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Glucocorticoid-Stimulated CCAAT/Enhancer-Binding Protein α Expression Is Required for Steroid-Induced G_1 Cell Cycle Arrest of Minimal-Deviation Rat Hepatoma Cells

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By genetic correlation with the growth-suppressible phenotype and direct functional tests, we demonstrate that the glucocorticoid-stimulated expression of the CCAAT/enhancer-binding protein α (C/EBPα) transcription factor is required for the steroid-mediated G_1 cell cycle arrest of minimal-deviation rat hepatoma cells. Comparison of C/EBPα transcript and active protein levels induced by the synthetic glucocorticoid dexamethasone in glucocorticoid growth-suppressible (BDS1), nonsuppressible receptor-positive (EDR1) and nonsuppressible receptor-deficient (EDR3) hepatoma cell proliferative variants revealed that the stimulation of C/EBPα expression is a rapid, glucocorticoid receptor-mediated response associated with the G_1 cell cycle arrest. Consistent with the role of C/EBPα as a critical intermediate in the growth suppression response, maximal induction of transcription factor mRNA occurred within 2 h of dexamethasone treatment whereas maximal inhibition of [^{3}H]thymidine incorporation was observed 24 h after steroid treatment. As a direct functional approach, ablation of C/EBPα protein expression and DNA-binding activity by transfection of an antisense C/EBPα expression vector blocked the dexamethasone-induced G_1 cell cycle arrest of hepatoma cells but did not alter general glucocorticoid responsiveness. Transforming growth factor β induced a G_1 cell cycle arrest in C/EBPα antisense transfected cells, demonstrating the specific involvement of C/EBPα in the glucocorticoid growth suppression response. Constitutive expression of a conditionally activated form of C/EBPα caused a G_1 cell cycle arrest of BDS1 hepatoma cells in the absence of glucocorticoids. In contrast, overexpression of C/EBPβ or C/EBPδ had no effect on hepatoma cell growth. Taken together, these results demonstrate that the steroid-induced expression of C/EBPα is necessary to mediate the glucocorticoid G_1 cell cycle arrest of rat hepatoma cells and implicates a role for this transcription factor in the growth control of liver-derived epithelial tumor cells.

Glucocorticoids, one class of steroid hormones, play a fundamental role in the physiological, proliferative, and developmental control of animal tissues by regulating a diversity of gene networks and complex pleiotropic processes (26, 38, 78, 84). The potent effects of glucocorticoids on the transcription of primary response genes occur by specific binding of the steroid-receptor complex to DNA transcriptional enhancer elements (9, 10, 76, 84, 86) and/or by enhancing or interfering with the action of other transcriptional regulators, such as the AP-1 complex or NF-κB, via direct protein-protein interactions (20, 45, 59, 73, 76). Moreover, the availability of particular sets of transcription factors can dramatically modulate the transcriptional effects of steroid receptors on specific target genes (56). It has been proposed that in the cellular responses to glucocorticoids, a select subset of primary response genes function to induce or repress the expression of secondary response target genes which can alter the production or activity of a wide variety of cellular components (30, 78).

Liver-derived normal and transformed cells provide experimental systems for uncovering glucocorticoid-regulated cellular cascades that ultimately target the genes that control proliferation and differentiation. Although not well understood, glucocorticoids can inhibit both the in vivo and in vitro growth of intact liver, primary hepatocytes, and some hepatoma cell lines (16, 33, 51, 75), as well as modulate the expression of a variety of genes involved in liver-specific functions, such as the acute-phase inflammation response (4, 6, 8). Conceivably, the glucocorticoid receptor-mediated signal transduction pathways that regulate liver cell growth and differentiation may be coordinately controlled or modulated by common transcriptional regulators. One candidate set of regulatory proteins is the members of the CCAAT/enhancer-binding protein (C/EBP) transcription factor gene family, which belong to a group of basic leucine zipper DNA-binding proteins (50, 66, 82). The C/EBP gene family includes the C/EBPα, C/EBPβ, and C/EBPδ isoforms, which can be expressed and regulated in a tissue- and cell line-specific manner (14, 18, 46, 69). In addition, in certain cell types, more than one molecular weight species of a given isoform can be detected. For example, transcriptionally active C/EBPα is expressed as 42- and 30-kDa forms in liver and adipocyte nuclei in vivo, as well as in the 3T3-L1 adipocyte cell line after differentiation (11, 13, 49). The expression of certain C/EBP isoforms can be inversely correlated with the proliferation of liver-derived cells and of other cell types such as adipocytes. For example, C/EBPα protein is rapidly down regulated during liver regeneration in vivo (23, 28) and is detected in reduced concentrations in a variety of proliferating hepatocytes, rat hepatoma cell lines in vitro, and adipocytes (7, 11, 13, 15, 49, 58, 83). Furthermore, C/EBPα is highly abundant in quiescent normal rat liver cells (22). Attempts to generate stable transfected cells that constitutively express C/EBPα have been unsuccessful (43, 77), suggesting

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the direct involvement of this transcription factor in the suppression of growth. Recently, several studies have demonstrated that overexpression of C/EBPα can suppress the growth of a variety of human and rat hepatoma cell lines (21, 43, 79) and delay tumor formation in nude mice transplanted with C/EBPα-transfected hepatoma cells (79), while the 42-kDa form of C/EBPα was shown to be antimitotic in transfected 3T3-L1 adipocytes (55).

C/EBP gene family members have also been proposed to assist in maintaining the terminally differentiated state and tissue-specific function of the liver and to act in other tissues by regulating tissue-specific gene expression (15, 29, 61). For example, C/EBPα and C/EBPβ help mediate the glucocorticoid-induced acute-phase inflammation response through transcriptional control of particular target genes (2, 7, 42, 72, 81). Specifically, glucocorticoid receptors and the α and β isoforms of C/EBP synergistically activate the promoter of the acute-phase protein alpha-1-acid glycoprotein through direct protein-protein interactions (7), as well as by their DNA-binding properties (63). Given the mechanistic relationship between glucocorticoids and C/EBPα in controlling normal liver cell function and growth, it is tempting to consider that the C/EBPα transcription factor may also be directly involved in growth suppression signaling pathways in glucocorticoid-responsive hepatic cells.

To analyze the mechanism of glucocorticoid-mediated growth suppression responses, we have isolated glucocorticoid-sensitive and -resistant hepatoma cell variants from minimal-deviation rat Reuber hepatoma-derived cell lines (16). Characterization of glucocorticoid-sensitive rat BDS1 hepatoma cells revealed that the synthetic glucocorticoid dexamethasone induces a G_1 block in cell cycle progression within one cell doubling time (75). Glucocorticoids did not affect cell viability, decrease total cell number, or induce an apoptotic response (16, 75). Two classes of glucocorticoid-resistant variants which were not susceptible to the antiproliferative effects of glucocorticoids were also identified (16). One class of nonsuppressible variants, represented by EDR1 cells, express wild-type levels of glucocorticoid receptors but fail to express a subset of steroid-responsive genes. EDR3 cells represent a second class of glucocorticoid-nonsuppressible hepatoma cell variants that produce less than 1% of the wild-type levels of glucocorticoid receptors and are completely glucocorticoid nonresponsive (16). Comparison of steroid-responsive genes in this panel of hepatoma cell variants revealed that expression of the glucocorticoid-stimulated acute-phase inflammation response gene product, alpha-1-acid glycoprotein, strikingly correlated with the growth-suppressible phenotype, although this protein did not inhibit hepatoma cell growth (16). The coordinate production of alpha-1-acid glycoprotein and growth inhibition of hepatoma cells suggests that factors regulating this gene may be linked mechanistically to the glucocorticoid growth suppression pathway. Candidate factors known to activate alpha-1-acid glycoprotein expression are members of the C/EBP gene family (2, 7, 48, 63, 72). To directly test this notion, these glucocorticoid growth-suppressible and -nonsuppressible hepatoma variants were used to demonstrate that the glucocorticoid stimulation of C/EBPα gene expression is a rapid response linked to the growth-suppressible phenotype and accounts for the lack of alpha-1-acid glycoprotein expression in glucocorticoid-nonsuppressible variants. Furthermore, ablation of C/EBPα expression by transfection of an antisense expression vector and constitutive expression of a conditionally activated form of C/EBPα directly demonstrated that the induced expression of this transcription factor is a required component of the glucocorticoid cell cycle arrest response of rat hepatoma cells.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's (DME)/F-12 (1:1) medium, fetal bovine serum, calcium- and magnesium-free phosphate-buffered saline (PBS), and trypsin-EDTA were supplied by BioWhittaker, Walkersville, Md. Dexamethasone, β-estradiol, and propidium iodide were obtained from Sigma Chemical Co., St. Louis, Mo. [^{3}H]thymidine (84 Ci/mmol), [^{14}C]chloramphenicol (40 to 60 mCi/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and [α-^{32}P]dATP (3,000 Ci/mmol) were obtained from DuPont NEN Products, Boston, Mass. Poly(dI-dC) · (dI-dC) was purchased from Pharmacia Biotech, Piscataway, N.J. Anti-C/EBPα, anti-C/EBPβ, anti-C/EBPδ, anti-c-*fos*, anti-CDK2, and anti-CDK4 antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, Calif. Transforming growth factor β was obtained from Gibco BRL, Gaithersburg, Md. Horseradish peroxidase-conjugated goat anti-rabbit antibodies were purchased from Bio-Rad, Hercules, Calif. The enhanced chemiluminescence (ECL) protein detection system and the Multiprime DNA-labeling kit were purchased from Amersham Corp., Arlington Heights, Ill. The chimeric pGRE-CAT reporter plasmid containing six glucocorticoid response elements linked to the chloramphenicol acetyltransferase (CAT) reporter gene was a generous gift from Keith R. Yamamoto, Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, Calif. The chimeric alpha-1-acid glycoprotein promoter CAT reporter plasmid pAGP3x(GRE)CAT, the C/EBPα eukaryotic expression vector pCD-mC/EBP, and the parental blank expression vector pCD were generously provided by Heinz Baumann, Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, N.Y., and have been described previously (5). Plasmid pMCETx-5/4, which constitutively expresses the chimeric C/EBPα-estrogen receptor ligand-binding domain (C/EBPα-ER) gene, was provided by Alan Friedman, Division of Pediatric Oncology, Johns Hopkins Oncology Center, Johns Hopkins University, Baltimore, Md., and was described previously (77). Plasmid pBCMG-AS, which constitutively expresses an antisense C/EBPα transcript, and the parental vector, pBCMGneo, were a kind gift of M. Daniel Lane, Department of Biological Chemistry, Johns Hopkins University, and have been described previously (53). Plasmids pCMV-NF-IL6 and pCMV-NF-IL6β which constitutively express the C/EBPβ and C/EBPδ isoforms, respectively, were generously provided by Shizuo Akira, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan, and have been described previously (1, 60). Plasmid pCNot was kindly provided by Francis Kern, Department of Biochemistry and Molecular Biology, Georgetown University, Washington, D.C., and has been described previously (32). All other reagents were of the highest purity available.

