C/EBPβ Participates in Regulating Transcription of the p53 Gene in Response to Mitogen Stimulation*

Kristy Boggs1 and David Reisman2
From the Department of Biological Sciences, University of South Carolina, Columbia, South Carolina 29208

The tightly regulated expression of p53 contributes to genomic stability, and transcription of the p53 gene is induced prior to cells entering S phase, possibly as a mechanism to ensure a rapid p53 response in the event of DNA damage. We have previously described the cloning of an additional 1000 bp of upstream p53 sequences that we have demonstrated play a role in the regulated expression of p53. As described in an earlier report, we preliminarily identified that a member of the CAAT/enhancer-binding protein (C/EBP) family of transcription factors may play a role in regulating p53. Here we have demonstrated that a particular C/EBPβ isoform, C/EBPβ-2, efficiently binds to the p53 promoter and induces its expression in a fashion that reflects the pattern of p53 expression seen as cells are induced to enter S phase and is absent from cells that are defective in proper p53 regulation. We conclude from these findings that C/EBPβ-2 plays a central role in the regulating of p53 transcription during the transition into S phase.

The p53 tumor suppressor gene plays a central role in the cell in maintaining genomic stability by functioning as a sequence-specific transcription factor that regulates the expression of genes required for cell cycle arrest or apoptosis in response to DNA damage (1, 2). Missense mutations in the p53 gene, which inactivate its DNA damage checkpoint and growth-suppressing activities, have been observed in >60% of all human tumors (2, 3). In many instances where the gene is not mutated, the complexing of p53 with overexpressed cellular proteins, such as MDM2 or Parc (4–9), has been shown to inactivate the protein. Loss of activity of wild-type p53 may also occur through mechanisms that prevent its entry into the nucleus (8, 10, 11) or through the loss of required p53 cofactors (12, 13). Finally, loss of p53 expression in some tumors has been shown to occur through inhibition of transcription of the p53 gene itself (14–18). These findings indicate that loss of wild-type p53 expression is an essential event in the genesis of cancer.

Given that the p53 protein is a critical regulator of cell growth, its expression must be tightly regulated for normal cell division as well as for its ability to function as a tumor suppressor. p53 levels must be suppressed for normal cell division to occur (19), which explains why p53 is found at low levels in normal dividing cells (2). When cells are stressed or damaged, p53 is rapidly induced. Several mechanisms exist to regulate p53 levels and function within the cell.

It is becoming more widely accepted that the transcriptional regulation of the p53 gene plays an important role in regulating the overall level of p53 protein. This was recently illustrated by Takaoka et al. (20), who demonstrate that interferon-α/β, an essential component leading to apoptosis in response to viral infections, activates p53 transcription, thus clearly implicating p53 in the interferon pathway. Likewise, it has been known for many years that the level of p53 is increased at the transcriptional level after growth factor stimulation of resting murine (21) and human (22) lymphocytes and resting fibroblasts (23–26). Induced expression of p53 prior to S phase may serve as a mechanism for providing a rapid response to DNA damage during S phase. Until recently, the molecular basis for this transcriptional regulation has not been investigated. In view of these observations, we have focused on defining the mechanisms controlling p53 expression in response to mitogen induction. In a previous report, we characterized an additional 1000 bp of upstream DNA sequences and identified a number of new positive and negative regulatory elements. One of these newly identified elements, located ~960 bp upstream from the transcription start site, was found to bind to a trans-acting factor in a cell cycle-regulated manner and found to be required for proper S phase expression (23). Here we have demonstrated that the transcription factor C/EBPβ (CAAT/enhancer-binding protein-β) binds to this regulatory site on the p53 promoter in response to mitogen stimulation and serves to increase p53 promoter expression as cells enter S phase. Because C/EBPβ is a transcription factor that is critical for normal proliferation and differentiation of a number of cell types (27–30) and has also been demonstrated to play a role in mitogen-stimulated induction of cell division (31), we have carried out a experiments to establish whether it also has a role in regulating p53.

EXPERIMENTAL PROCEDURES

Cell Lines—Murine fibroblast cells (Swiss3T3), generously provided by K. Miskimens (University of South Dakota), and mouse embryo fibroblasts prepared from wild-type and C/EBPβ (−/−) mice (32), generously provided by Peter Johnson (National Cancer Institute (NCI)), were maintained in Dulbecco’s modified Eagle’s medium. Murine breast cancer lines were provided by Esta Sterneck at NCI (HC11); Linda Sealy at Vanderbilt University (4T1); and Daniel Medina at Baylor Uni-
versity (FSK-3, TM40-A, TM-3). NuMuMg cells were purchased from the American Type Culture Collection (Manassas, VA). HC11 were grown in RPMI 1640 medium supplemented with 10 μg/ml insulin and 5 ng/ml epidermal growth factor. 4T1, FSK-3, TM40-A, TM-3, and MuMuMg were grown in Dulbecco’s modified Eagle’s medium supplemented with 10 μg/ml insulin and 5 ng/ml epidermal growth factor. All media contained 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of 6% CO2.

