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Analysis of ATF3, a Transcription Factor Induced by Physiological Stresses and Modulated by gadd153/Chop10

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We demonstrate that ATF3, a member of the ATF/CREB family of transcription factors, is induced in a variety of stressed tissues: mechanically injured liver, toxin-injured liver, blood-deprived heart, and postseizure brain. We also demonstrate that an ATF3-interacting protein, gadd153/Chop10, forms a nonfunctional heterodimer with ATF3: the heterodimer, in contrast to the ATF3 homodimer, does not bind to the ATF/cyclic AMP response element consensus site and does not repress transcription. Interestingly, ATF3 and gadd153/Chop10 are expressed in inverse but overlapping manners during the liver's response to carbon tetrachloride (CCl_4): the level of gadd153/Chop10 mRNA is high in the normal liver and greatly decreases upon CCl_4 treatment; the level of ATF3 mRNA, on the other hand, is low in the normal liver and greatly increases upon CCl_4 treatment. We hypothesize that in nonstressed liver, gadd153/Chop10 inhibits the limited amount of ATF3 by forming an inactive heterodimer with it, whereas in CCl_4-injured liver, the synthesis of gadd153/Chop10 is repressed, allowing the induced ATF3 to function.

Transcriptional regulation plays an important role in homeostasis. All cells exhibit changes in gene expression in response to extracellular signals such as peptide hormones, growth factors, cytokines, and stress signals (for a recent review, see reference 27). Differential screening of cDNA libraries has identified a set of genes, called immediate-early genes, that are induced by a variety of signals (for a review, see reference 26). Induction of these genes occurs in the absence of protein synthesis and requires only the modification of preexisting transcription factors. Importantly, many of these gene products are themselves transcription factors. Therefore, the response to extracellular signals entails, in many cases, a cascade of transcriptional regulations.

Responses to stress signals have long intrigued biologists, partly because some stress responses lead to cellular injuries and play important roles in many diseases (53). To date, many stress responses have been studied in tissue culture cells by using signals including heat shock (48), UV irradiation, cytokines, and protein synthesis inhibitors such as anisomycin or cycloheximide (16, 32, 37, 60). These studies indicate that a first step in stress responses is the posttranslational modification of certain transcription factors. As an example, upon UV irradiation or anisomycin treatment, two transcription factors, c-Jun and ATF2, are phosphorylated by the JNK/SAPK family of stress-induced kinases (15–17, 21, 32, 37, 39, 66).

Some candidate target promoters for c-Jun and ATF2 during the stress response are those of the immediate-early genes, because the induction of these genes requires only posttranslational modification of preexisting transcription factors. Consistent with this idea, the immediate-early gene c-*fos* (for reviews, see references 11 and 12) is induced in tissue culture cells by anisomycin, which activates the JNK/SAPK pathway (6, 42). In addition, it has been demonstrated that c-*fos* is induced in animals by stressors such as seizure (47, 56) and partial hepatectomy (29). The c-*fos* gene product (c-Fos) is a transcription factor containing a basic region-leucine zipper (bZip) DNA binding and dimerization domain. Although c-Fos by itself does not bind to DNA with high affinity, it can heterodimerize with other bZip proteins such as c-Jun to activate transcription (for reviews, see references 13 and 34). Therefore, c-Fos plays an important role in regulating genes during the early stage of stress response.

Although transcriptional activation has long been recognized to be important in regulating gene expression, only in recent years has transcription repression gained much appreciation in its role in transcriptional regulation. Thus far, it is not clear whether transcriptional repression plays a role in stress responses. Here, we report for the first time that ATF3, a transcriptional repressor, is induced by a variety of physiological stresses. ATF3 is a member of the ATF/CREB family of transcription factors (23; for reviews, see references 5 and 72). As a homodimer, it binds to the ATF/cyclic AMP response element (CRE) consensus sequence (TGACGTCA) and represses transcription (10). The physiological functions of ATF3 have not been elucidated. The following observations prompted us to speculate that ATF3 may be involved in responses to extracellular stimuli. First, ATF3 is expressed at low or undetectable levels in most cell lines examined and is induced by growth-stimulating signals such as serum and 12-*O*-tetradecanoylphorbol-13-acetate (9, 10, 29). Second, Hsu et al. isolated rat ATF3 (referred to as LRF-1) cDNA from a regenerating-liver library by subtractive hybridization and demonstrated by Northern (RNA) blot analysis that the level of ATF3 mRNA increases during liver regeneration (29). Third, ATF3 has extensive similarity to c-Fos in the bZip region, indicating that the two proteins might have evolved from a common ancestral gene and may have conserved functions (46). Fourth, the 3′ untranslated region of ATF3 mRNA contains several AUUUA sequences (10, 29), a characteristic of the mRNAs of many immediate-early genes. Taken together, these observations suggest that ATF3 is similar to the immediate-early genes in many aspects and may respond to extracellular signals.