Hepatoma cell lines and methods of culture. Glucocorticoid-sensitive BDS1 cells and glucocorticoid-resistant EDR1 and EDR3 cells are epithelial tumor cells minimally derived from rat Reuber hepatoma (16). The cell lines were routinely grown in DME/F-12–10% fetal bovine serum at 37°C in humidified air containing 5% CO_2. The as4, as3, and vector control cell lines were derived by single-cell subcloning of BDS1 cells transfected with the pBCMG-AS antisense C/EBPα expression vector or the pBCMGneo parental vector. These cell lines were maintained in DME/F-12–10% fetal bovine serum containing 200 μg of G418 per ml at 37°C in humidified air containing 5% CO_2. Cell culture medium was routinely changed every 48 h. Dexamethasone was added to a final concentration of 1 μM as indicated, and transforming growth factor β was added to a final concentration of 2 ng/ml.

Flow-cytometric analyses of DNA content. In all flow cytometry experiments, approximately 4×10^4 hepatoma cells were seeded onto Corning six-well tissue culture plates. After 24 h, dexamethasone was added to a final concentration of 1 μM to half of the wells on each plate. The cells were incubated for 48 h in the presence or absence of dexamethasone, washed twice with PBS, and hypotonically lysed in 1 ml of ice-cold DNA-staining solution (0.5 mg of propidium iodide per ml, 0.1% sodium citrate, 0.05% Triton X-100). Emitted nuclear fluorescence greater than 585 nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Approximately 10^4 nuclei were analyzed from each sample at a rate of 300 to 500 cells per s. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems.

Assay of DNA synthesis by [^{3}H]thymidine incorporation. Triplicate samples of asynchronously growing BDS1 cells were treated for the indicated times with dexamethasone, pulse-labeled for 2 h with 3 μCi of [^{3}H]thymidine (84 Ci/mmol), washed three times with ice-cold 10% trichloroacetic acid, and lysed with 300 μl of 0.3 N NaOH. Lysates (100 μl) were transferred directly into vials containing liquid scintillation cocktail, and radioactivity was quantitated by scintillation counting.

Isolation of poly(A)$^+$ RNA and Northern blot analysis of C/EBPα mRNA levels. At the indicated time points of dexamethasone treatment, poly(A)$^+$ RNA was isolated from hepatoma cells as previously described (75). For Northern (RNA) blot analysis, 5 μg of poly(A)$^+$ RNA was electrophoretically fractionated in 6% formaldehyde–1% agarose gels, transferred onto Nytran nylon membranes (Schleicher & Schuell, Keene, N.H.) and baked at 80°C for 1 h. Membranes were preannealed with 100 μg of denatured salmon sperm DNA per ml and subse-

quently hybridized with cDNA probes labeled with [α-^{32}P]dCTP by random-primer extension (Amersham). The membranes were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) twice at room temperature for 10 min and then by twice at 60°C for 2 h. The membranes were air dried and used to generate X-ray autoradiographs (1- to 5-day exposures at −80°C). C/EBPα expression was detected with a purified 700-bp *Pst*I insert from pMSV-C/EBP-wt (29), while alpha-1-acid glycoprotein expression was detected with a 900-bp *Pst*I insert of pIRL21 (3). β-Actin transcripts were detected with a 1.3-kb *Pvu*II insert of pRβA-1 (37).

Western blot (immunoblot) analysis of C/EBP isoform protein expression. Hepatoma cells were cultured in 100-mm tissue culture plates and were treated for either 0, 24, or 48 h with 1 μM dexamethasone. The cells were harvested in RIPA buffer (10 mM Tris HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate), and protein concentrations were determined by the Bradford protein assay (Bio-Rad). For each sample, 30 μg of protein was mixed with 15 μl of sample buffer (62.5 mM Tris HCl [pH 6.8], 8% glycerol, 5% β-mercaptoethanol, 3% SDS, 0.001% bromphenol blue) and fractionated on 12% polyacrylamide–0.1% SDS resolving gels by electrophoresis. The proteins were electrically transferred to nitrocellulose membranes (Micron Separations Inc., Westboro, Mass.) and blocked overnight at 4°C with 10 mM Tris HCl (pH 8.0)–150 mM NaCl–0.05% Tween 20 (TBS-T)–5% nonfat dry milk (NFDM). The blots were subsequently incubated overnight at 4°C with a 1:100 dilution of rabbit anti-C/EBPα, anti-C/EBPβ, or anti-C/EBPδ immunoglobulin G or a 1:400 dilution of rabbit anti-CDK2 immunoglobulin G in TBS-T–1% NFDM. The blots were washed three times at room temperature with TBS-T–5% NFDM for 20 min per wash and then incubated for 3 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G diluted to 10^{-4} in TBS-T–1% NFDM. The blots were washed twice at room temperature with TBS-T and once with TBS for 30 min per wash. They were treated with enhanced chemiluminescence reagents as specified by the manufacturer, and C/EBP isoforms or CDK2 were detected by autoradiography.

Transfection procedures. Logarithmically growing hepatoma cells were transfected by electroporation. Briefly, single-cell suspensions were washed twice with sterile PBS and resuspended in electroporation buffer (270 mM sucrose, 7 mM sodium phosphate buffer [pH 7.4], 1 mM $MgCl_2$). The cells (250 μl; 1×10^7 to 2×10^7 cells per sample) were dispensed into sterile cuvettes. In all transfection experiments, the cells and 30 μg of expression vector DNA were gently mixed, electrically pulsed five times (400-V square-wave pulse for 99 μs) with a BTX 800 Transfector apparatus (BTX Inc., San Diego, Calif.), and incubated on ice for 10 min. Transfected cells were plated into prewarmed DME/F-12–10% fetal bovine serum in 100-mm-diameter tissue culture dishes and propagated at 37°C. At 24 h after transfection, the cells were washed twice with PBS. For reporter gene assays, the cells were refed with fresh medium with or without 1 μM dexamethasone and harvested after 48 h. For the growth suppression assays, the cells were refed with medium supplemented with 400 μg of G418 per ml.

Electrophoretic mobility shift assays. Hepatoma cells were grown for 48 h in the presence or absence of dexamethasone, washed twice with PBS, scraped from the plates with a rubber policeman, and transferred to 15-ml conical Falcon tubes. The cells were centrifuged for 5 min at 500 × *g*, and the cell pellets were resuspended in 3 ml of hypotonic lysis buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6), 25 mM $MgCl_2$, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid (EGTA), 0.01% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 10 μg of aprotinin per ml, 2 μg of leupeptin per ml, and 2 μg of pepstatin per ml. Nuclear pellets were resuspended with 300 μl of nuclear extraction buffer containing 50 mM Tris HCl (pH 7.8), 420 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0), 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 2 μg of leupeptin per ml, and 2 μg of pepstatin per ml and incubated for 30 min at 4°C with gentle rocking. After centrifugation at $8.8 \times 10^4 \times g$ for 1 h at 4°C, the supernatant fractions were recovered and the protein content was determined by the Bradford assay. Equivalent amounts of nuclear lysates (3 μg of protein) were incubated for 10 min at room temperature with 2 μg of poly(dI-dC) · (dI-dC) in a reaction mixture containing 10 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. A double-stranded radiolabeled (2×10^4 cpm) DNA probe (5′-CTAGATGCAGATTGCGCAATCTGCAG-3′) containing a consensus C/EBP-binding site (underlined) (64) was added, and the reaction mixture was incubated for 30 min at room temperature. To demonstrate DNA specificity, identical reactions were performed except that unlabeled consensus C/EBP DNA-binding site or consensus STAT5 DNA-binding site (36) was added as the specific or nonspecific competitor DNA, respectively. To show C/EBP isoform-specific binding of the consensus C/EBP DNA-binding site, nuclear lysates were preincubated for 1 h at 4°C with rabbit anti-C/EBPα, anti-C/EBPβ, or anti-C/EBPδ or control antibodies (anti-c-*fos* or CDK4) prior to the addition of poly(dI-dC) · (dI-dC) and radiolabeled probe DNA. Protein-DNA complexes were resolved on a 5% neutral polyacrylamide gel containing 0.25× TAE (10 mM Tris acetate, 250 μM EDTA) and visualized by autoradiography.

CAT assays. For CAT assays, cells were harvested by being washed twice in PBS, resuspended in 100 mM Tris HCl (pH 7.8), and lysed by four cycles of freeze-thawing (alternating between an ethanol–dry-ice bath and a 37°C water bath, 5 min per cycle). Cell lysates were heated at 68°C for 15 min and centrifuged at $1.2 \times 10^4 \times g$ for 10 min, and the supernatant fractions were recovered. CAT activity in the cell extracts containing 20 to 50 μg of lysate protein was measured by thin-layer chromatography (31) or by a quantitative nonchromatographic assay (62). The enzyme assay was carried out with 20 mM Tris HCl (pH 8.0)–0.05 μCi of [^{14}C]chloramphenicol–0.8 mM acetyl coenzyme A for the chromatographic assay or with 100 mM Tris HCl (pH 7.8)–1 mM aqueous chloramphenicol–1 μCi of [^{3}H]acetyl coenzyme A (final reaction volume, 250 μl) for the nonchromatographic assay. For the chromatographic assay, reaction mixtures were applied to silica gels and chloramphenicol species were separated by chromatography with 5% methanol–95% chloroform. Chloramphenicol conversion to mono- and diacetylated species was visualized by autoradiography. For the quantitative assay, the reaction mixture was gently overlaid with 4 ml of Econofluor water-immiscible scintillation fluorochrome (DuPont NEN). CAT activity was monitored by direct measurement of radioactivity by liquid scintillation counting. Measurements of CAT activity were in the linear range of the assay as determined by a standard curve obtained with bacterial CAT enzyme (0.01 U; Pharmacia, Uppsala Sweden), the positive control for CAT enzymatic activity. The enzyme activity was expressed as a function of [^{3}H]acetylchloramphenicol produced per μg of protein present in corresponding cell lysates. For each assay procedure, reaction mixtures were incubated at 37°C for 3 to 8 h. Mock-transfected cells were used to establish the basal level of activity for both assays.

