub>19</sub> steroids to estrogens in a number of human cells and tissues, *e.g.* ovarian granulosa cell and skin and adipose fibroblasts, placental syncytiotrophoblast, bone, and the brain (10–12). P450arom expression in the adipose tissues is limited to undifferentiated fibroblasts and is not detected in significant quantities in the fully differentiated and lipid-filled adipocytes (10–12). Aromatase activity in adipose fibroblasts has long been implicated in the pathophysiology of breast cancer growth (13–16). Estrogen produced in breast adipose tissue acts locally to promote the growth of breast carcinomas (17). Thus, the relationship between adipose stroma and breast cancer is unique in that the adipose fibroblasts provide structural and functional support for cancer growth. O'Neill *et al.* demonstrated that the breast quadrant displaying the highest level of aromatase activity was consistently involved with tumor (15). Subsequently, we found that the highest levels of P450arom transcripts in adipose tissue from the quadrants bearing a tumor (12). The clinical relevance of these observations has been exemplified by the fact that aromatase inhibitors are now the most commonly used noncytotoxic drugs in the treatment of breast cancer.

Expression of P450arom is under the control of several distinct and partially tissue-specific promoters. The coding region of aromatase transcripts and thus the translated protein, however, are identical in each tissue site of expression (18, 19) (Fig. 1). Three of these promoters (I.4, I.3, and II) are used in adipose tissue. In disease-free breast adipose tissue, P450arom is usually expressed at low levels via a distal promoter (I.4), whereas in the adipose tissue of the breast bearing a tumor, P450arom expression is increased through the activation of two proximal promoters, II and I.3, which are within the 0.7-kb region upstream

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<sup>4</sup> The abbreviations used are: P450arom, aromatase P450; MCM, malignant breast epithelial cell-conditioned medium; CRE, cAMP response element; ATF-2, activating transcription factor-2; C/EBPβ, CCAAT/enhancer-binding protein-β; CBP, cyclic AMP-response element-binding protein-binding protein; Bt<sub>2</sub>cAMP, dibutyryl cyclic AMP; PDA, phorbol diacetate; BAF, breast adipose fibroblast; NaBu, sodium butyrate; DMEM, Dulbecco's modified Eagle's medium; ChIP, chromatin immunoprecipitation assay; FBS, fetal bovine serum; CREB, cAMP-response element-binding protein; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation.

## Promoter-specific Ablation of Aromatase by Na-Bu

to the common splice junction in the coding exon II (20–22) (Fig. 1). Because the activation of promoters I.3 and II is the critical molecular event responsible for the aberrant up-regulation of P450arom expression and thus local estrogen biosynthesis in breast tissues bearing a tumor, the possibility is presented then that promoter-specific inhibitors of P450arom could be developed to be used in the treatment of breast cancer.

There may be multiple potential mechanisms responsible for the activation of promoters II and I.3 in human breast adipose fibroblasts (BAFs). Treatment of BAFs in serum-free medium with a cAMP analogue (e.g.  $Bt_2cAMP$ ) switches the promoter use to II and I.3 (18, 19, 23). The stimulation of promoters II/I.3 by cAMP is potentiated by PDA, a protein kinase C activator (18). Simpson and co-workers (24) had also identified prostaglandin  $E_2$  as a potent factor that stimulates aromatase expression via promoters II/I.3. Prostaglandin  $E_2$  acts via E-prostanoid-2 ( $EP_2$ ) and  $EP_1$  receptor subtypes to stimulate both protein kinases A and C, respectively, and gives rise to strikingly high levels of promoter I.3- and II-specific P450arom transcripts (24). We and others had demonstrated that incubation of BAFs with malignant breast epithelial cell-conditioned medium induced aromatase expression via the activation of promoters I.3 and II (25, 26). Most recently, we demonstrated that malignant breast epithelial cell-conditioned medium (MCM) activated these promoters via a cAMP-independent pathway (25). The effects of tumor-conditioned medium appeared to be mediated by enhanced binding of transcription factor CCAAT/enhancer-binding protein (C/EBP) $\beta$  to an NF-IL6 site (–317/–304) in the promoter I.3/II region (25).

The factors secreted by malignant epithelial cells in MCM have not been identified yet (25). A mixture of factors in MCM seems to function in a redundant fashion to activate aromatase promoters I.3/II (25). We use MCM as a pathophysiologically relevant treatment, whereas  $Bt_2cAMP$  + PDA is also employed as a surrogate hormonal treatment to mimic the downstream effects of MCM.

Sodium butyrate (NaBu) is a four-carbon fatty acid, which is produced naturally in millimolar quantities during digestion by anaerobic bacteria in the cecum and colon (27). NaBu exerts potent positive effects on growth arrest and cell differentiation and induces apoptosis *in vitro* in various malignant tumor cell lines, including breast cancer cell lines (28). Its therapeutic potential in experimental cancer models in mice had also been well documented, and some derivatives of NaBu had been clinically evaluated in a phase I study after oral administration in patients with solid tumors (28, 29). NaBu has been shown to inhibit histone deacetylase activity and induce histone hyperacetylation (30). NaBu is also a potent inducer of a serine-threonine phosphatase (31). The mechanism responsible for the anti-neoplastic activity of NaBu, however, has not been well understood to date.

We used a model whereby NaBu was added to primary BAFs under various conditions to understand the molecular mechanism responsible for regulating aromatase expression from cancer-associated promoters II and I.3. We report here that the effects of NaBu on aromatase expression in breast tumor fibroblasts are mediated, at least in part, by a reduced phosphorylated state of ATF-2 at threonine 71 (Thr-71) and, consequently, disruption of a transcriptional complex containing phosphorylated ATF-2 (Thr-71), C/EBP $\beta$ , and CREB-binding protein (CBP) at the promoter II/I.3 regulatory region.

### EXPERIMENTAL PROCEDURES

**Cell Cultures**—Human adipose tissue samples were obtained at the time of surgery from women ( $n = 28$ ) undergoing reduction mammoplasty following a protocol approved by the Institutional Review Board

for Human Research of Northwestern University, Chicago. For primary human BAF cultures, adipose tissues were minced and digested with collagenase B (1 mg/ml) at 37 °C for 2 h. Single-cell suspensions were prepared by filtration through a 75- $\mu$ m sieve. Fresh cells were suspended in DMEM/F-12 containing 10% FBS in a humidified atmosphere with 5%  $CO_2$  at 37 °C. Twelve to 24 h after the attachment of fibroblasts, culture medium was changed at 48-h intervals until the cells became confluent. At confluence, the cells were placed in serum-free medium for 24 h to wash out the effects of serum and then maintained in serum-free DMEM/F-12 conditioned by MCF-7 breast cancer cells (MCF-7-conditioned medium, MCM) or DMEM/F-12 containing various pharmacologic agents for varying intervals as depicted in relevant figures.

We have employed human BAFs in primary culture since 1993 in this laboratory for studies related to regulation of aromatase expression (16, 25, 32–34). The cultured cells yield reproducible results for aromatase expression and do not demonstrate any significant subject-to-subject variation. Nevertheless, we repeated each experiment illustrated in Figs. 2–10 in cells from 3 to 6 different subjects and recorded reproducible results. Figs. 2–10 illustrate representative experiments. Cultures of T47D, Hep2G, and Jeg3 cell lines obtained from the ATCC and primary ovarian granulosa-lutein cells obtained during three *in vitro* fertilization cycles were performed as described previously (25, 35, 36).

Sodium butyrate (NaBu), cyclic AMP analogue ( $Bt_2cAMP$ ), protein kinase C activator (phorbol diacetate (PDA)), phosphatase inhibitor sodium orthovanadate, calyculin-A kinase inhibitor staurosporine, adenylyl cyclase inhibitor SB22,536, and transcription inhibitor actinomycin-D were purchased from Sigma. All other chemicals, unless indicated otherwise, were also purchased from Sigma.

MCF-7 cells (American Type Culture Collection, Manassas, VA) were grown in minimum Eagle's medium with 10% FBS. MCM was used subsequently to treat BAFs. To generate MCM, cells were initially grown to confluence and switched to DMEM/F-12 for a 12-h washout period; cells were then incubated in DMEM/F-12 for 24 h to allow accumulation of secreted factors in the medium. This was described in detail previously (25).

**Exon-specific RT-PCR Amplification**—Amplification of the untranslated 5'-ends of P450arom transcripts from BAFs under various treatments was accomplished with exon-specific oligonucleotide pairs as described previously and summarized below (25). Five  $\mu$ g of DNase I-treated total RNA were used for reverse transcriptase (RT) reaction. Five  $\mu$ l of the RT mix, 5'-end sense primer from coding exon II (5'-ATA CCA GGT CCT GGC TAC TG-3') and 3'-end antisense primer complementary to coding exon III (5'-TTG TTG TTA AAT ATG ATG GC-3'), were used to amplify the common coding region and thus determine the levels of total transcripts of P450arom. The distributions of P450arom mRNA species with unique promoter-specific 5'-untranslated ends were determined in the following manner. To amplify promoter-specific 5'-untranslated sequences, a sense primer from promoter II-specific sequence (5'-GCA ACA GGA GCT ATA GAT-3'), promoter I.3-specific sequence (5'-GTA AAG GTT CTA TCA GAC C-3'), or promoter I.4-specific sequence (5'-GTA GAA CGT GAC CAA CTG G-3') was used together with an antisense primer complementary to the coding exon III (5'-ATT CCC ATG CAG TAG CCA GG-3'). PCR conditions were as follows: denaturing at 95 °C for 30 s followed by annealing at 55 °C for amplification of promoter II-specific sequence, 57 °C for amplification of I.3-specific sequence, 60 °C for amplification of I.4-specific sequence, or 58 °C for amplification of coding region for 40 s; extension at 72 °C 40 s for 35–38 cycles. Glycerinaldehyde-3-phosphate dehydrogenase was chosen as an internal control to



ensure the equal usage of total RNA under different conditions. A 5'-end sense primer (5'-CGG AGT CAA CGG ATT TGG TCG TAT-3') and a 3'-end antisense primer (5'-AGC CTT CTC CAT GGT GGT GAA GAC-3') were used for amplifying a 306-bp-long sequence in glyceraldehyde-3-phosphate dehydrogenase mRNA. This RT-PCR method was described previously in greater detail (20, 25). Amplification of placental promoter I.1-specific mRNA was performed using primers and PCR conditions described previously (37).

