

Transcriptional Regulation of the Human Transferrin Gene by GADD153 in Hepatoma Cells

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The transcription factor CHOP/GADD153 is reportedly induced by cellular stresses such as UV light, genotoxic agents, and protein misfolding in the endoplasmic reticulum. However, the mechanism whereby induction of the *GADD153* gene is linked to a downstream pathway is still unclear. Previously, we observed that a synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4HPR) effectively impaired cell growth and survival (induction of growth arrest and apoptosis) in human hepatoma cells, which was accompanied by over expression of GADD153. Furthermore, *GADD153*-transfected Hep 3B cells were growth arrested and were sensitized to drug-induced apoptosis. Thus, in this study, we used suppression subtractive hybridization (SSH) to identify *GADD153* target genes that were up-regulated or down-regulated in the *GADD153* transfectants. We screened 614 sequence-verified clones by Northern blotting, of which 42 genes were scored as over expressed and 17 genes as under expressed in *GADD153* transfectants compared with control vector transfectants. Of those genes, 49 corresponded to known genes in public databases. Among them, we further verified that the expression of transferrin (Tf), which is a negative acute-phase protein and is essential to cell survival as a growth factor, was highly modulated by drug-induced GADD153 over expression or by *in vitro* transfection. GADD153 significantly antagonized the C/EBP (C/EBP- α , - β , and - δ)-mediated transcriptional activation of the *Tf* gene. In conclusion, *Tf* and other target genes identified may play a functional role in the downstream pathway of GADD153. (HEPATOLOGY 2003;38:745-755.)

The human *GADD153* gene encodes the nuclear protein C/EBP homologous protein 10 (CHOP), which can act as a negative modulator of C/EBP transcription factors that inhibits cell progression or can act as a positive regulator that activates target genes.¹ C/EBP genes encode transcription factors that participate in the process of terminal differentiation and growth ar-

rest in adipose tissue.^{2,3} They can form stable heterodimers with other C/EBP proteins and either prevent their binding to DNA or promote their binding to non-classical CCAAT sites.³ CHOP-C/EBP heterodimers are capable of recognizing novel DNA target sequences and thereby activating gene transcription.⁴ *GADD153* messenger RNA (mRNA) expression is very low in growing cells and is highly induced in response to a variety of cellular stresses, including glucose deprivation, oxidative stress, endoplasmic reticulum stress, and/or activation of acute phase response.⁵⁻⁹ In addition, the *GADD153* gene is highly inducible by genotoxic and by mutagenic agents,^{4,10,11} and its over expression is associated with the induction of growth arrest.² GADD153 contributes to the chemotherapy-induced apoptosis of myeloid cells treated with methylmethane sulfonate.¹² Other chemotherapeutic drugs, including paclitaxel, cisplatin, and etoposide, have been reported to induce GADD153 over expression, which was associated with cellular injury/apoptosis.¹³⁻¹⁵ More recently, GADD153 was reported to sensitize cells to endoplasmic reticulum stress through mechanisms that involve the down-regulation of Bcl-2 and enhance oxidant injury.¹⁶ Previously, we observed that a synthetic retinoid *N*-(4-hydroxyphenyl)retinamide

Abbreviations: CHOP, C/EBP homologous protein 10; mRNA, messenger RNA; 4HPR, *N*-(4-hydroxyphenyl)retinamide; cDNA, complementary DNA; SSH, suppression subtractive hybridization; Tf, transferrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; COUP-TF, chicken ovalbumin upstream promoter transcription factor; HNF-4, hepatocyte nuclear factor 4.

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(4HPR) effectively induced growth arrest and apoptotic cell death of hepatoma cells.¹⁷ During 4HPR treatment, GADD153 over expression was observed. Furthermore, GADD153-transfected Hep 3B cells were growth arrested and were sensitized to drug-induced apoptosis.¹⁸ Thus, to address the role of GADD153 over expression in hepatoma cells, it is necessary to identify GADD153 target genes.

The development of techniques such as serial analysis of gene expression^{19,20} and complementary DNA (cDNA) microarray technology^{21,22} has allowed for the generation of global expression profiles of various types of cancer cells. Although both serial analysis of gene expression and cDNA microarray approaches have proven to be powerful tools for the construction of gene indexes, they have a critical limitation in that both methods depend on the availability of previously cloned genes. Thus, we have used an alternative method called *suppression subtractive hybridization* (SSH)²³ because that method does not depend on the availability of previously cloned cDNA sets and allows the cloning of informative fragments of unknown sequences. SSH also allows for the normalization of frequent and rare cDNA and subtraction of cDNA common between 2 populations. Suppression PCR allows for the exponential amplification of differentially expressed genes and the suppression of sequences present in equal amounts in both cDNA populations. Using SSH and confirmatory Northern analysis, we identified 59 genes that are up-regulated or down-regulated in GADD153 transfectants. Among them, transferrin (*Tf*) was the most down-regulated gene in stable GADD153 transfectants. *Tf* is an iron transport protein and is essential to cell survival as a growth factor. Thus, we herein examined the mechanism of *Tf* down-regulation by GADD153.

Materials and Methods

Cell Lines and Reagents. Hep 3B hepatoma cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). 4HPR was kindly supplied by the Johnson Pharmaceutical Research Institute (Spring House, PA) and was dissolved in absolute ethanol at a concentration of 10^{-2} mol/L; it was stored in aliquots at -20°C for a maximum of 2 weeks.

Cell Culture and Transfections. Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in air containing 5% CO_2 . Transfection of the GADD153 gene into Hep 3B cells was performed using an expression plasmid vector encoding human GADD153 cDNA or control pcDNA3 as described previously.¹⁸ Transfections were carried out us-

ing lipofectin (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. Sequence-verified GADD153-transfected and neotransfected cells were selected in the presence of 600 $\mu\text{g}/\text{mL}$ G418 for 2 to 3 weeks. Finally, individual colonies were isolated using cloning rings and then expanded and assayed for expression of the transfected gene by Northern analysis and by Western analysis.

Generation of a Subtracted Library by SSH. SSH was performed between vector control cells (3B-C2, driver) and Hep 3B cells stably expressing GADD153 (3B-G5, tester) using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Tester and driver double-strand cDNA were synthesized from 2 μg of poly(A)⁺ RNA from 3B-G5 cells and from 3B-C2 cells, respectively. Tester cDNA was digested with *RsaI* at 37°C for 1.5 hours and then ligated to adaptors 1 and 2R in separate reactions at 16°C overnight. After ligation, for the first hybridization driver, cDNA was added to each of the tester samples, which were subsequently resuspended in the hybridization buffer, heat denatured, and then allowed to anneal at 68°C for 8 hours. For the second hybridization, driver cDNA was denatured at 98°C for 90 seconds and then added directly to the pooled mix of the 2 previous hybridizations and allowed to incubate at 68°C for 20 hours. It was necessary to alter the PCR conditions such that amplification of unwanted sequences was kept to minimum. All PCR and hybridization steps were performed on a Perkin-Elmer 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT).