Cell focus assay. To assay the effects of antisense C/EBPα sequences, BDS1 cells were transfected with 30 μg of the antisense C/EBPα expression vector, pBCMG-AS, which carries the neomycin resistance gene, or with the parental vector, pBCMGneo, without C/EBPα sequences. To assay the effects of various C/EBP isoforms on cellular proliferation, BDS1 cells were cotransfected with 27 μg of either pMCETx-5/4, which constitutively expresses an estrogen-activated form of C/EBPα, pCD-mC/EBP, which constitutively expresses C/EBPα, pCMV-NF-IL6, which constitutively expresses C/EBPβ, or pCMV-NF-ILβ, which constitutively expresses C/EBPδ, and 2.7 μg of pCNot, which carries the neomycin resistance gene, rendering cells resistant to G418. Hepatoma cells were also cotransfected with a control vector without a C/EBP gene and the pCNot neomycin resistance plasmid. At 24 h (antisense experiment) or 48 h (overexpression experiment) after transfection, the cells were washed twice with PBS and propagated with fresh medium containing G418 (400 μg/ml). Transfected cells were grown under G418 selection for 2 weeks. Single-cell suspensions were made by trypsinization, and either 10^4 cells were replated on 100-mm tissue culture dishes or 10^3 cells were replated on six-well tissue culture dishes. The transfected cells were cultured for 2 weeks in medium supplemented with G418 (200 μg/ml) in the presence or absence of the indicated hormones. The cells were washed twice with PBS, fixed, and stained with 10% formalin–0.5% crystal violet, and the number of foci with a diameter of at least 1 mm were counted.

RESULTS

Glucocorticoid-stimulated C/EBPα transcript levels genetically correlate with the phenotype of hepatoma cell growth variants that undergo a steroid-induced cell cycle arrest. We previously demonstrated that the glucocorticoid-stimulated production of secreted alpha-1-acid glycoprotein was highly correlated with the growth suppressibility of a panel of hepatoma cell proliferation variants, although this protein is not an inhibitor of cell proliferation (16). Other studies have shown that maximal activation of the alpha-1-acid glycoprotein promoter requires both functional glucocorticoid receptors and the C/EBPα or C/EBPβ transcription factor resulting from their adjacent and partially overlapping binding sites between bp −120 and −95 of the alpha-1-acid glycoprotein gene promoter (48, 63, 72). Glucocorticoid growth-suppressible BDS1 hepatoma cells, as well as its nonsuppressible EDR1 receptor-positive and EDR3 receptor-negative counterparts, provide the opportunity to genetically test the relationship between the dexamethasone-mediated cell cycle arrest and expression of both alpha-1-acid glycoprotein and C/EBPα. To monitor cell cycle regulation, nuclei isolated from BDS1, EDR1, and EDR3 hepatoma cells treated with 1 μM dexamethasone for 48 h or left untreated were analyzed for their nuclear DNA content after fluorescent propidium iodide staining. Flow cytometry profiles revealed that dexamethasone treatment altered the DNA content of the BDS1 cell population from an asynchronous growing population of cells in various phases of the cell cycle to one in which approximately 95% of dexamethasone-treated hepatoma cells exhibited a 2n-like DNA content (Fig. 1), indicating a G_1 block in cell cycle progression (75). In contrast, steroid treatment had no effect on the DNA content

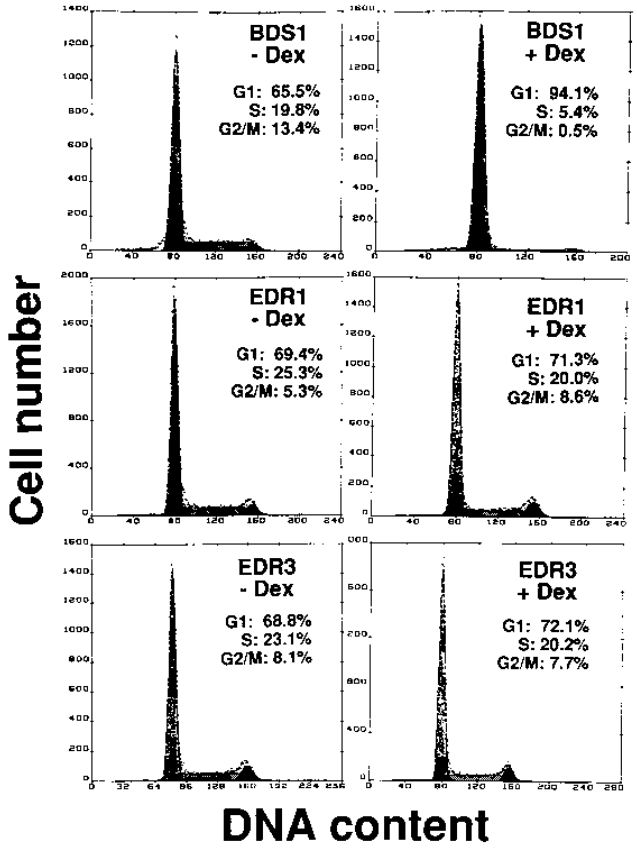

FIG. 1. Effects of dexamethasone (Dex) on the cell cycle phase distribution of glucocorticoid-sensitive and -resistant hepatoma cell variants. Glucocorticoid-sensitive (BDS1) and -resistant (EDR1 and EDR3) hepatoma cell variants were treated with 1 μM dexamethasone for 48 h, cell lysates were stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite laser. Approximately 10^4 nuclei were analyzed from each sample. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined with the Multicycle computer program as described in Materials and Methods.

of the nonsuppressible EDR1 and EDR3 cells, because in the presence or absence of dexamethasone, their growth profiles were typical of asynchronously proliferating cells. Approximately 70% of cells had a DNA content equivalent to the G_1 phase of the cell cycle, 20% of the cells had an S-phase DNA content, and 10% had a G_2/M DNA content (Fig. 1). The flow cytometry pattern of growth-suppressible BDS1 hepatoma cells was consistent with our previous results documenting that glucocorticoids cause a G_1 block in cell cycle progression in certain epithelial tumor cell lines (34, 75) whereas both the EDR1 and EDR3 hepatoma cell variants were defective in this steroid-induced cell cycle arrest.

In parallel with the flow cytometry studies, expression of C/EBPα mRNA and one of its transcriptional target genes, that encoding alpha-1-acid glycoprotein, were examined in each of the hepatoma proliferation variants. The hepatoma cell variants were treated with 1 μM dexamethasone for 24 h or left untreated, and Northern blots of electrophoretically fractionated poly(A)$^+$ RNA were probed for C/EBPα transcripts, as well as for alpha-1-acid glycoprotein mRNA. Dexamethasone stimulated a striking increase in C/EBPα transcript levels in growth-suppressible BDS1 cells under conditions in which alpha-1-acid glycoprotein mRNA was highly induced (Fig. 2A and B, BDS1). In contrast, dexamethasone failed to induce C/EBPα or alpha-1-acid glycoprotein transcript levels in either the receptor-positive (EDR1) or receptor-deficient (EDR3) glucocorticoid-nonsuppressible variants (Fig. 2A and B, EDR1 and EDR3). Although this Northern blot revealed some cell line-specific differences in the basal levels of C/EBPα mRNA, actin transcript levels remained constant in dexamethasone-treated and untreated cells (Fig. 2C) and served as a control for RNA quantitation. These results demonstrate that the dexamethasone stimulation of C/EBPα mRNA correlated with the glucocorticoid growth-suppressible phenotype.

Glucocorticoid stimulation in C/EBPα protein and transcriptional activity is associated with the growth-suppressible phenotype of hepatoma cells. Western blot analysis of cell extracts isolated from hepatoma cells treated with dexamethasone for 0, 24, or 48 h revealed that glucocorticoids strongly induced C/EBPα protein levels in growth-suppressible BDS1 cells but not in either the EDR1 or EDR3 nonsuppressible variants (Fig. 3). No C/EBPα protein was detected in EDR1 or EDR3 cells in the presence or absence of glucocorticoids, and therefore the lack of transcription factor production in the nonsuppressible cell lines was independent of any minor quantitative differences in basal levels of expressed C/EBPα transcripts (Fig. 2). At 24 and 48 h of dexamethasone treatment, the 42-kDa C/EBPα protein was produced at high levels in BDS1 hepatoma cells. A minor, lower-molecular-weight cross-reactive protein was also detected and is likely to be a smaller form of C/EBPα that has been detected in hepatic cells and adipocytes (11, 55, 65). These results demonstrate that the glucocorticoid induction of C/EBPα transcripts resulted in a

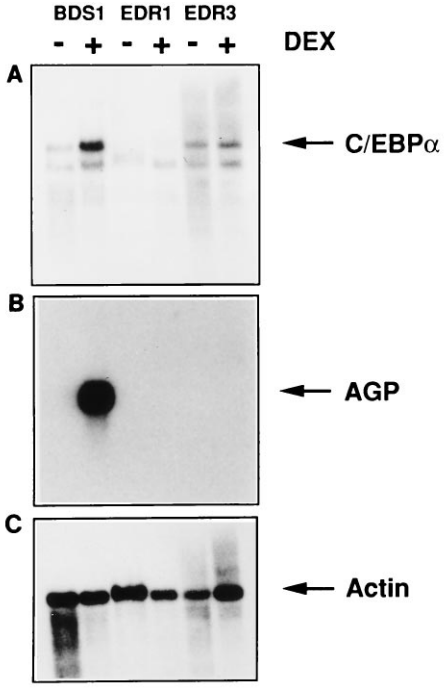

FIG. 2. Effects of dexamethasone (DEX) on the expression of C/EBPα and alpha-1-acid glycoprotein transcripts in growth-suppressible and -nonsuppressible hepatoma cell variants. Poly(A)$^+$ RNA isolated from untreated glucocorticoid growth-suppressible (BDS1), receptor-positive nonsuppressible (EDR1), and receptor-negative nonsuppressible (EDR3) hepatoma cells or parallel cultures treated with 1 μM dexamethasone for 48 h was electrophoretically fractionated, and Northern blots were probed for C/EBPα (A), alpha-1-acid glycoprotein (AGP) (B), or actin transcripts (C) as described in Materials and Methods. The lower transcript band observed in panel A is actin, which shows approximately equal loading in each lane.

