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Impaired Proliferation and Tumorigenicity Induced by CCAAT/Enhancer-binding Protein

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ABSTRACT

A plasmid containing the CCAAT/enhancer-binding protein (C/EBPα) gene transcriptionally controlled by the metallothionein promoter was constructed. The gene was transfected into the human hepatocellular carcinoma cell lines Hep3B and HepG2. When cultured *in vitro* in the presence of 100 μM $ZnSO_4$, *C/EBPα* expression caused reversible growth arrest. In soft agar clonogenic assays, *C/EBPα* expression decreased both the colony size and the total number of colonies compared with zinc-free controls. *C/EBPα* expressing cells s.c. implanted in CD-1 *nu/nu* mice were essentially nontumorigenic, whereas *C/EBPα* tumor cells implanted into immunodeficient SCID mice demonstrated a significantly delayed time of tumor appearance compared with cells transfected with a vector control plasmid. These studies suggest that the expression of endogenous genes normally associated with a quiescent, differentiated state, such as *C/EBPα*, can result in impaired proliferative activity and suppressed tumorigenicity of hepatoma cell lines.

INTRODUCTION

A selective and potentially nontoxic approach to cancer therapy may be to exploit the action of DNA transcriptional-associated proteins, which can, by definition, alter the transcription of endogenous genes in the tumor cells quantitatively and/or qualitatively. The transcriptional alteration of endogenous genes in a tumor cell may dominate and override the genetic lesions causing the neoplastic transformation. C/EBPα[2] is a model transcriptional-associated protein.

C/EBPα is a heat-stable, sequence-specific, DNA-binding protein expressed primarily in liver and adipose tissue but also found in adrenal gland, lung, small intestine, and skin (1). The C/EBPα protein is virtually absent in regenerating liver and hepatoma but is expressed at a high level in terminally differentiated, mature liver hepatocytes, suggesting that *C/EBPα* expression may be correlated inversely with cell proliferation (2, 3). C/EBPα binds to the CCAAT box and to viral regulatory DNA sequences (4) and is thought to play a major role in maintaining the differentiated state by *trans*-activation of tissue-specific genes involved in carbohydrate and fat metabolism [*e.g.*, serum albumin (3) and apolipoprotein B (5) in liver and 422 adipose P2 (6), insulin-dependent glucose transporter GLUT4 (7), and stearoyl-coenzyme A desaturase 1 (8) in adipocytes]. Furthermore, expression of the *C/EBPα* gene in 3T3-L1 or 3T3 cells is sufficient to trigger adipogenic differentiation (9, 10). Recent evidence has suggested that C/EBPα also may regulate genes that inhibit cell growth (*e.g.*, *c-fos*; Ref. 11) and that block differentiation (*e.g.*, *c-Myc*; Ref. 12). Overexpression of *C/EBPα* also may displace inactivating C/EBP isoforms (primarily C/EBPβ) from DNA-binding sites that control proliferation (11).

Antiproliferative effects of C/EBPα may not be tissue specific. Transfection of a gene encoding a C/EBPα-estrogen receptor fusion protein into 3T3-L1 adipoblasts caused transient growth arrest *in vitro* when cells were exposed to estrogen (1). Likewise, transient growth arrest was also observed *in vitro* when a *C/EBPα* expression vector was cotransfected with a *β-galactosidase* reporter gene into SaOS2 osteosarcoma cells, GM639 SV40-transformed skin fibroblasts, and HeLa cervical carcinoma and A1698 bladder carcinoma cells (13). Importantly, the SaOS2 cell line is deficient in normal *p53*, whereas the A1698 cell line contains an activated Ki-*ras* oncogene (14). Taken together, these data suggest that *C/EBPα* expression may be able to override tumor defects in growth regulation due either to the loss of tumor suppressor gene function or to the presence of activated oncogenes. Thus, the effects of C/EBPα on proliferation may have a broad tissue specificity.

Here, we report the transfection of an inducible rat *C/EBPα* gene into human hepatoma cell lines. Induction of the *C/EBPα* gene results in reversible arrest of proliferation of these cells. Furthermore, inhibition of tumorigenicity can be demonstrated *in vivo*.

