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gadd153/Chop10, a Potential Target Gene of the Transcriptional Repressor ATF3

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Recently, we demonstrated that the function of ATF3, a stress-inducible transcriptional repressor, is negatively regulated by a bZip protein, gadd153/Chop10. In this report, we present evidence that ATF3 can repress the expression of its own inhibitor, gadd153/Chop10. First, ATF3 represses a chloramphenicol acetyltransferase reporter gene driven by the gadd153/Chop10 promoter when assayed by a transfection assay in vivo and a transcription assay in vitro. Second, the gadd153/Chop10 promoter contains two functionally important binding sites for ATF3: an AP-1 site and a C/EBP-ATF composite site, a previously unidentified binding site for ATF3. The absence of either site reduces the ability of ATF3 to repress the promoter. Third, overexpression of ATF3 by transient transfection results in a reduction of the endogenous gadd153/Chop10 mRNA level. Fourth, as described previously, ATF3 is induced in the liver upon CCl_4 treatment. Intriguingly, we show in this report that gadd153/Chop10 mRNA is not present in areas where ATF3 is induced. Taken together, these results strongly suggest that ATF3 represses the expression of *gadd153/Chop10*. The mutual negative regulation between ATF3 and gadd153/Chop10 is discussed.

All cells exhibit alterations in gene expression under adverse environmental conditions, such as elevated temperature, lack of nutrients, and exposure to toxins (for a review, see reference 21). Because these stress signals can cause cellular damage, biologists have long sought to elucidate the molecular events involved in stress responses. Many lines of evidence indicate that stress signals initiate a cascade of phosphorylation events which in turn regulate the expression of a set of genes, termed immediate-early genes (for a review, see reference 23). Many immediate-early genes identified thus far encode transcription factors. Therefore, the response to stress signals entails a series of transcriptional regulations.

Recently, we demonstrated that ATF3, a transcriptional repressor, is induced by many physiological stress signals. By in situ hybridization, we demonstrated that the mRNA level of ATF3 greatly increases upon exposure to a variety of stresses: seizure, toxic chemicals, mechanical injury, ischemia, and ischemia coupled with reperfusion (ischemia-reperfusion) (7, 47). In each case, ATF3 is induced in the corresponding tissue that is exposed to the stress signal: it is induced in the brain under seizure, in the liver upon intoxication or hepatectomy, in the heart upon myocardial ischemia or ischemia-reperfusion (7), and in the kidney upon renal ischemia-reperfusion (47). Therefore, the induction of ATF3 is a common cellular response to many stress signals: it is neither tissue specific nor stress specific. This nonspecific induction by different stress signals in different tissues also applies to other immediate-early genes. Thus far, the best-characterized immediate-early genes are c-*fos* and c-*jun*. They have been demonstrated to be induced by many stress signals in a variety of tissues, such as seizure and ischemia in the brain (11, 35); ischemia and ischemia-reperfusion in the heart (for a review, see reference 9); mechanical and chemical injury in the liver (8); and axonal injury of the optic nerve (33), the sciatic nerve (13), and spinal motor neurons (45). Therefore, the initial genome response to stress signals appears to turn on a set of common genes, irrespective of the nature of the signals or the nature of the cell types that are exposed to the signals. Because these genes encode transcription factors, many questions arise. What are the target promoters of these gene products? Do they regulate the same sets or distinctive sets of genes in different tissues? Are there any tissue-specific responses to stress signals?

As a first step toward answering these questions, we sought to elucidate the target genes of ATF3. The following observations prompted us to hypothesize that one potential target gene for ATF3 is *gadd153/Chop10*. *gadd153/Chop10* encodes a transcription factor containing the basic region-leucine zipper (bZip) domain (14, 15, 34). It was first isolated from a hamster library by subtractive hybridization based on its ability to be induced by UV irradiation (14). It was subsequently shown to be induced by many signals including growth arrest, DNA damage (15), calcium ionophore (4), glucose deprivation (5), endoplasmic reticulum stress (44), and activation of the acute phase responses in vitro (39). Recently, we isolated a cDNA clone encoding gadd153/Chop10 by using its ability to interact with ATF3 (7). We also showed that the ATF3-gadd153 heterodimer fails to bind to the ATF consensus site and several ATF-related sites (7). These results suggest that gadd153/Chop10 inhibits ATF3 in functioning on the ATF or ATF-related sites. Interestingly, in the liver upon carbon tetrachloride (CCl_4) intoxication, gadd153/Chop10 and ATF3 are expressed in a reciprocal manner: the gadd153/Chop10 mRNA level greatly decreases whereas the ATF3 mRNA level greatly increases (7). Because ATF3 is a transcription repressor (6), and because the promoter of gadd153 contains an AP-1 site (29), a potential binding site for ATF3 (17), this reciprocal expression raises the possibility that ATF3 may repress the

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expression of gadd153/Chop10. In this report, we present evidence supporting the notion that *gadd153/Chop10* is a potential target gene of ATF3.

MATERIALS AND METHODS

Plasmids. Human ATF3 and ATF4 cDNAs were inserted into the following vectors for expression: pCG (40) for expression in mammalian cells and pTM1 (30) for expression in reticulocyte lysate or mammalian cells with T7 polymerase. p9000 (29), p9060 (16), and p9005 contain the chloramphenicol acetyltransferase (CAT) reporter driven by different regions of the hamster gadd153/Chop10 promoter: p9000 (−787 to +21), p9060 (−250 to +21), and p9005 (−225 to +21). pCA211B, a pGH56 derivative, contains the −454 to −141 region of the gadd153/Chop10 promoter. gadd153-CAT(ΔAP-1), generated by joining the appropriate promoter fragments together, contains the −810 to +21 region of the gadd153/Chop10 promoter with the −278 to −225 sequences deleted. gadd153-CAT(mC/EBP-ATF), generated by a four-primer-based PCR to introduce mutations, contains the −810 to +21 region of the gadd153/Chop10 promoter with the C/EBP-ATF site mutated from ATTGCATCA to CAGATCTCA (underline indicates the introduced *Bgl*II site). pC/EBP-ATF was generated by inserting the following double-stranded oligonucleotide (with the *Bam*HI compatible ends) into the *Bam*HI site of pGEM3: 5′-GATCCGGTTGCCAAACATTGCATCATCCA-3′ and 5′-GATCTGGATGATGCAATGTTTGGCAACCG-3′. The −342 to −319 region of the gadd153/Chop10 promoter, which contains the C/EBP–AP-1 site, is underlined.