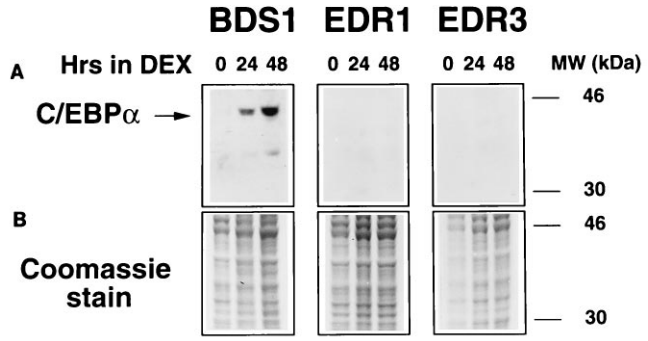

FIG. 3. Effects of dexamethasone on C/EBPα protein expression in growth-suppressible and -nonsuppressible hepatoma cell variants. (A) Cell lysates from untreated glucocorticoid growth-suppressible (BDS1), receptor-positive nonsuppressible (EDR1), and receptor-negative nonsuppressible (EDR3) hepatoma cells or parallel cultures treated with 1 μM dexamethasone for the indicated times were electrophoretically fractionated in SDS-polyacrylamide gels, and C/EBPα protein production was determined by Western blot analysis with a C/EBPα-specific antibody. The 42-kDa C/EBPα protein produced in hepatoma cells is delineated by the arrow. (B) Equivalent sample loading was determined by Coomassie blue staining. Molecular mass standards are ovalbumin and carbonic anhydrase (46 and 30 kDa, respectively).

corresponding augmentation of C/EBPα protein in growth-suppressible hepatoma cells but not in either of the two nonsuppressible variants. This observation implicates the glucocorticoid induction of C/EBPα as a biologically significant response associated with the growth regulation of hepatoma cells.

The coordinate regulation of the alpha-1-acid glycoprotein gene by glucocorticoids and C/EBP family members is a liver-specific process associated with the acute-phase inflammation response (2, 7, 42, 72, 81). To directly determine if glucocorticoids induce C/EBPα transcriptional activity in a manner that correlated with the growth-suppressible phenotype, the hepatoma cell variants were transiently cotransfected with the pAGP3x(GRE)-CAT reporter plasmid containing the glucocorticoid and C/EBP-responsive alpha-1-acid glycoprotein promoter (5), together with either a constitutive C/EBPα expression vector (pCD-mC/EBP) or the vector control (pCD). Cells were treated with 1 μM dexamethasone for 48 h or left untreated and were assayed for CAT activity. Dexamethasone strongly stimulated alpha-1-acid glycoprotein promoter activity in transiently transfected growth-suppressible BDS1 hepatoma cells but failed to activate the same promoter in transiently transfected nonsuppressible receptor-positive EDR1 hepatoma cells (Fig. 4A and B). Cotransfection of nonsuppressible EDR1 cells with both the C/EBPα expression vector and pAGP3x(GRE)-CAT partially rescued the defective dexamethasone-induced activation of the alpha-1-acid glycoprotein promoter (Fig. 4B, lanes pCD-mC/EBP). As a control for general glucocorticoid responsiveness, parallel sets of cells were transfected with the pGRE-CAT reporter plasmid. In growth-suppressible BDS1 cells, dexamethasone potently induced the transactivation of the pGRE-CAT reporter plasmid, whereas nonsuppressible EDR1 cells, the magnitude of dexamethasone stimulation of pGRE-CAT was distinctly less (Fig. 4A and B, lanes pGRE-CAT). Dexamethasone failed to induce either the pAGP3x(GRE)-CAT or pGRE-CAT reporter genes in the presence or absence of the C/EBPα expression vector in glucocorticoid receptor-deficient EDR3 cells, directly demonstrating the receptor dependency of these responses (Fig. 4C). Dexamethasone had no effect on the relatively high constitutive activity of a CAT reporter gene driven by the Rous sarcoma virus long terminal repeat in all three hepatoma cell lines (data not shown). Thus, C/EBPα transcriptional activity was highly associated with the growth-suppressible phenotype under conditions in which both C/EBPα transcripts and protein were strongly induced by glucocorticoids.

Dexamethasone stimulation of C/EBPα transcript levels is a rapid steroid response in growth-suppressible BDS1 hepatoma cells. To determine the kinetic relationship between glucocorticoid-stimulated C/EBPα gene expression and the cell cycle arrest of proliferating BDS1 cells, the rate of DNA synthesis and level of C/EBPα mRNA were monitored over a 48-h time course of dexamethasone treatment in parallel cell cultures. Northern blot analysis of isolated poly(A)$^+$ RNA demonstrated that dexamethasone maximally induced C/EBPα transcripts within 2 h of steroid treatment and remained at a high level during the remainder of the 48-h time course (Fig. 5A). Actin transcript levels remained constant during this entire period (Fig. 5B). Time course analysis of [^{3}H]thymidine incorporation revealed that dexamethasone gradually inhibited DNA synthesis in BDS1 hepatoma cells during the first 12 h of steroid exposure, with the magnitude of the response beginning to plateau by 24 h in dexamethasone (Fig. 5C). The asynchronous growing hepatoma cells required approximately 48 h of dexamethasone treatment for maximal inhibition of [^{3}H]thymidine incorporation. Importantly, glucocorticoid stimulation of C/EBPα transcripts represents an early steroid response relative to the time frame in which cell proliferation is arrested.

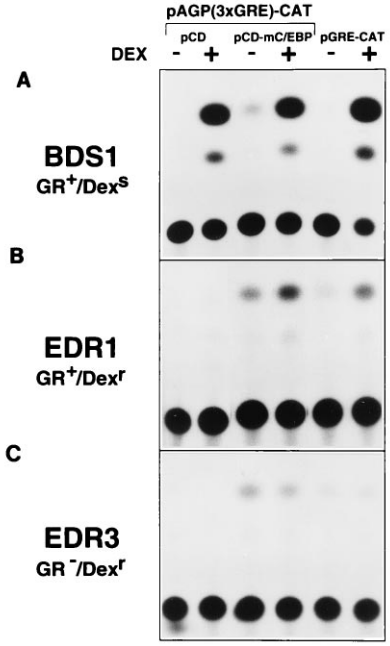

FIG. 4. Glucocorticoid responsiveness and C/EBPα regulation of alpha-1-acid glycoprotein promoter activity in growth-suppressible and -nonsuppressible hepatoma cell variants. Glucocorticoid growth-suppressible (BDS1), receptor-positive nonsuppressible (EDR1), and receptor-negative nonsuppressible (EDR3) hepatoma cells were transiently cotransfected with the pAGP3x(GRE)-CAT alpha-1-acid glycoprotein promoter reporter plasmid, containing the 5′-flanking sequences 127 bp upstream of the transcription start site which maintain both glucocorticoid receptor and C/EBP responsiveness linked to the CAT reporter gene, and either the pCD-mC/EBP expression vector for C/EBPα or the pCD vector control. Parallel sets of hepatoma cells were transfected with the pGRE-CAT reporter plasmid to assess general glucocorticoid responsiveness. Transfected cells were treated with 1 μM dexamethasone for 48 h and assayed for CAT activity (conversion of [^{14}C]chloramphenicol into [^{14}C]acetylchloramphenicol) by thin-layer chromatography.

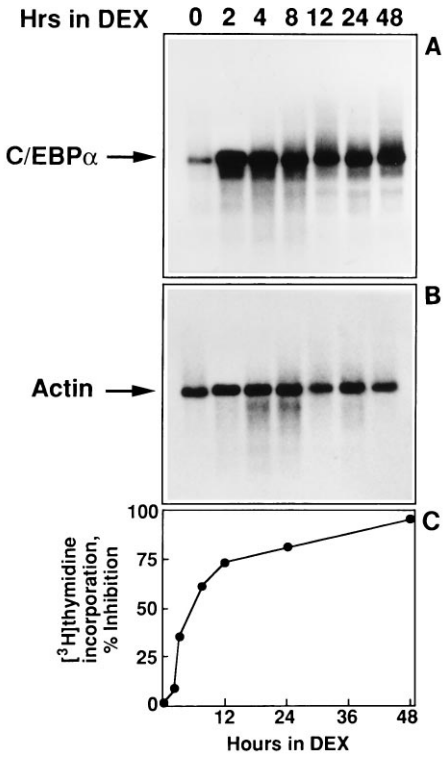

FIG. 5. Time course of glucocorticoid-stimulated C/EBPα transcript expression and inhibition of [^{3}H]thymidine incorporation. Growth-suppressible BDS1 hepatoma cells were treated with 1 μM dexamethasone (DEX) for the indicated times. From one set of cell cultures, poly(A)$^+$ RNA was isolated and electrophoretically fractionated, and Northern blots were probed for C/EBPα or actin transcripts (A and B), as described in Materials and Methods. A parallel set of BDS1 hepatoma cell cultures were plated at a density of 2×10^4 cells per well on 24-well multiwell plates, treated with 1 μM dexamethasone, and, at the indicated times, pulse-labeled with [^{3}H]thymidine for 2 h, and incorporation into DNA was determined by acid precipitation as described in the Materials and Methods. The percent inhibition of [^{3}H]thymidine incorporation at each time point was determined by dividing the radiolabel incorporated by the 0-h level (C). The reported values are an average of triplicate samples.

Transfection of an antisense C/EBPα expression vector blocks the glucocorticoid growth suppression response in BDS1 hepatoma cells. As an initial functional test to determine if expression of C/EBPα is a necessary component of the glucocorticoid-mediated cell cycle arrest in rat hepatoma cells, BDS1 hepatoma cells were transfected with an antisense C/EBPα expression vector or a vector control plasmid and assayed for a disruption of the glucocorticoid growth suppression response by a transient-cell-focus assay. Populations of positively transfected cells were recovered by their resistance to the cytotoxic effects of the neomycin derivative G418, and equal numbers of cells were plated onto 100-mm tissue culture dishes and propagated for 2 weeks in the presence or absence of dexamethasone. The resulting foci were fixed and visualized in formalin-crystal violet, and the final number of cell foci with diameters of at least 1 mm were counted in each plate. As shown in Fig. 6, dexamethasone had essentially no effect on the resulting number of cell foci in a cell population transfected with the antisense C/EBPα expression vector. For example, in a representative experiment of an antisense-transfected population of cells, 71 cell foci of at least 1 mm in diameter were detected in the absence of dexamethasone compared with 60 cell foci in the presence of dexamethasone. In contrast, on vector control plates (Fig. 6), dexamethasone treatment reduced the number of recovered cell foci (1 focus) compared with that in untreated cells (64 foci). The results of this transient-transfection assay indicate that the expression of C/EBPα is a necessary component of the glucocorticoid growth suppression of hepatoma cells.