Cloning into a TA Vector. After evaluation of the subtraction efficiency, the subtracted library cDNA was cloned into the pGEM-T vector (Promega, Madison WI). Approximately 100 ng PCR-amplified cDNA was ligated into 50 ng vector, and the ligation mixture was introduced into DH5 α cells by heat-shock treatment. The library was plated onto 15-cm agar plates containing 50 $\mu\text{g}/\text{mL}$ ampicillin, 100 $\mu\text{mol}/\text{L}$ isopropyl β -D-thioglucofuranoside, and 50 $\mu\text{g}/\text{mL}$ 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside. The plates were incubated at 37°C until small colonies were visible and then incubated a further 4°C until blue/white staining could be clearly distinguished. DNA sequences were determined using the automatic sequencer, ABI Prism 377 (Perkin Elmer, Norwalk, CT), according to the manufacturer's protocol. DNA and predicted amino acid sequences were searched against DNA and protein databases using the BLAST program available at the NCBI web site.

Total RNA Preparation and Northern Blot Analysis. Cells were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum until they reached

90% confluence, and total RNA was then extracted from treated or untreated cells using phenol and guanidine thiocyanate solution (Tri Reagent; Molecular Research Center, Inc., Cincinnati, OH). RNA was then fractionated by electrophoresis on 1.0% agarose gels containing formaldehyde and transferred to membranes. Blots were hybridized overnight with 2×10^6 cpm/mL of each cDNA probe of 614 individual clones (labeled with [32 P]dCTP [NEN, Boston, MA] by random priming), washed, and then exposed to x-ray film (AGFA, Agfa-Gevaert N.V., Belgium) at -70°C , as described previously.¹⁷ As a loading control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was detected using a *GAPDH* cDNA probe.

Luciferase Assay. A human transferrin promoter/enhancer-driven luciferase reporter construct pGLTf4000 and a human liver-specific transferrin enhancer-driven luciferase reporter construct pGLTf enhancer (pGLTfE) were gifts from Dr. Ronald H. Wenger (Physiologisches Institut der Universität Zürich, Switzerland).²⁴ For cotransfection experiments, the cells were cotransfected with the luciferase reporter constructs and GADD153 expression vector (pcDNA3-GADD153) using lipofectin (Gibco-BRL). Control experiments were performed using pGL3Basic or pGL3Control (Promega). Cells were plated at 2×10^4 cells per well in 24-well plates, and, 18 hours later, the cells were incubated at 37°C for 16 hours with 500 ng of pGLTf4000 or pGLTfE plasmids and various doses of pcDNA3-GADD153, as well as lipofectin. Following the transfection, the cells were replenished with complete medium and incubated for 36 hours. Cells were lysed in 120 μL reporter lysis buffer (Promega) and stored at -20°C until assayed. Normalizing the activity of the experimental reporter to the activity of the internal control minimizes experimental variability caused by difference in cell viability or transfection efficiency. Thus, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer's instructions. Cells were cotransfected with pRL-TK (Promega) and harvested. Firefly and renilla luciferase activities were measured for normalization using a luminometer (Lumat LB9507, Berthold, Germany).

Chloramphenicol Acetyltransferase Assays. Cells (10^6) were either transfected by the lipofectin method with 1 pmol of plasmid reporter DNA ($-125/+39$ Tf-chloramphenicol acetyltransferase; CAT) or were cotransfected with reporter DNA (0.5 pmol) and the C/EBP (C/EBP- α , - β , and - δ), chicken ovalbumin upstream promoter transcription factor (COUP-TF), or hepatocyte nuclear factor 4 (HNF-4) expression vectors (0.5 pmol).²⁵ Cell extracts of Hep 3B cells were analyzed for CAT activity by the method described previously.²⁶ Reaction

mixtures containing 30 μg cell extract, 0.2 μCi [^{14}C]chloramphenicol, and 4.4 mmol/L acetyl coenzyme A were incubated at 37°C for 3 hours. The reaction products were separated on thin layer plates, and the percentage of chloramphenicol substrate acetylates was determined by liquid scintillation counting. The expression plasmids C/EBP (C/EBP- α , - β , and - δ), COUP-TF, and the reporter plasmid Tf-CAT were provided by Dr. Schaeffer Evelyne (INSERM, Strasbourg Cedex, France).^{26,27} The expression plasmid HNF-4 was a gift of Dr. Gerhart U. Ryffel (Universitätsklinikum Essen, Essen, Germany).²⁸ To quantitatively compare the relative CAT activity, the pWH5 vector containing phosphoglucokinase-1 promoter and IRES (internal ribosomal entry site of the encephalomyocarditis virus)-*cat* sequence was used as a positive control.²⁹ The promoterless pBS-ECAT²⁹ and β -galactosidase expression (pCMV- β -gal) vectors were used as negative controls.

Immunofluorescence. Cells were grown on glass coverslips, fixed with 4% paraformaldehyde, permeabilized in phosphate-buffered saline containing 0.2% Triton, and blocked with 1% bovine serum albumin. Cells were incubated with rabbit GADD153 polyclonal antibody (R-20; Santa Cruz Biotechnology, Santa Cruz, CA) or with rabbit anti-human transferrin (A0061; DAKO, Glostrup, Denmark) overnight at 4°C , washed, and incubated with tetramethylrhodamine isothiocyanate isomer R-conjugated swine anti-rabbit immunoglobulin. After final washes, cells were stained for 15 minutes with 1 $\mu\text{g}/\text{mL}$ Hoechst 33258 to visualize nuclei and mounted with 50% glycerol in phosphate-buffered saline at 4°C . The cells were examined using a laser scanning microscope LCM510 (Carl Zeiss, Jena, Germany) with a $60\times$ oil immersion objective.

Quantification. Autoradiographs of Northern hybridizations were scanned using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA), and densitometric data were analyzed using the accompanying software on a Power Mac 6100 (Apple). The level of mRNA for *GAPDH* was determined in the same manner. Expression levels of each gene relative to the vector control were calculated by normalizing against the level of *GAPDH* mRNA and then calculating the ratio of mRNA from the transfectant cells. Statistical significance was assessed by Student's *t* test.