Transfection and CAT assay. Calcium phosphate transfections and CAT assays were carried out as described previously (6). For all repression experiments, 3 μg of reporter and 4 μg of effector DNAs were used. CAT activity was measured by a phase extraction method (1).

In vitro transcription. In vitro transcription using crude nuclear extracts was carried out as described previously (18). The CAT primer, 5′-GCCATTGGGATATATCAACGG-3′, is complementary to the +29 to +49 region of the CAT mRNA. Nuclear extracts were made from HeLa cells according to the method described in reference 10. In each reaction, an end-labeled *Eco*RI-*Hin*dIII polylinker fragment (55 bp) was added to the RNA sample to serve as a recovery control for the procedures.

DNase I footprinting and Maxam-Gilbert sequencing. DNase I footprinting was carried out as described previously (26) with histidine-tagged ATF3 (His-ATF3) purified from the vaccinia virus expression system and the *Bam*HI/*Pst*I fragment of pCA211B, which contains the −454 to −141 region of the gadd153/Chop10 promoter. The amount of His-ATF3 was empirically determined by titration of each batch of protein. Maxam-Gilbert sequencing was performed according to the method described in reference 36.

Expression of ATF3 with vaccinia virus. One liter of suspended HeLa cells (~10^6 cells/ml) was collected by centrifugation at room temperature for 15 min at 2,000 × *g*. The cell pellet was resuspended in 30 ml of minimal essential medium (Life Technologies, Inc.) and coinfected with vaccinia virus that encodes T7 polymerase (a gift from B. Moss) and virus that encodes His-ATF3 (10 PFU of each virus per cell). The mixture was incubated at 37°C for 2 h with gentle stirring to allow infection. The infected cells were then diluted with minimal essential medium to 5×10^5 cells/ml and incubated for 48 h at 37°C. The cells were collected as before and resuspended in 20 ml of Ni-nitrilotriacetic acid (NTA) buffer A (6 M guanidine-HCl, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0), stirred at 4°C for 30 min, sonicated, and spun at 4°C for 20 min at 18,000 × *g* to remove the insoluble debris. The supernatant was loaded on a 5-ml Ni-NTA agarose column (Qiagen) followed by sequential washes with 25 to 50 ml of Ni-NTA buffer B (8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0) and Ni-NTA buffer C (same as buffer B, except pH 6.3). His-ATF3 was eluted off the column by Ni-NTA buffer D (same as buffer B, except pH 5.0), supplemented with bovine serum albumin to a final protein concentration of 1 mg/ml, and dialyzed at 4°C against a series of buffers (0.1 M NaCl, 20 mM HEPES [pH 7.5], 10% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol) containing various concentrations of urea to renature the protein: 1, 0.1, and 0 M urea for 2 to 3 h each time. Any insoluble material was removed by centrifugation, and the soluble protein was frozen by liquid nitrogen and stored at −80°C until use.

In vitro protein synthesis and electrophoresis mobility shift assay (EMSA). ATF3 was synthesized by the TNT reticulocyte lysate system (Promega) with the pTM1 derivative. The binding reaction was carried out as described previously (19). The *Cla*I/*Hin*dIII fragment of p9060, which contains the −250 to +21 region of the gadd153/Chop10 promoter, was used as the AP-1 site. The *Eco*RI/*Hin*dIII fragment of pC/EBP-ATF, which contains the −342 to −319 region of the gadd153/Chop10 promoter, was used as the C/EBP-ATF site.

Primer extension. Primer extension was carried out as described previously (6) with the following modifications. HepG2 cells (10-cm-diameter plate) were transfected with 16 μg of the indicated expression plasmid with Lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. Total RNA was isolated with Trizol (Life Technologies, Inc.) according to the manufacturer's instructions. The gadd153/Chop10 primer, 5′-TCTGATTTTGGCTCTGTCGCTCG-3′, is complementary to the +52 to +74 region of the gadd153/Chop10 mRNA. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer, 5′-ATCCGTTGACTCCGACCTTC-3′, is complementary to the +31 to +51 region of the GAPDH mRNA. In each reaction, an end-labeled *Eco*RI/*Xba*I fragment (100 bp) was mixed with the RNA sample to serve as a recovery control for procedures. Extended products were resolved on a urea-polyacrylamide gel and analyzed by autoradiography.