Transfections and Reporter Gene Assays—The 0.7 kb fragment contained the 672-bp murine promoter fragment cloned into the HindIII site of the pGL3-basic luciferase vector (Promega). The 1.7 kbp p53 promoter contained a 1672-bp KpnI-HindIII fragment isolated from the murine genome and cloned into the KpnI and HindIII sites of the pGL3-basic luciferase vector (33). Five × 10⁴ cells in 24-well plates were transfected (TransFast Reagent; Promega) with the pGL3-basic luciferase reporter construct and 50 ng of Renilla driven by the HSV-TK promoter (pRL-TK vector) as an internal control. Eighteen hours after transfection, cells were grown in serum-depleted medium (0.1% FBS) for 24 h followed by serum stimulation (15% FBS). To assay wild-type full-length 1.7-kbp p53 promoter activity in the presence of C/EBPβ isoforms (34), exponentially growing Swiss3T3 and 6629 (C/EBPβ-null) cells were transfected with increasing 1.7-kbp p53 promoter pGL3-basic luciferase vector with or without co-transfection of 0.25 μg of C/EBPβ-2 and 50 ng of Renilla. Twenty-four hours after transfection, the cells were harvested, lysed, and assayed for luciferase activity. The results were normalized to TK-Renilla expression. To assay the activity of the 1.7 kb p53 promoter harboring a site-directed deletion or mutation within the −972/−953 site on the promoter, exponentially growing Swiss3T3 cells were transfected with an increasing concentration of the mutant 1.7-kbp promoter pGL3-basic luciferase vector with or without co-transfection of 0.25 μg of C/EBPβ-2 and 50 ng of Renilla. Cells were lysed at the indicated time points and assayed for luciferase activity using equal amounts of protein, as determined by Bradford assays. Reporter gene activity was normalized to TK-Renilla activity. Data are shown as means ± S.E.

Site-directed Mutagenesis—The −972/−953 cis-acting element carrying the C/EBPβ-binding site within the p53 promoter was either deleted or mutated using the GeneEditor in vitro site-directed mutagenesis system (Promega). The following mutant sense oligonucleotides were 5’-phosphorylated to generate the mutation: deletion (deleted nucleotides bracketed and in lowercase), 5’-CAAGTTTCTACTGCCTAAACCCAGGAATCTACAAAGGaAATAGCAATGTTTTTCATGTCTTAAATCTCCATAAAAG-3’; and mutation (altered nucleotides underlined), 5’-CAAGTTTCTACTGCCTAACCCAGGACTTACAAGGGCAAGCTCTGAAATAATGCAATGTTTTTCATGTCTTAAATCTCCATAAAAG-3’. In Vitro Transcribed/Translated C/EBPβ Isoforms—C/EBPβ-1, -2, and -3 cDNA were cloned into pcDNA3.1 expression vector containing a T7 RNA polymerase promoter in vitro transcribed/translated using the TNT T7 quick coupled transcription/translation protocol (Promega). Plasmid DNA template (0.5 μg) was transcribed/translated either in the presence of [35S]methionine (>1000 Ci/mmol) or with cold methionine. Non-programmed rabbit reticulocyte lysate was used as a negative control. Synthesized proteins were analyzed on 12% SDS-polyacrylamide gel and visualized after impregnating the gel with EN3HANCE and subjecting the dried gel to autoradiography for 2 h.

Electrophoretic Mobility Shift Assay (EMSA)—Cells were washed twice with cold phosphate-buffered saline and lysed on ice with a hypotonic lysis buffer (20 mM Hepes, pH 7.6, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/μl pepstatin, and 1 μg/μl aprotinin). Nuclei were resuspended at 2.5 × 10⁷ nuclei/ml in nuclear extraction buffer (identical to lysis buffer, except with 500 mM NaCl), gently rocked for 1 h at 4 °C, centrifuged at 10,000 revolutions/min for 10 min, and supernatant stored at −70 °C.

The binding reaction consisted of 10 fmol/reaction [γ-32P]ATP end-labeled double-stranded oligonucleotide, 2 μg of poly(dI-dC) in binding buffer TM.1 (50 mM Tris-HCl, pH 7.9, 0.1 mM KCl, 12.5 mM MgCl2, 1 mM EDTA, 20% glycerol and 1 mM dithiothreitol), and incubation on ice for 15 min followed by room temperature incubation for 15 min. The products were separated on a 4% polyacrylamide gel at 4 °C in 0.5× TBE (0.045 M Tris borate, 1 mM EDTA). Gels were dried and subjected to autoradiography for 30 min–6 h. To test for specificity, EMSA was performed with unlabeled specific and nonspecific competitors ranging from 10 to 50-fold molar excess of the labeled probe.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed using reagents in the ChIP-IT kit (Active Motif) on formaldehyde-fixed chromatin isolated from SWISS 3T3 cells. After shearing of the chromatin by sonication, samples were precleared with protein-G-agarose and incubated with antibodies. Complexes were immunoprecipitated and the DNA released, purified, and amplified by 36 cycles of PCR. Oligonucleotides used to amplify the putative C/EBP site in the p53 promoter were forward (5’-AGCGCTGGAGAATTCCTAGAGG-3’) and reverse (5’-CGAGATCTGTGTCTCGCAGC-3’) and yielded a 468-bp product assayed by electrophoresis through a 3% agarose (MetaPhor, Cambrex, Inc.). Controls included anti-RNApolII (positive), mouse IgG (negative), and a non-reactive anti-LAP (negative) for immunoprecipitations and random oligonucleotides for PCR.

