Xanthine oxidoreductase (XOR) catalyzes the formation of uric acid from xanthine and hypoxanthine and is recognized as a source of reactive oxygen and nitrogen species. Unexpectedly, XOR was found to play an essential role in milk secretion in the differentiating mammary gland, where it is an integral component of the milk fat globule. XOR gene expression in both mammary glands and differentiating mammary epithelial cells is in regulated by the lactogenic hormones prolactin and cortisol. Expression in mammary epithelial cells is also regulated by inflammatory cytokines and induced by cycloheximide. Cycloheximide was found to stimulate XOR gene expression in differentiating HC11 mouse mammary epithelial cells. Activation of XOR gene expression by both cycloheximide and inflammatory cytokines suggested that XOR may be regulated by stress-activated protein kinases, the MAPKs. We demonstrate here that XOR was induced in HC11 cells by low dose cycloheximide and that expression was blocked by inhibitors of p38 MAPK. Accumulation of phospho-p38 was stimulated by low dose cycloheximide. Low dose cycloheximide stress promoted phosphorylation and nuclear accumulation of the CCAAT/enhancer-binding protein-β (C/EBPβ) transcription factor, which was blocked by inhibition of p38. Furthermore, C/EBPβ was found to activate the mouse XOR promoter, and XOR promoter-C/EBPβ protein complexes were induced by low dose cycloheximide stress. These data demonstrate, for the first time, that mouse mammary epithelial cells XOR is regulated by p38 MAPK. They identify an essential function of the C/EBPβ transcription factor in mouse XOR expression and suggest a potential role for p38 MAPK activation of C/EBPβ in mammary epithelial cells.
mide stress. These data demonstrate the dependence of mouse XOR expression in mammary epithelial cells on the C/EBPβ transcription factor and suggest a potentially critical role for p38 MAPK activation of C/EBPβ in mammary epithelial cells.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Most reagents, buffers, substrates, PAGE supplies, SB203580, SB202190, U0126, insulin, prolactin, dexamethasone, cycloheximide, and puromycin were purchased from Sigma. The TOPO-II TA cloning vector, TRIZol reagent, and media for cell culture were obtained in powder form from Invitrogen. Antibodies to p38 family members and C/EBPβ were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-p38 and anti-phospho-C/EBPβ antisera were from Cell Signaling Technology, Inc. (Beverly, MA). Oligonucleotides were synthesized by Invitrogen or Integrated DNA Technologies, Inc. (Coralville, IA). Luciferase fusion plasmids and β-galactosidase expression plasmids were obtained from Promega Corp. (Madison, WI). O-Nitrophenyl β-d-galactopyranoside, poly(dI-dC), and restriction endonucleases were obtained from Roche Applied Science. Fetal bovine serum was from Gemini Bio-Products (Woodland, CA). Reverse transcription (RT)-PCR reagents were from Eppendorf Prime, Inc. (Boulder, CO).

Mammary Epithelial Cell Culture and Differentiation—HC11 mammary epithelial cells were grown in RPMI 1640 medium containing 2 mM l-glutamine, 2 g/liter sodium bicarbonate (pH 7.4), 1× antibiotic/antimycotic solution, 5 μg/ml insulin, 10 μg/ml epidermal growth factor (EGF), and 10% fetal calf serum (17). Cells were maintained at 37 °C in 95% air and 5% CO₂ fed every 2 days, and split 1:4 when at or near confluence. Cells are routinely differentiated by growing them to confluence in the presence of EGF and then shifting them into the above medium with 2% heat-inactivated fetal calf serum in the absence of EGF. Cells were exposed to cycloheximide or ethanol (vehicle) after 2 days of growth in EGF-free medium or shifted at this point into dexamethasone-insulin/prolactin medium as described (17).

Protein synthesis was determined as described (18). Briefly, HC11 cells were grown to confluence on 6-well plates in the presence of EGF and then shifted to medium with 2% heat-inactivated fetal calf serum in the absence of EGF for an additional 48 h. Cells were washed with phosphate-buffered saline (PBS) and incubated for 10 min with methionine-free RPMI 1640 medium. Subsequently, cells were incubated for 2 h with the same medium supplemented with 15 Ci/ml [35S]methionine in the presence or absence of cycloheximide or puromycin at the indicated concentrations. At the end of the labeling period, cells were washed twice with PBS on ice and twice with 5% trichloroacetic acid and then solubilized in 0.5 ml of 0.25 N NaOH. Equal aliquots were counted by liquid scintillation.

RNA Quantitation—Quantitative fluorescence RT-PCR was conducted as described previously (10) with the following modifications. RNA was isolated from PBS-washed HC11 cells using TRIZol reagent. 50 ng of total RNA was reverse-transcribed for 55 min at 45 °C in a 20-μl reaction mixture containing 1 μg of oligo(dT), 2 μl of 10 mM dNTPs, and 0.5 μl of RNase inhibitor (Eppendorf-5 Prime, Inc.) in 1× RT buffer using avian myeloblastosis virus reverse transcriptase (C master kit, Eppendorf-5 Prime, Inc.). 2 μl of cDNA was then used for each PCR. RT-PCRs were quantitated on an Applied Biosystems 310 genetic analyzer as described (10). RT-PCR experiments were performed in triplicate and normalized to the β-actin control. Data are expressed as the mean normalized fluorescence ± S.E. Independent RT-PCRs for XOR and β-actin were examined by agarose gel electrophoresis and photographed. Negative controls for cDNA synthesis were run without template or without reverse transcriptase. The primers used in RT-PCR were as follows: XOR, 5′-Hex-GCCGCTTGACGGC-TATAGACC-3′ (forward) and 5′-CTCTTGGTACCTTAGAT-GCG-3′ (reverse); and β-actin, 5′-carboxfluorescein-GGCGCA-GAGACAGATGATC-3′ (forward) and 5′-CACGACAGC-CTGGATGCTAC-3′ (reverse).