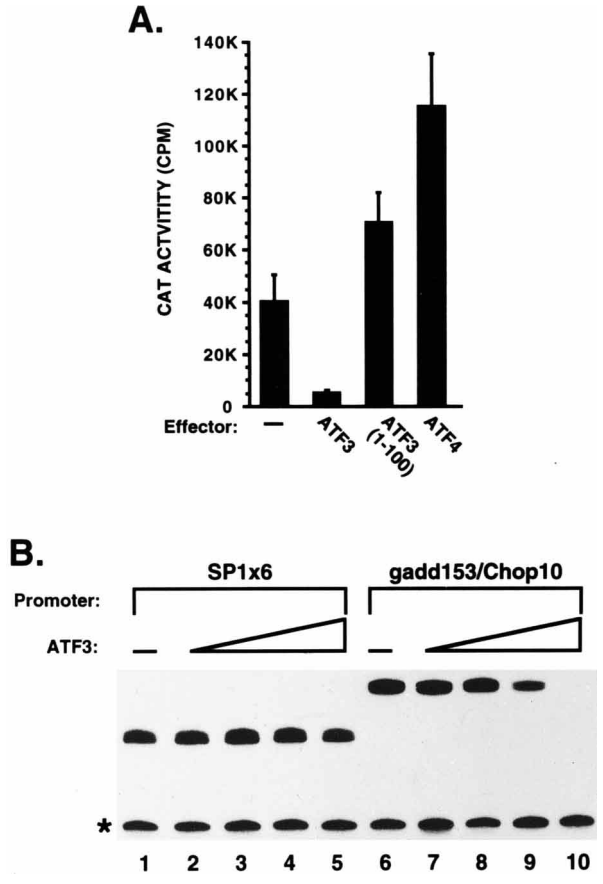

FIG. 1. ATF3 represses transcription of the gadd153/Chop10 promoter in vivo and in vitro. (A) A CAT reporter driven by the gadd153/Chop10 promoter was cotransfected into HeLa cells with plasmids expressing ATF3, ATF3(1-100), or ATF4. The vector (pCG) carrying the cytomegalovirus promoter was included in some experiments to ensure that each transfection mix contained the same amount of promoter. The average CAT activities from five experiments are shown. (B) In vitro transcription of the CAT reporter driven by the gadd153/Chop10 promoter (lanes 6 to 10) or six tandem SP1 sites (lanes 1 to 5) in the absence (lanes 1 and 6) or presence of increasing amounts (1, 2, 3, and 4 μl) of His-ATF3 purified from the vaccinia virus expression system (lanes 2 to 5 and 7 to 10). CAT mRNAs were analyzed by primer extension. The asterisk indicates a 55-bp labeled DNA fragment that serves as a recovery control for various manipulations.

Animal experiments. All procedures were performed at Zivic-Miller Laboratories, Inc. (Zelienople, Pa.). Eight-week-old male Sprague-Dawley rats were lightly anesthetized with ether and intragastrically injected with either 0.08 ml of saline or 0.08 ml of 100% carbon tetrachloride (CCl_4), a dose that causes death in 20% of rats (32). The rats were sacrificed 1.5 h later, and the livers were collected and frozen by the isopentane method (22).

In situ hybridization and analysis of ATF3 protein in crude liver extracts. Assays were carried out as described previously (6, 7).

RESULTS

ATF3 represses transcription from the gadd153/Chop10 promoter. To test whether ATF3 represses the transcription of the gadd153/Chop10 promoter, we cotransfected into HeLa cells a plasmid expressing ATF3 (pCG-ATF3) with a plasmid containing a CAT reporter driven by the gadd153/Chop10 promoter. Figure 1A shows the results from five experiments: ATF3 repressed the transcription of the gadd153/Chop10 pro-

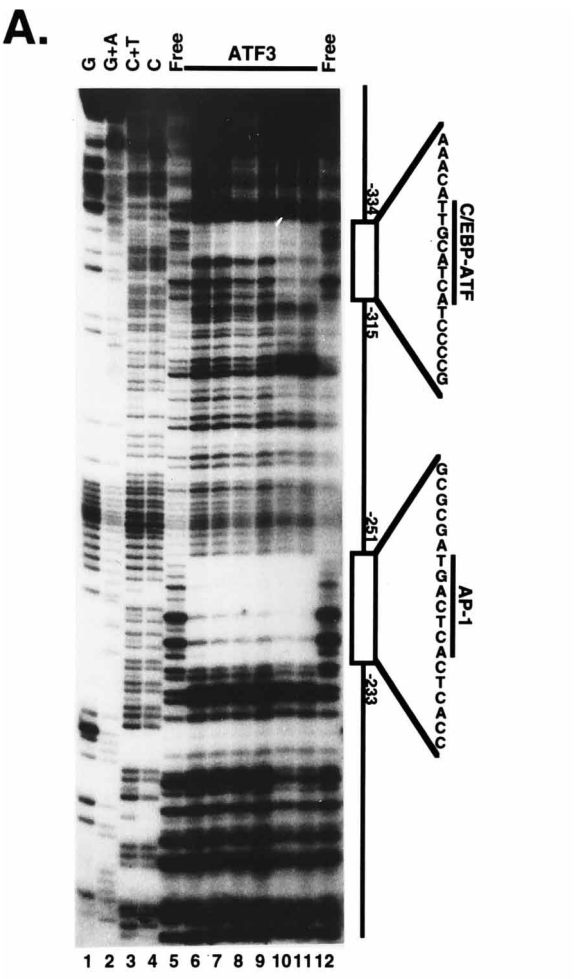

FIG. 2. ATF3 binds to the AP-1 and the C/EBP-ATF composite sites in the gadd153/Chop10 promoter. (A) DNase I footprint analysis of the gadd153/Chop10 promoter. The end-labeled promoter fragment (−141 to −40) was analyzed in the absence (lanes 5 and 12) or presence (lanes 6 to 11) of His-ATF3 (5 μl) purified from the vaccinia virus expression system with different amounts of poly(dI · dC): 1 (lanes 6 and 7), 3 (lanes 8 and 9), and 5 (lanes 10 and 11) μg. Arrow, transcription start site (+1). Maxam-Gilbert sequence reactions of the free probe are shown in lanes 1 to 4. (B) A portion of the gadd153/Chop10 promoter sequence is shown, and the regions protected by ATF3 are indicated by stippled boxes.

moter eightfold. This repression was dependent on the DNA-binding activity of ATF3, because ATF3(1-100), a truncated form of ATF3 which lacks the bZip DNA-binding domain, did not repress this promoter. The slight activation by ATF3(1-100) is consistent with our previous observation that ATF3 may sequester inhibitory cofactors away from the promoter when not bound to the promoter (6). Furthermore, the repression of the gadd153/Chop10 promoter by ATF3 was not a general characteristic of the ATF/CREB family of transcription factors, because ATF4, a *trans*-activator (27), activated the transcription of this promoter.