**Aromatase Assay**—The aromatase activity of cultured adipose fibroblasts was measured by [<sup>3</sup>H]water release assay, which is routinely used in this laboratory (38). In each well, 60 pmol of [<sup>3</sup>H]androstenedione (PerkinElmer Life Sciences) and 240 pmol of cold androstenedione (Sigma) were added to 3 ml of serum-free DMEM/F-12 that covered BAFs in culture dishes. Experiments were conducted when cells reached 80% confluency. In order to study the effect of MCM, cAMP, or NaBu on aromatase activity in BAFs, the cells were incubated first in serum-free DMEM/F-12 medium for 12 h followed by treatment with MCM alone, NaBu (15 mM) alone, MCM + NaBu (15 mM), cAMP (0.5 mM) + PDA (100 nM), or cAMP (0.5 mM) + PDA (100 nM) + NaBu (15 mM). Each treatment was performed in triplicate. Treatments were carried out for 24 h at 37 °C in 95% air and 5% CO<sub>2</sub>. After 18 h of treatment, the mixture of labeled and cold androstenedione was added to each well, and cells were incubated for another 6 h. [<sup>3</sup>H]Androstenedione conversion to [<sup>3</sup>H]estrogen was stopped by adding 10% (weight/volume) trichloroacetic acid. Steroidal compounds containing unconverted [<sup>3</sup>H]androstenedione were removed from the mixture by first mixing with 4 ml of chloroform followed by centrifugation at 3000 rpm. The upper aqueous layer was removed and mixed with dextran-coated charcoal (1% weight/volume). Charcoal was precipitated by centrifugation. From each tube, 2 ml of clear solution was taken into 10 ml of scintillation vial and counted in a scintillation counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA).

**Transient Transfections and Luciferase Assay**—BAFs in primary culture were transfected using Lipofectamine Plus™ (Invitrogen) with the following plasmids: (i) 1 μg of modified PGL<sub>3</sub>-basic luciferase reporter plasmid that contains wild-type or site-directed mutants of the P450arom promoter II/I.3 region; (ii) 5 ng of pRL-CMV *Renilla* luciferase control reporter vectors that contain the cDNA encoding *Renilla* luciferase (Promega, Madison, WI) as an internal control for transfection efficiency. Selective mutations of the two NF-IL6 sites (−350/−337 and −317/−304) and CRE (−211/−199) in the PGL<sub>3</sub>B construct containing the −517/−16-bp 5'-flanking region of the P450arom gene harboring TATA boxes of both promoters II and I.3 have been described previously (25, 39) (see Fig. 1).

The day before transfection, BAFs in primary culture were seeded into 35-mm dishes at 2 × 10<sup>5</sup> cell/dish. At the time of transfection, BAFs were 80% confluent. The transfection solution was made of 200 μl of OPTI-MEM I reduced serum medium containing PLUS reagent (8 μl), pre-complexed DNA (1.2 μg), and 5 μl of Lipofectamine reagent. After transfection for 6 h in transfection solution at 37 °C in 5% CO<sub>2</sub>, medium was changed to antibiotic-free DMEM/F-12 containing 10% FBS for overnight recovery. Cells were then switched to treatment conditions for another 48 h. Luciferase and *Renilla* luciferase (internal control) assays were performed in 10 μl of cell lysates using a dual-luciferase reporter assay system kit (Promega, Madison, WI). Luminescence activities were measured using a LUMAT LB9507 luminometer (Berthold GmbH, Bad Wildbad, Germany). Results are presented as the average of data from triplicate replicates and expressed as the ratio to the internal standard *Renilla* luciferase. The empty luciferase vector PGL<sub>3</sub>-basic was arbitrarily assigned a unit of 1, and the rest of the results were expressed

as multiples of the PGL<sub>3</sub>-basic vector. Both experiments were repeated in BAFs from three different subjects with reproducible results.

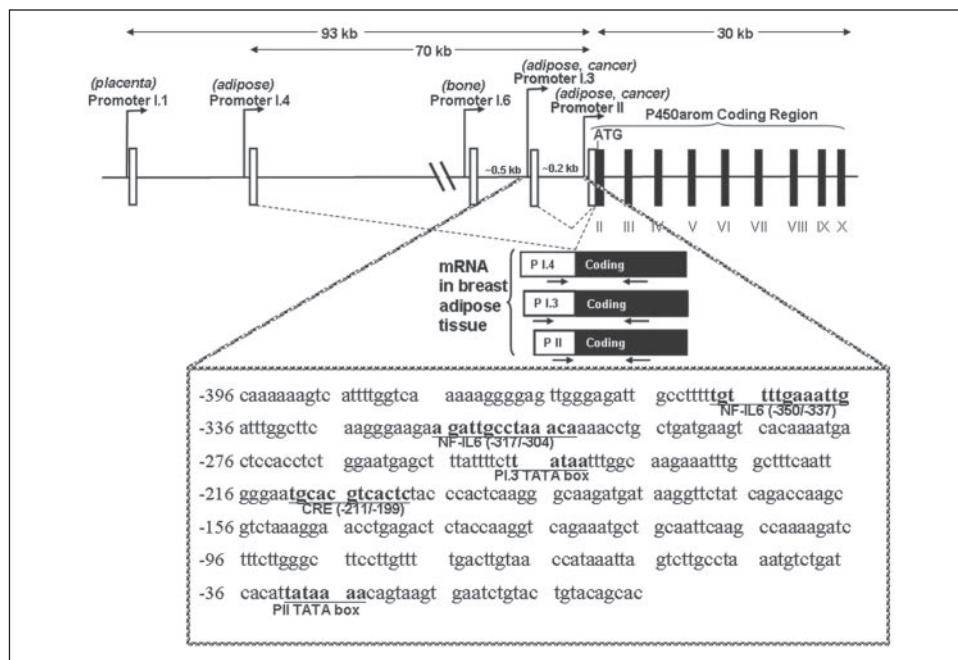
**Electrophoretic Mobility Shift Assay (EMSA)**—The nuclear extracts used for EMSA were prepared using NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce) according to the product instruction provided by the vendor. Briefly, treated cells were scraped and pelleted by centrifugation at 500 × g for 3 min. Ice-cold CER-I containing 1× protease inhibitor mixture was added to the cell pellet and incubated on ice for 10 min followed by addition of ice-cold CER-II for another 1 min. The cell nuclei were pelleted by centrifugation at 16,000 × g for 5 min and extracted with ice-cold NER containing 1× protease inhibitor mixture for 40 min. Nuclear debris was removed by centrifugation at 16,000 × g for 30 min. The nuclear protein was assayed for concentration using BCA-200 protein assay reagents and stored at −80 °C.

The double-stranded oligonucleotide was obtained through annealing sense and antisense sequences. The double-stranded oligonucleotide probe was end-labeled with [<sup>γ</sup>-<sup>32</sup>P]ATP using T4 kinase. One μg of nuclear extract was incubated with cold probe competitor and a binding buffer containing 20 mM HEPES (pH 7.6), 75 mM KCl, 0.2 mM EDTA, 20% glycerol, and 2 μg of poly(dI-dC)-poly(dI-dC) as a nonspecific competitor on ice for 1 h. The incubation was followed by addition of 30,000 cpm of the radiolabeled double-stranded oligonucleotide and incubated at room temperature for 20 min. Protein-DNA complexes were resolved on 6% nondenaturing polyacrylamide gels. Antibody-mediated depletions were performed by incubating 1.5 μl of the specific antibody with nuclear protein on ice for 1 h before the addition of radiolabeled probe. The probe (5'-GAA TGC ACG TCA CTC TAC CCA CT-3') represents an identical 23-bp-long sequence (−214/−192) within the promoter II regulatory region of the P450arom gene and contained an imperfect CRE (−211/−199). PAGE-purified oligonucleotides were obtained from Invitrogen.

**Chromatin Immunoprecipitation Assay (ChIP)**—The *in situ* binding of specific transcription factors to the promoter II/I.3 region was analyzed using ChIP, as detailed elsewhere (40, 41). BAFs were cultured in 10-cm plates. After reaching confluence, BAFs were maintained either in serum-free DMEM/F-12, serum-free DMEM/F-12 containing 15 mM of NaBu, MCM, or MCM containing 15 mM of NaBu. Treatment with MCM lasted 12 h. For the treatment with MCM plus NaBu, NaBu was added 24 h prior to the addition of MCM, and BAFs were incubated in MCM plus NaBu for an additional 12-h period. ChIP assay was performed according to the instructions provided by the vendor (Upstate Biotechnology, Inc., Lake Placid, NY) with minor modifications. Briefly, treated cells were cross-linked by adding formaldehyde directly to culture plates at a final concentration of 1% and incubating for 10 min at 37 °C. Cells were washed once using ice-cold phosphate-buffered saline containing 1× protease inhibitor mixture (Sigma) and pelleted for 4 min at 700 × g at 4 °C. SDS lysis buffer was added to resuspend the cell pellet for 10 min on ice. Cell lysate was sonicated on ice to an average DNA length of 200–500 bp using a Branson sonifier 250 (G. Heineemann, Schwäbisch Gmünd, Germany) at a constant output of 20% for 10 s. This was repeated four more times. Lysate was then centrifuged for 10 min at 13,000 rpm at 4 °C to remove cell debris. The supernatant fraction was adjusted to 25 units/ml at A<sub>260</sub>. Five hundred-μl quantity of each sample was diluted by 10-fold in ChIP dilution buffer containing 1× protease inhibitors mixture (Sigma) and pre-cleared with 80 μl of salmon sperm DNA/protein A-agarose slurry for 30 min at 4 °C with agitation. Antibodies against acetylated histone H3, acetylated histone H4, acetylated lysine, histone H3, and CBP were purchased from Upstate Biotechnology, Inc. Antibodies against ATF-2, C/EBPβ, and phosphorylated ATF-2 (Thr-71) were purchased from Santa Cruz Bio-

## Promoter-specific Ablation of Aromatase by Na-Bu

**FIGURE 1. The structure of the human *P450arom* gene.** The coding region contains nine translated exons (II–X, closed bars). The 5'-flanking region of the *P450arom* gene contains a number of alternatively used and partly tissue-specific promoters that give rise to splicing of untranslated first exons (open bars) onto a common junction upstream of the ATG translation start site. The coding regions of *P450arom* transcripts and thus the translated protein, however, are identical in each tissue site of expression. Three promoters are used in breast adipose tissue. In benign tissue, I.4 is primarily used for transcription of the *P450arom* gene at low levels. In breast tissue that bears a malignant tumor, promoters I.3 and II are markedly up-regulated in breast adipose fibroblasts severely increasing total mRNA levels. Positions of primers used for PCR amplification of promoter-specific mRNA species are indicated by arrows. Promoters I.3 and II are coordinately regulated by a number of cis-acting elements responsive to a cAMP-dependent pathway. TATA boxes of these promoters lie within a 215-bp region upstream to the common splice junction. Some of the cis-acting elements that regulate aromatase expression in adipose fibroblasts are indicated (22, 31–34, 42).