MATERIALS AND METHODS

Plasmids with Metallothionein Promoter. pPC18 (Fig. 1) is a 7.9-kbp plasmid containing a zinc-inducible, human metallothionein IIA promoter-SV40 polyadenylation signal expression cassette with unique *Xba*I and *Kpn*I cloning sites for insertion of genes. It also contains a neomycin phosphotransferase gene for G418 selection.

pMSV-C/EBP-wt (a kind gift from Alan D. Friedman, The Johns Hopkins Hospital, Baltimore, MD; Ref. 3) contains the rat *C/EBPα* gene, which can be removed as a 1.1-kb *Bam*HI-*Bss*HII fragment. This fragment was treated with the Klenow fragment of DNA polymerase and deoxynucleotide triphosphates to generate a blunt-ended fragment, which was ligated into the *Sma*I site of pBS− (Stratagene, Inc., La Jolla, CA). The plasmid pPC20 contains the *C/EBPα* gene in an orientation such that the *Xba*I site of pBS− is 5′ of the *C/EBPα* coding sequence, and the *Kpn*I site of pBS− is 3′ of the gene.

The 1.1-kbp *Xba*I-*Kpn*I fragment from pPC20 was ligated into pPC18 digested with *Xba*I and *Kpn*I to generate pPC22 (Fig. 1). The vector contains a unique *Xmn I* site, which was used to linearize the plasmid for transfections.

Transfection into Cells by Lipofection or Electroporation. The *C/EBPα*-containing plasmid PC22 and the *C/EBPα*-deficient control plasmid PC18 were transfected into the human hepatocellular carcinoma cell line Hep3B (HB 8064; American Type Culture Collection, Rockville, MD; Ref. 15) and the human hepatoblastoma cell line HepG2 (HB 8065; American Type Culture Collection; Ref. 15) by either electroporation in phosphate-buffered sucrose using a Bio-Rad Gene Pulsar apparatus (Bio-Rad Laboratories, Melville, NY) or with N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) transfection reagent (Boehringer Mannheim, Indianapolis, IN), both according to the recommendations of the manufacturers. Following transfections by either method, cells were grown in GM supplemented with 10% FBS for 24 h, then selected for resistance to Geneticin (antibiotic G418 sulfate; GIBCO-BRL, Gaithersburg, MD), 500 μg/ml for Hep3B cells and 1 mg/ml for HepG2 cells. Stable G418-resistant transfectants were single-cell cloned by limiting dilution. Growth kinetics of resulting clones were determined in SFM in the presence or absence of 100 μM $ZnSO_4$.

Medias and Culture Conditions. GM was a 1:1 mixture of DMEM and Ham's F-12 medium (JRH Biologicals, Lenexa, KS) supplemented with 10% FBS (heat-inactivated FBS; HyClone, Logan, UT), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 0.5 mM sodium pyruvate, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (JRH). SFM was a modification of Lanford's medium for baboon hepatocytes (16):

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[2] The abbreviations used are: C/EBPα, CCAAT/enhancer-binding protein; kbp, kilobase pair; BS, Bluescript; GM, growth medium; FBS, fetal bovine serum; SCID, severe combined immunodeficiency; SFM, serum-free medium.

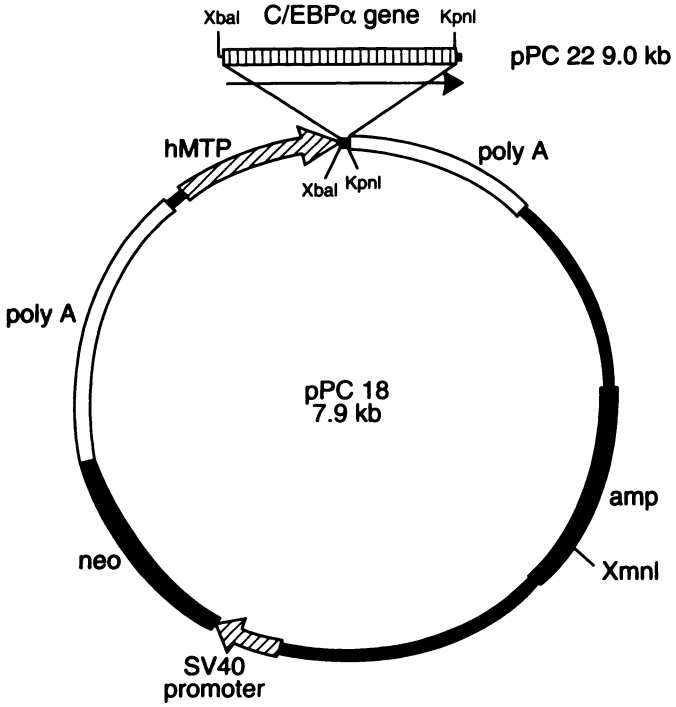

Fig. 1. *C/EBPα* plasmid construct. The plasmid pPC22 contains the 1.1-kbp *C/EBPα* gene inserted between the zinc-inducible human metallothionein IIA promoter and the SV40 polyadenylation signal of pPC18.