Western Transfer Analysis—Serum-treated Swiss3T3 cells were washed twice in phosphate-buffered saline and lysed in radioimmunoprecipitation assay buffer (10 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA, pH 8.0) containing phosphatase inhibitors (0.1 mM Na3VO4, 50 mM NaF) and protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 1 μg/ml phenylmethylsulfonyl fluoride) followed by sonication. Protein concentrations were determined by Bradford assays, and equal amounts of protein (35 μg) were mixed with 2× sample buffer (140 mM Tris, pH 6.8, 22% glycerol, 6% SDS, 0.02% bromphenol blue, and 10%
C/EBPβ Regulates p53 Transcription

**FIGURE 1.** An illustration of murine p53 promoter and C/EBPβ isoforms. A, the cloned promoter and regulatory regions are contained on a 1.7-kbp fragment. A number of previously identified regulatory sites within the well characterized first 700-bp promoter are shown. The sequence of the regulatory site shown to bind to C/EBPβ maps −960 bp upstream of the transcription start site within a newly cloned upstream region of the p53 gene. B, illustration of C/EBPβ isoforms. Three isoforms are formed by alternative translation because of three in-frame methionines. C/EBPβ-1 is full-length. C/EBPβ-2 lacks 21 amino acids at the N-terminal transactivation domain. C/EBPβ-3 completely lacks the N-terminal transactivation domain (13).

β-mercaptoethanol) and heated at 100 °C for 2 min. Protein samples were separated by electrophoresis in a 12% SDS-polyacrylamide gel in 1× running buffer (25 mM Tris, 0.2% glycine, and 0.1% SDS) and electrophoretically transferred to Hybond-P membranes (Amersham Biosciences). After blocking with 5% powdered milk in 1× TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) overnight at 4 °C, the membrane was incubated with 0.1 μg/ml primary antibodies (either polyclonal anti-C/EBPβ antibody (Geneka), anti-p53 antibody pAB421, or polyclonal anti-actin antibody) for 1 h followed by incubation with a 1:5000 dilution of anti-mouse or -rabbit Ig antibody conjugated to horseradish peroxidase for 1 h at room temperature. The membranes were then washed extensively with 1× TBST, and the proteins were visualized by ECL (Amersham Biosciences) and by subjecting the films to exposure for 3–15 min. The separated RNA was transferred to a nylon membrane, baked, and prehybridized at 42 °C (2:1 ratio of Ambion ULTRAHyb and 50% formamide, 5× saline/sodium phosphate/EDTA, 5× Denhardt’s medium (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 μg/ml denatured salmon sperm DNA, and 0.1% SDS). Hybridization was to 32P-labeled cDNA overnight at 42 °C. Membranes were washed with 1× saline/sodium phosphate/EDTA/0.1% SDS (150 mM NaCl, 10 mM NaH2PO4·H2O, and 1 mM 0.5 M EDTA, pH 8.0), 0.5× saline/sodium phosphate/EDTA/0.1% SDS and 0.1× saline/sodium phosphate/EDTA/0.1% SDS at 60 °C. Membrane was stripped in boiling 0.1× SSC (15 mM NaCl and 1.5 mM sodium Citrate) and 0.1% SDS prior to reprobing with GAPDH cDNA as a loading control. In Fig. 7, equal RNA loading was determined by staining the gels with ethidium bromide and visualizing 18 and 28 S ribosomal RNA. Filters were subjected to autoradiography for 1–3 days.

**RESULTS**

**In Vitro Translated C/EBPβ Binds to the p53 Promoter—** Having previously shown (23) that a nuclear factor that binds to a site on the p53 promoter at −972/−953 (Fig. 1A) contributes to cell cycle-regulated transcription of p53, we carried out a data base search for transcription factors that may bind the p53 promoter at this site. To identify the factor(s) that may be binding to this element, we entered the 20-bp sequence into the Genomatix MatInspector transcription factor data base. As described previously (23), three potential candidates (C/EBPβ, RBP-Jκ (CBF1), and Ikaros-2) were identified as potential candidates, but only oligonucleotides specific for C/EBPβ were able to block binding of this factor to the promoter. In view of these findings, we further examined the role of C/EBPβ in regulating p53 transcription.

C/EBPβ is critical for the normal growth and differentiation of various cell types (27,28,35). Three protein isoforms of C/EBPβ are formed by alternative translation of three in-frame initiation sites on C/EBPβ mRNA (34,36,37) (Fig. 1B). C/EBPβ-1 is the full-length form of the protein (38 kDa) that contains an intact N-terminal transactivation domain and C-terminal DNA-binding domain. C/EBPβ-2 (35 kDa) differs from C/EBPβ-1 by only 21 amino acids at the N terminus; however, the N-terminal transactivation domain is still functional. Both C/EBPβ-1 and C/EBPβ-2 are transactivators, although only recently have studies addressed their functional differences. C/EBPβ-1 is the only isoform detected in normal human
C/EBPβ regulates p53 transcription

C/EBPβ antibody, specific for the C-terminal DNA-binding domain, when included in the DNA-binding assays resulted in a supershift of the bound complex (Fig. 2B). A C/EBPβ neutralizing peptide, which blocks the ability of the C/EBPβ antibody to bind, prevented the supershift and demonstrated the specificity of the anti-C/EBPβ antibody. Also, a supershift was not seen in the presence of anti-p53 antibody when used as a negative control. Furthermore, chromatin immunoprecipitation with anti-C/EBPβ antibody demonstrated the association of C/EBPβ with the p53 promoter (Fig. 2C). These results indicate that endogenous C/EBPβ in Swiss3T3 nuclear extracts binds to the −972/−953 site on the p53 promoter. Because the anti-C/EBPβ antibody recognizes all three isoforms of C/EBPβ, these experiments cannot distinguish which form is binding. As described below, our results led us to conclude that it is likely to be the C/EBPβ-2 isoform.