SDS-PAGE and Western Immunoblot Analysis—HC11 cells were grown in 6-well plates until confluent, switched to 2% heat-inactivated serum in the absence of EGF for 48 h, and then treated with cycloheximide for the indicated times. Cells were washed once with ice-cold PBS, resuspended in cell lysis buffer A (2 mM dithiothreitol, 2.0% SDS, 25 mM β-glycerophosphate, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, and a 1:5000 dilution of protease inhibitor mixture set III (Calbiochem)), sonicated for 15 s, and kept on ice. The protein concentration in the supernatant was determined as described (4). Aliquot containing 50 μg of protein were incubated with equal amounts of loading buffer (5% β-mercaptoethanol and 95% Laemmli loading dye) for 10 min at 37 °C and then boiled for 2 min. Samples were separated by electrophoresis on 7.5% SDS-polyacrylamide gel for 40 min at 75 V, transferred to nitrocellulose membranes (GE Osmonics, Minnetonka, MN), and blocked overnight in 5% nonfat dried milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween at 4 °C. Membranes were then incubated with antibodies against C/EBPβ, phospho-C/EBPβ, p38α, p38β, and phospho-p38 MAPK. Antibigen-antibody complexes were detected by reaction with an ECL Western blotting detection kit (Amersham Biosciences) according to the manufacturer’s instruction. Each experiment was run in triplicate, and representative immunoblots are shown.

Mouse XOR Promoter Cloning, Plasmid Construction, and Mutagenesis—An in-frame fusion to the luciferase translational start site of the luciferase expression vector pGL3-Basic (Promega Corp.) was constructed exactly as indicated previously (19). Briefly, upstream DNA to be cloned into pGL3-Basic was amplified by PCR, combining sequences from the mouse XOR cDNA and chromosomal locus (20, 21). This region of the mouse genome has been cloned on bacterial artificial chromosome RPCL-23 (clone RP23-170391, GenBank™ accession number AC101679.8, GE33147367), which served as a template for PCR. The resulting PCR product was cleaved with XhoI and Ncol and cloned in the forward orientation into pGL3-Basic. The mouse promoter fusion clone (pMXOR-B1) comprises 1000 bp of the XOR upstream DNA fused to the translational start site to luciferase. This region contains the functional mouse proximal promoter identified previously and 700 bp of additional upstream DNA (21, 22). Luciferase fusion constructs were confirmed by DNA sequence analysis as described (19). Fluorescence sequence analysis was performed using the dyeoxynucleotide chain termination system from PerkinElmer Life Sciences. Reactions were carried out using the ABI PRISM™ dye terminator cycle sequencing ready reaction kits (PerkinElmer Life Sciences). Sequence reactions were fractionated on an ABI PRISM 310 DNA sequencer equipped with a 47-cm microcapillary (PerkinElmer Life Sciences). All sequences were determined from both directions, and sequence data were compiled manually.3

The putative C/EBP-binding site (nucleotides −140 to −170) (see Fig. 8) identified in mouse XOR upstream DNA by sequence analysis (11) was mutated by sequence exchange using PCR amplification of pMXOR-B1. The C/EBP core-binding site (5′-ATTGTGCAA-3′) was replaced with 5′-AAGGCGCAA-3′ as follows. The C/EBP forward primer (5′-CTGGTCCCCTTGGAGGAGGACCCACCTTGACT-CTTG-3′) was paired with a distant primer (5′-GGAGCTGAC-
TGGGGTGAAGGC-3') in one PCR, whereas the C/EBP reverse primer (5'-CAAGAGTCACAGTTGGGCTTCCAGGAAGG-ACCAG-3') was paired with a distant primer (5'-CAGGTCAGGGG-GAGGTTGTTG-3'). PCR products were cleaved with BamHI and ApaI, gel-purified, ligated with T4 DNA ligase, and transformed into F' negative TOP10 cells (Invitrogen). Candidate plasmids were screened for retention of single NcoI and KpnI sites as well as BamHI and ApaI sites. Appropriate clones were sequenced across the entire 1000 bp of the mouse XOR upstream region to confirm the exchange and the absence of unanticipated changes in sequence. The C/EBP exchange construct is called pMXOR-mut17.

**Transient Transfection and Luciferase Reporter Assay**—Cells to be transfected were grown to 50–70% confluency in 6-well plates and shifted to 2 ml of fresh medium 1 h prior to transfection. Transfections were conducted using FuGENE 6 (Roche Applied Science) essentially as described by the supplier. 1.7 μg of total plasmid was mixed with FuGENE 6 in 0.1 ml of RPMI 1640 medium containing sodium bicarbonate with or without 10% fetal bovine serum as indicated below. After mixing, the transfecting solution was held at room temperature for 30 min and then applied to cells in a single well of a 6-well plate. Cells were harvested for analysis after 24 or 48 h of incubation. Cytoplasmic extracts were analyzed for luciferase activity in cell culture lysis reagent (Promega Corp.) using a BMG LABTECH Lumistar luminometer. As reported previously (22), uniformity of data and transfection efficiency were determined by a minimum of six independent transfections because, in all cases, β-galactosidase cotransfection plasmids suppressed the activity of the XOR reporter. Luciferase activity was normalized to total cytoplasmic protein as determined spectrophotometrically using the Lowry assay (4). Each individual transfection was assayed in quadruplicate, and each individual transfection was repeated six times; thus, each value reported represents 24 biochemical assays for each replicate, and each individual transfection was repeated six times; thus, thereby, in all cases, S.D. were calculated for each group, and in most cases, S.D. values were no greater than 10% of the mean value. Comparisons between groups were made using Student's t test.