Williams' E media (GIBCO-BRL), 4.1 μg/ml ethanolamine, 10 μg/ml bovine pituitary extract, 1 μM thyrotropin-releasing hormone, 1 μM hydrocortisone, 20 ng/ml liver growth factor, 4 μg/ml glucagon, 1 μl/ml bovine cholesterol-rich lipids (Sigma Chemical Co., St. Louis, MO), 10 μl/ml insulin, transferrin and selenous acid premix culture supplement (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA), glutamine, nonessential amino acids, HEPES buffer, sodium pyruvate, penicillin, and streptomycin (JRH). Cell lines were free of mycoplasma contamination (Mycotrim TC; Irvine Scientific, Santa Ana, CA).

Preparation of Cell Lysates. For cell lysis, preheated 2× SDS sample buffer [0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% β-mercaptoethanol] was added to cell pellets to yield an approximate protein concentration of 2 mg/ml, passaged through an 18-gauge needle, heated at 100°C for 2 min, sheared through the needle again, and cooled on ice. Nucleic acids were digested by adding 0.1 volume of 1× DNase/RNase buffer [0.5 mM Tris-HCl (pH 6.8), 50 mM $MgCl_2$, 1 mg/ml DNase I, and 0.5 mg/ml RNase A] and incubating for 2 min on ice. The digestion step was repeated, an equal volume of water was added, and the lysate was vortexed and frozen at −70°C until electrophoretic analysis.

Gel Electrophoresis and Western Blot Analysis. Acrylamide, SDS, ammonium persulfate, tetramethylethylenediamine, β-mercaptoethanol, prestained molecular weight standards, and Tween 20 were obtained from Bio-Rad (Richmond, CA). Samples of cell lysates (10 μg) or conditioned media (50 μg protein or 25 μl 1:1 dilution of medium in 2× SDS sample buffer) were electrophoresed on 10% discontinuous SDS polyacrylamide gels according to the method of Laemmli (17) and transferred to nitrocellulose (Hybond enhanced chemiluminescence; Amersham International plc, Buckinghamshire, United Kingdom) using the method of Towbin *et al.* (18). C/EBPα was visualized following overnight incubation with rabbit antirat CEBPα antibody (1:80; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C and detection by enhanced chemiluminescence (Amersham) according to the protocol of the manufacturer using the appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000).

***In Vitro* Growth and Soft Agar Assays and *in Vivo* Tumorigenicity Assays.** Growth kinetics in SFM was determined by either cell counts using trypan blue dye exclusion or by using the tetrazolium mitochondrial dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, described elsewhere (19).

A standard soft agar colony formation assay (20) was used to assess anchorage-independent growth. A basal layer of 0.5% SeaPlaque agarose (FMC BioProducts, Rockland, ME) in GM ± 100 μM $ZnSO_4$ was poured in 35-mm dishes and allowed to solidify at room temperature. Cells were seeded at 1000 or 3000 cells/dish in media plus 0.3% agarose, layered over the basal layer, and incubated at 37°C for 2–4 weeks, at which time colony formation and growth were evaluated.

Cell lines were s.c. implanted into CD-1 *nu/nu* mice and immunodeficient CB 17 SCID mice.

RESULTS

Induction of *C/EBPα* Expression. Plasmids pPC22 and pPC18 were transfected stably into Hep3B and HepG2 cells. Subsequent to selection with G418 in serum-containing medium, Western blot analysis was used to confirm the zinc-inducible expression of *C/EBPα*. The protein was detected only in Hep3B/PC22 and HepG2/PC22 cell samples exposed to 100 μM $ZnSO_4$ (Fig. 2). No C/EBPα was detected in Hep3B/PC18 or HepG2/PC18 cells cultured with or without 100 μM $ZnSO_4$.