Results

Ectopic GADD153 Over Expression in Hep 3B Cells. We introduced the *GADD153* gene into Hep 3B cells because these cells express low constitutive levels of *GADD153*. Two types of Hep 3B cells (3B-G1 and 3B-

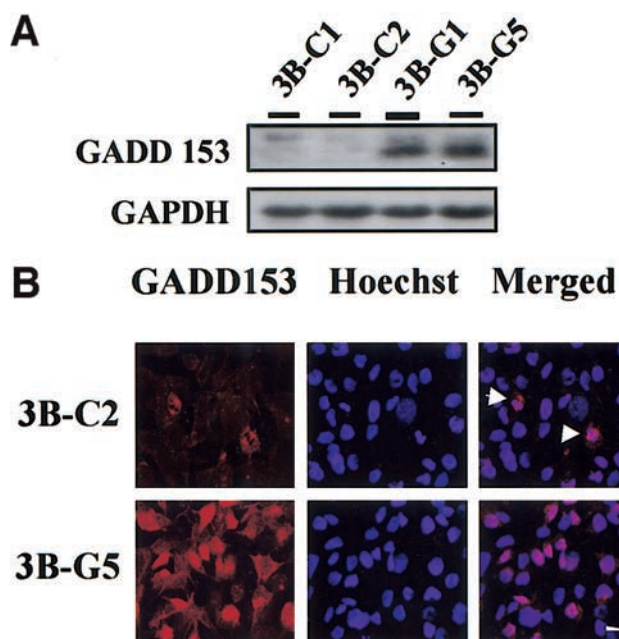


Fig. 1. Stable expression of GADD153 in Hep 3B cells. (A) Northern blot analysis of *GADD153* mRNA expression in stable transfectants (3B-G1 and 3B-G5 cells) compared with vector control cells (3B-C1 and 3B-C2 cells). Total RNA from the cells was fractionated by electrophoresis on 1.0% agarose gels containing formaldehyde and was then transferred to membranes. Blots were hybridized overnight with 2×10^6 cpm/mL *GADD153* cDNA probe labeled with [32 P]dCTP by random priming, washed, then exposed to x-ray film at -70°C . (B) Immunofluorescence microscopy analysis of GADD153 protein expression in 3B-G5 cells or in the vector control 3B-C2 cells. Cells were fixed in 4% paraformaldehyde then processed for indirect immunofluorescence with antibodies directed against GADD153 (red, left panels) and stained with 1 $\mu\text{g}/\text{mL}$ Hoechst 33258 to visualize the nuclei (blue, middle panels). The cells were examined using a laser scanning microscope LCM510. Both images of GADD153 and nuclei of the same cells are merged (right panels). Arrowheads indicate the cells with mitotic figure. Scale bar = 10 μm .

G5), which stably express human *GADD153* mRNA, were isolated (Fig. 1A). We used 3B-G5 cells and 3B-C2 cells as stable *GADD153* transfectants and vector control cells, respectively, for this study. Immunoreactivity for GADD153 showed stronger cytoplasmic signals in nearly all 3B-G5 cells, along with increased nuclear staining, than in vector control 3B-C2 cells (Fig. 1B). These findings are consistent with results previously reported.³⁰ Intriguingly, some cells with mitotic figures revealed high immunopositivity for GADD153 in both 3B-G5 and 3B-C2 cells.

Confirmation of the Expression of Selected Genes by Northern Hybridization. PCR-generated products from subtractive hybridization were inserted in the pGEM-T plasmid vector, and a subtractive cDNA library containing 924 clones (504 forward subtracted clones and 420 reverse subtracted clones) was constructed. Sequence analysis of these clones revealed that 614 individual clones

contained a unique gene. Three hundred sixty-five clones were derived from the forward subtracted cDNA library and 249 clones from the reverse subtracted library. We screened the 614 sequence-verified clones by Northern blotting and found 42 different genes that were over expressed (Fig. 2A) and 17 different genes that were under expressed in *GADD153* transfectants compared with vector control cells (Fig. 2B). Up-regulated or down-regulated genes were classified in terms of function by reference to the literature and to Web databases as shown in Tables 1 and 2.

GADD153-Mediated Down-regulation of Tf Expression by 4HPR. Previously, we showed that the *GADD153* gene is preferentially expressed in apoptotic Hep 3B cells treated with 4HPR for 72 hours, and we determined that the GADD153 protein expression correlated with *GADD153* mRNA induction.¹⁸ In the present study, 4HPR induced the expression of GADD 153 protein in a time-dependent manner (Fig. 3A). Simultaneously, the expression of *Tf* mRNA decreased along the same time course. These results suggest that GADD153 protein expression inversely correlates with *Tf* mRNA regulation. To investigate this hypothesis, we examined whether the expression of *Tf* mRNA changed in *GADD153*-transfected cells. Interestingly, cells stably expressing *GADD153* mRNA showed decreased expression of *Tf* mRNA, whereas the vector control cells expressed it abundantly (Fig. 3B). Next, to determine whether treatment with 4HPR decreased transferrin protein expression in Hep 3B cells, we examined transferrin expression in those cells by immunofluorescence laser scanning microscopy. Treatment with 4HPR (10 $\mu\text{mol}/\text{L}$ for 48 hours) effectively reduced the number of Hep 3B cells expressing transferrin protein compared with untreated cells (Fig. 4A). Furthermore, the expression of transferrin protein also decreased in stable *GADD153* transfectants compared with vector control cells (Fig. 4B). These results suggest that 4HPR down-regulates Tf protein expression by modulating GADD153 expression.

Transcriptional Regulation of Tf by 4HPR. To examine whether the *cis*-acting elements of *Tf* are functional in Hep 3B cells treated with 4HPR, we performed transient expression studies using pGLTf4000, a $-4,000$ to $+39$ *Tf* promoter/enhancer DNA fragment inserted upstream of a promoter-less luciferase reporter pGL3Basic. We also used pGLTfE, the liver-specific *Tf* enhancer, from nucleotide position -3.6 kb to -3.3 kb relative to the transcriptional start site downstream of a luciferase reporter gene driven by a heterologous SV40 promoter. Following transient transfection into Tf-expressing Hep 3B cells with the 4.0-kb *Tf* promoter/enhancer or the *Tf* enhancer (promoterless), 4HPR decreased luciferase ex-