**Chromatin Immunoprecipitation (ChIP), PCR, and Quantitation**—ChIP analysis was performed essentially as described (23–25) using the EZ-ChIP kit from Upstate (catalog no. 17-371) and the modifications indicated here. HC11 cells were grown in T75 flasks as described above and treated with vehicle (control cells) or 10 μg/ml cycloheximide or pretreated with 20 μM SB202190 and exposed to 10 μM SB202190 and exposed to 10 μg/ml cycloheximide 45 min later. Cells from three T75 flasks (8 × 107 cells) were prepared for ChIP 5 h after exposure to cycloheximide. After refreshing the low serum EGF-free medium, cells were cross-linked with 1% freshly prepared formaldehyde for 10 min at room temperature. Glycine was added to 125 mM, and cells incubated for 5 min at room temperature. Cells were then washed twice with ice-cold PBS containing fresh protease inhibitors, scraped into PBS, and sedimented. Pelleted cells were lysed for 10 min on ice in cell lysis buffer B (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Nuclei were harvested by sedimentation and lysed in a total volume of 3.2 ml for 10 min on ice in nuclear lysis buffer (50 mM Tris (pH 8.1), 10 mM EDTA, 1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Nuclei lysates were sonicated in 400-μl aliquots on ice for six bursts of 20 s using a Branson sonicator at a setting of 50 with an amplitude of 20 and a tuned setting of 40. Cells were held on ice for at least 1 min between sonications. Debris was sedimented at 11,000 × g for 10 min at 4 °C, and chromatin was snap-frozen in liquid nitrogen and stored at −80 °C. 5 × 10⁹ nuclei eq were preclared in a total volume of 8 ml for each immunoprecipitation with 20 μl of protein G-Sepharose for 30 min at 4 °C in chromatin dilution buffer (16.7 mM Tris (pH 8.1), 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). After sedimentation of the Sepharose beads, 1.60 ml from each tube was removed for analysis of input DNA, and chromatin was then incubated overnight without or with 10 μg of either anti-C/EBP β antibody (C-19, Santa Cruz Biotechnology catalog no. sc-150) or isotype control antibody at 4 °C. As an additional control, a no-chromatin sample was also precipitated with 10 μg of antibody C-19. Immunoprecipitates were collected with 60 μl of protein G-Sepharose for 1 h at 4 °C, and beads were washed sequentially with low salt, high salt, LiCl, and Tris/EDTA as recommended by Upstate. Chromatin was eluted from the beads in a total of 400 μl of elution buffer (1% SDS and 0.1 mM NaHCO₃) in two steps and frozen at −80 °C. Cross-links were reversed, and samples were treated with RNase A and precipitated with 2 volumes of ethanol. Samples were resuspended in 100 μl of water and treated with proteinase K as recommended by Upstate, and DNA was purified on spin columns. DNA was eluted from the spin columns in a volume of 100 μl, extracted in phenol/chloroform/isoamyl alcohol, reprecipitated, and suspended in 50 μl of Tris/EDTA. Cross-link reversal, RNase A and proteinase K treatment, and DNA purification of input chromatin were conducted in an identical fashion.

5 or 10 μl of ChIP DNA or 1.0 μl of input DNA (2 × 10⁹ nuclear eq) was amplified by PCR in the presence of 2.5 mM MgCl₂ using AmpliTaq polymerase (Applied Biosystems). PCR conditions were as follows: 1) one cycle at 95 °C for 1 min; 2) six cycles at 95 °C for 30 s, 65 °C for 30 s decreasing 1°C cycle, and 72 °C for 30 s; 3) 31 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C, for 30 s; and 4) one cycle at 72 °C for 2 min 30 s, and then cooling to 4 °C. PCR primers were designed to span 284 bp of mouse XOR upstream DNA comprising nucleotides −1 through −284 and including the proximal promoter. The sequences of the upstream and downstream primers were 5′-AGAATTCCAGTCATCTGTCGCC-3′ and 5′-CGTACGCGGGTCAAGGTG-3′, respectively. PCR products were analyzed by agarose gel electrophoresis. Ethidium bromide-stained gels were photographed under ultraviolet illumination using a Kodak Gel Logic 200 imaging system, scanned, and quantitated using Kodak 1D image analysis software (Version 3.6).

**Preparation of Nuclei and Nuclear Proteins**—Nuclei were prepared from hypotonically swollen cells by lysis in 0.1% Nonidet P-40 and differential centrifugation as described previously (26) with the present addition of 2 mM sodium vanadate and 1 mM NaF in each buffer. Proteins were isolated from isolated and washed nuclei by incubation in 320 mM potassium buffer as described (26). Nuclear preparations were routinely followed microscopically and were estimated to contain >95% nuclei with no more than 5% cellular contamination. Following sedimentation at 10,000 × g to remove extracted nuclei, protein solutions were stored at −70 °C in high salt buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 320 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethlysulfonyl fluoride, 0.5 mM dithiothreitol, 25% glycerol, 1 mM NaF, and 2 mM Na₃VO₄.