***In Vitro* Growth Kinetics.** C/EBPα caused a decrease in cell growth. Clones transfected with *C/EBPα* were screened *in vitro* for inducible growth arrest. One Hep3B and one HepG2 clone demonstrating the best inducible growth arrest were selected for further study. Fig. 3*A* illustrates the growth arrest of Hep3B/PC22 cells when *C/EBPα* is induced with zinc. Control Hep3B/PC18 cells were not growth inhibited in the presence of zinc (Fig. 3*B*). Similar results were observed with HepG2 transfectants (Fig. 3, *C* and *D*), validating further the correlation between *C/EBPα* induction and growth arrest.

The effect of different concentrations of $ZnSO_4$ on metallothionein-regulated expression of *C/EBPα* and growth kinetics of Hep3B/PC22 cells was investigated. Hep3B/PC22 and PC18 cells were cultured in the presence of 100, 75, 50, 25, or 0 μM $ZnSO_4$, and cell growth was monitored for 10 days. Fig. 4 illustrates the zinc dose response of *C/EBPα*-induced growth inhibition, with the highest concentrations of the inducer yielding the most pronounced antiproliferative effects. Similarly, HepG2/PC22 cells demonstrated a clear dose response to $ZnSO_4$, with 60, 80, and 100 μM all arresting growth completely, 40 μM slowing growth, and 20 μM and control SFM having no antiproliferative effect on these transfected cells (data not shown). As seen previously in Fig. 3, *B* and *D*, 100 μM zinc was nontoxic to pPC18 transfectants.

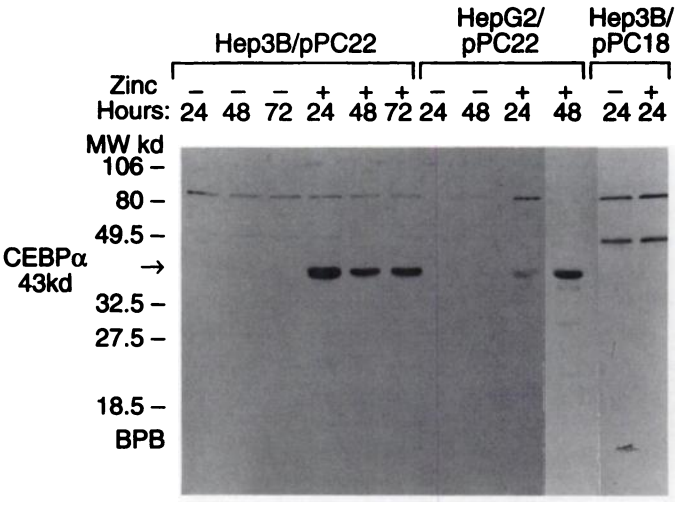

Fig. 2. Western blot analysis of C/EBPα in Hep3B/PC22 cells, HepG2/PC22 cells, and Hep3B/PC18 cells cultured continuously in medium without zinc (−) or medium with 100 μM $ZnSO_4$ (+). Whole-cell lysates were prepared as described in "Materials and Methods."

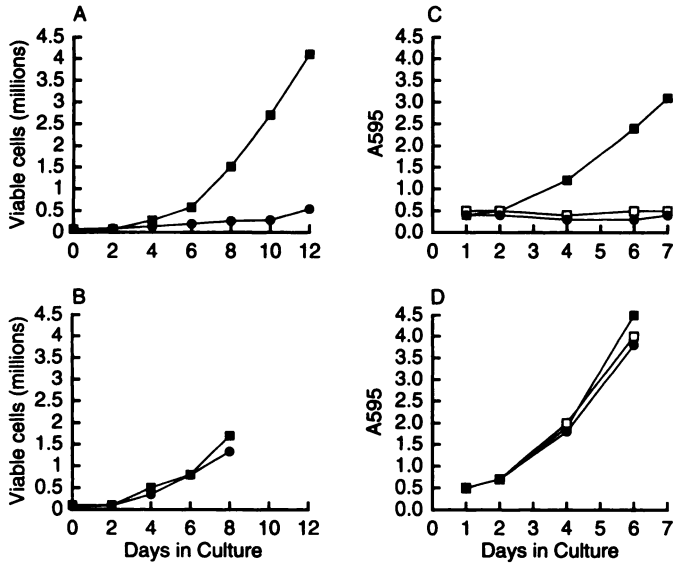

Fig. 3. Effect of zinc on hepatoma cell growth *in vitro*. Hep3B (*A* and *B*) and HepG2 cells (*C* and *D*) were transfected with the *C/EBPα*-containing plasmid PC22 (*A* and *C*) or with the vector control plasmid PC18 (*B* and *D*). Tumor cell growth was assayed *in vitro* by viable cell counts with trypan blue (Hep3B cells) or by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide mitochondrial dye (HepG2 cells). ■, zinc free control; □, 80 μM zinc; ●, 100 μM zinc.