Establishment and characterization of a glucocorticoid receptor-positive C/EBPα-deficient hepatoma cell subclone from the population of antisense C/EBPα-transfected cells. The hepatoma cells transfected with the antisense C/EBPα expression vector probably include a heterogeneous population of individual cell clones that range in their phenotypes from one in which C/EBPα expression is completely ablated to a wild-type phenotype of unimpeded glucocorticoid-regulated stimulation of C/EBPα. Therefore, to directly determine if C/EBPα expression is required for the glucocorticoid G_1 cell cycle ar-

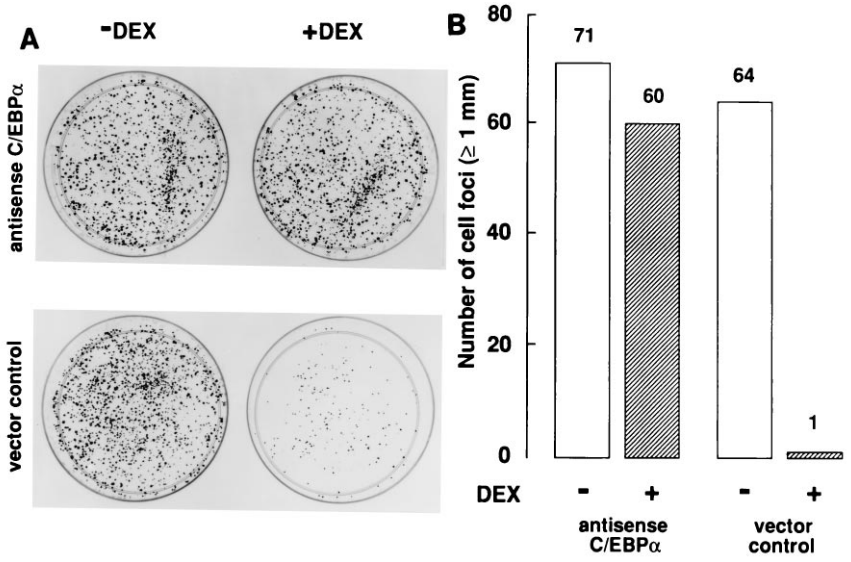

FIG. 6. Analysis of the effects of C/EBPα ablation on the glucocorticoid growth suppression of hepatoma cells by a cell focus assay. (A) BDS1 hepatoma cells were transfected with an antisense C/EBPα expression vector or with vector control DNA. Approximately 10^4 cells were replated on 100-mm tissue culture dishes and cultured for 2 weeks in the presence or absence of 1 μM dexamethasone (DEX), washed, and stained with formalin-crystal violet. (B) The number of foci that were at least 1 mm in diameter was counted and is illustrated graphically.

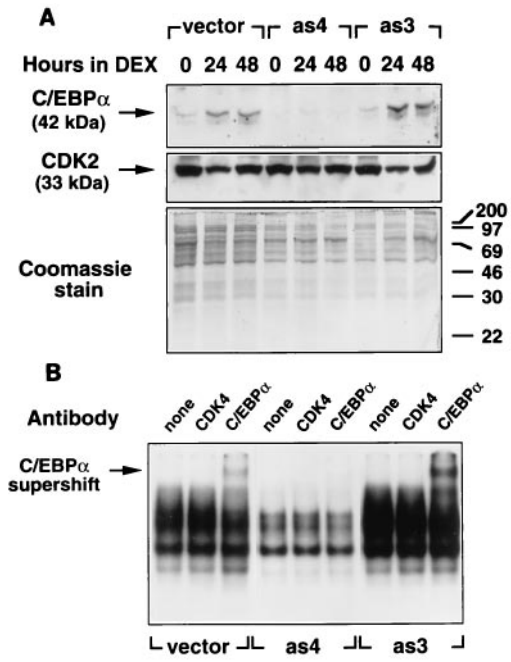

FIG. 7. Effects of antisense C/EBPα expression on glucocorticoid-induced endogenous C/EBPα protein levels and DNA-binding activity in individual subclones derived from BDS1 hepatoma cells. (A) Cell lysates were isolated from antisense C/EBPα-transfected BDS1 subclones (as4 and as3) or vector control-transfected BDS1 hepatoma cells treated with 1 μM dexamethasone for the indicated hours. Total-cell extracts were electrophoretically fractionated in SDS-polyacrylamide gels, and C/EBPα protein production was determined by Western blot analysis with a C/EBPα specific antibody. The 42-kDa C/EBPα protein produced in hepatoma cells is delineated by an arrow. The specificity of the glucocorticoid effect and equivalent sample loading was determined by using CDK2 Western blot analysis and Coomassie blue-stained gels. Molecular mass standards are myosin (200 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (22 kDa). (B) Gel shift assay for C/EBPα-specific DNA-binding activity. Nuclear lysates were prepared from dexamethasone-treated vector control, as4, or as3 cells and incubated with a radiolabeled oligonucleotide corresponding to the consensus C/EBP-binding site in the presence of antibodies specific for either C/EBPα or CDK4 or with no added antibodies. The protein-DNA complexes were electrophoretically resolved in a 6% nondenaturing polyacrylamide gel and visualized by autoradiography. The C/EBPα-DNA complexes supershifted by C/EBPα-specific antibodies are indicated by the arrow.

rest of hepatoma cells, it was crucial to recover and characterize individual antisense transfected derivatives of BDS1 hepatoma cells that fail to express C/EBPα. Random subclones from the population of cells transfected with either the antisense C/EBPα expression vector or a vector control plasmid were isolated after 2 weeks of selection in 200 μg of G418 per ml. Approximately 71% of the isolated subclones (10 of 14) transfected with the antisense C/EBPα expression vector failed to be growth arrested by dexamethasone. Two individual subclones from the antisense transfections, as4 cells, which were disrupted in their glucocorticoid growth suppression response, and as3 cells, which have a wild-type phenotype, as well as one vector-transfected control cell line, were further characterized for C/EBPα protein levels, DNA-binding ability, general glucocorticoid responsiveness, and the dexamethasone-induced cell cycle arrest. Western blot analysis of cells treated for 0, 24, or 48 h with dexamethasone revealed that in the as4 antisense transfected cell line, the basal level of C/EBPα protein was significantly reduced and glucocorticoids failed to stimulate higher levels of this transcription factor (Fig. 7A). Thus, transfection of the antisense C/EBPα expression vector prevented C/EBPα protein synthesis in this cell line; the other independently recovered subclones with a disruption in their glucocorticoid growth suppression response had the same phenotype. In contrast, as3 antisense transfected cells produced C/EBPα protein levels upon dexamethasone stimulation comparable to those in hormone-treated vector control cells and therefore are representative of clones within the transfected-cell population in which the antisense expression vector failed to shut down transcription factor production. In all three of the cell clones tested, the expression of CDK2 protein remained at approximately the same level and was unaffected by dexamethasone treatment, which suggests that C/EBPα ablation in as4 cells by antisense C/EBPα expression is not due to a global reduction of protein synthesis (Fig. 7A). The Coomassie blue-stained gel demonstrates equivalent protein loading of each sample (Fig. 7A).

To test if ablation of C/EBPα resulted in a loss of C/EBPα function, nuclear extracts from as4, as3, and vector control cells were incubated with a radiolabeled oligonucleotide probe encoding a consensus C/EBP DNA-binding sequence and DNA-binding capability was monitored by a gel shift assay. The presence of C/EBPα in the protein-DNA complex was monitored by the ability of C/EBPα-specific antibodies to induce a supershift of this complex. As shown in Fig. 7B, incubation with a C/EBPα-specific antibody induced a supershift of the protein-DNA complex formed with nuclear extracts from vector control and as3 cells, both of which express glucocorticoid-inducible C/EBPα protein. Control incubations with CDK4 antibodies did not affect protein-DNA complex formation. In contrast, the C/EBPα antibody failed to supershift the protein-DNA complexes formed with nuclear extracts from the as4 subclone, which indicates the lack of functional C/EBPα (Fig. 7B). The composition of the residual protein-DNA complexes is not known, although the complexes are likely to contain C/EBPβ (see the description of the gel shift analysis, below). The protein-DNA complexes formed in this assay were specific for the C/EBPα recognition site oligonucleotide because the gel-shifted complexes were inhibited by excess unlabeled oligonucleotide but not by a nonspecific sequence (data not shown).

It is conceivable that the absence of C/EBPα expression in as4 cells was not due to the antisense ablation of C/EBPα expression per se but resulted from the fortuitous recovery of a cell line during the subcloning process that is defective in general glucocorticoid receptor responsiveness. To rule out this possibility, as3, as4, and vector control hepatoma cells were transiently transfected with the GRE-CAT glucocorticoid responsive reporter plasmid that contains six consensus glucocorticoid response elements. As shown in Fig. 8, in each of these transfected cell subclones, 48-h dexamethasone treatment markedly enhanced CAT activity. Thus, the lack of C/EBPα expression in as4 cells was due not to a general defect in glucocorticoid receptor responsiveness but, rather, to the specific ablated production of this transcription factor.