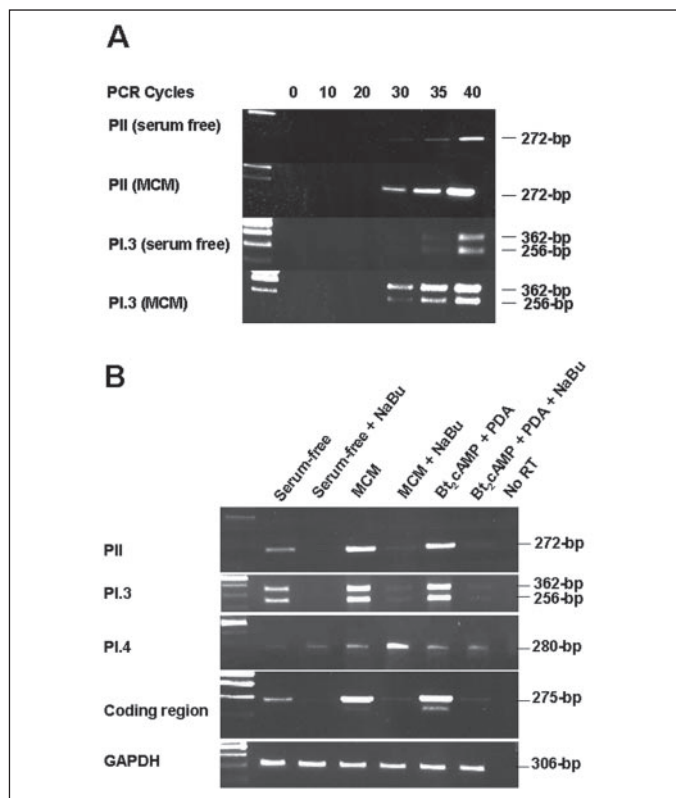
technology (Santa Cruz, CA). Normal rabbit IgG was also obtained from Santa Cruz Biotechnology as a negative antibody control. Five- $\mu\text{g}$  quantity of each antibody was added to 1 ml of chromatin solution and incubated overnight at 4 °C with rotation. The immune complexes were collected with 60  $\mu\text{l}$  of salmon sperm DNA/protein A-agarose slurry for 1 h at 4 °C with rotation. The beads were pelleted by centrifugation and washing sequentially using 1 ml of each of the buffers as listed below: low salt immune complexes wash buffer; high salt immune complexes wash buffer; LiCl immune complex wash buffer; and 1 $\times$  TE buffer. To reduce the background, the last washed buffer of IgG control was monitored by measuring the OD. The immune complex was eluted twice by using 250  $\mu\text{l}$  of elution buffer. Part of the eluate was saved and checked for the targeted protein immunoprecipitated using Western blotting. Twenty- $\mu\text{l}$  quantity of 5 M NaCl was added to the eluates to reverse cross-links at 65 °C for 4 h. Ten  $\mu\text{l}$  of 0.5 M EDTA, 20  $\mu\text{l}$  of 1 M Tris-HCl (pH 6.5), and 2  $\mu\text{l}$  of 10 mg/ml proteinase K were added to the eluate and incubated for 1 h at 45 °C. The eluate was extracted once using phenol/chloroform/isoamyl alcohol and precipitated with ethanol. DNA was resuspended in 50  $\mu\text{l}$  of TE buffer and used for PCR amplification of -511/-194-bp sequence of promoter II/I.3 region. A 5'-end sense primer (5'-CGG AGT CAA CGG ATT TGG TCG TAT-3') and a 3'-end antisense primer (5'-AGC CTT CTC CAT GGT GGT GAA GAC-3') were used for amplifying a 318-bp-long sequence in promoter II/I.3 regulatory region for 35 cycles.

**Immunoprecipitation (IP)-Immunoblotting Assays**—IP with an anti-pATF-2 or anti-CBP antibody was performed with cytosolic preparations of BAFs treated with MCM, Bt<sub>2</sub>cAMP + PDA, or NaBu, individually or in combination. The cells were scraped in IP buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 0.5 mM sodium orthovanadate, 40 mM NaF, 10 mM  $\beta$ -glycerophosphate, complete protease inhibitors). Cytosols were immunoprecipitated with antibodies against human pATF-2 or CBP. Incubation was carried out overnight at 4 °C. Protein antibody complexes were recovered by protein A-Sepharose or anti-rabbit IgG; the isolated immune complexes were washed three times with IP buffer and fractionated on SDS-PAGE. Proteins transferred to nitrocellulose membrane were probed with antibodies against pATF-2, nonphosphorylated ATF-2, and C/EBP $\beta$ . Anti-

bodies against pATF-2, nonphosphorylated ATF-2, or C/EBP $\beta$  were purchased from Santa Cruz Biotechnology. Anti-CBP antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Intensities in specific bands were quantified by Luminescent Image Analyzer, LAS-3000, Fujifilm, Tokyo, Japan.

**Immunoblotting**—Total protein used for Western blotting was prepared by using M-PER<sup>TM</sup> mammalian protein extraction reagent (Pierce). Pretreated cells were pelleted by centrifugation at 2,500  $\times g$  for 10 min. M-PER<sup>TM</sup> reagent was added to the cell pellet containing 1 $\times$  protease inhibitor mixture and gently mixed for 10 min. Cell debris was removed by centrifugation at 27,000  $\times g$  for 15 min. Protein concentration was determined using BCA-200 protein assay reagents (Pierce). Twenty five  $\mu\text{g}$  of total protein was mixed 1:1 with Laemmli sample buffer (Bio-Rad) and heated at 95 °C for 5 min. Samples were subjected to 12% SDS-PAGE. Equal protein loading was confirmed by staining in a parallel gel. Proteins were then transferred to nitrocellulose filters in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). Filters were blocked with phosphate-buffered saline containing 5% nonfat dried milk for 1 h and then incubated with 1  $\mu\text{g}/\text{ml}$  specific antibody for 1 h at room temperature. After washing with TTBS (0.1 M Tris-HCl (pH 7.5), 0.9% sodium chloride, 0.05% Tween 20) four times for 15 min, filters were incubated with 1:10,000 horseradish peroxidase-labeled second antibody for 1 h at room temperature and washed with TTBS four times for 15 min. Immunodetection was performed using SuperSignal West Femto maximum sensitivity substrate (Pierce). Filters were stripped with 0.2 M NaOH for 5 min and re-used for other antibody hybridizations. Antibodies against ATF-2 and phosphorylated Thr-71 ATF-2 (p-ATF-2) were obtained from Santa Cruz Biotechnology. Antibody against phosphorylated CREB-1 was purchased from Sigma. The phosphorylated CREB-1 antibody was reactive with serine 133-phosphorylated CREB-1 and correspondingly phosphorylated CREM-1 and ATF-1, as defined by the vendor. Intensities in specific bands were quantified by Luminescent Image Analyzer, LAS-3000, Fujifilm, Tokyo, Japan.

**Statistical Analysis**—Statistical analysis for comparison of treatment groups was performed by paired *t* test. A *p* value <0.05 was considered



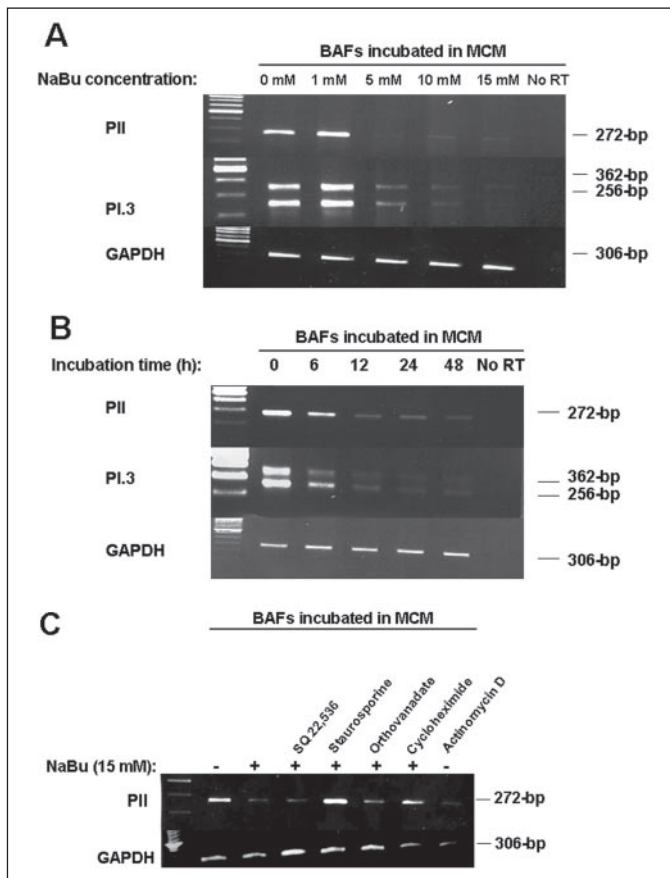
**FIGURE 2. NaBu inhibits P450arom expression via selectively silencing P450arom promoters I.3 and II in BAFs.** *A*, PCR yields of promoter II- or I.3-specific P450arom transcripts showed linear increases in parallel to PCR amplification cycles using RNA from BAFs incubated in the presence or absence of MCM. *B*, both MCM and Bt<sub>2</sub>cAMP plus PDA stimulated P450arom transcript levels via activation of promoters II and I.3. The addition of NaBu strikingly decreased P450arom transcript levels via silencing of promoters II/I.3 but not I.4. The increase in I.4-specific transcript levels in response to NaBu did not give rise to a significant increase in total P450arom transcript levels as measured by amplification of the common coding region. After reaching confluence, BAFs did not give rise to a significant increase in total P450arom transcript levels as measured by amplification of the common coding region. After reaching confluence, BAFs were placed in serum-free medium for 24 h to remove the effects of serum. BAFs were then incubated under various conditions for a total of 48 h. Control, DMEM/F-12 only; NaBu, DMEM/F-12 + NaBu (15 mM); MCM, MCF-7 cell-conditioned medium; MCM + NaBu, MCM + NaBu (15 mM); Bt<sub>2</sub>cAMP + PDA, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM); Bt<sub>2</sub>cAMP + PDA + NaBu, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM) + NaBu (15 mM); no RT, no reverse transcriptase reaction mixture added as a negative control. NaBu was added 24 h prior to the treatment with MCM or Bt<sub>2</sub>cAMP plus PDA, and treatments with NaBu lasted for another 24 h together with other treatments (MCM or Bt<sub>2</sub>cAMP plus PDA) before harvesting the cells for RNA isolation. Total RNA was subjected to exon-specific RT-PCR to amplify promoter-specific untranslated first exons or the common coding region (for total P450arom transcript levels). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to control the integrity of RNA.

significant. All values are given as the mean, with the bars showing mean  $\pm$  S.E.

## RESULTS

**Bt<sub>2</sub>cAMP + PDA or MCM Induced but NaBu Selectively Silenced P450arom Promoters I.3 and II in BAFs**—Aromatase expression in human tissues is under the control of alternatively used and partially tissue-specific promoters (18, 19). The coding region of P450arom transcripts and thus the translated protein, however, are identical in each tissue site (18, 19) (Fig. 1). In the breast adipose tissue of disease-free women, P450arom is expressed at low levels via a distal promoter I.4, whereas in the adipose tissue of breast bearing a tumor, P450arom expression is increased through activation of promoters I.3 and II (20–22).