Reporter Gene Assays Indicate C/EBPβ-2 Enhances p53 Promoter Activity—To characterize the role of C/EBPβ in regulating p53 promoter activity, several approaches were taken. In one approach, we assayed the effect of the different C/EBPβ isoforms on the expression of p53 promoter in transient transfection assays. Exponentially growing Swiss3T3 cells were co-transfected with the 1.7-kbp p53 promoter and either C/EBPβ-1, -2, or -3 (Fig. 3). When C/EBPβ-1 was co-transfected with the 1.7-kbp p53 promoter, it acted as a weak positive regulator and led to an enhancement of p53 promoter activity by ~2.5-fold. The expression of C/EBPβ-3, a putative transcriptional repressor, resulted in a slightly reduced p53 promoter activity. Of all three isoforms, co-transfection of C/EBPβ-2 with the 1.7-kbp p53 promoter had the most dramatic effect on promoter activity. When compared with cells only transfected with the 1.7-kbp p53 promoter, co-transfection of C/EBPβ-2 resulted in an elevation of p53 promoter activity by ~15-fold (Fig. 3). Interestingly, increasing the amount of reporter plasmid in the transfection resulted in a gradual elimination of activation by C/EBPβ-2, presumably because of the presence of a vast excess of introduced target sequences that could not be bound by a limiting amount of input C/EBPβ-2.

To determine whether the enhanced p53 promoter activity required the C/EBPβ recognition site at −972/−953, we tested the activity of a 1.7-kbp p53 promoter that harbored either a deletion or a mutation within this site. Prior to assaying the promoter activity, the −972/−953-deleted and -mutated sites were tested for their ability to bind C/EBPβ from Swiss3T3 nuclear extracts by EMSA. Both the mutated and the deleted mammary tissue, although in breast cancer cells, C/EBPβ-1 is absent (34, 38). C/EBPβ-3 (21 kDa) completely lacks the N-terminal transactivation domain and is thought to repress transcription by complexing with C/EBPβ-1 or -2 and inhibiting their ability to transactivate target genes (34, 36, 37).

To confirm whether the factor binding to the −972/−953 cis-acting regulatory element may be one of the C/EBPβ isoforms, we in vitro transcribed/translated the three isoforms of C/EBPβ from pcDNA3.1 C/EBPβ cDNAs encoding C/EBPβ-1, -2, and -3. The in vitro synthesized proteins were used in EMSA to assay binding to the −972/−953 site (Fig. 2A). Although equivalent amounts of the individual proteins, as measured by Western transfer, were introduced into the DNA-binding reactions, C/EBPβ-2 demonstrated greater binding activity, as compared with either C/EBPβ-1 or -3. Anti-C/EBPβ antibodies are specific for the C-terminal DNA-binding domain, which is present and functional in all three C/EBPβ isoforms. In the presence of anti-C/EBPβ antibody, all three isoforms supershifted, and as above, the supershifted complex containing C/EBPβ-2 was much more prominent than either of the other C/EBPβ isoforms (Fig. 2A). The intensity of in vitro synthesized C/EBPβ-2 binding as compared with the other two isoforms indicates that C/EBPβ-2 interacts more effectively to the p53 promoter than the other two isoforms of C/EBPβ.

Endogenous Nuclear C/EBPβ Binds to the p53 Promoter—Having determined that in vitro synthesized C/EBPβ can bind the p53 promoter, we proceeded to test for binding by endogenous C/EBPβ from exponentially growing Swiss3T3 cells. Anti-
C/EBPβ Regulates p53 Transcription

FIGURE 3. C/EBPβ-2 enhances p53 promoter activity. Exponentially growing Swiss3T3 cells were co-transfected with increasing amounts of 1.7-kbp p53 promoter and 0.25 μg of pcDNA3.1 expression vector harboring either C/EBPβ-1, -2, or -3 cDNA. The promoter activity is reported relative to the promoter activity of Swiss3T3 cells transfected with only the 1.7-kbp promoter (assigned a value of 1). All experiments were done in duplicate, and the results were normalized to TK-Renilla. Solid bars, C/EBPβ-1; hatched bars, C/EBPβ-2; mosaicked bars, C/EBPβ-3.

FIGURE 4. p53 promoter with a mutated/deleted −972/−953 site fails to respond to C/EBPβ-2. Exponentially growing Swiss3T3 cells were co-transfected with 0.25 μg of C/EBPβ-2 expression vector and increasing amounts of the p53 promoter harboring a mutated −972/−953 site (A) or 1.7-kbp p53 promoter harboring a deletion of the −972/−953 site (B). Cells were harvested 24 h after transfection and assayed for luciferase activity. Solid bars, without C/EBPβ; hatched bars, in the presence of C/EBPβ. All experiments were performed in duplicate, and the results were normalized to TK-Renilla expression.

sites eliminated binding of C/EBPβ (data not shown) (23), and as a result, C/EBPβ-2 expression had no significant effect on either the mutated or deleted p53 promoter (Fig. 4). These results are in stark contrast to the 15-fold increase seen with the co-transfection of the wild-type full-length 1.7-kbp p53 promoter and C/EBPβ-2 (Fig. 3), indicating that the −972/−953 regulatory element on the p53 promoter is critical for p53 promoter activation by the C/EBPβ-2 protein.