The repression of the gadd153/Chop10 promoter by ATF3 can be recapitulated in an in vitro transcription assay (Fig. 1B): increasing amounts of recombinant ATF3 repressed the activity of the gadd153/Chop10 promoter. In a control experiment, a nonspecific promoter composed of six tandem SP1 sites was not inhibited by ATF3. Furthermore, ATF1, another member of the ATF/CREB family of transcription factors, did not repress the gadd153/Chop10 promoter (data not shown). Therefore, we conclude that ATF3 represses the transcription of the gadd153/Chop10 promoter both in vitro and in vivo.

ATF3 binds to the AP-1 site and the C/EBP-ATF composite site of the gadd153/Chop10 promoter. To investigate the regions recognized by ATF3, we performed DNase I footprint analysis of the gadd153/Chop10 promoter with ATF3 purified from a vaccinia virus expression system. Figure 2A shows that ATF3 protected two regions, the −240 and the −320 regions. The −240 region contains a consensus AP-1 site, a potential binding site for ATF3 (17). The −320 region contains the sequence TTGCATCA, a previously unidentified binding site for ATF3. Close inspection of the sequence reveals that the site is similar to both the C/EBP consensus (TTGCGCAAT) and the ATF/CRE-like sequence (TGACATCA). For the convenience of discussion, we will refer to this site as a C/EBP-ATF composite site. We note that protection of this site by ATF3 was enhanced by the presence of nonspecific competitor DNA (Fig. 2A, lanes 6 to 11); the reasons for this enhancement remain to be determined. Figure 2B summarizes the results.

To determine whether ATF3 can bind to the AP-1 site and the C/EBP-ATF composite site individually, we performed EMSA with DNA fragments containing either the AP-1 site or the composite site from the gadd153/Chop10 promoter. As shown in Fig. 3, and ATF3 bound to each site individually and specifically: ATF3 bound to each fragment and the binding was competed by unlabeled ATF consensus sequence (lanes 3 to 5) but not by a nonspecific sequence (lanes 6 to 8); in addition, unprogrammed reticulocyte lysate did not result in any binding (data not shown). The presence of ATF3 in the protein-DNA complex was indicated by the supershift of the complex by an antibody against ATF3 (lane 9) but not by a nonspecific antibody (lane 10).

Taken together, the footprint and EMSA results indicate that ATF3 can bind to the AP-1 and the C/EBP-ATF composite sites on the gadd153/Chop10 promoter. Significantly, binding of ATF3 to these two sites on the gadd153/Chop10 promoter renders the DNA between these two sites more sensitive to DNase I (Fig. 2A), suggesting a conformational change of the DNA between the binding sites. However, further studies

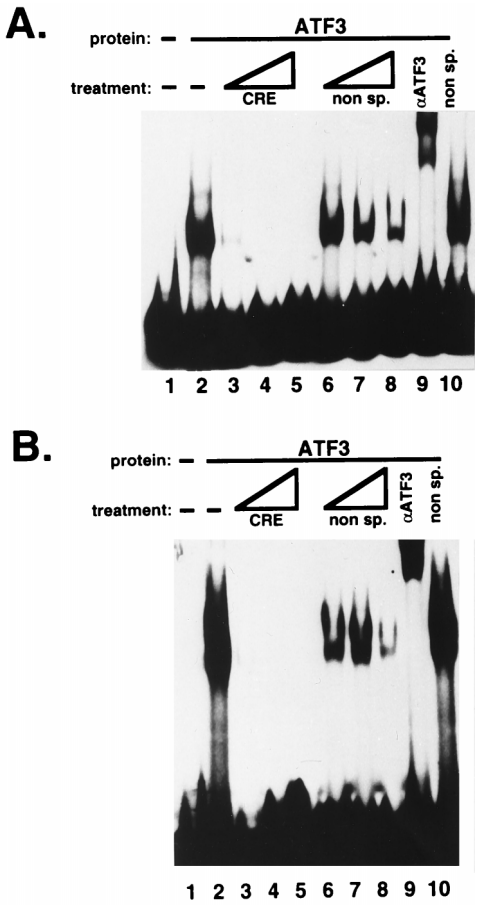

FIG. 3. ATF3 binds to the AP-1 and C/EBP-ATF sites independently of each other. (A) ATF3 binds to the AP-1 site. A radiolabeled DNA fragment containing the AP-1 site from the gadd153/Chop10 promoter (−250 to +21) was analyzed by EMSA in the absence (lane 1) or presence (lanes 2 to 10) of ATF3 generated by reticulocyte lysate (2 μl). Competition experiments were carried out with increasing amounts of DNA fragment containing the ATF/CRE consensus sequence (TGACGTCA) (lanes 3 to 5) or a nonspecific sequence derived from the multicloning region of pGEM3 (lanes 6 to 8). The protein-DNA complex was shifted by the anti-ATF3 antibody (lane 9) but not by a nonspecific antibody (lane 10). (B) ATF3 binds to the C/EBP-ATF composite site. A radiolabeled DNA fragment containing the C/EBP-ATF composite site from the gadd153/Chop10 promoter (−343 to −322) was analyzed by EMSA as described for panel A.

are required to elucidate the mechanisms for this DNase I hypersensitivity.