**Electrophoretic Mobility Shift Assay (EMSA) Analysis**—A synthetic double-strand oligonucleotide corresponding to mouse XOR nucleotides −140 through −170 and spanning the consensus C/EBP-binding site of the proximal promoter (21, 22) was used for EMSA analysis. 60 ng of double-strand oligonucleotide probe was labeled with 50 μCi of [γ-32P]ATP and polynucleotide kinase (Roche Applied Science) according to the manufacturer’s specifications. Probes were extracted in phe-
nol/chloroform/isoamyl alcohol (24:24:1), precipitated in ethanol, and resuspended in Tris/EDTA at 0.5 ng/μl. EMSA binding reactions were conducted as described (26) and contained 17.8 μg of nuclear protein, 5 μg of poly(dI-dC), and 0.5 ng of labeled probe, and the final reaction buffer was composed of 10 mM Tris (pH 7.9), 50 mM NaCl, 1.0 mM dithiothreitol, 1.0 mM EDTA, and 5% glycerol. Double-strand oligonucleotide competitors were included at 50- or 100-fold molar excess as indicated in the figure legends. Supershift experiments with antisera to C/EBPβ were conducted at a 19.6:1 dilution of antisera. Isotype antisera were used to control for nonspecific effects of the antisera. Binding reactions were assembled in the following order: H2O, buffer, nuclear protein solution, poly(dI-dC), competitor oligonucleotide, and labeled probe. Competitor oligonucleotides were derived from a consensus C/EBP-binding site (Santa Cruz Biotechnology, Inc.) or the mouse XOR proximal promoter region corresponding to a near consensus C/EBPβ-binding site (21). Antisera were added 5 min before probe addition.
Binding reactions were conducted on ice for 45 min as described (27). Following addition of Ficoll dye, 15 µl of the binding reaction was electrophoresed on 4% native polyacrylamide gels in 25 mM Tris (pH 8.5), 0.38 M glycine, and 2 mM EDTA. Gels were run in the cold at 4 °C and 125 V for ~4 h (27) and were subsequently dried and exposed to Kodak XAR autoradiographic film.

Statistical Analyses—Data are expressed as means ± S.E. and were assessed for significance using Student’s t test. A p value <0.05 was considered significant.

RESULTS

XOR Is Induced by Cycloheximide but Not by Puromycin—Previous Northern blot analysis demonstrated cycloheximide induction of XOR in HC11 cells (11). We observed that the steady-state XOR mRNA concentration was dose-dependently increased by cycloheximide 24 h following treatment (Fig. 1, A and B). Increased XOR expression was observed at a low concentration of cycloheximide (0.01 µg/ml) and increased further at concentrations between 0.1 and 1.0 µg/ml. A high

FIGURE 2. Puromycin does not stimulate XOR expression. HC11 cells were grown as described in the legend to Fig. 1 and treated for 24 h with increasing concentrations of puromycin. XOR and β-actin mRNAs were subsequently assayed by quantitative fluorescence RT-PCR (A) and examined by agarose gel electrophoresis (B). The level of protein synthesis inhibition by puromycin was determined as described in the legend to Fig. 1 (C). We observed that 100 µg/ml puromycin was required to inhibit HC11 protein synthesis to the same extent as 10 µg/ml cycloheximide.
concentration of cycloheximide (10 \mu g/ml) decreased XOR expression 24 h after exposure in EGF-starved cells grown in reduced serum. We determined the effect of cycloheximide on HC11 protein synthesis (Fig. 1C). Protein synthesis was largely abrogated at 10 \mu g/ml, was stimulated at 0.01 \mu g/ml, and was unaltered at 0.1 \mu g/ml. XOR mRNA was induced by ~3-fold at cycloheximide concentrations between 0.01 and 0.1 \mu g/ml, at which net protein synthesis was not inhibited (Fig. 1C). XOR mRNA was induced by ~6-fold in the presence of 1.0 \mu g/ml cycloheximide, a concentration at which residual protein synthesis was 75% of the control. In contrast, puromycin, which inhibits protein synthesis by a different mechanism, failed to induce XOR at any concentration (Fig. 2, A and B), even when protein synthesis was reduced to 30% of the control (Fig. 2C). Induction of XOR mRNA by cycloheximide was evident as early as 2 h following stimulation and continued over the course of 10 h, with marked induction occurring by 10 h at both high and low concentrations of cycloheximide (Fig. 3). Prolonged growth in high dose cycloheximide resulted in loss of XOR expression, perhaps reflecting the stringent conditions of growth in reduced serum.

**XOR Induction by Cycloheximide Is Blocked by Inhibitors of p38 MAPK**—The observation that XOR was induced by cycloheximide (but not by puromycin) in a concentration-dependent manner suggested that induction was not likely to represent simply the translational inhibition of a labile repressor protein. Furthermore, low dose cycloheximide stress was shown to induce expression of some genes by activation of p38 MAPK (18). We treated HC11 cells with the MAPK inhibitors SB202190 and SB203580 in the presence of cycloheximide and quantitated XOR expression by RT-PCR. Both p38 MAPK inhibitors reduced XOR expression when examined 10 h after exposure to cycloheximide (Fig. 4). At a concentration of 20 \mu M, the effect of SB202190 was more pronounced than that of SB203580, completely abolishing the XOR signal at the low concentration of cycloheximide. The MEK1/2 inhibitor U0126 produced no effect on cycloheximide-stimulated XOR expression.

**Cycloheximide Promotes Rapid p38 Phosphorylation in Cultured Mammary Epithelial Cells**—p38 MAPK could mediate the effects of cycloheximide in several ways, including an increase in p38 expression, phosphorylation, and/or nuclear accumulation. Furthermore, several isoforms of p38 may be expressed in HC11 cells and be responsible for XOR induction. We examined the levels of p38α and p38β immunoreactive protein and phosphorylation status because these two isoforms are expressed in other mammary epithelial cells (28). We observed that HC11 cells express both p38α and p38β isoforms, and the levels of each protein remained constant throughout the 60-min period of exposure to cycloheximide (Fig. 5, A and B). The levels of phospho-p38 were determined following treatment with cycloheximide. Whereas untreated HC11 cells expressed some phospho-p38, a marked increase in phospho-p38 was evident after 15 min of cycloheximide treatment and continued to rise throughout the 60-min exposure period (Fig. 5, A and B). Exposure of HC11 cells to 1 M sorbitol, a hypertonic stress that induces rapid p38 phosphorylation (28), identified the upper band in Fig. 5A as specifically phospho-p38 (Fig. 5, B and C), and this was the band most notably increased by cycloheximide.