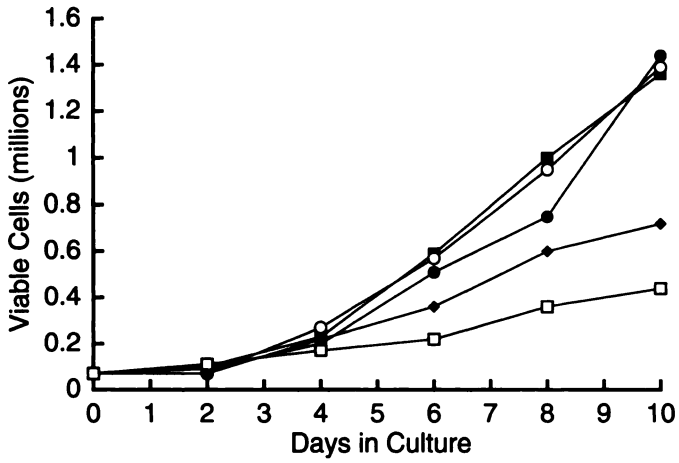

Fig. 4. Effect of varying levels of $ZnSO_4$ on *C/EBPα*-induced growth patterns. Hep3B/PC22 cells were cultured continuously in the presence of SFM plus zinc, and cell numbers were determined every other day by trypan blue viability counts. ■, zinc-free control; ○, 25 μM zinc; ●, 50 μM zinc; ♦, 75 μM zinc; □, 100 μM zinc.

The growth arrest associated with zinc-induced *C/EBPα* expression was reversible (Fig. 5). Hep3B/PC22 cells were cultured in the presence of 100 μM $ZnSO_4$, then zinc was removed, and the growth rate was determined. *C/EBPα* induction via the metallothionein promoter was accompanied by complete growth arrest. However, the cells resumed their basal growth pattern consistently 1–2 days after removal of the exogenously supplied zinc inducer.

Anchorage-independent Growth. A recognized characteristic of transformed cells is the ability to proliferate in an anchorage-independent manner (21). To assess the effect of *C/EBPα* expression on this characteristic, we investigated the ability of the HepG2 transfectants to form colonies in soft agar. As shown in Table 1, there is no effect from zinc on the number of colonies observed from HepG2/PC18 cells. However, zinc-induced expression of *C/EBPα* in HepG2/PC22 cells resulted in a 51-fold reduction in colony formation. In contrast, the expression of *C/EBPα* in Hep3B/PC22 cells did not affect the total number of colonies with respect to cells without zinc; rather, exposure to zinc affected the size of colonies that arose and their rate of appearance. Whereas the Hep3B/PC22 colonies without zinc were ~1 mm in size and became visible 10 days after initial plating, the colonies that arose in the presence of zinc were not visible until 3–4 weeks in culture and were very small (data not shown).

***In Vivo* Tumorigenicity of Hep3B/PC22 and Hep3B/PC18 Cells in CD-1 *nu/nu* Mice.** Because anchorage-independent growth correlates so closely with cellular tumorigenicity (22), it was of interest to determine whether the protein would exert a similar effect on the *in vivo* tumor phenotype. Of the 20 CD-1 *nu/nu* mice implanted with Hep3B/PC22 cells, only 1 developed a measurable tumor, which was detectable 44 days after implantation (Fig. 6). C/EBPα was not detectable by Western analysis of this tumor (data not shown). Seven of 10 mice with pPC18 cells developed tumors. Exogenous zinc was not added to the drinking water for these mice.