Both the AP-1 and the C/EBP-ATF composite sites are important for efficient repression of the gadd153/Chop10 promoter by ATF3. To test whether the AP-1 site and the C/EBP-ATF composite sites are functionally important, we examined the ability of ATF3 to repress transcription on mutant promoters. Figure 4A shows the constructs used in this experiment, and Fig. 4B shows the results from five experiments. ATF3 failed to repress the −225 construct, indicating that the region upstream from −225, which contains both C/EBP-ATF and AP-1 sites, is necessary for the repression. To delineate the importance of each binding site, we first deleted the AP-1 site. The lower activity of the mutant promoter is consistent with previous reports that the AP-1 site is important for the overall promoter activity (16, 39). Although ATF3 still repressed this promoter, it was not as efficient (fourfold, compared to eightfold repression on the wild-type promoter), indicating that the AP-1 site is required for efficient repression. In a complementary experiment, we mutated the C/EBP-ATF site. Similar to the AP-1 deletion, this mutant promoter has a lower activity. However, addition of ATF3 did not result in any obvious repression of this promoter. Taken together, these results indicate that both the C/EBP-ATF site and the AP-1 site are important for the efficient repression of the gadd153/Chop10 promoter by ATF3. However, the C/EBP site appears to play a more important role than the AP-1 site, because the C/EBP mutation had a greater effect than the AP-1 deletion. A similar result was observed in another model system with PC12 cells (11a).

Overexpression of ATF3 results in a reduced level of endogenous gadd153/Chop10 mRNA in HepG2 cells. The above results suggest that ATF3 can bind to the gadd153/Chop10 promoter and repress its activity. However, the promoter used in the in vivo transfection and in vitro transcription assays contains the −778 to +21 region of the gadd153/Chop10 promoter; it is not clear whether ATF3 can repress the endogenous gadd153/Chop10 promoter. To address this issue, we transfected a plasmid expressing ATF3 (pCG-ATF3) into HepG2 hepatocytes and examined the endogenous gadd153/Chop10 mRNA level by primer extension. As shown in Fig. 5, the level of endogenous gadd153/Chop10 mRNA was readily detectable in control HepG2 cells transfected with pGEM3

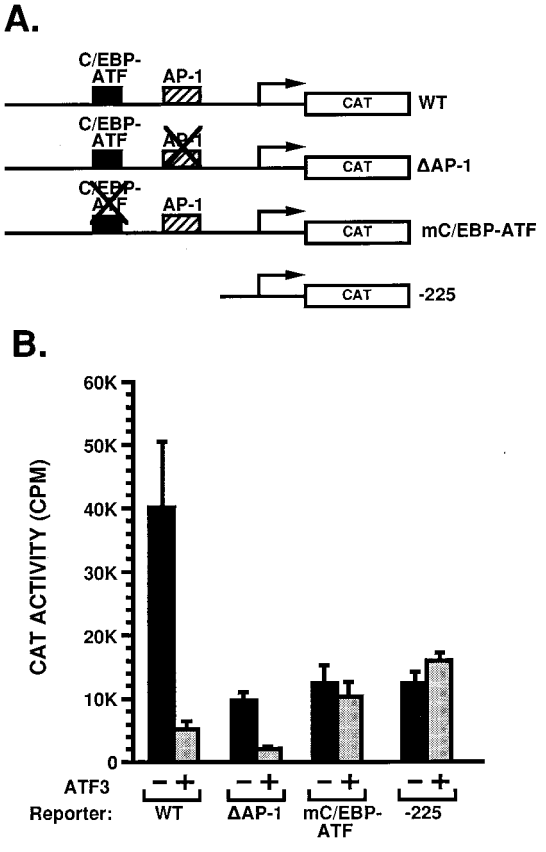

FIG. 4. The AP-1 and the C/EBP-ATF sites are required for the efficient repression of the gadd153/Chop10 promoter by ATF3. (A) Schematic representation of the CAT reporters used in panel B. (B) HeLa cells were transfected with CAT reporters driven by the wild-type (WT) or mutant gadd153/Chop10 promoters in the absence (−) or presence (+) of DNA expressing ATF3 (pCG-ATF3). The pCG vector was included in the experiment without the DNA to ensure that each transfection mix contained the same amount of promoter. The average CAT activities from five experiments are shown.

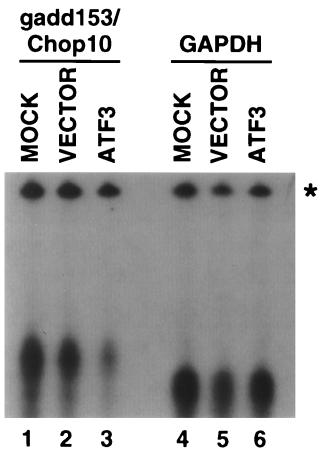

FIG. 5. Overexpression of ATF3 reduces the level of the endogenous gadd153/Chop10 mRNA in HepG2 cells. HepG2 cells were transfected with pGEM3 (mock [lanes 1 and 4]), pCG (vector [lanes 2 and 5]), or pCG-ATF3 (ATF3 [lanes 3 and 6]). The levels of gadd153/Chop10 mRNA (lanes 1 to 3) and GAPDH mRNA (lanes 4 to 6) were determined by primer extension. The asterisk indicates a 100-bp labeled DNA fragment that serves as a recovery control for various manipulations. Signals in each lane were standardized and calculated as described in the text. In three separate experiments, ATF3 reduced the level of gadd153/Chop10 mRNA from two- to fourfold. Shown is the fourfold reduction.

(mock) or pCG vector (lanes 1 and 2). Significantly, the level of gadd153/Chop10 mRNA was lower in cells transfected with pCG-ATF3 than in the control cells (lane 3 versus lanes 1 and 2). The fold of repression was calculated by first standardizing the gadd153/Chop10 signal against the recovery control (indicated by an asterisk) in each lane. The recovery control represents a radiolabeled DNA fragment added to the mRNA preparation before primer extension; thus it controls for sample recovery after various manipulations. The resulting gadd153/Chop10 signal was then divided by the standardized internal control GAPDH signal, which controls for the quantity and the quality of mRNA, to give rise to the true signals. The calculated reduction of gadd153/Chop10 true signals in cells transfected with ATF3 versus those in cells transfected with pCG vector ranged from two- to fourfold in three separate experiments. Importantly, this range of repression underrepresents the real repression, because less than 50% of cells were transfected, as indicated by immunofluorescent analysis of ATF3 protein in a parallel experiment (data not shown). Therefore, expression of the endogenous gadd153/Chop10 in more than 50% of the cells stayed unaffected, resulting in a less apparent repression.