Reporter Gene Assays Demonstrate that p53 Promoter Activity Is Reduced in C/EBPβ-null Cells—As another measure of the role of C/EBPβ in contributing to p53 promoter activity, we asked whether cells devoid of C/EBPβ expression were deficient in p53 promoter activity. Mouse embryo fibroblast cells prepared from wild-type C/EBPβ (+/+) and C/EBPβ-null (−/−) mice (32) were used in reporter gene assays to further characterize the role of C/EBPβ in regulating p53 promoter activity. Transfection of the 1.7-kbp p53 promoter into 6362 (C/EBPβ+/+) cells resulted in a 5–7-fold higher promoter activity as compared with the 1.7-kbp promoter activity in 6629 (C/EBPβ-null) cells (Fig. 5A). Likewise, p53 expression was undetectable in C/EBPβ-null cells, even in response to serum treatment and cell cycle induction (Fig. 5C). These results further support C/EBPβ being a positive regulator of p53 expression.

Given that 6629 (C/EBPβ-null) cells have reduced p53 promoter activity in comparison to their wild-type counterpart 6632 (C/EBPβ+/+), we assayed the p53 promoter activity in the presence of ectopic C/EBPβ-2 expression in the 6629 (C/EBPβ-null) cells. The cells were co-transfected with increasing amounts of 1.7-kbp p53 promoter and constant amounts of either C/EBPβ-1, -2, or -3 (Fig. 5B). In the presence of C/EBPβ-1 and -3, promoter activity was not significantly affected in comparison to the 1.7-kbp promoter activity in the absence of C/EBPβ isoforms (Fig. 5B). Co-transfection of C/EBPβ-2 resulted in a >4-fold induction in p53 promoter activity relative to cells only transfected with the 1.7-kbp promoter. Taken together, these results indicate that C/EBPβ expression contributes to enhanced p53 promoter activity by binding to the regulatory site at position −972/−953.

EMSA Demonstrates C/EBPβ Binds the Promoter in a Cell Cycle-dependent Manner—Because we previously demonstrated differential binding of trans-acting factors within the −972/−953 element on the p53 promoter in a cell cycle-regulated manner (Fig. 6A) (23) that contributes to the regulation of p53 gene expression during the cell cycle and found that C/EBPβ binds to this critical regulatory element and enhanced p53 promoter activity in exponentially growing cells, we examined the binding pattern of C/EBPβ in arrested cells stimulated to re-enter the cell cycle.

To assay for C/EBPβ binding the p53 promoter during the cell cycle, we tested nuclear extracts from arrested and serum-treated Swiss3T3 cells for the presence of C/EBPβ by EMSA both in the presence and absence of anti-C/EBP antibodies. Upon serum-depletion, there was a decrease in C/EBPβ binding to the promoter as denoted by the decrease in intensity of the complex seen at 0 h. By 3 h, post-serum stimulation binding of C/EBPβ increased substantially and coincided with the
increased endogenous p53 mRNA levels and an increase in p53 promoter activity at 3 h of post-serum stimulation (Fig. 6D) (23).

Having established that C/EBPβ is capable of differentially binding the p53 promoter during the cell cycle, we tested for the presence of C/EBPβ protein during the cell cycle to determine whether changes in binding activity are reflected by changes in the abundance of C/EBPβ protein. Swiss3T3 cells were serum-depleted for 24 h followed by serum stimulation prior to harvesting. Western blot analysis of the serum-treated cells detected the presence of the C/EBPβ protein with protein levels at their highest in exponentially growing cells of 0, 3, and 8 h of post-serum stimulation (Fig. 6C). At 18 h of post-serum stimulation, the levels of C/EBPβ begin to decrease to some extent. Although increased binding of the protein to the p53 promoter is seen at 3 h of post-serum stimulation, there is no apparent change in the level of C/EBPβ during this period. These results indicate that C/EBPβ protein is present throughout the cell cycle and that post-translational modification of the protein or interactions with additional regulatory partners at 3 h of post-serum stimulation may be responsible for its enhanced binding to the promoter.

Cells Defective in Regulated p53 Expression Lack C/EBPβ DNA-Binding Activity—Swiss 3T3 cells as well as other cell lines expressing wild-type p53, such as NIH 3T3 and Balb 3T3, have been demonstrated to undergo a regulated p53 response upon growth arrest and induced re-entry into the cell cycle (23, 25). The overall pattern observed is that the level of p53 mRNA significantly reduces by 18–24 h of post-serum depletion and then increases in response to mitogen treatment. First detectable by 3–6 h post-mitogen treatment, the level of p53 RNA generally is maximal by 18 h, after which it tends to return to the level observed in exponentially growing cells. As shown above, the appearance of C/EBPβ DNA-binding activity generally parallels this response. To determine whether cells that do not undergo the normal p53 cell cycle response may have a detectable alteration in C/EBPβ expression or activity, we assayed a series of murine breast carcinoma cell lines for their response to cell cycle arrest and mitogen stimulation. All cells were grown exponentially, transferred to medium containing 0.1% FBS for 18 h, and then supplemented with 15% FBS. Samples were taken from exponentially growing cells, serum-depleted cells, and cells stimulated with serum for 18 h and assayed for the level of p53 and C/EBP mRNA and for C/EBPβ DNA-binding activity. As shown in Fig. 7, upper panel, four of the cell lines tested, NuMuMg, FSK-3, TM40-A, and TM-3, all show an elevation in p53 expression in response to treatment with serum. Two cell lines, HC11 and 4T1, show no increase in p53. HC11 cells express very low levels of p53 mRNA that do not change during the course of the experiment, whereas 4T1 cells express p53 mRNA that decreases upon growth arrest but fails to increase by 18 h post-serum treatment.