Previously, we have shown that MCM stimulated aromatase expression via the promoter I.3/II-specific genomic region in BAFs (25). Therefore, we used the same *in vitro* system to identify potential pro-



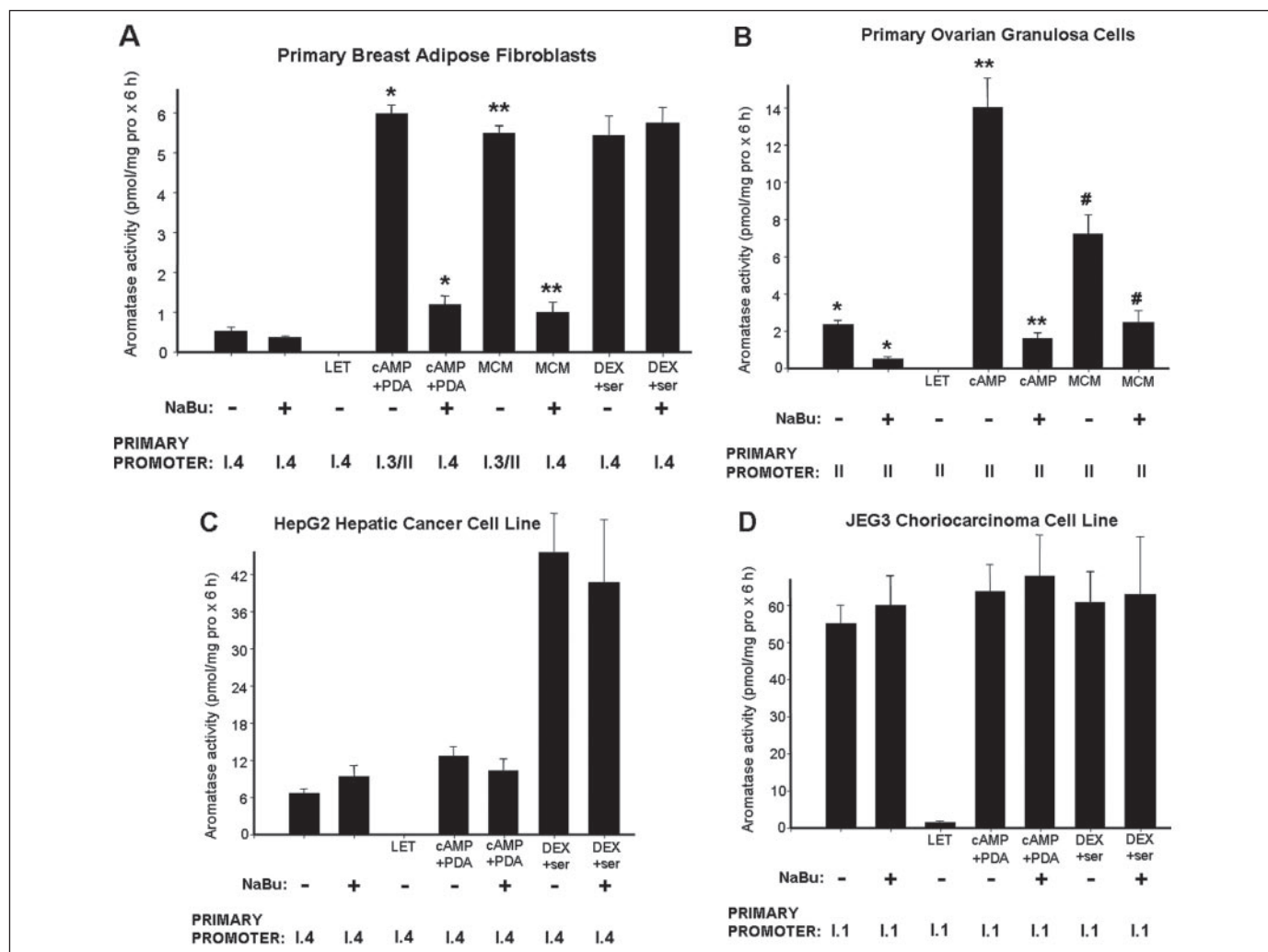
**FIGURE 3. Time kinetics and dose response for the inhibitory effects of NaBu.** BAFs were incubated in MCM for 24 h. We then added 15 mM NaBu and continued incubation for various periods, or we maintained these cells in MCM containing various concentrations of NaBu for another 24 h. Total RNA was isolated and subjected to exon-specific RT-PCR to amplify promoter-specific untranslated first exons of P450arom transcripts. *A*, the time kinetics of inhibitory effects of NaBu on promoters II and I.3. *B*, NaBu decreased promoter II- and I.3-specific transcripts in a dose-dependent manner. *C*, staurosporine (10 nM) or cycloheximide (30  $\mu$ M) reversed the inhibitory effect of NaBu, whereas neither SQ22,536 (100  $\mu$ M) nor sodium orthovanadate (5  $\mu$ M) altered the NaBu effect. As expected, actinomycin D (5  $\mu$ g/ml) decreased MCM-stimulated mRNA levels. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

motor-specific regulators of P450arom promoters I.3 and II. Because the use of each alternative promoter gives rise to a P450arom transcript with an untranslated 5'-end unique for that particular promoter, we used exon-specific RT-PCR to determine total and promoter-specific transcript levels in BAFs in primary culture. Specific primers that anneal to sequences in promoter-specific first exons were employed (see Fig. 1). To standardize PCR conditions for promoter I.3/II-specific transcripts, PCR yields under different number of cycles using RNA from BAFs treated with or without MCM were determined (Fig. 2A). In subsequent experiments, we performed 35 cycles of PCR to remain within the linear range (Fig. 2A).

Because NaBu has been implicated in regulating cancer progression, we determined promoter II-, I.3-, and I.4-specific as well as total P450arom transcript levels in BAFs treated with MCM or Bt<sub>2</sub>cAMP + PDA in the absence or presence of NaBu. MCM was generated from one of the breast cancer cell lines MCF-7 or T47D. Either cell line gave rise to similar results. Each experiment using MCF-7 or T47D cell-conditioned media was reproducibly repeated at least three times. For consistency, we illustrate results from MCF-7-conditioned media in Figs. 2–10.

MCM or Bt<sub>2</sub>cAMP + PDA stimulated P450arom transcript levels via activation of promoters II and I.3 (Fig. 2B). The addition of NaBu strik-





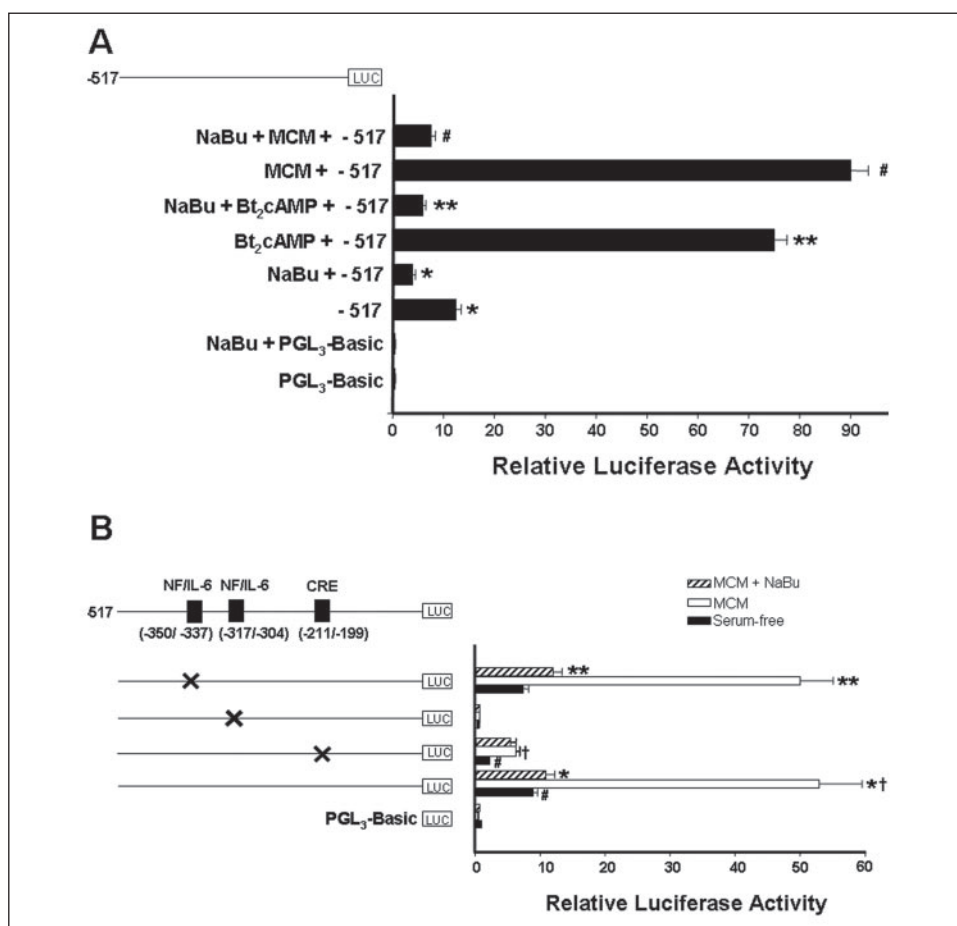
**FIGURE 4. Regulation of aromatase activity and promoter usage by various hormones or NaBu in four distinct human cell types that use alternative promoters for aromatase expression.** Cells were maintained in serum-free DMEM/F-12 medium for 12 h and then incubated with the labeled substrate androstenedione under various conditions. Control, DMEM/F-12; LET, letrozole  $10^{-8}$  M; cAMP + PDA,  $Bt_2cAMP$  (0.5 mM) + PDA (100 nM) in DMEM/F-12; MCM, MCF-7 cell-conditioned DMEM/F-12; DEX + ser, dexamethasone  $5 \times 10^{-7}$  M and 10% fetal bovine serum in DMEM/F-12; NaBu, NaBu (15 mM). Assay conditions are described under "Experimental Procedures." Enzyme activity in the absence of a treatment is taken as 100%. The error bars represent means  $\pm$  S.E. for each treatment that was carried out in triplicate replicates. Primary promoter usage was determined by untranslated first exon-specific RT-PCR applied to total RNA isolated from cells incubated under identical conditions. Aromatase activity in each cell type treated with the aromatase enzyme inhibitor letrozole (LET) was undetectable. Statistically significant differences between treatment pairs were indicated as follows. A, \*,  $p < 0.001$ ; \*\*,  $p < 0.001$ . B, \*,  $p < 0.01$ ; \*\*,  $p < 0.01$ ; #,  $p < 0.05$ . C and D, NaBu did not regulate aromatase activity significantly in HepG2 or JEG3 cell lines, in which promoters I.3/II are not important regulators of the P450arom gene.

ingly decreased promoter I.3/II-specific basal P450arom mRNA levels in untreated BAFs or in BAFs incubated with MCM or  $Bt_2cAMP$  + PDA, whereas promoter I.4-specific mRNA species increased slightly upon treatment with NaBu. This NaBu-induced increase in I.4-specific P450arom mRNA levels was physiologically insignificant, because NaBu gave rise to a robust decrease in total P450arom mRNA levels as determined by amplification of the common coding region or aromatase enzyme activity (Fig. 2B, see Fig. 4). Because of low copy numbers of promoter I.4-specific transcripts, we used 38 cycles of PCR to amplify I.4-specific transcripts, whereas 35 cycles were used for total, I.3-, or II-specific transcripts. NaBu showed no toxic effects on the viability of BAFs as assessed by trypan blue dye exclusion test (data not shown). These results suggested that NaBu could suppress the levels of both base line- and MCM-induced promoter II- and I.3-specific P450arom transcripts.

In the presence of increasing concentrations of NaBu, levels of promoter I.3/II-specific transcripts in MCM-stimulated cells

decreased in a dose-dependent fashion with a maximum inhibition at 15 mM (Fig. 3A). A significant decrease in promoter I.3/II-specific transcript levels in BAFs incubated in the presence of 15 mM of NaBu was evident after 6 h, and a maximal inhibition was observed after 12 h of incubation. The inhibition lasted up to the 48-h time point (Fig. 3B). In an independent experiment, the inhibitory effects of NaBu (15 mM) on P450arom transcript levels in MCM-treated BAFs lasted up to 72 h (data not shown).