**Cycloheximide Promotes p38-dependent C/EBPβ Phosphorylation**—C/EBPβ is a complex transcription factor expressed as full-length, liver enriched activator protein (LAP), and liver enriched inhibitor protein (LIP) isoforms, the latter constituting a truncated and potentially inhibitory form (29). C/EBPβ contains a highly conserved p38 MAPK phosphorylation site corresponding to Thr286 in murine LAP and Thr277 in murine LIP (30) and was previously demonstrated to be a substrate of p38 both in vitro and in vivo (31). To determine the effect of cycloheximide and p38 MAPK on the levels of phospho-C/EBPβ, we treated cells with cycloheximide for var-
p38- and C/EBPβ-mediated XOR Activation

FIGURE 4. XOR induction by cycloheximide is blocked by p38 MAPK inhibitors. HC11 cells were differentiated for 48 h, treated with the MAPK inhibitors (20 μM) indicated for 40 min, and then exposed to a high or low concentration of cycloheximide (Chx) for 10 h. XOR and β-actin mRNA levels were subsequently analyzed by quantitative fluorescence RT-PCR (A), and agarose gels of RT-PCR products were photographed (B). Both p38 MAPK inhibitors blocked cycloheximide-mediated XOR induction; and at low a concentration of cycloheximide, XOR induction was completely abolished by SB202190.

ious times up to 10 h in the absence or presence of SB203580 and analyzed whole cell extracts by Western immunoblotting. The levels of C/EBPβ protein were largely unchanged throughout the 10-h time course, although a small increase in the LIP isoform was apparent after 30 min and gradually returned to control levels (Fig. 6, A and B). Untreated control cells revealed a low level of phospho-C/EBPβ that was markedly increased after a 30-min cycloheximide treatment, with the full-length, LAP, and LIP isoforms all showing an increase in the levels of the phosphorylated isoforms (Fig. 6, C and D). The full-length, LAP, and LIP isoforms of phospho-C/EBPβ remained elevated throughout the 10 h of exposure, but showed a gradual decline after 30 min. When HC11 cells to be treated with cycloheximide were pretreated with increasing concentrations of SB202190 for 45 min and subsequently with 1.0 μg/ml cycloheximide, phospho-C/EBPβ accumulation was dose-dependently inhibited. Furthermore, the cycloheximide-induced increase in the full-length, LAP, and LIP isoforms of phospho-C/EBPβ was inhibited by pretreatment with SB202190 (Fig. 6, E and F). Essentially identical results were obtained using SB203580 (data not shown), whereas pretreatment with the MEK1/2 inhibitor U0126 failed to inhibit cycloheximide-induced phospho-C/EBPβ (Fig. 6, E and F).

Cycloheximide Activation of Mouse XOR Is Mediated by C/EBPβ—A functional promoter was identified in the mouse XOR upstream DNA from nucleotides −1 to −300 relative to the translational start site, and the DNA sequence of this region contains a potential C/EBP-binding site (Fig. 7A) that could contribute to XOR activation (21, 22). DNA-protein complexes were formed between mouse XOR upstream DNA nucleotides −140 to −170 (spanning the consensus C/EBP-binding site) and nuclear proteins prepared from cycloheximide-treated cells and analyzed by EMSA. As shown in Fig. 7B, a major complex was formed with the XOR upstream DNA that was efficiently competed using an unlabeled consensus oligonucleotide competitor for C/EBP; the complex could be supershifted using anti-C/EBPβ antisera; and the complex failed to form in cells treated with SB202190 prior to exposure to cycloheximide. Thus, C/EBPβ from cycloheximide-treated cells formed specific protein complexes with the mouse XOR promoter DNA.

To confirm that mouse XOR is activated by C/EBPβ and to determine whether the putative C/EBP-binding site mediates cycloheximide activation, we cloned 1000 bp of XOR 5′-flanking DNA into the pGL3-Basic vector as described (21) and mutagenized the C/EBP-binding site by nucleotide exchange (Fig. 7A). The resulting mutant (pMXOR-mut17) and the wild type (pMXOR-B1) were tested in transfection analyses (Fig. 7C). pMXOR-mut17-transfected cells exhibited marked reduction in luciferase expression in the absence of cycloheximide stimulation. HC11 cells transfected with pMXOR-B1 or pMXOR-mut17 were grown to confluency, shifted to EGF-free medium for 24 h, and then exposed to increasing concentrations of cycloheximide in the presence or absence of SB202190 (Fig. 7C). The pMXOR-mut17 mutation suppressed cycloheximide activation, and both native and pMXOR-mut17 expression were blocked by preincubation with SB202190. These data demonstrate that mouse XOR expression is dependent on C/EBPβ and that low dose cycloheximide stress-induced expression is blocked by pMXOR-mut17 and inhibited by SB202190.

Confirmation that increased C/EBPβ was indeed bound to XOR promoter DNA following cycloheximide treatment was obtained by in vivo ChIP. C/EBPβ-specific ChIP (23–25) was performed on HC11 cells that were untreated (control), exposed to 10 μg/ml cycloheximide for 5 h, or treated with 20 μM SB202190 for 45 min and then exposed to cycloheximide for 5 h. PCR products obtained from C/EBPβ-specific immunoprecipitation (antibody C-19) showed magnesium and DNA concentration dependence, and PCR band intensities were increased
by 2.2–8-fold by cycloheximide (Fig. 7D). We obtained no detectable PCR product from cells pretreated with SB202190 or from nonspecific immunoprecipitation or mock immunoprecipitation. These data indicate that increased C/EBPβ associates with the XOR promoter following cycloheximide treatment and that the association is blocked by SB202190.