Delayed Tumorigenicity of Hep3B/PC22 and HepG2/PC22 Cells in SCID Mice. To corroborate the antitumor effect of *C/EBPα* expression in tumor cells further and to avoid possible intervention by an immune response, *C/EBPα*-containing cells were tested in immunodeficient SCID mice (23). Hep3B/PC22 and Hep3B/PC18 cells as well as HepG2/PC22 and HepG2/PC18 cells were each implanted s.c. into groups of 10 immunodeficient CB 17 SCID mice. Although tumors grew in all of the mice that received implants, the timing of tumor appearance was significantly different between cells transfected with pPC22 and pPC18. There was a 27-day delay (Fig. 7*A*) in the appearance of palpable tumors from Hep3B/PC22 cells (mean day of appearance, 56.6 ± 1.9) compared with tumors from Hep3B/PC18 cells (mean day of appearance, 28.2 ± 0.6) and a 21-day delay (Fig. 7*B*) in the appearance of palpable tumors from HepG2/PC22 cells (mean day of appearance, 51.8 ± 1.9) compared with tumors from HepG2/PC18 cells (mean day of appearance, 31.0 ± 2.7). Kaplan-Meier survival analysis indicated that *C/EBPα* expression had a significant impact on tumor formation (Fig. 7; $P < 0.0001$ by log-rank

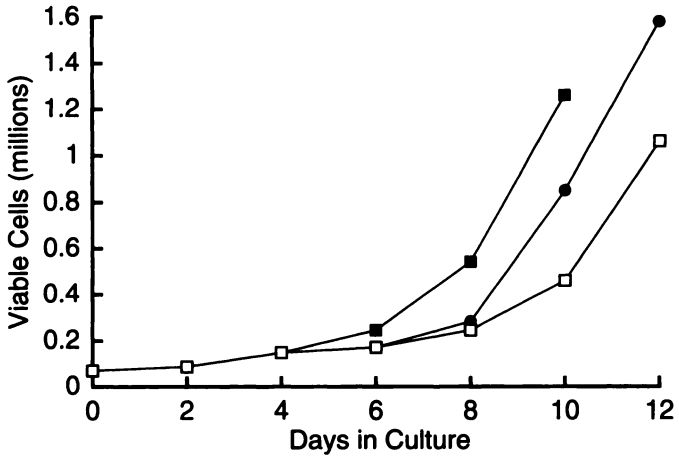

Fig. 5. Reversal of *C/EBPα*-induced growth arrest by removal of zinc. Cultures of Hep3B/PC22 cells were exposed continuously to 100 μM $ZnSO_4$ until removal on day 4, ■; day 6, ●; or day 8, □.

Table 1 *Anchorage-independent growth of HepG2 transfectants*

See "Materials and Methods" for experimental procedures

Cell type	Average no. of colonies/well[a]	
	No $ZnSO_4$	+ 100 μM $ZnSO_4$
HepG2/PC18	161 ± 23	159 ± 26
HepG2/PC22	308 ± 24	6 ± 3

[a] Numbers represent the average of three wells with SD between individual wells.

test). It was observed further that Hep3B/PC18 tumors demonstrated the same growth rate in both CD-1 and SCID mice (data not shown).

When palpable tumors did appear, the SCID mice were separated into groups to receive either drinking water with 10 mM zinc or control drinking water with no zinc. There was virtually no difference between the overall growth rates for the pPC22 tumors in mice treated with zinc when compared with the tumors in control mice that received no zinc in the drinking water. Similarly, there was no difference between the two groups with pPC18 tumors (data not shown). C/EBPα was not detectable by Western analysis of the delayed PC22 tumors (data not shown).

DISCUSSION

The current study demonstrates that expression of the *C/EBPα* gene in hepatocellular carcinoma cells can alter the phenotype and the tumorigenicity of these cells. Baker *et al.* (24) and Runnebaum *et al.* (25) observed similar results with wild-type *p53* expressed in colorectal carcinoma cells and breast carcinoma cells, respectively, demonstrating strong tumor suppressor effects with inhibited proliferation *in vitro*, reduced colony formation in soft agar, and impaired tumorigenicity in nude mice. Likewise, in our study, expression of *C/EBPα* was followed by complete, but reversible, arrest of the proliferative state. The growth-arrested cells were viable and metabolically active but were incapable of significant growth while under the regulatory control of the C/EBPα protein. In the soft agar, colony-forming assay, proliferative capacity was impaired for HepG2 and Hep3B cells expressing the transfected *C/EBPα*.

Tumorigenicity studies in which the *C/EBPα*-transfected cells were implanted into two different strains of mice confirmed the antitumor potential of *C/EBPα* expression in tumor cells. Even in the absence of zinc, endogenous factors may have acted on the metallothionein promoter to induce tumor delays. Both glucocorticoid hormones and IFNs have been shown to induce transcription from the human metallothionein IIA promoter (26). We conclude from the current studies that *C/EBPα* expression can suppress growth of hepatoma cells in CD-1 *nu/nu* mice.