We next assayed whether C/EBPβ activity is present in these cells, and as shown in Fig. 7, lower panel, although the cells undergoing an induction in p53 expression upon serum treatment exhibited C/EBPβ DNA-binding activity, the two lines, HC11 and 4T1, which did not express p53 in response to mitogen treatment, also did not harbor any detectable C/EBPβ DNA-binding activity (Fig. 7). In view of these results, we conclude that C/EBPβ is essential for the proper regulation of p53 transcription during the transition from growth arrest to entry into the cell cycle.

**DISCUSSION**

Although it has been known for quite some time that the p53 gene is induced upon mitogenic stimulation of murine fibroblasts (26) and human lymphocytes (22), the molecular mechanism responsible for this regulation has remained unexplored. Because elevated levels of p53 protein have been shown to lead to either growth arrest or apoptosis in response to DNA damage, it might seem anomalous that transcription of the p53 gene induced upon induction with mitogens, with a peak in transcription prior to the onset of DNA synthesis. This type of response has been suggested to be important for a rapid p53-induced arrest in DNA synthesis in response to DNA damage at a time when cells are synthesizing DNA and thus would be most

---

**FIGURE 5. C/EBPβ-2 enhances p53 promoter activity in C/EBPβ-null cells.** A, exponentially growing 6362 (wild-type C/EBPβ, solid bars) and 6629 (C/EBPβ-null, hatched bars) mouse embryo fibroblast cells were transfected in duplicate experiments with increasing amounts of p53 promoter. Twenty-four hours after transfection, the cells were harvested and assayed for luciferase activity. The results were normalized to TK-promoter activity in 6629 cells transfected with only the p53 promoter (assigned a value of 1). The experiments were done in duplicate and normalized to TK-luciferase expression. C/EBPβ-2 enhances p53 promoter activity. The promoter activity is related to the promoter activity in 6629 cells transfected with only the p53 promoter (assigned a value of 1). The experiments were done in duplicate and normalized to TK-luciferase expression. C, levels of p53 protein were assayed by Western blot analysis on 6629 cells that were either exponentially growing (E) or serum-depleted for 24 h (0 h) and then serum-stimulated and harvested 3, 8, and 24 h post-serum stimulation. Murine SVT2 cells serve as a positive control for p53 expression. +/+ wild-type C/EBPβ−/− C/EBPβ-null.
susceptible to DNA-damaging events. In fact, Mosner et al. (25) have demonstrated an exceptionally rapid accumulation of active p53 protein in response to DNA damage in synchronized cell populations in mid-S phase. Increased synthesis of p53 during this phase of the cell cycle would be predicted to provide p53 protein poised to act and serve as a type of “rapid response” system for preventing the replication of damaged DNA. Therefore, a description of the mechanisms responsible for regulating p53 transcription will have implications with respect to our understanding of the normal response to DNA damage as well as to how perturbations in this response could contribute to oncogenesis.

Endogenous levels of p53 mRNA and p53 promoter activity are significantly reduced in cells that are serum-depleted for 24 h and begin to increase at 3 h of post-serum stimulation. During different phases of the response, we have shown differential binding of a trans-acting factor to the −972/−953 region of the p53 promoter. Binding was reduced after cells were serum-depleted for 24 h and increased at 3 h of post-serum stimulation. This pattern of binding parallels the expression pattern seen previously with the promoter and the endogenous p53 mRNA levels (23).

Previous work has indicated that binding to the −972/−953 positive cis-acting element is necessary for maximal p53 promoter activity in exponentially growing cells as well as for the appropriate response to mitogen treatment (23). A data base search to identify candidate transcription factors that may bind the p53 promoter within the −972/−953 region as well as some preliminary DNA-binding assays revealed one factor (C/EBPβ) as a likely candidate. C/EBPβ is a CCAAT enhancer-binding protein and is critical for normal proliferation and differentiation of mammary epithelial cells (29, 30, 34, 38) as well as numerous other cell types (27, 28). It has also been shown to transactivate the c-fos serum response element upon activation of the Ras-dependent signaling pathway in response to mitogenic stimulation (31). C/EBPβ is found to be constitutively expressed in the liver, intestine, lung, adipose tissue, spleen, and kidney (39–43) and plays an important role in adipocyte (40, 44), hepatocyte (45), and mammary gland differentiation (29), as well as in ovulation (32, 46). C/EBPβ knock-out mice (43) have alteration in glucose homeostasis (47), immunological defects (48), develop lymphoproliferative disorders (49, 50) and defects in female reproduction and infertility (32). The participation of C/EBPβ in so many diverse pathways suggests that multiple levels of regulation contribute to its various biological functions.