C/EBPα expression is required for the G_1 cell cycle arrest by glucocorticoids. To determine if C/EBPα was required for the glucocorticoid G_1 cell cycle arrest, the DNA content of the antisense C/EBPα (as4 and as3) and vector control transfectant subclones were analyzed by flow cytometry. In as4 cells, in which expression of the antisense C/EBPα sequences ablated the expression of endogenous C/EBPα protein, dexamethasone failed to induce a G_1 cell cycle arrest (Fig. 9, second row). In the presence or absence of dexamethasone, the as4 subclone displayed a DNA content profile typical of an asynchronously growing population of cells. In contrast, in vector control or as3 cells, in which glucocorticoids enhance the level of C/EBPα

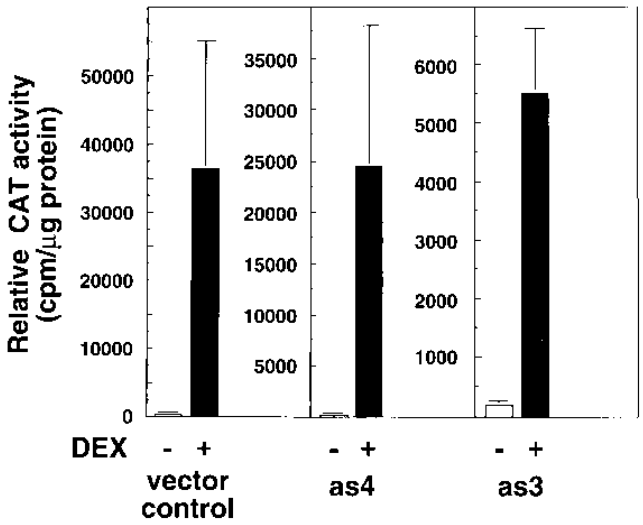

FIG. 8. Analysis of general glucocorticoid responsiveness in antisense C/EBPα and vector control-transfected hepatoma cells. Antisense C/EBPα-transfected BDS1 subclones (as4 and as3) and vector control-transfected BDS1 hepatoma cells were transiently transfected with the pGRE-CAT reporter plasmid to assess glucocorticoid responsiveness. Transfected cells were treated with or without 1 μM dexamethasone (DEX) for 48 h and assayed for CAT activity by a quantitative method which measures the conversion of [^{3}H]acetyl coenzyme A into [^{3}H]acetylchloramphenicol. The *y* axis represents relative CAT specific activity for steroid-treated transfected cells in comparison with untreated transfected cells. The reported values are means of results from three experiments with duplicate samples.

protein, dexamethasone efficiently induced a G_1 cell cycle arrest (Fig. 9, first and third rows). TGF-β has potent antiproliferative effects on BDS1 hepatoma cells and other liver-derived cell types (27). To test the specificity of the requirement for C/EBPα in the steroid-mediated cell cycle arrest, as4, as3, and vector control cells were treated with TGF-β for 48 h and the DNA content was examined by flow cytometry. As also shown in Fig. 9 (third column), TGF-β induced a G_1 cell cycle arrest in each of these hepatoma subclones regardless of whether C/EBPα expression was ablated. Most importantly, in as4 cells, TGF-β treatment caused a significant increase in the number of nuclei with a G_1-like DNA content. These results demonstrate that glucocorticoid-inducible C/EBPα protein is a necessary and specific component of the steroid-mediated G_1 cell cycle arrest of rat hepatoma cells.

Activation of overexpressed C/EBPα in the absence of glucocorticoids is sufficient to suppress rat hepatoma cell growth. To directly test if overexpression of C/EBPα in the absence of glucocorticoids is sufficient to inhibit hepatoma cell growth, BDS1 cells were transfected with a vector that constitutively expresses a conditionally activated form of this transcription factor. In the C/EBPα-ER vector, the coding sequences of the estrogen receptor ligand-binding domain are linked to the 3′ side of C/EBPα gene (77). After transfection of this expression plasmid, C/EBPα function can be selectively tested by activation with β-estradiol because the growth of these hepatoma cells is not affected by estrogen. A parallel set of hepatoma cells was transfected with a control expression vector devoid of

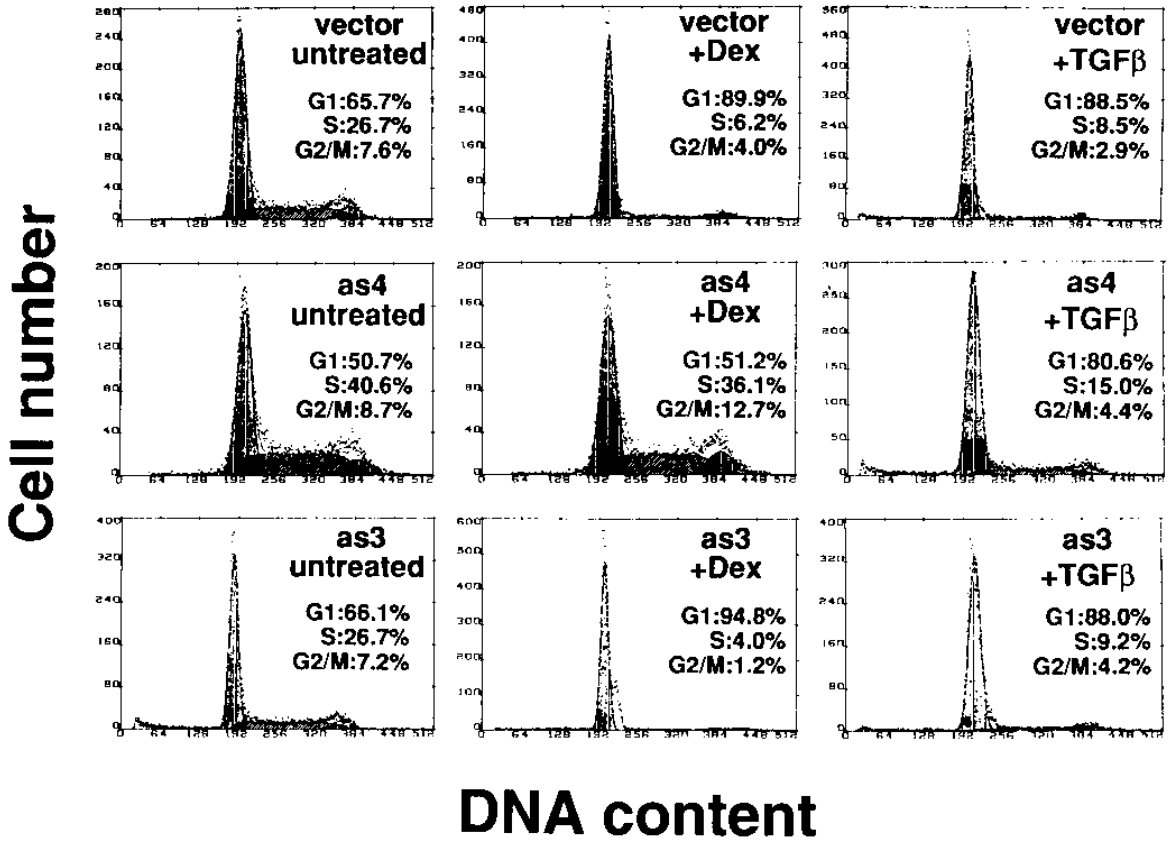

FIG. 9. Specific disruption of the glucocorticoid G_1 cell cycle arrest of BDS1 hepatoma cells by ablation of C/EBPα expression. Nuclei from untreated, dexamethasone (Dex)-treated, or TGF-β-treated antisense C/EBPα-transfected (as4 and as3) or vector control-transfected BDS1 cells were stained with propidium iodide and analyzed for DNA content by flow cytometry with a Coulter Elite laser. A total of 10^4 cells from each sample were analyzed. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined with the Multicycle computer program as described in Materials and Methods.

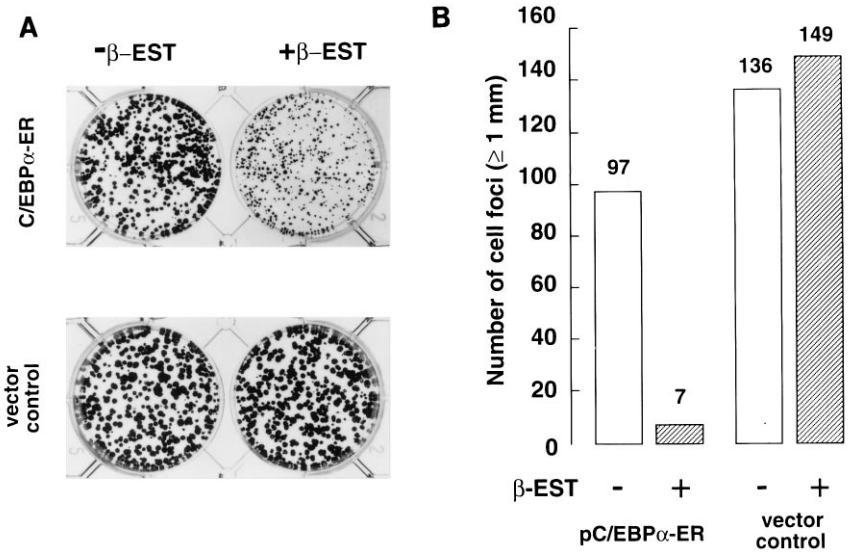

FIG. 10. Effects of activation of constitutively expressed C/EBPα-ER on the growth of BDS1 hepatoma cells. (A) BDS1 cells were transfected with a constitutive expression vector encoding the C/EBPα-ER fusion protein or with vector control DNA. From stably transfected populations of cells, 10^3 cells were replated on 6-well tissue culture dishes and cultured for 2 weeks with no hormone addition or 1 μM β-estradiol. The cells were washed and stained with formalin-crystal violet, and the number of foci at least 1 mm in diameter were counted. (B) Graphic quantitation of the resulting cell foci.

the C/EBPα gene. Along with C/EBPα-ER or the control vector, hepatoma cells were cotransfected with an expression vector (pCNot) for the neomycin resistance gene. Equal numbers of transfected cells which survived 2 weeks of propagation in 400 μg of G418 per ml were replated in the presence or absence of 1 μM β-estradiol and assayed for cell growth by the transient-cell-focus assay. The final number of cell foci at least 1 mm in diameter was counted in each plate. In hepatoma cells transfected with C/EBPα-ER, treatment with β-estradiol alone induced a significant growth suppression of the resulting hepatoma cell foci (Fig. 10). In vector-transfected controls, β-estradiol had no effect on hepatoma cell growth, because the number of observed cell foci was approximately the same in the presence or absence of this steroid (Fig. 10). Transfection of the C/EBPα expression vector or the control vector did not affect the cell phenotype, because dexamethasone effectively suppressed the growth of both sets of transfected-cell populations (data not shown).

Comparison of C/EBP isoform expression, DNA-binding activity, and growth effects in BDS1 hepatoma cells. It is well established that the C/EBP gene family contains several isoforms that can be expressed and regulated in a tissue-specific manner (14, 18, 46, 69). To compare the expression of C/EBPα with that of C/EBPβ and C/EBPδ, cell extracts isolated from BDS1 hepatoma cells treated with dexamethasone for 0, 24, or 48 h were electrophoretically fractionated and Western blots were probed with antibodies to these three C/EBP isoforms, as well as to CDK2 as a gel loading control. As shown in Fig. 11A, dexamethasone stimulated the level of the 35-kDa form of C/EBPβ in a manner similar to that observed for C/EBPα, although with a somewhat lower magnitude of induction. Under conditions in which both C/EBPα and C/EBPβ were induced, no C/EBPδ protein was detected whereas the level of CDK2 remained unchanged during the 48 h of dexamethasone treatment. A gel shift analysis with a radiolabeled consensus C/EBP DNA-binding sequence as a probe and nuclear extracts from dexamethasone-treated or untreated BDS1 hepatoma cells demonstrated the glucocorticoid-induced formation of both C/EBPα and C/EBPβ protein-DNA complexes. As shown in Fig. 11B, the inclusion of antibodies to either C/EBPα or C/EBPβ in the reaction mixture caused a supershift of the protein-DNA complex, whereas, control c-*fos* antibodies had no effect on the mobility of protein-DNA complexes. This result suggests that the residual protein-DNA complex observed in the antisense expressing as4 cells that are depleted of C/EBPα protein is due to the formation of C/EBPβ-DNA complexes (Fig. 7). Consistent with the inability to detect C/EBPδ by Western blot analysis, antibodies to this C/EBP isoform failed to cause a protein-DNA complex supershift (Fig. 11B).