The inhibitory effects of NaBu on promoters I.3 and II (data for I.3 not shown) were reversed by the kinase inhibitor staurosporine but not by the phosphatase inhibitor sodium orthovanadate or the adenyl cyclase inhibitor SQ22,536 (Fig. 3C). Reversal by cycloheximide indicated that the NaBu effect was dependent on new protein synthesis (Fig. 3C). NaBu also decreased basal promoter I.3/II-specific P450arom mRNA levels in BAFs not incubated in MCM (data not shown). Experiments illustrated in Figs. 2 and 3 were reproducibly repeated using BAFs from six different subjects.



**FIGURE 5. Regulation of the P450arom promoter I.3/II region by Bt<sub>2</sub>cAMP + PDA, MCM, and NaBu in BAFs.** Luciferase plasmids containing the -517/-16 bp 5'-flanking region containing P450arom promoters II and I.3 were transfected into BAFs. pCMV *Renilla* was used as an internal control for transfection efficiency. Promoter II/I.3 activity was normalized to pCMV *Renilla* and was represented as the average of data from triplicate replicates  $\pm$  S.E. The empty luciferase vector PGL<sub>3</sub>-basic was arbitrarily assigned a unit of 1, and the rest of the results were expressed as multiples of the PGL<sub>3</sub>-basic vector. **A**, statistically significant differences were observed between the following treatment pairs: \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ ; #,  $p < 0.001$ . **B**, selective mutations of the NF-IL6 site at -317/-304 or CRE at -211/-199 significantly decreased basal promoter activity and induction by MCM. #,  $p < 0.01$ ; †,  $p < 0.001$ ; \*,  $p < 0.005$ ; \*\*,  $p < 0.005$ .

**NaBu Inhibited Induction of Aromatase Enzyme Activity Regulated by Promoters I.3 and II but Not by I.4 or I.1 in Various Cell Types**—We and others demonstrated previously that MCM or Bt<sub>2</sub>cAMP + PDA markedly induced aromatase activity regulated via promoters I.3/II (25, 39). Here we showed that the addition of NaBu to either MCM or Bt<sub>2</sub>cAMP + PDA treatment eliminated the induction of aromatase activity. On the other hand, NaBu did not inhibit dexamethasone plus serum-induced aromatase activity that is regulated by promoter I.4 (Fig. 4A). The experiment illustrated in Fig. 4A is representative of three experiments performed on BAFs from three subjects undergoing reduction mammoplasty.

Untreated, Bt<sub>2</sub>cAMP-treated, or MCM-treated ovarian granulosa cells in primary culture use primarily promoter II for aromatase expression or activity. NaBu inhibited aromatase activity in granulosa cells under all these conditions (Fig. 4B). In contrast, NaBu did not inhibit aromatase activity of HepG2 liver cancer cells that use promoter I.4 or Jeg3 cells that use promoter I.1 for aromatase expression (Fig. 4, C and D). These results are significant because they indicate that regardless of cell type, NaBu inhibits only promoter II or I.3-mediated aromatase activity. Failure to inhibit aromatase activity in HepG2 or Jeg3 cells ruled out the possibility that NaBu directly inhibited the aromatase enzyme. Experiments illustrated in Fig. 4, B–D, were repeated three times.

**Induction of P450arom Promoter I.3/II Activity by MCM or Bt<sub>2</sub>cAMP + PDA Was Conferred by a CRE at -211/-199 bp**—To determine further the target of NaBu, we performed transfection-based reporter assays. NaBu inhibited basal, Bt<sub>2</sub>cAMP-induced, or MCM-induced activity of a P450arom-promoter I.3/II-Luciferase vector (Fig. 5A). Selective mutations of the -211/-199-bp CRE or the -317/-304-bp

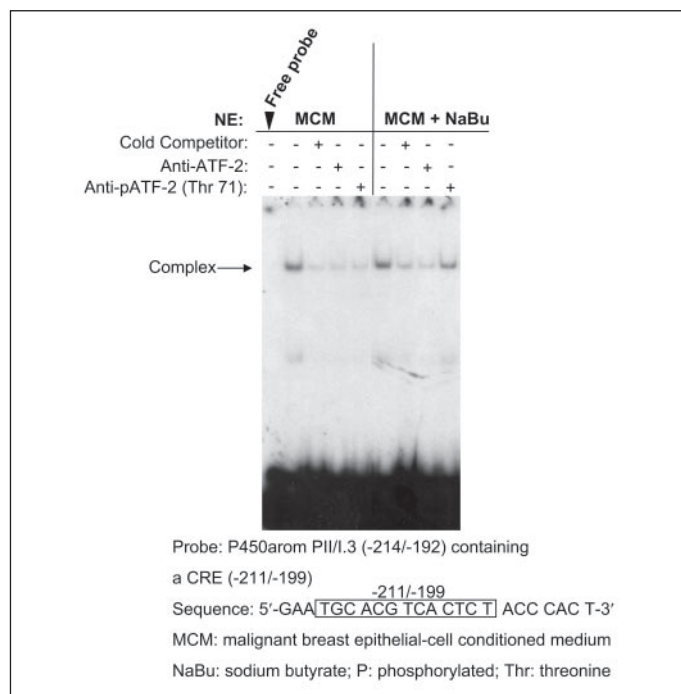
NF-IL6 site in this -517-bp promoter I.3/II region significantly reduced basal or MCM-induced activity (Fig. 5B). On the other hand, mutation of another NF-IL6 site at -350/-337 bp did not alter promoter activity (Fig. 5B).

**Phosphorylated ATF-2 Bound to a CRE in the Promoter I.3/II Region**—We demonstrated previously that C/EBP $\beta$  bound to the -317/-304 bp-NF-IL6 in the P450arom promoter I.3/II region in response to MCM (25). Here we characterized the -211/-199-bp CRE, because its mutation also abolished promoter II/I.3 activity (Fig. 5B). By using a radiolabeled double-stranded DNA probe containing this sequence and nuclear extracts from BAFs, we demonstrated a specific DNA-protein complex (Fig. 6).

We employed antibodies against CREB-1, CREB-2, ATF-1, and ATF-2 (data not shown). In BAFs incubated with MCM, only antibodies against both nonphospho-specific ATF-2 and phospho-specific ATF-2 phosphorylated at Thr-71 (pATF-2) depleted this complex. In the presence of MCM plus NaBu, however, the anti-pATF-2 antibody failed to deplete this complex. In conclusion, both phosphorylated and nonphosphorylated ATF-2 bound to the -214/-192-bp region of promoter I.3/II that contains a CRE in MCM-treated BAFs. NaBu selectively inhibited binding of pATF-2 to this motif (Fig. 6). This experiment was reproducibly repeated using BAFs from three different subjects.

**Binding Activities of Phosphorylated ATF-2 (pATF-2), C/EBP $\beta$ , and CBP to the Promoter I.3/II Regulatory Region in BAFs Were Enhanced by MCM or Bt<sub>2</sub>cAMP + PDA and Abolished by NaBu**—We used ChIP-PCR to evaluate binding of a number of transcription factors and coregulators to the -511/-194-bp 5'-flanking region of the P450arom gene. We chose this region because it contains the two critical cis-acting



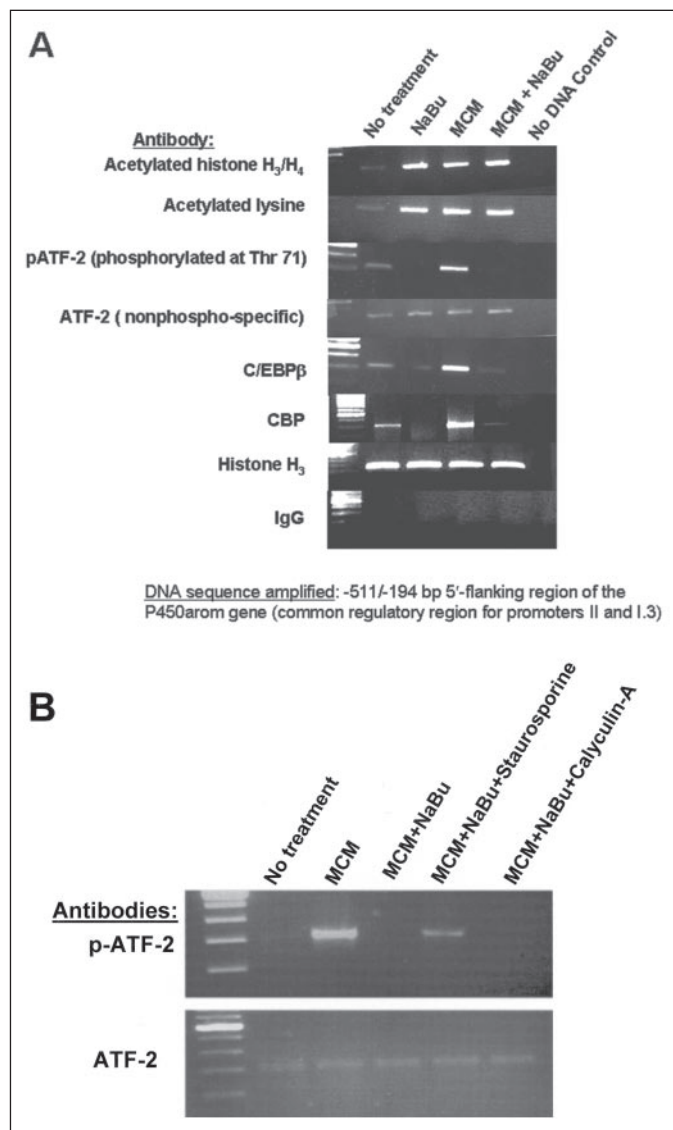


**FIGURE 6. MCM enhances and NaBu reduces recruitment of phosphorylated ATF-2 by a CRE (-211/-199) at promoter I.3/II region.** EMSA was performed using an oligonucleotide probe (-214/-192 bp) containing the -211/-199-bp CRE, 1  $\mu$ g of nuclear extract (NE) from BAFs incubated with MCM or MCM plus NaBu (15 mM), and antibodies against nonphospho-specific ATF-2 or phosphorylated ATF-2 at Thr-71 (pATF-2). We identified a specific complex as verified by a cold competitor probe. The complex had a similar size in BAFs treated with MCM in the presence or absence of NaBu. Antibodies against ATF-2 (nonphospho-specific) or pATF-2 depleted the complex in BAFs treated with MCM, indicating the presence of nonphosphorylated and phosphorylated ATF-2 in this complex. On the other hand, the complex in BAFs treated with MCM plus NaBu was depleted only by the anti-ATF-2 antibody (nonphospho-specific) indicating the absence of phosphorylated ATF-2 in the complex.

elements (-211/-199-bp CRE and -317/-304 C/EBP site), which we showed to be essential for MCM or  $Bt_2cAMP$  + PDA induction of promoter I.3/II activity (Fig. 5) (25).