Mouse XOR Is Activated by Ectopic Overexpression of C/EBPβ—To confirm that mouse XOR is activated by C/EBPβ, pMXOR-B1 was transfected into HC11 cells in the presence of increasing concentrations of a C/EBPβ cDNA expression clone, pc/EBPβ (27). C/EBPβ protein was observed to rise in concentration in direct proportion to the amount of pc/EBPβ cDNA used in transfection (Fig. 8, A and B). Concomitantly, EMSA analysis showed that nuclear protein binding complexes formed with the mouse XOR −140/−170 C/EBPβ oligonucleotide were efficiently competed by either the consensus C/EBPβ oligonucleotide or the mouse XOR C/EBPβ oligonucleotide. Furthermore, these complexes were shifted by antisera to C/EBPβ, but not by nonspecific isotype antisera (Fig. 8C). Luciferase expression by the mouse XOR 5′-flanking DNA in pMXOR-B1 was increased in a concentration-dependent manner upon activation with the concentration of pc/EBPβ cDNA used in transfection (Fig. 8D). Finally, C/EBPβ concentration-dependent increases in luciferase expression were blocked in pMXOR-mut17 (Fig. 8E). Thus, ectopic overexpression of C/EBPβ stimulated XOR expression, and C/EBPβ-induced expression was blocked by mutagenesis of the putative C/EBPβ-binding site in the mouse XOR proximal promoter.

Cycloheximide-independent Activation of C/EBPβ by p38 MAPK and the Activating Kinase MKK6b in HC11 Cells—We sought evidence that p38 activation would promote C/EBPβ nuclear accumulation and XOR DNA-protein complex formation in HC11 cells in the absence of cycloheximide stress. Notably, p38 is the primary substrate of the upstream activating kinase MKK6b (16), and cDNA expression clones have been developed in the pcDNA-3 vector for both p38α and MKK6b (32). In addition, an inactive derivative of p38α (p38α-AF) has been generated and cloned into the same expression vector. p38α-AF remains inactive after exposure to MKK6b (32). HC11 cells were cotransfected with cDNA expression plasmids for p38α and MKK6b, included at concentrations of 0–1.0 μg. Transfected cells were fractionated into nuclear and cytoplasmic fractions, and Western blot and EMSA analyses were performed on the resulting protein extracts. We observed C/EBPβ in both the nuclear and cytoplasmic fractions in the presence of p38α even in the absence of MKK6b (Fig. 9, A and B). Nonetheless, C/EBPβ was found to increase in the nuclear fraction and to decrease in the cytoplasmic fraction as a function of the amount of pMKK6b used in transfection (Fig. 9, A and B), and nuclear accumulation was inhibited in the presence of p38α-AF (Fig. 9, C and D). Furthermore, DNA-protein binding complexes formed with the XOR C/EBPβ oligonucleotide were
increased in the nuclear fraction in direct proportion to the level of activating MKK6b kinase cDNA used in the transfection. Concomitantly, the capacity to form complexes with cytoplasmic proteins was decreased with increasing concentrations of the MKK6b cDNA (Fig. 9). C/EBPβ containing complexes formed with the XOR −140/−170 C/EBPβ oligonucleotide using nuclear protein extracts were supershifted with antisera to C/EBPβ or β-actin. C/EBPβ −170 C/EBPβ-binding site oligonucleotide derived from mouse XOR (lane 5). Thus, overexpression of MKK6b and p38α promoted nuclear accumulation of C/EBPβ that formed specific protein complexes with the mouse XOR proximal promoter.