Because C/EBPβ exists as three different isoforms, the binding of the different C/EBPβ isoforms to the p53 promoter was assayed to determine the activity of each isoform on the p53 promoter. In vitro synthesized C/EBPβ isoforms to the p53 promoter was assayed by Western blot analysis on Swiss 3T3 cells that were either growing exponentially or serum-depleted for 24 h (0 h) and then serum-stimulated and harvested at 3, 8, 18, and 24 h of post-serum stimulation. Extracts were probed with the labeled −972/−953 regulatory site. **, nonspecific binding (C/EBP complex). B, nuclear extracts from serum-stimulated cells (explained above) were tested for the presence of C/EBPβ by adding 4 μl of 0.6 μg/μl of anti-C/EBPβ in the binding reaction. C/EBPβ supershifted complexes are indicated by an arrow. **, nonspecific binding. Exposure of autoradiograms was carried out for 3 and 2 h, respectively. C, levels of C/EBPβ protein was assayed by Western blot analysis on Swiss 3T3 cells that were either exponentially growing (Exp) or serum-depleted for 24 h (0 h) and then serum-stimulated and harvested at 3, 8, 18, and 24 h of post-serum stimulation. Actin is shown as a loading control. D, mRNA levels of p53 and GAPDH in exponentially growing cells (E), serum-depleted cells (D), and serum-stimulated cells harvested at 3, 8, 18, and 24 h of post-serum stimulation.

FIGURE 6. EMSA of the region of the p53 promoter that spans −972/−953 demonstrates C/EBPβ DNA-binding activity is induced as cells are released from growth arrest. A, the nuclear extracts used were from Swiss 3T3 cells that were either growing exponentially (Exp) or serum-depleted for 24 h (0 h) and then serum-stimulated and harvested at 3, 8, 18, and 24 h of post-serum stimulation. Extracts were probed with the labeled −972/−953 regulatory site. **, nonspecific binding (C/EBP complex). B, nuclear extracts from serum-treated cells (explained above) were tested for the presence of C/EBPβ by adding 4 μl of 0.6 μg/μl of anti-C/EBPβ in the binding reaction. C/EBPβ supershifted complexes are indicated by an arrow. **, nonspecific binding. Exposure of autoradiograms was carried out for 3 and 2 h, respectively. C, levels of C/EBPβ protein was assayed by Western blot analysis on Swiss 3T3 cells that were either exponentially growing (Exp) or serum-depleted for 24 h (0 h) and then serum-stimulated and harvested at 3, 8, 18, and 24 h of post-serum stimulation. Actin is shown as a loading control. D, mRNA levels of p53 and GAPDH in exponentially growing cells (E), serum-depleted cells (D), and serum-stimulated cells harvested at 3, 8, 18, and 24 h of post-serum stimulation.
C/EBPβ Regulates p53 Transcription

The results further support that C/EBPβ was incubated with the labeled promoter. These results demonstrate that C/EBPβ promoter activity is regulated. ChIP assays will demonstrate the cell cycle regulation of the p53 promoter during later stages of the cell cycle.

Of the three C/EBPβ isoforms, C/EBPβ-2 not only exhibits the greatest binding activity on the p53 promoter, it likewise has the greatest effect on the promoter activity showing a 15-fold induction of the p53 promoter in co-transfection assays. These results further support that C/EBPβ-2 binds to and regulates promoter expression during the cell cycle. C/EBPβ-2 up-regulated the p53 promoter in C/EBP-null cells >4-fold, whereas C/EBPβ-1 and -3 had no effect on the promoter. These results demonstrate that C/EBPβ-2 has a role in regulating p53 promoter activity.

Taken together, these findings provide insight as to how p53 gene expression is regulated during the cell cycle; however, several avenues of research need to be pursued to further elucidate p53 regulation during the cell cycle. First, identification of additional regulatory factors binding the promoter will provide further insight as to how p53 promoter activity is regulated. ChIP assays will demonstrate in vivo binding of the identified regulatory factors to the p53 promoter. Also, once additional proteins binding the p53 promoter have been identified, protein-protein interactions with C/EBPβ can be demonstrated via co-immunoprecipitations and/or yeast two-hybrid assays. Ultimately, experiments utilizing small interfering RNA to inhibit C/EBPβ expression will be invaluable in elucidating the role C/EBPβ and any other identified trans-acting factors play in the cell cycle. Therefore, the knowledge gained from these and future results will have implications with respect to our understanding of the normal response to DNA damage as well as to how perturbations in this response contribute to oncogenesis.

Acknowledgments—We thank P. Johnson, D. Medina, K. Miskimins, L. Sealy, and E. Sterneck for providing us with many of the cell lines used in this study. We thank L. Sealy and E. Eaton for providing constructs carrying C/EBPβ cDNAs. We also thank M. Coggins for technical assistance on the ChIP assays and S. Thomas and S. B. L. Volate for critical reading of the manuscript.

REFERENCES
1. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Nature 408, 307–316
2. Vousden, K. H., and Lu, X. (2002) Nat. Rev. Cancer 2, 594–606
3. Hussain, S. P., and Harris, C. C. (1998) Cancer Res. 58, 4023–4037
4. Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998) Nature 395, 124–125
5. Bond, G., Hu, W., Bond, E., Robins, H., Lutzker, S., Arva, N., Bargonetti, J., Bartel, F., Taubert, H., Wuel, P., Onel, K., Yip, L., Hwang, S., Strong, L., Lozano, G., and Levine, A. (2004) Cell 119, 591–602
6. Freedman, D. A., and Levine, A. J. (1999) Cancer Res. 59, 1–7
7. Gosh, M., Huang, K., and Berberich, S. J. (2003) Biochemistry 42, 2291–2299
8. Nikolaev, A. Y., Li, M., Puskas, N., Qin, J., and Gu, W. (2003) Cell 112, 29–40
9. Sherr, C. J. (1998) Genes Dev. 12, 2984–2991
10. Moll, U., LaQuaglia, J., Benard, J., and Riou, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4407–4411
C/EBPβ Regulates p53 Transcription