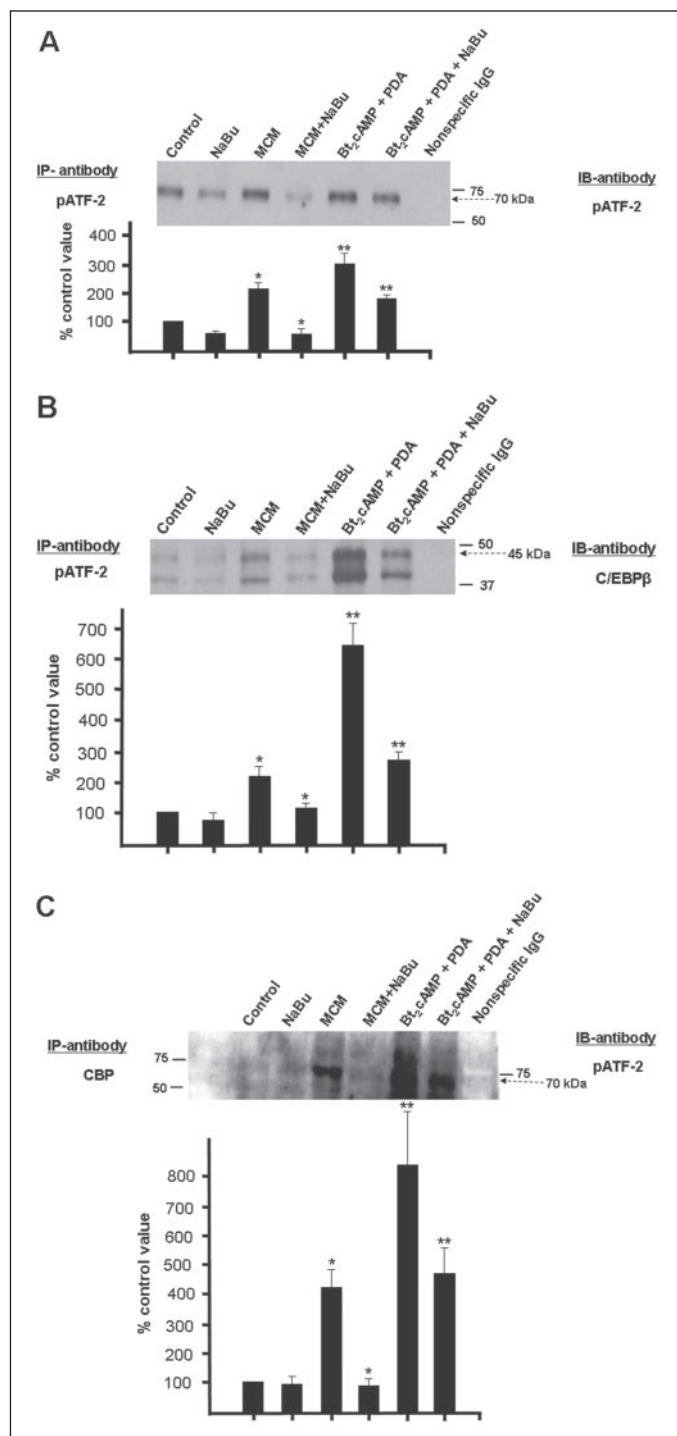
As illustrated in Fig. 7A, acetylation of histones and other DNA-binding proteins across the -511/-194-bp promoter regulatory region as detected by either anti-acetylated histone H3/H4 or anti-acetylated lysine antibody was strikingly enhanced upon treatment by MCM or  $Bt_2cAMP$  + PDA. Among its various functions, NaBu inhibits class I and II histone deacetylases. Apparently, increased histone acetylation *per se* did not account for the activation of promoters I.3/II because NaBu also enhanced histone hyperacetylation of this regulatory region (Fig. 7A). NaBu, however, silenced both promoters II and I.3 (see Figs. 2-4). Thus, we hypothesized that binding of a multimeric complex containing specific transcription factors rather than the histone acetylation status was important to activate promoters I.3/II, and NaBu might block formation of such an activating complex.

We next investigated whether the recruitment of a stable complex composed of transcription factors (pATF-2 and C/EBP $\beta$ ) and a coactivator CBP was regulated by MCM and NaBu. We determined the recruitment of C/EBP $\beta$  because it was shown previously to be essential for MCM induction of promoters I.3/II (25). We also just uncovered that pATF-2 was a key transcription factor for this induction. CBP is a commonly recruited coactivator that interacts with members of the CREB/ATF family transcription factors and C/EBPs. As we predicted, MCM strongly enhanced the recruitment of pATF-2, C/EBP $\beta$ , and CBP to the -511/-194-bp common regulatory region of promoters I.3/II, on the other hand, NaBu disrupted strikingly the recruitment of each of

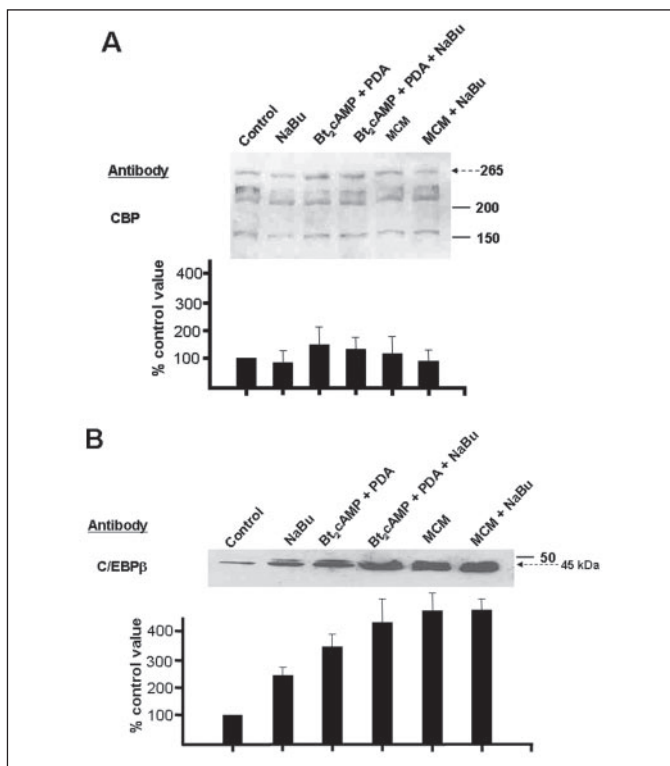


**FIGURE 7. Binding of phosphorylated ATF-2 (Thr-71), C/EBP $\beta$ , and CBP to promoter I.3/II regulatory region in BAFs is enhanced by MCM and abolished by NaBu.** Findings depicted in Fig. 6 suggested that MCM increased and NaBu decreased the phosphorylated state of ATF-2 that occupies a CRE in the promoter I.3/II region. We confirm these observations by ChIP, and we also demonstrate that pATF-2 likely stabilizes a complex composed of pATF-2, C/EBP $\beta$ , and CBP at this promoter region. BAFs were maintained in serum-free DMEM/F-12 for 24 h and then incubated under various conditions for a total of 36 h (no treatment, DMEM/F-12 only; NaBu, DMEM/F-12 + NaBu (15 mM); MCM, MCF-7 cell-conditioned medium; MCM + NaBu, MCM + NaBu (15 mM)). Treatments lasted for 12 h. For the treatment with MCM plus NaBu, NaBu was added 24 h prior to the addition of MCM, and incubation in MCM plus NaBu lasted for another 12 h. Cells were cross-linked with 1% formaldehyde followed by immunoprecipitation using various antibodies. *A*, anti-histone H3 and normal rabbit IgG were used as positive and negative controls for the ChIP assay, respectively. We amplified by PCR the -511/-194-bp sequence in the P450arom promoter II/I.3 regulatory region (see Fig. 1). The intensities of PCR fragments correlate with the *in situ* binding affinities of these factors to the -511/-194-bp region. Both MCM and NaBu increased the quantities of acetylated histones, which were detected by both anti-acetylated histone H3/H4 and anti-acetylated lysine antibodies. In contrast to acetylated histone H3/H4, binding affinities of phosphorylated ATF-2 (Thr-71), C/EBP $\beta$ , and CBP to the promoter II/I.3 regulatory region were specifically enhanced by MCM and inhibited by NaBu. On the other hand, binding affinities of factors that could be immunoprecipitated by a nonphospho-specific ATF-2 was not altered by these treatments. *B*, staurosporine (10 nM) reversed NaBu-dependent inhibition of pATF-2 binding to the P450arom promoter, whereas calyculin-A (80 nM) did not alter this effect.

these factors (Fig. 7A). These findings are further indicative that stable recruitment of C/EBP $\beta$  and CBP is dependent on binding of pATF-2 because these treatments did not change the binding activity of ATF-2 detected by a nonphospho-specific antibody (Fig. 7A).



**FIGURE 8. Association of phosphorylated ATF-2 with CBP and C/EBP $\beta$  in BAFs in response to treatments with MCM and NaBu.** Immunoprecipitation (IP) with an anti-pATF-2 (A and B) or anti-CBP (C) antibody was performed using whole cell preparations of BAFs treated in a manner described below. Control, DMEM/F-12 only; NaBu, DMEM/F-12 + NaBu (15 mM); MCM, MCF-7 cell-conditioned medium; MCM + NaBu, MCM + NaBu (15 mM); Bt<sub>2</sub>cAMP + PDA, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM); Bt<sub>2</sub>cAMP + PDA + NaBu, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM) + NaBu (15 mM). A and B, whole cell extracts were immunoprecipitated (IP) with a mouse monoclonal IgG against human pATF-2. After washing, immunoprecipitates were separated in 8% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted (IB) with a rabbit polyclonal antibody against pATF-2 (A) or C/EBP $\beta$  (B). C, whole cell extracts were immunoprecipitated with a rabbit polyclonal anti-human CBP. Protein-antibody complexes were recovered by an anti-rabbit IgG and resolved in 8% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and probed with an antibody against human pATF-2. Controls: nonspecific IgG, immunoprecipitation with a nonspecific mouse (A and B) or rabbit (C) IgG. Significance of differences between paired treatments are as follows: A, \*,  $p < 0.001$ ; \*\*,  $p < 0.05$ . B, \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ . C, \*,  $p < 0.001$ ; \*\*,  $p < 0.05$ .