Complementary expression of ATF3 and gadd153/Chop10 in rat liver. The above primer extension result indicates that overexpression of ATF3 reduces the level of endogenous gadd153/Chop10 mRNA in cultured hepatocytes. To examine whether this also occurs in the liver, we treated rats with carbon tetrachloride (CCl_4), which induces ATF3 in the liver (7), and examined the mRNA levels of both ATF3 and gadd153/Chop10 by in situ hybridization. Figure 6C shows that ATF3 mRNA was readily detectable in areas immediately around central veins (central zone) and areas further out (midzone) at 1.5 h after CCl_4 treatment. As described previously (7), ATF3 mRNA was not detectable in any areas in the saline-treated liver (Fig. 6A). In contrast, gadd153/Chop10 mRNA was

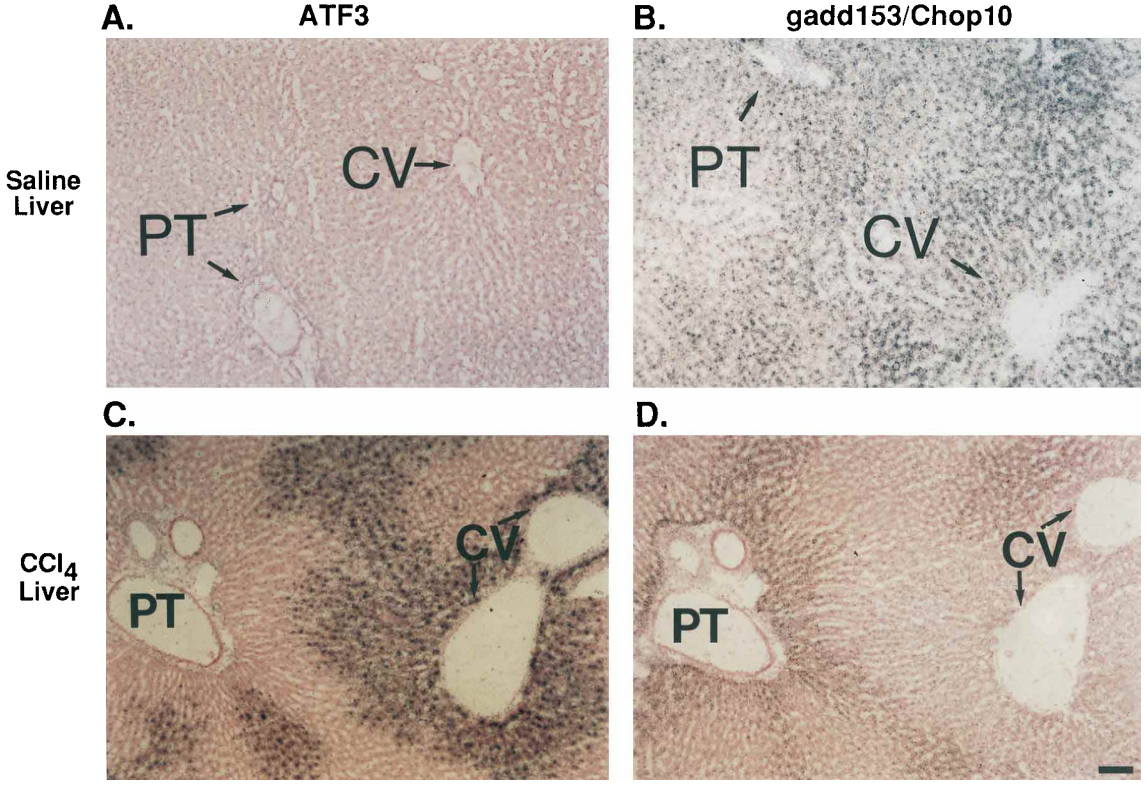

FIG. 6. Complementary expression of ATF3 and gadd153/Chop10 in rat livers treated with CCl_4. Liver sections from rats treated with saline (A and B) or CCl_4 for 1.5 h (C and D) were analyzed by in situ hybridization with ATF3 (A and C) or gadd153/Chop10 (B and D) antisense RNA as a probe. Panels C and D represent adjacent tissue sections. The signals were detected by autoradiographic emulsion with a 10-day exposure. Pictures were generated by bright-field photography, which gives black signals on hematoxylin- and eosin-stained sections (bar = 80 μm). CV, central vein. PT consist of the portal vein, artery, and bile duct. The hepatocytes in the lobule are arbitrarily divided into zones: central (around central vein), mid-, and periportal (around PT) zones.