DISCUSSION

Previous experiments demonstrated that XOR expression is activated by both cycloheximide and inflammatory cytokines in mammary epithelial cells (11, 15). The combination of cycloheximide stress and inflammatory cytokine activation suggests that XOR may be regulated by stress-activated protein kinases that could specifically mediate the physiological stress of low dose cycloheximide (16, 34). In the present experiments, we identified, for the first time, a critical role of p38 MAPK and the C/EBPβ transcrption factor in the activation of mouse XOR by cycloheximide stress. XOR was rapidly activated by low dose cycloheximide, and activation was suppressed by inhibitors of p38 MAPK. The
FIGURE 7. Cycloheximide activation is blocked by mutagenesis of a putative C/EBPβ-binding site present in the XOR core promoter. A, a potential C/EBPβ-binding site was identified by DNA sequence analysis of the XOR core promoter. B, an oligonucleotide corresponding to mouse XOR upstream DNA nucleotides −140 to −170 and spanning the consensus C/EBP-binding site was used in EMSA analysis. DNA-protein complexes were formed using nuclear protein extracts from HC11 cells treated for 10 h with 1.0 μg/ml cycloheximide. Lane 1, probe alone; lanes 2 and 3, complexes formed with nuclear proteins from two independent experiments; lane 4, complexes formed in the presence of a 50-fold excess of the unlabeled consensus C/EBP-binding site; lane 5, complexes formed in the presence of a 100-fold excess of the unlabeled consensus C/EBP-binding site; lane 6, complexes formed in the presence of anti-C/EBPβ antisera; lane 7, complexes formed from cells pretreated with 20 μM SB202190 45 min prior to treatment with cycloheximide. C, the potential C/EBPβ-binding site was mutagenized as illustrated in A to create pMXOR-mut17. pMXOR-B1 or pMXOR-mut17 was independently transfected into HC11 cells, and transfected cells were grown to confluency and shifted into medium with 2% heat-inactivated fetal calf serum and 5 μg/ml insulin along with the indicated concentrations of cycloheximide. Parallel cultures were exposed to 20 μM SB202190 45 min prior to treatment with cycloheximide. Cells were harvested 24 h after treatment with cycloheximide and assayed for luciferase expression. Cells treated with SB202190 are indicated (horizontal line). Gray bars, cells transfected with pMXOR-B1; black bars, cells transfected with pMXOR-mut17. R. L. U., relative light units. D, the results from in vivo ChIP were analyzed by agarose gel electrophoresis. Panel a, PCR analysis of 10 μl of DNA from ChIP; panel b, PCR analysis of 5 μl of DNA from ChIP; panel c, PCR analysis of 1.0 μl of input DNA without ChIP. Lanes 1, 100-bp DNA ladder; lanes 2, control, antibody C-19; lanes 3, cycloheximide treatment, antibody C-19; lanes 4, SB202190 followed by cycloheximide, antibody C-19; lanes 5, cycloheximide treatment, nonspecific isotype antibody; lanes 6, cycloheximide treatment, no antibody; lanes 7, no chromatin, antibody C-19; lanes 8, 100-bp DNA ladder.
FIGURE 8. The XOR promoter is activated by a cotransfected C/EBPβ cDNA expression clone. A, whole cell extracts from cells transfected with the indicated concentrations of pC/EBPβ cDNA were analyzed by Western immunoblotting. B, the densitometric scan is shown. C, nuclear protein complexes were formed using the mouse XOR proximal promoter C/EBP-binding site oligonucleotide from cells transfected with 1.0 μg of C/EBPβ cDNA. EMSA analysis was conducted described under “Experimental Procedures.” Lane 1, probe alone; lane 2, complexes formed with nuclear proteins and no competitor; lanes 3 and 4, 20- and 50-fold molar excesses of a consensus C/EBP-binding site competitor, respectively; lane 5, 50-fold molar excess of the mouse proximal promoter C/EBP-binding site competitor; lane 6, supershifting monoclonal antibody (ab) to mouse C/EBPβ; lane 7, isotype control antibody. D, HC11 cells were transfected with pMXOR-B1 alone or cotransfected with increasing concentrations of pC/EBPβ (0 – 1.0 μg). Both the pGL3-Basic (pGL3B) parent vector and pMXOR-B1 were transfected at a concentration of 0.725 μg. Luciferase activity was quantitated from six independent transfection experiments 24 h following transfection. R. L. U., relative light units. E, pMXOR-mut17-transfected cells expressed markedly reduced levels of luciferase and showed reduced response to cotransfected C/EBPβ cDNA. HC11 cells were cotransfected with pMXOR-B1 and increasing amounts of C/EBPβ cDNA (0 – 1.0 μg) or with pMXOR-mut17 and increasing amounts of C/EBPβ cDNA (0 – 1.0 μg). After 24 h, cells were harvested and lysed, and luciferase activity was quantitated as described for D.
FIGURE 9. MAPK activation promotes C/EBPβ nuclear accumulation and XOR promoter complex formation. A, HC11 cells were transfected with cDNA expression clones for p38α and the cDNA expression clone for the activating kinase MKK6bE at the indicated concentrations. Western blotting was performed on the cytoplasmic and nuclear fractions. B, the densitometric scan is shown. C, Western blots were also performed on cytoplasmic and nuclear fractions from cells transfected with p38α-AF and the indicated concentrations of pMKK6bE. D, the densitometric scan is shown. Protein loading for both cytoplasmic and nuclear Western blots was normalized to β-actin as described (33). E, HC11 cells were transfected with 1.0 μg of the cDNA expression clone for p38α and the indicated concentrations of the pMKK6bE expression clone, and EMSA analysis was performed on nuclear and cytoplasmic fractions using the mouse XOR −140/−170 C/EBPβ oligonucleotide. F, HC11 cells were transfected with 1.0 μg of the cDNA expression clone for p38α and cotransfected with the cDNA for MKK6bE at a concentration of 0.1 μg. EMSA complexes were then formed with the mouse XOR C/EBPβ oligonucleotide using nuclear extracts. Lane 1, probe alone; lane 2, complexes formed with nuclear proteins and no competitor; lanes 3 and 4, 20- and 50-fold molar excesses of a consensus C/EBP-binding site competitor, respectively; lane 5, 50-fold molar excess of the mouse proximal promoter C/EBP-binding site competitor; lane 6, supershifting monoclonal antibody (ab) to mouse C/EBPβ; lane 7, isotype control antibody.
previously identified mouse proximal promoter contains a potential C/EBPβ-binding domain, and we observed that the proximal promoter was indeed activated by C/EBPβ. Furthermore, C/EBPβ was activated by the MKK6b kinase in the presence of p38 and by low dose cycloheximide, which promoted nuclear accumulation and the capacity to form C/EBPβ protein complexes with the mouse XOR upstream DNA.

We have demonstrated that XOR was induced in a concentration-dependent manner by cycloheximide but not by puromycin. Because puromycin and cycloheximide inhibit protein synthesis by different means, it was unlikely that cycloheximide induction represented only the loss of an unstable inhibitor, as has been observed for other genes (35–37). Furthermore, we found no evidence of ERK activation by low dose cycloheximide in HC11 cells, as was found for cycloheximide-induced cytosolic phospholipase A2 activation in mouse macrophage cell lines (38). These results, in conjunction with the well described activation of XOR by inflammatory cytokines (15), suggest the involvement of the stress/cytokine-activated MAPKs that mediate cycloheximide activation of the α-epithelial Na+ channel (18). Indeed, XOR induction by low dose cycloheximide was blocked by two different inhibitors of p38 MAPK, SB202190 and SB203580. Cycloheximide induction of XOR was not blocked by the MEK1/2 inhibitor U0126.