11. Tao, W., and Levine, A. J. (1999) Proc. Nat. Acad. Sci. U. S. A. 96, 6937–8941
12. Garkavtsev, I., Grigorian, I. A., Ossovskaya, V. S., Chernov, M. V., Chumakov, P. M., and Gudkov, A. V. (1998) Nature 391, 295–298
13. Sui, G., Affar, E. B., Shi, Y., Brignone, C., Wall, N. R., Yin, P., Donohoe, M., Luke, M. P., Calvo, D., Grossman, S. R., and Shi, Y. (2004) Cell 117, 859–892
14. Hodge, D. R., Peng, B., Cherry, J. C., Hurt, E. M., Fox, S. D., Kelley, J. A., Munroe, D. J., and Farrar, W. L. (2005) Cancer Res. 65, 467–4682
15. Kang, J. H., Kim, S. J., Noh, D. Y., Park, I. A., Choe, K. J., Yoo, O. J., and Kang, H. S. (2001) Lab. Invest. 81, 573–579
16. Phan, R. T., and Dalla-Favera, R. (2004) Nature 432, 635–639
17. Raman, V., Martensen, S. A., Reisman, D., Evron, E., Odenwald, W. F., Jaffee, E., Marks, J., and Sukumar, S. (2000) Nature 405, 974–978
18. Stuart, E. T., Haffner, R., Oren, M., and Gruss, P. (1995) EMBO J. 22, 5638–5645
19. Choi, J., and Donehower, L. A. (1999) Cell. Mol. Life Sci. 55, 38–47
20. Takaoka, A., Hayakawa, S., Yanai, H., Stoiber, D., Negishi, H., Kikuchi, H., Sasaki, S., Imai, K., Shubue, T., Honda, K., and Taniguchi, T. (2003) Nature 422, 516–523
21. Milner, J., and Milner, S. (1981) Virology 112, 785–788
22. Reed, J. C., Alpers, J. D., Nowell, P. C., and Hoover, R. G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3982–3986
23. Boggs, K., and Reisman, D. (2006) Oncogene 25, 555–565
24. Ginsberg, D., Oren, M., Yaniv, M., and Piette, J. (1990) Oncogene 5, 1285–1290
25. Mosner, J., Mummenbrauer, T., Bauer, C., Sczakiel, G., Grosse, F., and Deppert, W. (1995) EMBO J. 14, 4442–4449
26. Reich, N. C., and Levine, A. J. (1984) Nature 308, 199–201
27. Johnson, P. F. (2005) J. Cell Sci. 118, 2545–2555
28. Ramji, D., and Foka, P. (2002) Biochem. J. 365, 561–575
29. Robinson, G. W., Johnson, P. F., Hennighausen, L., and Sterneck, E. (1998) Genes Dev. 12, 1907–1916
30. Seagroves, T. N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G. L., and Rosen, J. M. (1998) Genes Dev. 12, 1917–1928
31. Hanlon, M., and Sealy, L. (1999) J. Biol. Chem. 274, 14224–14228
32. Sterneck, E., Tessaroloo, L., and Johnson, P. F. (1997) Genes Dev. 11, 2153–2162
33. Reisman, D. E., Eaton, E., McMillin, D., Doudican, N., and Boggs, K. (2001) Gene 274, 129–137
34. Eaton, E. M., Hanlon, M., Bundy, L., and Sealy, L. (2001) J. Cell. Physiol. 189, 91–105
35. McKnight, S. L. (2001) Cell 107, 259–261
36. Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E., and Schibler, U. (1990) Genes Dev. 4, 1541–1551
37. Ossipow, V., Descombes, P., and Schibler, U. (1993) Proc. Nat. Acad. Sci. U. S. A. 90, 8219–8223
38. Sealy, L., and Bundy, L. M. (2003) Oncogene 22, 869–883
39. Akira, S., Ishihara, H., Sugita, H., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) EMBO J. 9, 1897–1906
40. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) Genes Dev. 5, 1538–1552
41. Descombes, P., and Schibler, U. (1991) Cell 67, 569–579
42. Katz, S., Kowenz-Leutz, E., Muller, C., Meese, K., Ness, S. A., and Leutz, A. (1993) EMBO J. 12, 1321–1332
43. Poli, V., Mancini, F. P., and Cortese, R. (1990) Cell 63, 643–653
44. Darlington, G. J., Ross, S. E., and MacDougald, O. A. (1998) J. Biol. Chem. 273, 30057–30060
45. Diehl, A. M. (1998) J. Biol. Chem. 273, 30843–30846
46. Pall, M., Hellberg, P., Brannstrom, M., Mikuni, M., Peterson, C. M., Sundfeldt, K., Norden, B., Hedin, L., and Enerback, S. (1997) EMBO J. 16, 5273–5279
47. Croniger, C., Trus, M., Lysek-Stupp, K., Cohen, H., Liu, Y., Darlington, G. J., Poli, V., Hanson, R. W., and Reshef, L. (1997) J. Biol. Chem. 272, 26306–26312
48. Tanaka, T., Akira, S., Yoshida, K., Umemoto, M. M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. (1995) Cell 80, 353–361
49. Chen, X., Liu, W., Ambrosino, C., Ruocco, M. R., Poli, V., Romani, L., Quinto, I., Barbieri, S., Holmes, K. L., Venuta, S., and Scala, G. (1997) Blood 90, 156–164
50. Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, K., Sellitto, C., Scarpa, S., Bellavia, D., and Lattanzio, G. (1995) EMBO J. 14, 1932–1941