To determine if the C/EBP-mediated growth suppression of hepatoma cells is isoform specific, BDS1 cells were cotransfected with vectors that constitutively express C/EBPα, C/EBPβ, or C/EBPδ and the neomycin resistance expression vector, pBCMGneo. Cells transfected with the neomycin resistance expression vector pBCMGneo served as the no-addition control. After selection of positively transfected cells by propagation for 2 weeks in 400 μg of G418 per ml, equal numbers of cells were replated and assayed for cell growth by the transient-cell-focus assay. Consistent with our results with cells transfected with the conditionally activated form of C/EBPα, the hepatoma cell population transfected with the C/EBPα constitutive expression vector was strongly growth suppressed (Fig. 11C and D). In contrast, hepatoma cells transfected with expression vectors for either C/EBPβ or C/EBPδ displayed cell foci levels similar to those for the no-addition control cells, demonstrating that neither of these C/EBP isoforms had antiproliferative effects on BDS1 hepatoma cells (Fig. 11C and D). Therefore, the C/EBP-mediated growth suppression of rat BDS1 hepatoma cells appears to be specific to the C/EBPα isoform, even though the levels of both C/EBPα and C/EBPβ proteins were induced by glucocorticoids.

Overexpression of C/EBPα induces a G_1 cell cycle arrest of hepatoma cells that mimics the glucocorticoid growth suppression response. To test if overexpression of C/EBPα can induce a G_1 cell cycle arrest similar to that observed with dexamethasone (Fig. 1), hepatoma cells transfected with the conditionally activated C/EBPα-ER expression vector were se-

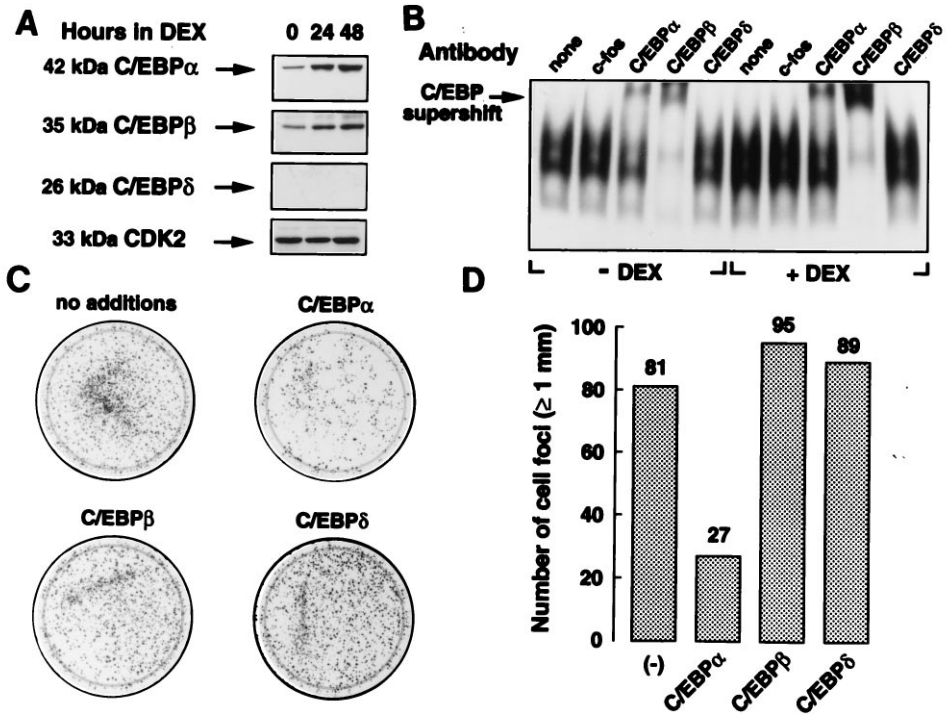

FIG. 11. Analyses of C/EBP isoform expression, DNA-binding activity, and growth effects in BDS1 hepatoma cells. (A) Western blot analysis of C/EBP isoform expression. Cell lysates from BDS1 hepatoma cells treated for 0, 24, or 48 h with 1 μM dexamethasone (DEX) were electrophoretically fractionated in SDS-polyacrylamide gels, and C/EBP isoform production was determined by Western blot analysis with C/EBPα, C/EBPβ-, or C/EBPδ-specific antibodies. As a gel-loading control, a fourth Western blot was probed with CDK2 antibodies. Specific proteins expressed in hepatoma cells are delineated by arrows. (B) Gel shift analysis of C/EBP isoform DNA-binding activity by an antibody supershift assay. Nuclear lysates were prepared from dexamethasone-treated or untreated BDS1 hepatoma cells and incubated with a radiolabeled oligonucleotide corresponding to the consensus C/EBP-binding site in the presence of antibodies specific for either C/EBPα, C/EBPβ, C/EBPδ, or c-*fos* or with no added antibodies. The protein-DNA complexes were electrophoretically resolved in a 6% nondenaturing polyacrylamide gel and visualized by autoradiography. The C/EBP-DNA complexes supershifted by the corresponding antibodies are indicated by the arrow. (C and D) Effect of constitutive expression of C/EBP isoforms on hepatoma cell growth. BDS1 cells were cotransfected with the pBCMGneo neomycin resistance expression plasmid and with constitutive expression vectors encoding either C/EBPα, C/EBPβ, or C/EBPδ or with no additions. From stably transfected cell populations, 10^4 cells were replated on 100-mm tissue culture dishes and cultured for 2 weeks. The cells were washed and stained with formalin-crystal violet, and the number of foci at least 1 mm in diameter was counted (C). Also illustrated is a graphic quantitation of the resulting cell foci (D).

lected for 4 weeks in G418 and the DNA content of β-estradiol-treated and untreated cells was analyzed by flow cytometry. In C/EBPα-ER-expressing cells, activation of C/EBPα-ER with 1 μM β-estradiol induced a significant increase in the percentage of cells with a G_1-like DNA content from 64.8% with a mean and standard error of 67.5% ± 3.8% to 87.0% with a mean and standard error of 83.0% ± 1.76% (Fig. 12, top row). This effect on DNA content is specific for cells transfected with the conditionally activated form of C/EBPα because β-estradiol had no effect on the flow cytometry profiles of vector transfected control cells (Fig. 12, bottom row). Untreated cells transfected with either the control expression vector (mean and standard error, 67.1% ± 2.4%) or C/EBPα-ER (mean and standard error, 67.5% ± 3.8%) had similar growth profiles, indicating that activation of C/EBPα-ER is required to observe the growth-inhibitory function of this transcription factor. Furthermore, dexamethasone suppressed the growth of C/EBPα-ER-transfected cells to the same extent as it suppressed the growth of vector control cells, showing that transfection of C/EBPα-ER did not disrupt the glucocorticoid responsiveness of these cells (Fig. 12, third column). Apoptotic cells were not observed under any of these conditions, indicating that the relative increase in G_1-phase cells by activation of C/EBPα was due to a block in cell cycle progression and not to the decreased viability of cells in the other phases of the cell cycle. These results demonstrate that C/EBPα can act as a direct inhibitor of hepatoma cell growth and, together with the antisense transfection data, show that this transcription factor is a key component of the glucocorticoid-regulated pathway leading to G_1 cell cycle arrest of hepatoma cells.

DISCUSSION

An intricate network of growth-inhibitory and -stimulatory signals transduced from the extracellular environment converge on specific sets of cell cycle components which, through their concerted action, either drive cells through critical cell cycle transitions or inhibit cell cycle progression (24, 25, 44). Depending on the cell type and growth regulatory conditions, different "checkpoints" throughout the cell cycle have been uncovered (41, 47, 52, 67). In mammalian cells, most characterized hormone signaling pathways that regulate proliferation target the G_1 phase or G_1/S boundary of the cell cycle (67). We have previously established that one such hormone-stimulated restriction point that exists in minimal-deviation rat hepatoma cells and in certain rat mammary tumor cells is a glucocorticoid-inducible early G_1 block in cell cycle progression (75). Given the known mechanism of action of steroid hormones (10, 35, 68, 78, 86), the glucocorticoid cell cycle arrest is likely to be mediated by cellular cascades induced by glucocorticoid receptor interactions with specific DNA recognition sites and/or sets of transcription factors governing the expression of

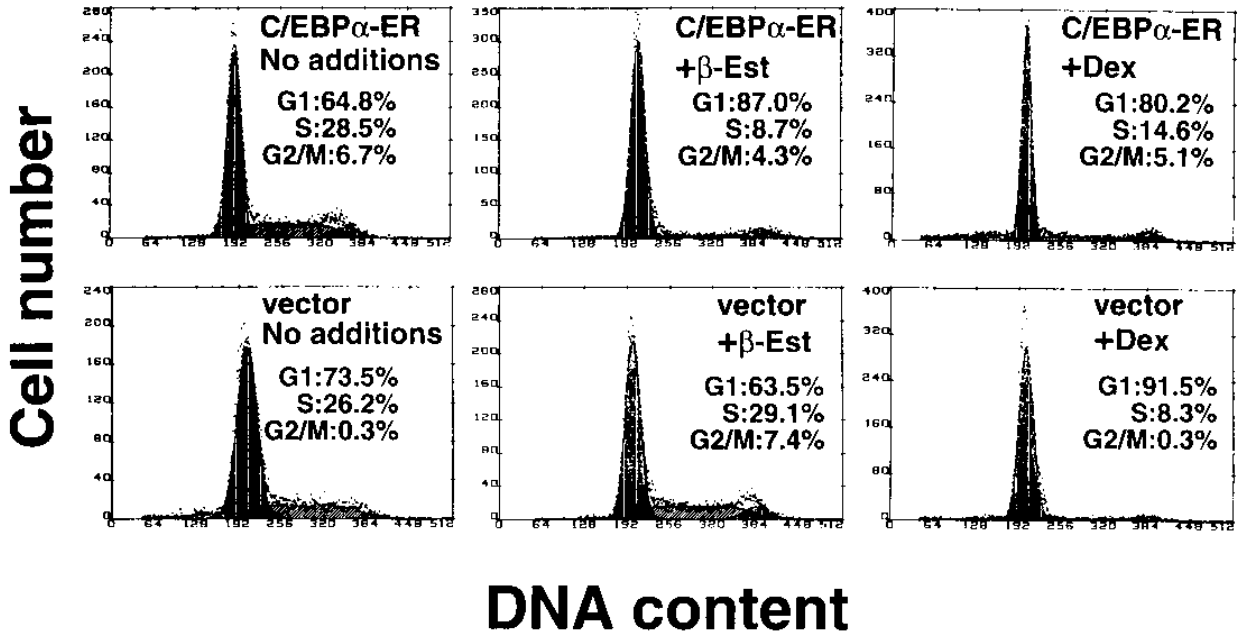

FIG. 12. Effects of C/EBPα-ER activation on the cell cycle phase distribution of BDS1 hepatoma cells. C/EBPα-ER-transfected and vector control-transfected BDS1 hepatoma cells were treated with 1 μM β-estradiol (β-Est), 1 μM dexamethasone (Dex), or no steroid additions for 48 h. Cell lysates were stained with propidium iodide, and approximately 10^4 nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite laser. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined with the Multicycle computer program as described in Materials and Methods.