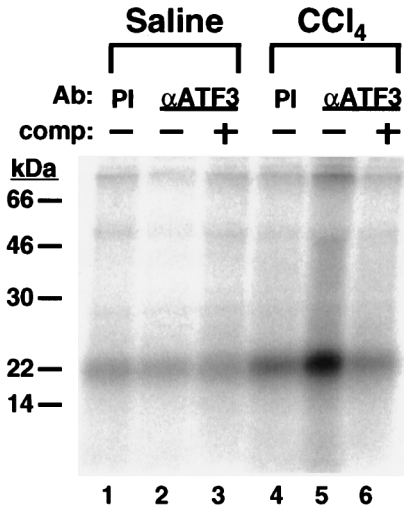

FIG. 7. ATF3 protein was present in the liver at 1.5 h after CCl_4 treatment but not in saline-treated liver. Equal amounts of liver nuclear extracts derived from saline-treated (lanes 1 to 3) or 1.5-h CCl_4-treated (lanes 4 to 6) rats were partially purified with a phosphocellulose column. The 0.5 M salt eluate which contained ATF3 was radiolabeled with heart muscle kinase in the presence of [γ-^{32}P]ATP and immunoprecipitated with a preimmune antibody (PI; lanes 1 and 4) or affinity-purified ATF3 antibody in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of an excess amount of cold ATF3. Pellets from immunoprecipitation were resolved on a sodium dodecyl sulfate–15% polyacrylamide gel and analyzed by a PhosphorImager. Size markers in kilodaltons are indicated on the left. Ab, antibody; comp, competitor.

readily detectable in the normal liver throughout the liver lobule (Fig. 6B) but greatly decreased upon CCl_4 treatment (7). As shown in Fig. 6D, at 1.5 h after treatment gadd153/Chop10 mRNA, although still detectable, was much reduced and restricted to areas around portal triads (PT) (periportal zone). Intriguingly, the areas expressing gadd153/Chop10 mRNA are precisely complementary to the areas expressing ATF3: Fig. 6C and D show a representative result of adjacent tissue sections from three experiments.

To make sure that ATF3 protein was synthesized when its mRNA was induced in the CCl_4-treated liver, we examined ATF3 protein as follows. We partially purified ATF3 from liver extract by a phosphocellulose column, radiolabeled ATF3 in vitro with the catalytic subunit of protein kinase A, and immunoprecipitated ATF3 by affinity-purified anti-ATF3 antibodies. As shown in Fig. 7, ATF3 protein was detectable in CCl_4-treated liver (lane 5) but not in saline-treated liver (lane 2). The weak band in lane 2 was due to nonspecific immunologic reactions because preimmune serum gave rise to a band of similar intensity (lanes 1 and 4). Furthermore, the signal in lane 5 was competed off by excess amounts of cold ATF3 protein (lane 6), confirming the specificity of the band. Therefore, we conclude that ATF3 protein was produced in the liver when its mRNA was induced. Although many events happen upon CCl_4 treatment, in light of the results of DNA binding, transcription, transfection, and primer extension (presented above), the best explanation for the complementary expression pattern of ATF3 and gadd153/Chop10 is that ATF3 represses the expression of gadd153/Chop10. Therefore, we conclude that *gadd153/Chop10* is a potential target gene of ATF3.

DISCUSSION

The C/EBP versus ATF families of proteins. In this report, we demonstrate that the gadd153/Chop10 promoter contains a previously unidentified ATF3 binding site: the C/EBP-ATF composite site. ATF and C/EBP are both bZip families of proteins and were considered to be distinct from each other because (i) they were thought to bind only to their respective binding sites and (ii) there was no evidence indicating any heterodimer formation between these two families of proteins. Recent evidence, however, indicates that members of these two families can bind to the consensus binding sites of the other family (for an example, see reference 41) and that they can form heterodimers with each other (for examples, see references 7, 42, and 43). Therefore, a much greater interplay exists between these two families of proteins than previously expected. The presence of the C/EBP-ATF composite site and the finding that ATF3 binds to this site further reinforce this notion. In contrast, some ATF proteins such as ATF1 and ATF3, despite sharing the name ATF, do not form heterodimers (19). Furthermore, most ATF proteins are quite different from each other in their amino acid sequences; the only similarities they have are the conserved residues in the basic region and the leucine zipper motif. These ATF proteins are as different from each other as they are from C/EBP proteins. Therefore, the names of these bZip proteins reflect the history of discovery but not necessarily their similarity.

A potential target promoter of ATF3. As described in the introduction, ATF3 is induced in a variety of tissues by different stress signals (7). Because ATF3 is a transcription factor, it is necessary to identify its target promoters in order to understand the physiological significance of this induction. Results described in this report suggest that *gadd153/Chop10* is a potential target of ATF3. First, ATF3 represses a CAT reporter gene driven by the gadd153/Chop10 promoter as evidenced by both in vivo transfection assay and in vitro transcription assay. Second, the gadd153/Chop10 promoter contains binding sites for ATF3 as shown by EMSA and footprint analysis. Third, these binding sites are functionally important, because mutant promoters with either site deleted are not as efficiently suppressed by ATF3 as the wild-type promoter. Fourth, overexpression of ATF3 in HepG2 hepatocytes decreased the level of the endogenous gadd153/Chop10 mRNA as assayed by primer extension. In light of the results from DNA binding, transcription, and transfection, we favor the idea that overexpression of ATF3 decreases the level of gadd153/Chop10 mRNA by suppressing the transcription of the gadd153/Chop10 promoter.

The precise complementarity between the expression pattern of ATF3 and that of gadd153/Chop10 provides a compelling argument for the ability of ATF3 to repress gadd153/Chop10 expression in a physiological setting. It is reminiscent of the stripe formation in *Drosophila* embryos. Many lines of evidence indicate that in *Drosophila* embryos transcriptional repressors such as even-skipped, hairy, and Krüppel turn off the expression of their target genes in segments in which they themselves are expressed (for a review, see reference 31), resulting in a complementary expression between these repressor genes and their target genes. Therefore, the complementary expression of ATF3 and gadd153/Chop10 is consistent with the notion that gadd153/Chop10 is a target of ATF3, a transcriptional repressor. To further confirm this notion, we are currently making stable cell lines and transgenic mice expressing ATF3 in an inducible manner, in order to test whether gadd153/Chop10 is turned off when ATF3 is turned on.