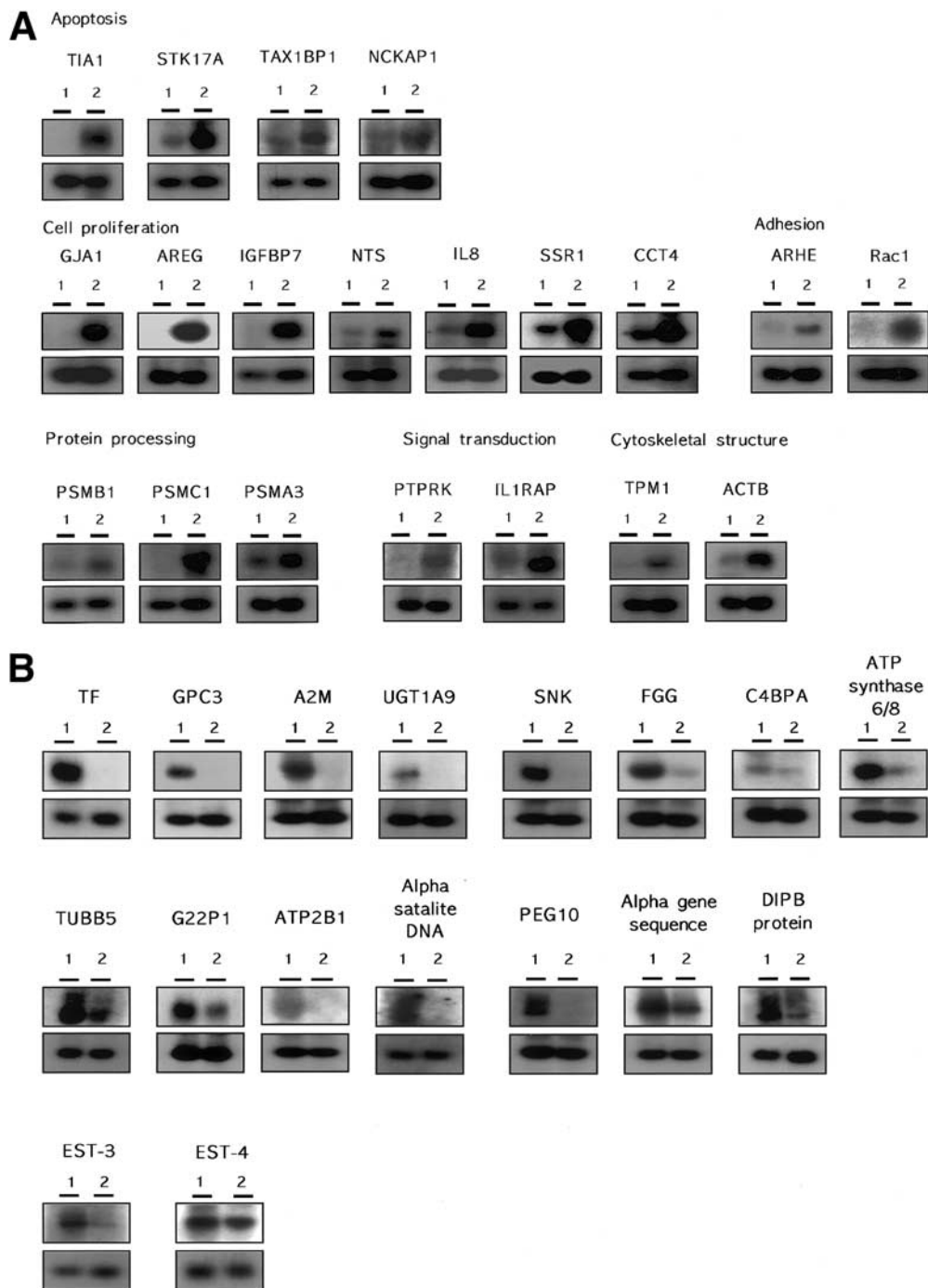


Fig. 2. Differentially expressed genes analyzed by Northern blot analysis. (A) Differentially up-regulated genes that relate to apoptosis, cell proliferation, adhesion, protein processing, signal transduction, and cytoskeletal structure in stable *GADD153* transfectants. (B) Differentially down-regulated genes in stable *GADD153* transfectants. Total RNAs from the 3B-G5 or 3B-C2 cells were fractionated by electrophoresis on 1.0% agarose gels containing formaldehyde and then transferred to membranes. Blots were hybridized overnight with 2×10^6 cpm/mL cDNA probe labeled with [P^{32}]dCTP by random priming, washed, then exposed to x-ray film at -70°C . The blots were stripped and sequentially hybridized with a probe for *GAPDH* cDNA as a loading control (lane 1, 3B-C2; lane 2, 3B-G5).

pression in a time-dependent manner. This inhibition was up to $23\% \pm 3\%$ (mean \pm SE, $P < .01$) of the control within 48 hours (Fig. 5A). Thus, 4HPR responsiveness seems to be coupled to liver-specific *cis*-acting elements present within this *Tf* promoter/enhancer DNA fragment. Next, we examined whether the $-3,600/-3,300$ -bp liver-specific *Tf* enhancer might also be involved in the 4HPR responsiveness of the *Tf* gene. The luciferase activity did not decrease in Hep 3B cells trans-

fectected with pGLTfE alone, similarly to Hep 3B cells transfected with the pGL3Control plasmid, which matches the promoter of the enhancer-driven plasmid (SV40 promoter and enhancer) and expresses the same luciferase (Fig. 5B). These results suggest that 4HPR may down-regulate *Tf* transcription through a *cis*-acting enhancer/promoter element and that the 300-bp enhancer alone may be insufficient to confer 4HPR responsiveness to Hep 3B cells.

Table 1. Summary of cDNA Clones Generated by Suppression Subtractive Hybridization: Differentially Up-regulated Genes in Stable GADD153 Transfectants (3B-G5) Versus Vector Control Cells (3B-C2)