specific subsets of steroid-regulated genes. The pleiotropic nature of the growth suppression response suggests that the most rapidly regulated steroid-induced or -inhibited genes within these cascades that initiate the growth inhibitory signals are regulatory in nature in that they subsequently alter the expression and/or activities of specific sets of target genes downstream in the pathway. The genetic and functional evidence uncovered by our study has defined a specific requirement for C/EBPα gene expression in the steroid-mediated G_1 cell cycle arrest of hepatoma cells and has shown that overexpression of C/EBPα can suppress hepatoma cell growth in the absence of glucocorticoids. Thus, in addition to the well-established role of the C/EBP family of transcription factors in normal liver function, our results have demonstrated that C/EBPα functions as a crucial intermediate in the glucocorticoid-stimulated antiproliferative cascade that governs the cell cycle of liver-derived epithelial tumor cells.

A genetic approach that utilized three classes of hepatoma cell proliferative variants showed that dexamethasone strongly stimulated C/EBPα transcript and protein levels, as well as transactivation activity, only in cells that undergo a G_1 cell cycle arrest. In growth-suppressible BDS1 hepatoma cells, dexamethasone maximally stimulated the expression of C/EBPα transcripts in proliferating cells within 2 h of steroid treatment. The temporal pattern of glucocorticoid-stimulated C/EBPα expression is consistent with a regulatory role for this transcription factor in initiating the cell cycle arrest, since dexamethasone maximally stimulated C/EBPα mRNA levels before exerting any detectable inhibitory effects on DNA synthesis. In contrast, dexamethasone failed to induce C/EBPα gene expression in glucocorticoid receptor-positive EDR1 hepatoma cells, which failed to be growth arrested by steroid treatment. Moreover, this steroid had no effect on C/EBPα gene expression in glucocorticoid receptor-deficient EDR3 cells, establishing the receptor dependency of this response. The glucocorticoid receptor-dependent stimulation of C/EBPα expression accounts for the striking correlation we observed previously between production of secreted alpha-1-acid glycoprotein and the growth-suppressible phenotype of the hepatoma cells (16), because this transcription factor targets the glucocorticoid-responsive alpha-1-acid glycoprotein gene promoter (2, 7). Ablation of C/EBPα protein synthesis by transfection of an antisense C/EBPα expression vector blocked the ability of glucocorticoids to induce the G_1 cell cycle arrest of BDS1 hepatoma cells. This result demonstrated directly that the steroid-stimulated expression of C/EBPα is a key intermediate event necessary for the growth suppression response in hepatoma cells. Moreover, this effect appears specific for the glucocorticoid receptor signaling pathway, because TGF-β induced a cell cycle arrest in transfected cells expressing antisense C/EBPα sequences. Estrogen activation of a C/EBPα-ER fusion protein or constitutive expression of C/EBPα was sufficient to suppress the growth of hepatoma cells by mediating an apparent G_1 arrest of the cell cycle in the absence of glucocorticoids. In contrast, overexpression of C/EBPβ or C/EBPδ did not affect the growth of BDS1 cells, indicating that the cell cycle arrest was specific for the C/EBPα isoform in this minimal-deviation rat hepatoma cell line.

Previous studies have shown an inverse correlation between C/EBPα expression and proliferation of liver-derived cells (7, 22, 23, 28, 29, 58, 83). C/EBPα gene expression was repressed in proliferating hepatocyte cultures, as well as in regenerating liver (39, 58, 71). In liver cells, C/EBPα and the mitogen-stimulated early response genes are reciprocally expressed, with maximal levels of C/EBPα detected following the growth period induced by partial hepatectomy (39, 58). The capacity for multiple C/EBP isoforms to make homodimeric and heterodimeric complexes accounts, in part, for the gene-specific DNA binding and activation by this transcription factor. For example, the DNA-binding activity of C/EBPα-C/EBPβ heterodimers decreases while the DNA-binding activity of C/EBPβ homodimers slightly increases as hepatocytes progress through

the G_1 phase of the cell cycle following partial hepatectomy (71). Furthermore, tissue, cell line, and species differences probably account for the antiproliferative activities of the various C/EBP isoforms and may explain previously reported discrepancies in the relative antiproliferative effects of C/EBPα versus C/EBPβ. In this regard, consistent with our results, C/EBPα, but not C/EBPβ or C/EBPδ, was shown to inhibit the growth of human Hep3B2 hepatoma cells, 639 fibroblast, or Saos2 osteosarcoma cells (43). Moreover, C/EBPα-transfected hepatoma cells had an increased latency period prior to tumor detection in athymic mice given transplants of C/EBPα expression vector-transfected HepG2 and Hep3B hepatoma cells when compared with that in transplanted "control" HepG2 and Hep3B cells (79). In contrast, Buck et al. reported that C/EBPβ, but not C/EBPα, blocked cell cycle progression of HepG2 cells presumably near the G_1/S phase border (12). Other studies have shown that C/EBPα can suppress the growth of hepatocytes, hepatoma cell lines, intestinal epithelial cells, fibroblasts, cervical and bladder carcinoma cells, and adipocytes from a variety of species (13, 43, 77, 79). One possible explanation for these observations that is related to cell-type-specific responses is that the quiescent versus proliferative state of a given cell line may be differentially modulated by precise ratios of expressed C/EBPβ (liver-activating protein) and liver inhibitory protein, a naturally occurring dominant negative form of C/EBPβ (19). Also, the microenvironment in which tissues and cells grow governs cell behavior and may therefore contribute to the regulated expression and ratios of the various C/EBP isoforms. For example, in primary hepatocytes, C/EBPα expression decreased in cells isolated from normal quiescent liver when cultured on rat tail collagen, a condition which promotes hepatocyte proliferation, whereas C/EBPα levels were increased in hepatocytes that were growth suppressed by being cultured on an extracellular matrix derived from the Englebreth-Holm-Swarm tumor (70). Our study provides the first direct evidence that one such environmental cue, glucocorticoids, induces a G_1 cell cycle arrest of rat BDS1 hepatoma cells and identifies C/EBPα as a key factor in this response.

The mechanistic relationships between C/EBPα function and the glucocorticoid control of growth and terminal differentiation of liver cells is reminiscent of those observed in adipocyte differentiation. For example, in liver-derived cells, C/EBP family members and glucocorticoids coordinately control the expression of liver-specific genes such as alpha-1-acid glycoprotein and other gene products associated with the acute-phase reaction (2, 7, 42, 63, 72, 81), as well as that of phosphoenolpyruvate kinase (56), a key gluconeogenic enzyme. In an analogous manner, in differentiated adipocytes and adipose tissue, C/EBPα mediates the 3T3-L1 adipocyte differentiation program induced by glucocorticoids, insulin, and methylisobutylxanthine (54, 77, 85). Transfection with an antisense C/EBPα expression vector blocked the expression of differentiation-specific genes and delayed density-dependent growth arrest of 3T3-L1 adipocytes (53), whereas overexpression of C/EBPα was sufficient to induce terminal differentiation of 3T3-L1 preadipocytes in the absence of hormonal stimuli (54). In adipose tissue, glucocorticoids transiently inhibit C/EBPα and stimulate C/EBPδ gene expression (57), whereas our results show that in rat hepatoma cells, dexamethasone stably induced the expression of C/EBPα transcripts and protein. The kinetic and expression differences between hepatoma cells and adipocytes suggest that glucocorticoids mediate their effects through a different set of transcriptional regulators in each cell type. Given these findings, it is conceivable that in both liver and adipocytes, growth suppression and terminal differentiation are governed by distinct pathways coordinately regulated by common extracellular cues whose intracellular signals converge, in part, on the C/EBP transcription factor family.

The loss of cell cycle control in G_1 has been implicated in the uncontrolled proliferation observed in many types of neoplastically transformed cells (17, 40). By this view, the effects of antiproliferative signals on a transformed cell type, such as rat hepatoma cells, may result in part from the regulated expression or activation of G_1-associated genes that define specific restriction points within this phase of the cell cycle. Conceivably, as part of the glucocorticoid-induced growth arrest in hepatoma cells, C/EBPα alone or in combination with other transcriptional regulators may selectively alter expression of certain G_1-acting cell cycle components. For example, glucocorticoid-inducible C/EBPα may inhibit the transcription of components necessary for cell cycle progression, such as the cyclins or cyclin-dependent kinases (CDK), or stimulate the expression of one or more of the various cell cycle inhibitors that block cell cycle progression by inhibiting CDK activity. In murine P1798 T-lymphoma cells, for example, glucocorticoids cause a dramatic decrease in CDK4 mRNA and protein levels and a moderate decrease in CDK2 expression (74). Other extracellular signals that act through members of steroid/thyroid hormone receptor family have been shown to stimulate the expression or activity or both of certain classes of CDK inhibitors. For example, estrogen stimulates the growth of human MCF7 cells, and anti-estrogen treatment of MCF7 human breast cancer cells increases the expression of $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ after an approximately 12-h time lag (80). Currently, we are exploiting the glucocorticoid growth-suppressible and -nonsuppressible hepatoma variants and stably transfected cells to uncover cell cycle-regulated target genes of C/EBPα that mediate the steroid antiproliferative response in liver-derived tumor cells.

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