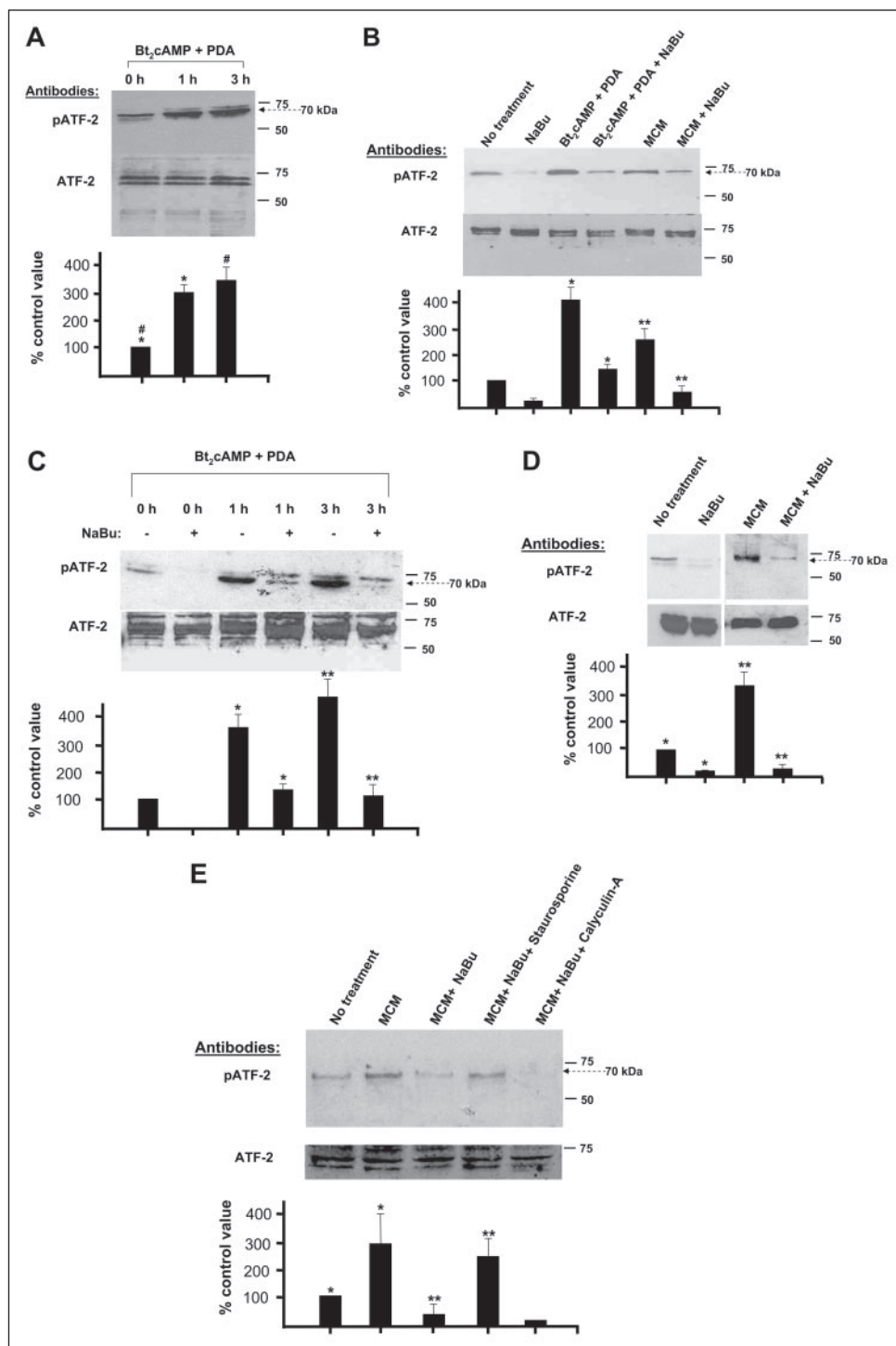
**FIGURE 9. NaBu does not decrease levels of CBP or C/EBP $\beta$ .** BAFs were maintained in serum-free medium for 24 h and then incubated under various conditions for a total of 36 h (no treatment, DMEM/F-12 only; NaBu, DMEM/F-12 + NaBu (15 mM); Bt<sub>2</sub>cAMP + PDA, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM); Bt<sub>2</sub>cAMP + PDA + NaBu, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM) + NaBu (15 mM); MCM, MCF-7 cell-conditioned medium; MCM + NaBu, MCM + NaBu (15 mM)). Bt<sub>2</sub>cAMP + PDA or MCM treatments lasted for 12 h. NaBu was added 24 h prior to the treatments with MCM or Bt<sub>2</sub>cAMP + PDA and maintained in the presence of these treatments for another 12 h. Total protein was subjected to immunoblotting using an anti-CBP or anti-C/EBP $\beta$  antibody. A, the antibody against CBP recognized three isoforms with molecular masses of 265, 210, and 165 kDa. No obvious changes in protein abundance were observed under various conditions. B, BAFs cultured in serum-free condition exhibited a base-line level of C/EBP $\beta$ , which was markedly enhanced by treatment with Bt<sub>2</sub>cAMP + PDA or MCM. NaBu increased the protein level of C/EBP $\beta$ . (NaBu, however, inhibited the binding of C/EBP $\beta$  to the -511/-194 bp region; see Fig. 5.)

The use of anti-histone H3 antibody not selective for acetylated form (positive control) and nonspecific IgG (negative control) demonstrated that these findings were specific (Fig. 7A). This experiment was reproducibly repeated using BAFs from three separate subjects. These findings were suggestive that the binding of ATF-2 phosphorylated at Thr-71 but not nonphosphorylated ATF-2 was critical for MCM-induced activation of promoters I.3/II. They also suggested that NaBu interferes with the formation of this enhancer complex. Consistent with the effects of NaBu and signaling inhibitors on P450arom mRNA levels, the negative effect of NaBu on the recruitment of pATF-2 to the promoter I.3/II region was reversed by the kinase inhibitor staurosporine but not by the phosphatase inhibitor calyculin-A (Fig. 7B).

**MCM or Bt<sub>2</sub>cAMP + PDA Enhanced Formation of a Protein Complex Composed of pATF-2, C/EBP $\beta$ , and CBP, Which Was Disrupted by NaBu**—We performed IP by using antibodies against pATF-2 and CBP followed by immunoblotting using antibodies against pATF-2 or C/EBP $\beta$ . To determine the specific IP of the protein complex associated with pATF-2, we incubated proteins from BAFs treated with MCM, Bt<sub>2</sub>cAMP + PDA, or NaBu with a mouse monoclonal anti-pATF-2-agarose conjugate. Proteins recovered from such immunoprecipitates were analyzed by immunoblotting using a rabbit anti-pATF-2 antibody.

**FIGURE 10. Breast cancer cell-conditioned medium, Bt<sub>2</sub>cAMP plus PDA and NaBu regulate levels of phosphorylated ATF-2 (Thr-71).**

BAFs were maintained in serum-free medium for 24 h and then incubated under various conditions. **A**, Bt<sub>2</sub>cAMP + PDA, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM) incubated for 0, 1, and 3 h, respectively. Upon treatment with Bt<sub>2</sub>cAMP plus PDA, a 70-kDa immunoreactive band was recognized specifically by an antibody raised against ATF-2 phosphorylated at threonine 71 (pATF-2). Total ATF-2 levels as recognized by a nonphospho-specific antibody, however, did not change as a result of these treatments. \*,  $p < 0.001$ ; #,  $p < 0.005$ . **B**, no treatment, DMEM/F-12 only; NaBu, DMEM/F-12 + NaBu (15 mM); Bt<sub>2</sub>cAMP + PDA, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM); Bt<sub>2</sub>cAMP + PDA + NaBu, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM) + NaBu (15 mM); MCM, MCF-7 cell-conditioned medium; MCM + NaBu, MCM + NaBu (15 mM). MCM or Bt<sub>2</sub>cAMP plus PDA strikingly induced levels of phosphorylated ATF-2 (Thr-71). On the other hand, NaBu significantly decreased both base-line and induced levels of phosphorylated ATF-2 (pATF-2). Total ATF-2 protein levels did not vary under these conditions. \*,  $p < 0.005$ ; \*\*,  $p < 0.01$ . MCM or Bt<sub>2</sub>cAMP plus PDA treatment lasted for 12 h. NaBu was added 24 h prior to treatments with MCM or Bt<sub>2</sub>cAMP plus PDA and maintained in the presence of these treatments for another 12 h. **C**, Bt<sub>2</sub>cAMP + PDA, DMEM/F-12 + Bt<sub>2</sub>cAMP (0.5 mM) + PDA (100 nM), incubated for 0, 1, or 4 h in the presence or absence of 15 mM NaBu. NaBu decreased the phosphorylated state of ATF-2 in the presence of Bt<sub>2</sub>cAMP plus PDA. \*,  $p < 0.01$ ; \*\*,  $p < 0.01$ . **D**, no treatment, DMEM/F-12 only; NaBu, DMEM/F-12 + NaBu (15 mM); MCM, MCF-7-cell-conditioned medium; MCM + NaBu, MCM + NaBu (15 mM). MCM treatment lasted for 12 h. NaBu was added 24 h prior to the treatment of MCM and maintained in the presence of MCM for another 12 h. Immunoblotting using nuclear extracts verified the inhibitory effects of NaBu on levels of phosphorylated ATF-2 (pATF-2). \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ . (Experiments illustrated in A–C and E were performed using whole cell extracts.) **E**, staurosporine (10 nM) and calyculin-A (80 nM) were also used. Samples of total protein or nuclear protein were subjected to immunoblotting using antibodies against phosphorylated ATF-2 (Thr-71) and non-phospho-specific ATF-2. Staurosporine reversed the effect of NaBu on the phosphorylated state of ATF-2, whereas calyculin-A did not alter this effect. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



Higher levels of pATF-2 were detected in BAFs treated with MCM or Bt<sub>2</sub>cAMP + PDA, as expected (Fig. 8A). Treatment with NaBu decreased the levels of pATF-2 (Fig. 8A). Stripping and reprobing the blot with an anti-C/EBP $\beta$  antibody showed that complex pulled down by a mouse monoclonal anti-pATF-2 also contained C/EBP $\beta$  protein (Fig. 8B). Treatment with MCM or Bt<sub>2</sub>cAMP + PDA caused remarkable increases of C/EBP $\beta$  protein in the complex, whereas NaBu decreased significantly the interaction between pATF-2 and C/EBP $\beta$  (Fig. 8B).

As shown in Fig. 8C, immunoprecipitates generated by anti-CBP antibodies also contained pATF-2. CBP-pATF-2 complexes were detected in BAFs treated with MCM or Bt<sub>2</sub>cAMP + PDA, whereas

NaBu significantly inhibited formation of such complexes (Fig. 8C). These experiments were reproducibly repeated using BAFs from three different subjects.