In this context, it is important to note that, in the normal liver, gadd153/Chop10 is expressed at a higher level relative to ATF3 throughout the liver as indicated by in situ hybridization (Fig. 6) and Northern blot analysis (7). Time course analysis indicates that the level of gadd153/Chop10 mRNA starts to decrease at 30 min after CCl_4 treatment when ATF3 mRNA is

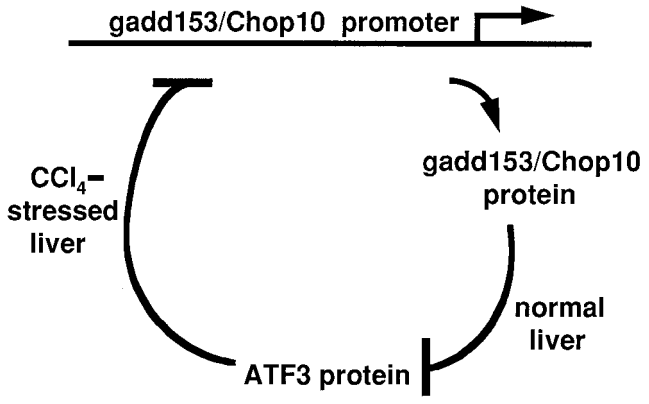

FIG. 8. A schematic model of mutual negative regulation between ATF3 and gadd153/Chop10. In the normal liver, the concentration of ATF3 is not readily detectable, while that of gadd153/Chop10 is. Under this condition, gadd153/Chop10 inhibits the function of any basal amounts of ATF3; therefore, expression of its own gene is not subjected to inhibition by ATF3. Upon stress induction, the concentration of ATF3 increases, resulting in a shift of the equilibrium and the formation of ATF3 homodimers, which in turn maintains or augments the repression of the *gadd153/Chop10* gene initiated by another factor(s).

not yet readily detectable (data not shown). This observation suggests that some factor(s) other than ATF3 initiates the repression of the *gadd153/Chop10* gene, and ATF3 probably maintains or further augments the repression. Consistent with this notion, at 1.5 h post-CCl_4 treatment, the level of gadd153/Chop10 mRNA around PT is noticeably lower than that in the normal liver. Because ATF3 is not induced around PT (Fig. 6C), the reduction of the gadd153/Chop10 mRNA level in this area also suggests that some factor(s) other than ATF3 initiates the repression of gadd153/Chop10.

The mutual negative regulation between ATF3 and gadd153/Chop10. Previously, we demonstrated that gadd153/Chop10 can functionally inhibit ATF3 by forming a heterodimer that does not bind to the ATF or ATF-related site (7). Therefore, ATF3 and gadd153/Chop10 appear to mutually inhibit each other (Fig. 8): gadd153/Chop10 inhibits the function of ATF3, whereas ATF3 inhibits the expression of gadd153/Chop10. This mutual inhibition allows fine-tuning of gene expression based on the relative concentrations of the molecules. As an example, in the normal liver the concentration of ATF3 is not detectable, while that of gadd153/Chop10 is (7). We suspect that under this condition gadd153/Chop10 inhibits the function of any basal amounts of ATF3; therefore, expression of its own gene is not subjected to inhibition by ATF3. Upon stress induction, the concentration of ATF3 increases (7). Although the signal transduction pathways for this induction are not well defined currently, one potential pathway is the JNK/SAPK pathway (28). An increase of ATF3 concentration will shift the equilibrium, resulting in the formation of ATF3 homodimers, which as proposed above can either maintain or augment the repression of the *gadd153/Chop10* gene initiated by another factor(s).

The ability of transcription factors to regulate the expression of their own inhibitors is a common strategy for gene regulation and has been observed in many cases, although the details may vary. As an example, p53 up-regulates the expression of the *mdm-2* gene (3, 46), which encodes a protein that promotes the degradation of p53 protein (20, 24). Therefore, p53 activates the expression of its own inhibitor, contributing to the maintenance of low p53 concentration in normal cells (24); at the same time, *mdm-2* promotes the degradation of its own transcriptional activator, thus autoregulating its gene expression (46). Another example is the ability of NF-κB to activate the expression of IκB (25, 37, 38), an inhibitor of NF-κB (for a review, see reference 2). The situation between ATF3 and gadd153/Chop10 differs from these two cases in that ATF3 is a transcriptional repressor which negatively, instead of positively, regulates the expression of its own inhibitor.

Clearly, the model proposed here requires further investigation. In addition, more work is needed to find out whether the reciprocal expression of ATF3 and gadd153/Chop10 is a tissue- and/or stress-specific event. Numerous previous studies have shown that gadd153/Chop10 is itself stress inducible (4, 5, 12, 15, 16, 29, 39, 44). As an example, gadd153/Chop10 expression is markedly elevated in rat liver during the acute phase response to lipopolysaccharide treatment (39). These results appear to contradict our current data that gadd153/Chop10 expression decreases, rather than increases, after CCl_4 treatment. One explanation for this apparent discrepancy is that gadd153/Chop10 responds differently to different stresses. Another explanation is that induction of gadd153/Chop10 by stress signals usually occurs over a longer time course relative to the repression of gadd153 by CCl_4. In the lipopolysaccharide experiment, gadd153/Chop10 induction did not occur until 8 h following treatment. Therefore, it is possible that the gadd153/Chop10 level could decrease at the initial stage of the stress response and increase later. Consistent with this notion, we have recently observed an initial decrease followed by an increase in gadd153/Chop10 expression after arsenite treatment (11a). Analyses of the gadd153/Chop10 promoter indicated that both the AP-1 site and the C/EBP-ATF site (via interaction with C/EBPβ) are functionally important for its induction by stress signals (12, 16, 39). The finding described in this report that ATF3 also binds to these promoter sites to inhibit gadd153/Chop10 expression points to a complicated network of gene regulation involving multiple protein complexes binding at similar sites. Further investigation is needed to delineate the components of these regulatory complexes and how they might change with various stresses, with time following treatments, and with different cell types.

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