Function	Accession No.	Sequence Identity*	Frequency†	Differential Expression‡	
Apoptosis	NM_022173	Cytotoxic granule-associated RNA binding protein (TIAI), transcript variant 2	1	54.7	
	AB011420	Serine/threonine kinase 17a (STK17A or DRAK1)	2	2.5	
	NM_006024	Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BPI)	1	1.7	
	NM_013436	NCK-associated protein 1 (NCKAP1)	1	1.5	
Cell proliferation	NM_000165	Gap junction protein, α 1, 43 kd (connexin 43) (GJA1)	49	69.7	
	NM_001657	Amphiregulin (schwannoma-derived growth factor) (AREG)	6	30.5	
	NM_001553	Insulin-like growth factor-binding protein 7 (IGFBP7)	5	8.7	
	NM_006183	Neurotensin (NTS)	1	3.6	
	XM_031289	Interleukin 8 (IL-8)	4	3.5	
	AF156965	Translocon-associated protein α subunit (SSR1)	1	2.3	
	NM_006430	Chaperonin containing TCP1, subunit 4 (δ) (CCT4)	1	1.7	
Adhesion	S82240	Ras homolog gene family, member E (ARHE)	1	3.1	
	BC004247	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP-binding protein Rac1)	1	2.6	
Protein processing	NM_002793	Proteasome (prosome, macropain) subunit, β type, 1 (PSMB1)	2	2.1	
	L02426	Proteasome (prosome, macropain) 26S subunit, ATPase, 1 (PSMC1)	1	1.9	
	NM_002788	Proteasome (prosome, macropain) subunit, α type, 3 (PSMA3)	1	1.5	
Signal transduction	NM_002844	Protein tyrosine phosphatase, receptor type, K (PTPRK)	1	4.9	
	NM_002182	Interleukin 1 receptor accessory protein (IL1RAP)	2	3.2	
Cytoskeletal structure	M19715	Tropomyosin 1 (α) (TPM1)	2	4.5	
	NM_001101	Actin, β (ACTB)	3	3.3	
Miscellaneous	AF139768	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 5 (CLECSF5)	1	15.2	
	NM_012198	Grancalcin, EF-hand calcium-binding protein (GCA)	1	14.8	
	U12709	Neutrophil-activating ENA-78 prepeptide gene or SCYB5	3	4.9	
	X65965	SOD-2 gene for manganese superoxide dismutase (SOD-2)	1	3.4	
	AF346992	Mitochondrion cytochrome c oxidase subunit and tRNA-Lys and ATP synthase 6 (8592-8198)	1	3	
	BC023990	Annexin A2 (ANXA2)	1	2.8	
	XM_010822	Putative L-type neutral amino acid transporter (KIAA0436)	2	2.8	
	XM_042066	Mitogen-activated protein kinase kinase kinase 1 (MAP3K1)	1	2.5	
	AF047033	Sodium bicarbonate cotransporter 3 (SLC4A7)	1	2.4	
	NM_001343	Disabled homolog 2, mitogen-responsive phosphoprotein (<i>Drosophila</i>) (DAB2)	1	2.2	
	NM_015577	Retinoic acid induced 14 (RAI14)	1	1.7	
	U87279	Splicing factor SRp30c gene, exons 3 and 4	1	1.6	
	X77494	RNA-binding motif, single-stranded interacting protein 1 (RBMS1)	1	1.5	
	NM_001154	Annexin A5 (ANXA5)	1	1.5	
	Unknown	AL080084	LOC50999 CGI-100 protein	1	6.4
		AF107454	C7orf2 chromosome 7 open-reading frame 2	1	5.4
		XM_047970	LOC92912	1	4.1
XM_085177		LOC145577	1	3.8	
		EST-1	1	3.2	
AF113020		PRO2463 protein	3	2.4	
AL137648		DKFZp434J1813 (from clone DKFZp434J1813)	1	2.4	
		EST-2	1	1.9	

*Sequence identity based on comparison with GeneBank/EMBL database.

†The number of clones with same gene identity that appeared in the analysis of forward subtracted 504 clones.

‡Relative expression folds of mRNA based on Northern analysis of 3B-G5 cells and 3B-C2 cells. Values attained by densitometry and normalized by comparison with GAPDH.

Transcriptional Regulation of Tf by GADD153 Protein. We observed that 4HPR effectively induced GADD153 over expression and simultaneously down-regulated Tf expression. Thus, we suggest that 4HPR-mediated GADD153 induction may relate to down-regulation of Tf expression as suggested by our SSH and Northern data. Thus, we examined whether *Tf* is a

downstream target gene of GADD153. The GADD153-mediated down-regulation of *Tf* was analyzed by an *in vitro* transfection assay. Cells were cotransfected with the GADD153 expression plasmid (pcDNA3-GADD153) and the 4.0-kb pGLTf4000. As expected, GADD153 suppresses expression of the *Tf* enhancer/promoter coupled with the reporter luciferase in Hep 3B cells in a

Table 2. Summary of cDNA Clones Generated by Suppression Subtractive Hybridization: Differentially Down-regulated Genes in Stable GADD153 Transfectants (3B-G5) Versus Vector Control Cells (3B-C2)

Function	Accession No.	Sequence Identity*	Frequency†	Differential Expression‡
Iron binding	NM_001063	Transferrin (TF)	38	-26.9
Cell growth and maintenance	NM_004484	Glypican 3 (GPC3)	5	-24.5
Proteinase inhibitor	NM_000014	α -2-Macroglobulin (A2M)	15	-22.1
Metabolism	NM_021027	UDP glycosyltransferase 1 family, polypeptide A9 (UGT1A9)	4	-9.5
Signaling molecule	NM_006622	Serum-inducible kinase (SNK)	1	-7.3
Cell adhesion	NM_000509	Fibrinogen, γ polypeptide (FGG)	5	-4.2
Complement system	M31452	Complement 4-binding protein, α (C4BPA)	1	-2.3
Oxidative phosphorylation	AF347015	ATP synthase 6/8	8	-2.3
Cytoskeletal structure	NM_032525	Tubulin β -5 (TUBB5)	1	-2.0
DNA binding	XM_066628	Thyroid autoantigen 70 kd (Ku antigen) (G22P1)	1	-1.4
Calcium transporting	NM_001682	ATPase, Ca ⁺⁺ transporting, plasma membrane 1 (ATP2B1)	2	-1.2
Miscellaneous	Z12006	α Satellite DNA	4	-8.1
	NM_015068	Paternally expressed 10 (PEG10)	16	-7.6
	AF203815	α Gene sequence	1	-4.9
	XM_030301	DIPB protein (LOC116020)	1	-2.1
Unknown		EST-3	1	-4.7
		EST-4	1	-1.2

*Sequence identity based on comparison with GeneBank/EMBL database.

†The number of clones with the same gene identity that appeared in the analysis of reverse subtracted 462 clones.

‡Relative expression folds of mRNA based on Northern analysis of 3B-G5 and 3B-C2 cells. Values attained by densitometry and normalized by comparison with GAPDH.

dose-dependent manner (Fig. 6A). Hep 3B cells transfected with 2.5 μ g of GADD153 expression plasmid inhibited the relative luciferase activity to 34% \pm 2% of control ($P < .01$). In contrast, GADD153 expression did not inhibit luciferase activity in Hep 3B cells transfected with pGL3Control plasmid. As expected, GADD153 did not inhibit luciferase activity in Hep 3B cells transfected with pGLTfE alone (Fig. 6B). Thus, these results suggest that GADD153 may down-regulate *Tf* transcription through a *cis*-acting enhancer/promoter element in Hep 3B cells.