In the immunodeficient SCID mouse model, there was significant tumor delay in the absence of zinc. Western analysis suggests that *C/EBPα* was lost during this period of tumor delay, resulting in the outgrowth of *C/EBPα*-negative cells over time. Accordingly, once tumor growth was initiated, proliferation was not affected by zinc supplementation in the drinking water, and the pPC22 tumors grew at the same rate as the pPC18 tumors. We are investigating currently whether the metallothionein promoter is maintained in the SCID mouse milieu, and whether *C/EBPα* is indeed lost over time *in vivo*.

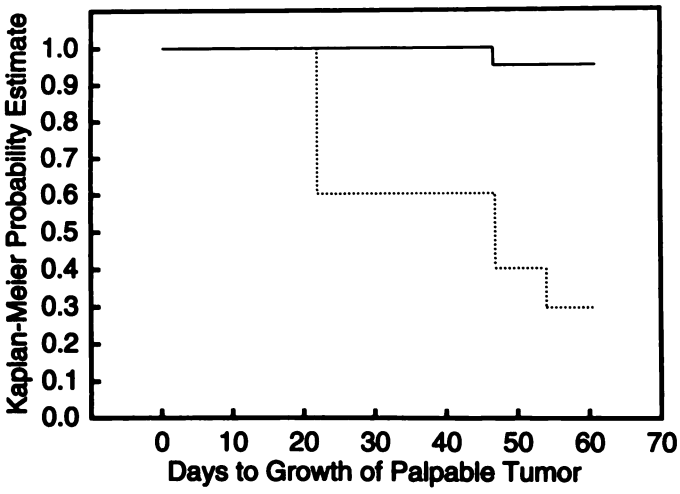

Fig. 6. Kaplan-Meier probability estimate of tumorigenicity of Hep3B/PC22 (—) and Hep3B/PC18 cells (·····) implanted s.c. in CD-1 *nu/nu* mice. Twenty mice were each implanted in the right flank with 1×10^7 pPC22 cells. Ten mice were implanted similarly with 1×10^7 pPC18 cells each. By day 62, 70% of the mice with pPC18 cells produced measurable tumors *versus* 5% of the mice inoculated with pPC22 cells. A measurable tumor was defined as having a weight of >75 mg.

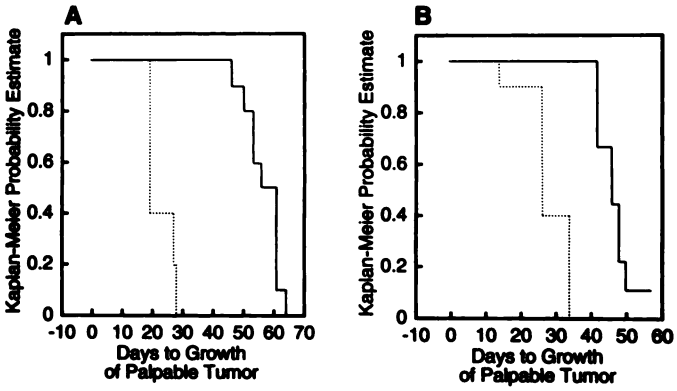

Fig. 7. Kaplan-Meier probability estimate of tumorigenicity of cells implanted s.c. in immunodeficient SCID mice. One × 10^7 cells/mouse were implanted in groups of 10 mice/cell line. *A*, Hep3B/PC22 (—) and Hep3B/PC18 (·····) cells; *B*, HepG2/PC22 (—) and HepG2/PC18 (·····) cells. A palpable tumor was defined as having a weight of >75 mg.

C/EBPα may regulate growth either by inducing a cascade of transcriptional events to redifferentiate tumor cells to a more normal phenotype or by inducing cytostatic growth arrest. Our *in vitro* studies have demonstrated that *C/EBPα* expression is capable of altering tumor pathogenicity and, therefore, should be able to modulate tumor progression. Because *C/EBPα* is itself an endogenous gene expressed in normal quiescent cells, transduction of proliferating tumor cells with *C/EBPα* DNA or with the C/EBPα protein should produce very little systemic toxicity. Therefore, *C/EBPα* could provide a selective approach to gene-based therapy for human cancer.

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