*The Inhibitory Effects of NaBu on the Binding of CBP or C/EBP $\beta$  to the Promoter I.3/II Region Were Not Mediated via Decreases in the Total Protein Levels of These Transcription Factors*—To understand the mechanism underlying the variable occupancy of the promoter II/I.3 region by CBP and C/EBP $\beta$  under different treatments (see Fig. 7), we determined the protein abundance of CBP and C/EBP $\beta$  in BAFs using immunoblotting (Fig. 9). The antibody against CBP recognized three isoforms with molecular masses of ~265, ~210, and ~165 kDa. No



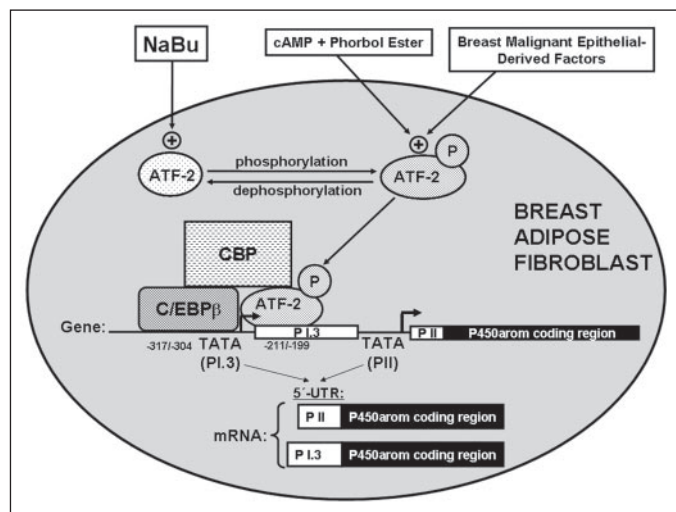


FIGURE 11. Model that depicts the effects of malignant epithelial derived factors or a surrogate hormonal treatment (cAMP + PDA) on the activation of two coordinately regulated P450arom promoters, TATA boxes of which lie within 215 bp from each other. Malignant epithelial cell-derived factors or cAMP + phorbol ester give rise to an increased phosphorylated state of ATF-2 and P450arom expression via promoters I.3 and II. NaBu inhibits transcription via these two promoters by reducing phosphorylation status of ATF-2. 5'-UTR, 5'-untranslated region.

obvious changes in the abundance of CBP protein were observed under different treatments (Fig. 9A). BAFs cultured in serum-free medium exhibited a base-line expression of C/EBP $\beta$  (~45 kDa), which was markedly enhanced by treatments with Bt<sub>2</sub>cAMP plus PDA or MCM (Fig. 9B). NaBu also enhanced the expression of C/EBP $\beta$  (Fig. 9B), although it inhibited the binding of C/EBP $\beta$  to -511/-194 region (see Fig. 7). These experiments were reproducibly repeated using BAFs from three different subjects.

*MCM, Bt<sub>2</sub>cAMP + PDA, or NaBu Regulates Levels of Phosphorylated ATF-2 (Thr-71) but Not Other Members of ATF/CREB Family*—We used immunoblotting to determine the levels of total and phosphorylated members of the CREB/ATF family in BAFs under various treatments (Fig. 10). Upon treatment with Bt<sub>2</sub>cAMP + PDA, an ~70-kDa immunoreactive band recognized by an antibody against pATF-2 (Thr-71) was strikingly enhanced (Fig. 10A). The levels of total ATF-2 were not regulated by Bt<sub>2</sub>cAMP + PDA (Fig. 10A). Antibodies against CREB-1 or ATF-1 did not show any changes in levels of these proteins in response to the same treatments (data not shown).

Next, we incubated BAFs under various conditions in the presence or absence of MCM and/or NaBu. As in the case with Bt<sub>2</sub>cAMP + PDA treatment, MCM also induced levels of pATF-2 most strikingly (Fig. 10B). The BAFs cultured in serum-free medium exhibited a base-line level of pATF-2, which was enhanced by Bt<sub>2</sub>cAMP + PDA or MCM (Fig. 9B). Most importantly, NaBu significantly inhibited both constitutive and induced levels of pATF-2 but not total ATF-2 (Fig. 10B).

Because it has been reported that phosphorylation of members of the CREB/ATF family in response to a cAMP analogue occurs within the first several hours of treatment, we determined the effects of NaBu on phosphorylation of ATF-2 within hours (42) (Fig. 10C). ATF-2 was rapidly phosphorylated within 1 h and remained phosphorylated at 3 h after treatment with Bt<sub>2</sub>cAMP + PDA, whereas phosphorylation was markedly abolished by the addition of NaBu at both time points (Fig. 10C). Immunoblotting employing nuclear proteins gave rise to similar results (Fig. 10D). (Experiments illustrated in Fig. 10, A–C, were performed using whole cell extracts.) This negative effect of NaBu on the phosphorylation status of ATF-2 was reversed by the addition of the kinase

## Promoter-specific Ablation of Aromatase by Na-Bu

inhibitor staurosporine but not the phosphatase inhibitor calyculin-A (Fig. 10E).

These results together indicate that NaBu regulates phosphorylation status of ATF-2, which in turn plays a critical role in promoter-selective activation of the P450arom gene expression. It appears that this effect of NaBu is dependent on new protein synthesis and phosphorylation as demonstrated by experiments illustrated in Figs. 3C, 7B, and 10E.

## DISCUSSION

Aromatase inhibitors are the most commonly used and effective means of nontoxic treatment of estrogen-responsive breast cancer (1–3). Current aromatase inhibitors, however, block aromatase activity indiscriminately in all tissues and deny estrogen to the whole body. Thus, it is desirable to develop novel promoter-specific inhibitors that target breast cancer. Although this concept has been entertained before, this report represents the first demonstration of promoter-specific inhibition of aromatase expression in a disease model (9). NaBu inhibits aromatase expression by targeting signaling pathways that determine phosphorylation of ATF-2 and its binding activity to the P450arom promoters II/I.3 (Fig. 11).

Investigators from at least four different laboratories have demonstrated strikingly increased levels of aromatase activity or P450arom mRNA in breast adipose tissue containing a tumor compared with breast tissue from disease-free women (15, 16, 20–22). These investigators also consistently reported that up-regulation of promoters II and I.3 by breast tumor was responsible for increased aromatase expression in breast cancer (15, 16, 20, 21). We showed that undifferentiated adipose fibroblasts isolated from normal breast tissue exposed to a malignant environment expressed increased levels of aromatase activity and P450arom mRNA (25).

Here we provide evidence that protein kinase A (cAMP) and protein kinase C (phorbol diacetate) analogues or MCM induces aromatase expression in breast adipose fibroblasts by enhancing phosphorylation and binding of ATF-2 to the proximal promoter (II/I.3) region of the P450arom gene. These treatments also enhance binding of C/EBP $\beta$  and CBP to the same regulatory region and protein-protein interactions in the multimeric complex composed of pATF-2 (Thr-71), C/EBP $\beta$ , and CBP. On the other hand, NaBu decreased aromatase expression and enzyme activity by decreasing the phosphorylated state of ATF-2 and disrupting this multimeric complex at the promoter II/I.3 region (Fig. 11).

Several critical cis-elements, including two CREs (-211/-199 bp and -292/-285 bp), a C/EBP site (-317/-304 bp), a GATA site (-183/-163 bp), and a nuclear receptor half-site (-136/-124 bp), had been identified and characterized to be responsible for the regulation of promoters I.3/II in BAFs or breast cancer cell lines (23, 43–46). These suggested that there might be multiple potential pathways triggered by a diverse array of extracellular stimuli to activate or inactivate P450arom promoters I.3/II. We chose to characterize binding of pATF-2 to the -211/-199 bp-CRE by EMSA because this site was essential for promoter I.3/II activation by cancer cell-conditioned medium as defined by site-directed mutagenesis experiments.

CREB/ATF family members are important transcriptional regulators linking extracellular stimuli, e.g. hormones and growth factors, to alterations of gene expression. CREB/ATF family consists of a number of isoforms, including CREB-1, CREM, ATF-1, ATF-2, ATF-3, and ATF-4, which function through binding to a CRE (42, 47). Phosphorylation of CREB/ATF transcription factors stimulated by cAMP, Ca<sup>2+</sup>, growth factors, and stress signals allows recruitment of CBP, which is sufficient to transactivate a number of target genes (48).

## Promoter-specific Ablation of Aromatase by Na-Bu

In the present study, we identified ATF-2 as the major phosphorylated isoform of the CREB/ATF family in cultured BAFs (49). Our findings are indicative that ATF-2 is the essential convergence point downstream of diverse pathways and serve to activate promoters I.3/II under various hormonal stimuli. Down-regulation of the phosphorylation status of ATF-2 by NaBu disrupted its property to form a complex with C/EBP $\beta$  and recruit CBP. In this complex, the presence of C/EBP $\beta$  might be essential for pATF-2 to recruit CBP because interaction of C/EBP isoforms with either ATF-2 or CBP has been reported previously (42, 50–52). In fact, various groups reported heterodimer formation between C/EBP isoforms and ATF-2 (42, 50, 51). The interaction of C/EBP $\beta$  with pATF-2 in this enhancer complex is further supported by our previously published findings that C/EBP $\beta$  is a critical transcription factor mediating MCM-induced activation of promoter I.3/II in BAFs (25).

The reduction of ATF-2 phosphorylation by NaBu or its interaction with P450arom promoter II/I.3 region did not appear to be mediated by its potential inhibition of histone deacetylase (HDAC) activity, because the acetylation status of histones at the P450arom promoter II/I.3 was not altered by NaBu in adipose fibroblasts treated with malignant cell-conditioned media. The inhibitory effect of NaBu was mediated by its capability to disrupt a transcriptional enhancer complex occupying this promoter region. This notion is further supported by the fact that other HDAC inhibitors trichostatin-A or FK228 did not decrease basal or MCM-induced aromatase enzyme activity of breast adipose fibroblasts.<sup>5</sup> Moreover, the elevated histone acetylation in response to NaBu-only treatment did not seem to affect selectively the incorporation of any of the factors into the enhancer transcriptional complex, *i.e.* phosphorylated ATF-2, C/EBP $\beta$ , or CBP.

The mechanism responsible for the NaBu-mediated regulation of the phosphorylation status of ATF-2 is not clear. Because no changes were noted in the histone acetylation status of the P450arom promoters I.3/II in MCM-stimulated cells in the presence or absence of NaBu, this is not a likely mechanism. The effects of NaBu on a number of cellular functions have been reported previously to be reversed by various kinase or phosphatase inhibitors (31, 53–55). For example, NaBu treatment was reported to give rise to activation of a type 1 protein phosphatase (31, 55). On the other hand, kinase inhibitors were also shown to reverse various effects of NaBu under different cellular and molecular conditions (54, 56, 57). However, we did not observe a reversal of the NaBu effect on ATF-2 phosphorylation by the phosphatase inhibitors orthovanadate or calyculin-A. On the other hand, the kinase inhibitor staurosporine consistently reversed the negative effects of NaBu on promoter I.3/II-specific P450arom mRNA levels, ATF-2 phosphorylation, and binding of phosphorylated ATF-2 to the promoter I.3/II region. This is consistent with a recent publication (57) reporting the reversal of NaBu-dependent differentiation of colon cancer cells by staurosporine. In summary, our findings are suggestive that NaBu-dependent silencing of aromatase promoters I.3/II is complex and involves new protein synthesis and phosphorylation before eventually decreasing the phosphorylated state of ATF-2.

The results presented here are encouraging enough to warrant a clinical evaluation of the potential therapeutic effects of NaBu with respect to aromatase inhibition in estrogen-dependent disorders such as breast cancer and endometriosis (58). The observation that NaBu or its derivatives exert potent effects on growth arrest and cell differentiation of various malignant tumor cells both *in vitro* and *in vivo* had initiated their

use as a novel therapeutic strategy with broad applications in oncology (28). It remains to be seen whether the dose range of NaBu found to be effective *in vitro* in this study (5–15 mM) is compatible with the clinical settings. In one study, patients with ulcerative colitis tolerated a dose of oral NaBu at 4 mg/day for 6 weeks very well (59). Tissue-selective inhibition of aromatase with NaBu may offer an effective therapeutic mechanism permitting patients with breast cancer to be treated with this natural compound for prolonged periods. More importantly, these findings may lead development of new strategies that target the enhancer transcriptional complex occupying the tumor-related aromatase promoter region selectively activated in estrogen-responsive disorders (58).

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