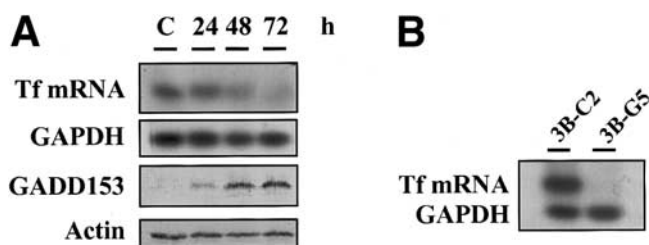


Fig. 3. GADD153-mediated down-regulation of *Tf* by 4HPR. (A) Northern blot analysis of *Tf* mRNA down-regulation in Hep 3B cells treated with 10 μ mol/L 4HPR at the indicated time interval. Total RNA from the cells was fractionated by electrophoresis on 1.0% agarose gels containing formaldehyde then transferred to membranes and autoradiographed. The blots were stripped and sequentially hybridized with a probe for GAPDH cDNA as a loading control. Simultaneously, the expression of GADD153 protein was examined by Western blot analysis. Thirty micrograms of extracted proteins were resolved by 13% SDS-PAGE and transferred to the membrane. The blot was probed with a polyclonal antibody to GADD153 (R-20) and then stripped and reprobed with a monoclonal antibody to actin as a loading control. (B) Northern blot analysis of *Tf* mRNA in 3B-G5 cells compared with 3B-C2 cells.

Inhibition of C/EBP-Mediated *Tf* Transactivation by GADD153 Protein.

The transient CAT activity was apparent in cells transfected with the pWH5 vector, whereas no CAT activity was detectable in the extract of control cells transfected with pBS-ECAT or pCMV- β -galactosidase (pCMV- β -Gal) as described previously.²⁹ Furthermore, the expression of GADD153 did not change CAT activity in Hep 3B cells transfected with pWH5 plasmid (Fig. 7). Liver-specific expression of the *Tf* gene is governed by the combination of 2 elements, primarily by HNF-4 and, to a lesser extent, by COUP-TF on promoter region I, and by C/EBP, which acts on promoter region II.²⁷ Thus, we determined the mechanism whereby GADD153 protein modulates transcription of the *Tf* gene. Hep 3B cells were cotransfected with equal amounts (0.5 pmol) of C/EBP- α , C/EBP- β , C/EBP- δ , COUP-TF, or HNF-4 expression vectors and the -125/+39 *Tf* CAT reporter vector, containing the promoter regions I and II. The cotransfection experiments in Hep 3B cells revealed that mostly C/EBP and HNF-4 stimulates *Tf* transcription driven by the -125/+39 region as described previously.²⁸ However, C/EBP-dependent transactivation was only repressed by the simultaneous presence of GADD153. The most dramatic antagonistic effects were observed with C/EBP- α (91% reduction) and C/EBP- δ (88.5% reduction) and to a lesser extent with C/EBP- β expression vectors (75% reduction) in the presence of GADD153. Thus, our data suggest that GADD153 protein expression may be effectively and specifically ($P < .01$) antagonized by C/EBP-stimulated *Tf*

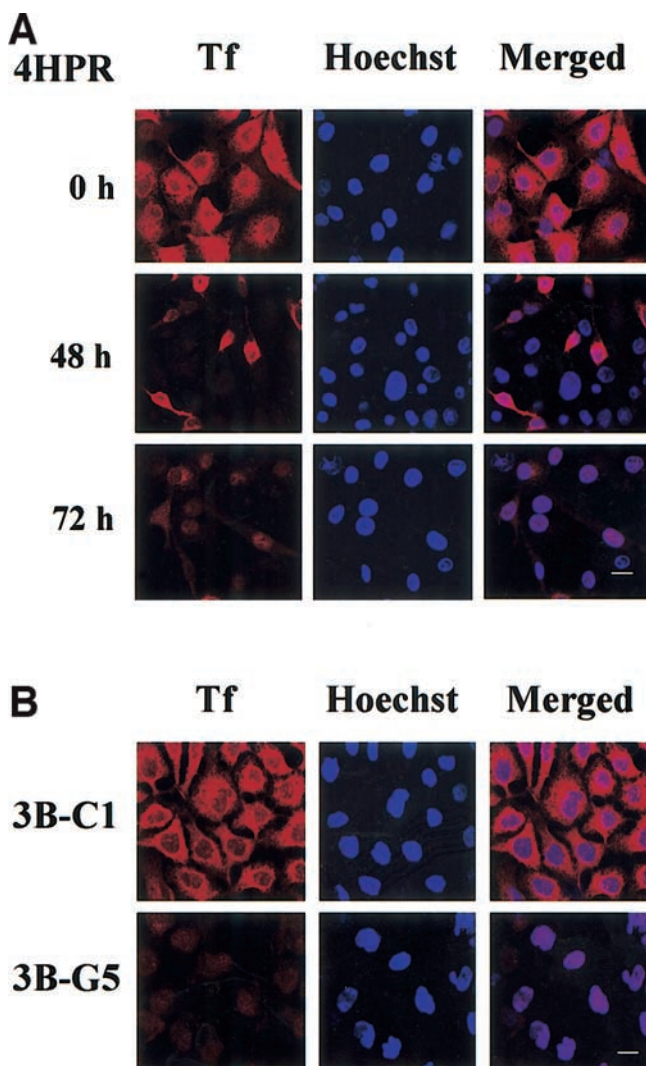


Fig. 4. Immunofluorescence microscopy analysis of Tf protein expression in Hep 3B cells. (A) Tf expression in Hep 3B cells treated with 10 $\mu\text{mol/L}$ 4HPR (left panels) for the indicated time intervals. Cells were fixed in 4% paraformaldehyde then processed for indirect immunofluorescence with antibodies directed against Tf (red) and stained with 1 $\mu\text{g/mL}$ Hoechst 33258 to visualize the nuclei (blue). The cells were examined using a laser scanning microscope LCM510. (B) Tf expression in stable GADD153 transfectants (upper panels) or in vector control cells (lower panels). Cells were fixed in 4% paraformaldehyde then processed for indirect immunofluorescence with antibodies directed against Tf (red) and stained with 1 $\mu\text{g/mL}$ Hoechst 33258 to visualize the nuclei (blue). The cells were examined using a laser scanning microscope LCM510. Both images of GADD153 and nuclei of the same cells are merged (right panels). Scale bar = 10 μm .

gene transactivation and that 4HPR- and GADD153 responsiveness may localize in part to the promoter of Tf gene.