Our experience with cycloheximide induction differs from previously published experiments in one respect. High dose cycloheximide (10 µg/ml) promotes XOR mRNA accumulation 24 h after treatment when cells are cultivated in serum-rich medium (11). However, in the present experiments, cells were cultivated in 2% heat-inactivated serum in the absence of EGF for 48 h prior to treatment with cycloheximide. This resulted in accumulation of XOR mRNA throughout the initial 10-h period by either high or low dose cycloheximide; however, by 24 h, a clear decline in XOR mRNA was evident when cells were exposed to high dose cycloheximide. Although the basis for this difference is unclear, we imagine that the stringent conditions imposed by serum starvation may be responsible for the decline observed in the presence of high dose cycloheximide, where residual protein synthesis was not more than 20% of the untreated cells.

We considered whether C/EBPβ might mediate the effect of low dose cycloheximide stress on XOR expression. C/EBPβ has an important role in regulating mammary gland function and development (29, 39) and mediates lactogenic hormone induction of β-casein (40, 41). We observed that cycloheximide promoted nuclear translocation and phosphorylation of C/EBPβ and that cycloheximide-induced phosphorylation was blocked by inhibitors of p38 MAPK, as was activation of XOR. This suggested that cycloheximide activated p38 MAPK, which subsequently promoted C/EBPβ phosphorylation and nuclear translocation, where it could contribute to XOR activation. To confirm that C/EBPβ contributes to XOR transcription, we cloned 1000 bp of the mouse XOR upstream DNA into the pGL3-Basic luciferase reporter gene and transfected this construct into HC11 cells along with a cDNA expression clone for full-length C/EBPβ (23). We observed a C/EBPβ concentration-dependent increase in luciferase expression mediated by the XOR upstream DNA, and this was associated with the capacity to form C/EBPβ-specific complexes with mouse XOR upstream DNA. Mutagenesis of the mouse XOR C/EBPβ site blocked expression both in the absence of C/EBPβ cDNA and in the presence of increasing concentrations of C/EBPβ cDNA. Furthermore, in vivo ChIP analysis of cycloheximide-treated cells revealed increased association of C/EBPβ with XOR promoter DNA, which was blocked by pretreatment with SB202190. Thus, C/EBPβ is essential for XOR expression and regulation in mouse mammary epithelial cells, and its association with the XOR upstream DNA can be blocked by inhibition of p38 MAPK. These data are consistent with results for rat XOR activation by C/EBPβ in NIH3T3 cells (42) and amplify a recent report that revealed increased XOR mRNA accumulation in mouse neuronal cells following transfection with a C/EBPβ cDNA expression clone (43). Thus, C/EBPβ may be essential for XOR regulation in many different cells.

We confirmed that p38 activation in the absence of cycloheximide promotes C/EBPβ activation by transfecting HC11 cells with cDNA expression clones for p38α in the presence of increasing concentrations of a CDNA expression clone for MKK6b, a kinase found to activate p38 (32). We observed MKK6b concentration-dependent nuclear translocation of C/EBPβ and the concomitant loss of C/EBPβ from the cytoplasm, in conjunction with the formation of XOR promoter-specific complexes. Thus, C/EBPβ activation in HC11 cells is associated with its nuclear translocation and capacity to form specific DNA complexes with XOR promoter DNA. It remains to be determined whether C/EBPβ is a direct substrate of p38 in HC11 cells. C/EBPβ bears a p38 phosphorylation sequence; it can serve as a direct substrate for p38 (in vitro) and both p38 and C/EBPβ are required for adiocyte differentiation of 3T3-L1 fibroblasts (28). Nonetheless, in HC11 cells, unidentified effector kinases may mediate the effect of p38 on C/EBPβ activation.

Deletion analysis of human and rat XOR upstream DNAs revealed complex protein binding that maintains XOR in a state of repression that presumably must be released before XOR can be activated (22). Although derepressing or activating transcription factors were not specifically identified in these studies, many different proteins that bind to human and rat promoters have been identified (22, 44). Although the present data reveal a role for C/EBPβ in the activation of XOR in mouse mammary epithelial cells, additional proteins potentially involved in C/EBPβ-mediated activation have not yet been identified. For example, XOR transcriptional activation by prolactin and dexamethasone is blocked by inhibitors of the JAK2/STAT5 and glucocorticoid pathways (11) and by inhibitors of the MEK1/2 and ERK1/2 pathways (10). Thus, JAK2/STAT5 and the glucocorticoid receptor may also contribute to XOR activation in the mammary epithelial cell and perhaps contribute to activation mediated by C/EBPβ (41, 45). Nonetheless, binding of STAT5 or the glucocorticoid receptor to human or rat XOR promoters has not been identified so far, and additional studies will determine the degree to which these other activators may contribute to C/EBPβ-mediated activation.

In the mammary gland, lactogenic hormones regulate XOR expression coordinately with other gene products that compose the MFG. However, activation of XOR by stress/cytokine-activated MAPKs reveals an additional level of complexity to the regulation of XOR not described for other genes encoding proteins of the MFG. XOR plays critical but largely unknown functions in development, pregnancy, and lactation, and it is an important marker of mammary gland development and differentiation. Activation of XOR by p38 in mammary epithelial cells suggests a potentially critical role for p38 in mammary gland development and lactation as well. Although this role has not been defined, our experiments have indicated that C/EBPβ and p38 MAPK are fundamentally involved in XOR expression and may mediate the critical role played by XOR in lactation and mammary gland development. We note as well that XOR is also activated in inflammatory leukocytes (4), where C/EBPβ also plays a critical role (23, 46, 47), and we imagine that the regulation of XOR by MAPKs may underlie the complex roles played by XOR in these different biological settings. As an essential component of innate immunity, XOR is responsive to the complex and evolving setting of inflammation, where MAPKs integrate diverse signals from cytokine activation, adhesion, and integrin activa-
p38- and C/EBPβ-mediated XOR Activation

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