Discussion

CHOP/GADD153 is a stress-inducible bZIP transcription factor and is also expressed in hepatocytes and hepatoma cells in response to acute stress.^{7,31-33} In this

study, we examined the influence of GADD153 expression on downstream gene expression in stable GADD153 transfectants. Changes in patterns of gene expression can be used to identify and possibly target specific genes involved in cell growth and survival. Therefore, we have used SSH to generate a profile of GADD153-induced genes.

CHOP not only functions as a negative or a positive regulator of CEBP target genes but also, when tethered to AP-1 factors, can activate AP-1.¹ In addition, recent reports have described a new mechanism of GADD153-mediated apoptosis through a nontranscriptional regulation, *i.e.*, CHOP, as well as c-Fos, interacts with a ribosomal protein (FTE/S3a) whose down-regulation results in the induction of apoptosis.^{34,35} In the present study, we identify GADD153 target genes that may be directly or indirectly associated with growth arrest and apoptotic cell death. Forty-two genes were differentially over expressed in stable GADD153 transfectants. They can be classified based on their functions, which include apoptosis, cell proliferation, adhesion, protein processing, signal transduction, cytoskeletal structure, miscellaneous, and unknown. *TIA1*³⁶ and *STK17A/DRAK1*³⁷ genes were reported to be involved in the induction of apoptosis. However, TAX1BP1³⁸ and NCKAP1³⁹ are considered to be antiapoptotic proteins. Therefore, the functional role of TAX1BP1 or NCKAP1 induction in GADD153-mediated apoptosis should be further elucidated. Seven genes related to cell proliferation were up-regulated in the stable GADD153 transfectants. Among them, GJA1 is responsible for contact growth inhibition of tumor cells⁴⁰; AREG is a 1,25(OH)2D3 target gene, and its induction may contribute to the growth inhibitory effects of 1,25(OH)2D3,⁴¹ and IGFBP-7 is a potential tumor suppressor protein.⁴²

In the present study, GADD153 attenuates the expression of 17 genes in stable GADD153 transfectants. Among them, we focused attention on the down-regulation of the *Tf* gene because Tf is an iron transport protein and is essential to cell survival as a growth factor. It is mainly produced by the liver and is secreted into the bloodstream to various cells in the body.⁴³ Previously, Hep 3B cells were reported to express high levels of the *Tf* gene.⁴⁴ In the present study, 4HPR effectively induced apoptosis (data not shown), which was accompanied by the induction of GADD153 expression along the time course. Simultaneously, 4HPR reduced the expression of *Tf* mRNA and Tf protein assessed by Northern blot analysis and by immunofluorescence.

A previous report characterized the *cis*-acting elements involved in the liver-specific expression of the human transferrin gene.^{44,45} The regulatory functions of se-

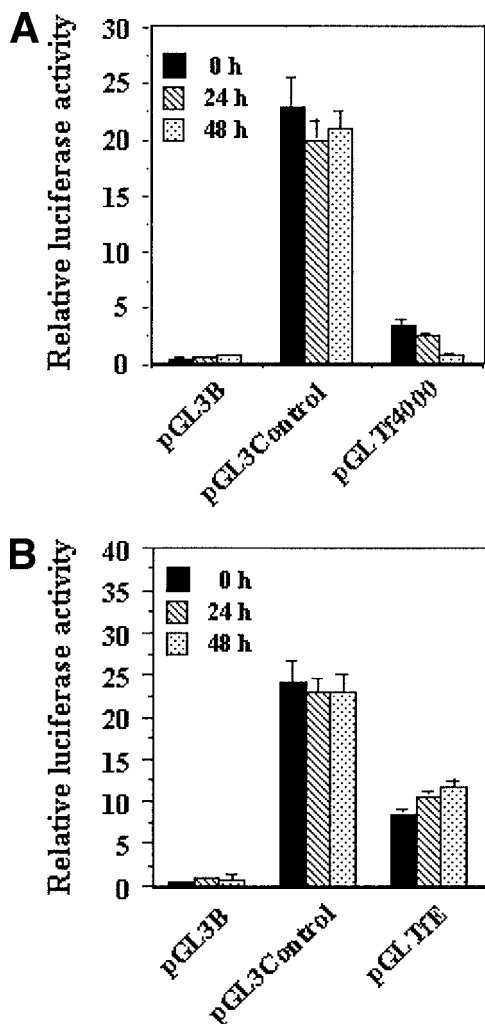


Fig. 5. 4HPR-mediated transcriptional regulation of *Tf* in 4HPR-treated hepatoma cells. (A) Luciferase activity measured in Hep 3B cells transiently transfected with pGLTf4000 (Tf promoter/enhancer) or control pGL3Control. Hep 3B cells were treated with 10 μ mol/L 4HPR for the indicated time intervals. **Vertical bars** represent the means \pm SE of 3 experiments performed in duplicate. (B) Luciferase activity measured in Hep 3B cells transiently transfected with pGLTfE (Tf enhancer) or control pGL3Control. Hep 3B cells were treated with 10 μ mol/L 4HPR for the indicated time intervals. **Vertical bars** represent the means \pm SE of 3 experiments performed in duplicate. Cells were cotransfected with pRL-TK (Promega) and harvested. Firefly and renilla luciferase activities were measured for normalization using a luminometer.

quences extending over 4 kb of the 5'-flanking region of the *Tf* gene were analyzed. Deletion analysis of the 5' sequence of the gene have defined 3 functionally different regions: (1) A cell type-specific promoter located between positions -125 and $+1$, which interacts with 2 nuclear factors and is sufficient for liver specific expression; (2) a negative-acting region between $-1,000$ and -620 base pairs, which down-regulates transcription from the *Tf* promoter; (3) an enhancer located between $-3,600$ and $-3,300$ base pairs, which is more active in hepatoma cells.⁴⁴ In the present study, we observed that treatment

with 4HPR induced GADD153 over expression and that this induction subsequently might down-regulate *Tf* expression in Hep 3B cells through a *cis*-acting enhancer/promoter.

The liver-specific expression of the *Tf* gene is governed by the $-125/+39$ promoter region. Cotransfection analyses in hepatoma Hep 3B cells indicated that C/EBP- α , C/EBP- δ , and HNF-4 stimulate transcription driven by the $-125/+39$ region. C/EBPs are characterized by a conserved leucine zipper domain in the carboxyl-terminal region of the protein, which is adjacent to a basic region

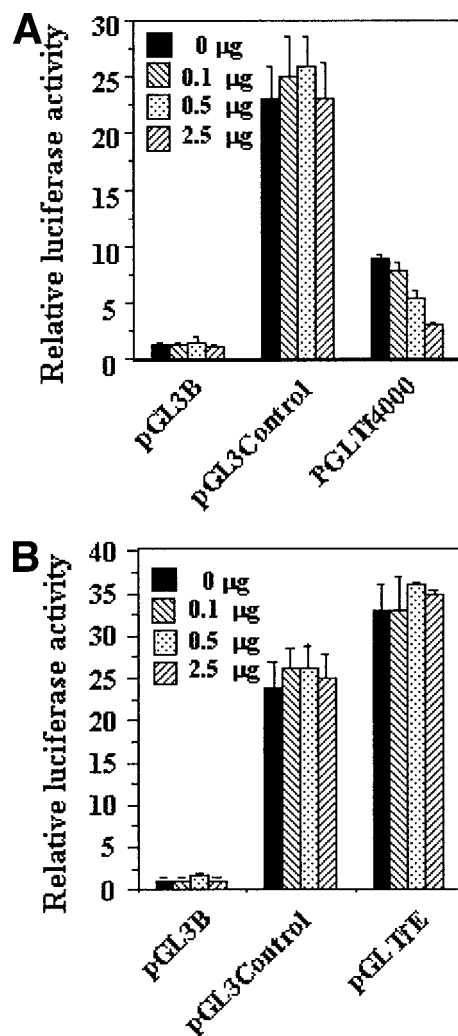


Fig. 6. GADD153 transcriptionally down-regulates *Tf* expression. (A) Luciferase activity measured in Hep 3B cells transiently transfected with pGLTf4000 or control pGL3Control. Hep 3B cells were cotransfected with GADD153 expression plasmid at the indicated concentrations. **Vertical bars** represent the means \pm SE of 3 experiments performed in duplicate. (B) Luciferase activity measured in Hep 3B cells transiently transfected with pGLTfE or control pGL3Control. Hep 3B cells were cotransfected with the GADD153 expression plasmid at the indicated concentrations. **Vertical bars** represent the means \pm SE of 3 experiments performed in duplicate. Cells were cotransfected with pRL-TK (Promega) and harvested. Firefly and renilla luciferase activities were measured for normalization using a luminometer.

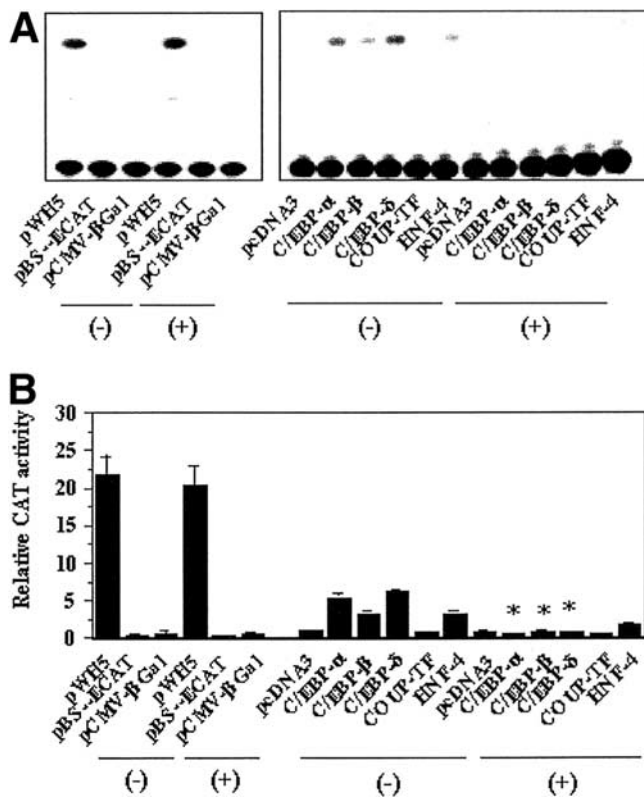


Fig. 7. Functional effect of GADD153 on C/EBP- α , C/EBP- β , C/EBP- δ , COUP-TF, and HNF-4-mediated *Tf* gene regulation. (A) Autoradiogram of 1 typical CAT assay. Cotransfection assays were performed in Hep 3B cells with the -125/+39 Tf-CAT reporter construct, together with the expression vectors in the presence (+) or absence (-) of GADD153 plasmid. One picomole of each vector was used. pWH5 containing the *cat* gene linked with the IRES under control of the phosphoglucokinase-1 promoter was used as a positive control. The promoterless pBS-ECAT and pCMV- β -gal vectors were used as negative controls. (B) The bar graphs report the CAT activities determined as described in the Material and Methods section. They represent the means \pm SE of at least 3 separate transfections (* P < .01).

involved in DNA sequence recognition.⁴⁶ The basic region of GADD153 deviates significantly from the consensus defined by other members of the C/EBP family in that it contains proline and glycine substitutions in conserved residues believed to be essential to the interaction of these proteins with DNA-binding sites.^{47,48} Dimerization of monomeric proteins through the leucine zipper domain is necessary for sequence-specific binding to DNA. C/EBP- α , C/EBP- β , and C/EBP- δ are all capable of forming homodimers as well as heterodimers with other C/EBPs.⁴⁹ GADD153 is a member of the C/EBP family; thus, it can heterodimerize with other members of C/EBP family. CHOP-C/EBP heterodimers fail to bind several known C/EBP sites *in vitro*. When over expressed in cells, GADD153 attenuates the ability of other C/EBP proteins to activate promoters containing such sites.¹¹ Thus, it has been suggested that GADD153 acts as a dominant neg-

ative inhibitor of other C/EBPs. In support of this hypothesis, over expression of GADD153 prevents C/EBP-regulated gene activation and blocks adipocyte differentiation in cultured cells.^{3,50} In the present study, the GADD153 protein, through its interaction with C/EBP, is likely to participate in the down-regulation of the *Tf* gene expression. However, because the deletion analysis has only ruled out the transcriptional regulation of the upstream enhancer and because the promoter is weakly responsive to C/EBP isoforms (for which GADD153 is a known inhibitor), the main effect of 4HPR does not seem to be confined to the promoter region.

In summary, the GADD153 target genes identified that are directly or indirectly regulated by GADD153 may further understanding of the mechanisms underlying the involvement of GADD153 in the inhibition of cell growth and survival. Furthermore, expression of the *Tf* gene, one of the target genes, seems to be down-regulated by GADD153 induction through the negative inhibition of C/EBP.

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