To test this hypothesis, we examined the expression of ATF3 by in situ hybridization in rats after a variety of treatments. We used in situ hybridization, instead of Northern blotting, in

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order to reveal the subtissue distribution of ATF3 mRNA. In this report, we demonstrate that ATF3 is induced in a variety of stressed tissues: mechanically injured liver, toxin-injured liver, blood-deprived heart, and postseizure brain. We further demonstrate that an ATF3-interacting protein, gadd153/Chop10 (gadd stands for growth arrest and DNA-damaging; Chop stands for C/EBP-homologous protein), functions as a negative regulator of ATF3 by forming an inactive heterodimer with it. Significantly, ATF3 and gadd153/Chop10 are expressed in inversely but overlapping manners during the liver's response to the hepatoxin carbon tetrachloride (CCl_4). We discuss a hypothesis to explain these observations.

MATERIALS AND METHODS

Isolation of cDNAs encoding ATF3-interacting proteins. A COS-1 cell cDNA expression library was constructed in the λEX*lox* T7 expression vector (Novagen) by using a Riboclone cDNA synthesis kit (Promega). The primary library was amplified in *Escherichia coli* ER1647, and the amplified library was introduced into *E. coli* BL21 (DE3/LysS) to express the T7 gene 10 fusion protein and screened by radiolabeled HisK-ATF3 fusion protein as described previously (31). HisK-ATF3 was expressed by using the pET-HisK vector (8), which provides tandem phosphorylation sites for heart muscle kinase (HMK) and labeled by heart muscle kinase (Sigma) in vitro in the presence of [γ-^{32}P]ATP (4). pλEX-gadd153 containing partial gadd153 cDNA was isolated from this screening.

Plasmids. pET (63), pTM1 (49), pTM1-GST, pBSSK (Stratagene), and pCG (65) derivatives containing human ATF1, human ATF3, human gadd153/Chop10, rat ATF3, rat gadd153/Chop10, and rat c-Jun cDNAs were constructed by inserting DNA fragments containing the corresponding open reading frame in the vectors. Insert DNA was generated from corresponding cDNA clones or by a combination of reverse transcription and PCR (RT-PCR), using *Pfu* polymerase (Stratagene).

Protein-protein blot analysis. ATF1, ATF3(bZip), ATF3(1-100), and gadd153/Chop10 proteins were produced by expressing pET-HisK derivatives containing the corresponding human cDNAs in *E. coli* BL21(DE3/LysS) (63). Proteins were analyzed by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to a nitrocellulose membrane, denatured, renatured, and incubated with radiolabeled ATF3 as in the library screening method.

In vitro protein synthesis and band shift analysis. ATF3 and gadd153/Chop10 were synthesized by the TNT reticulocyte lysate system (Promega), using pTM1 derivatives containing the corresponding human cDNAs. Band shift analysis was carried as described previously (23).

Transfection and CAT assay. Calcium phosphate transfection and the chloramphenicol acetyltransferase (CAT) assay were carried out as described previously (10). CAT activity was measured by a phase extraction method (2).

Immunofluorescence. HeLa cells (10^5) in a two-chamber slide (Nunc) were transfected with 2 μg of pCG-ATF3, using Lipofectamine as instructed by the manufacturer (Gibco BRL). At 24 h after incubation, the cells were fixed for 10 min with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS), and blocked with normal goat serum. The cells were treated with 1:500 dilution of an ATF3 antibody (Santa Cruz Biotechnology) for 1 h, washed with PBS, treated with a 1:200 dilution of a Texas red-conjugated anti-rabbit immunoglobulin G antibody (Jackson ImmunoResearch, West Point, Pa.), and analyzed by using a 535- to 580-nm-diameter filter.

Glutathione *S*-transferase (GST) selection and immunoprecipitation. HeLa cells (2×10^7) in two plates (10-cm diameter) were infected at 10 PFU per cell with vaccinia virus vTF 7-3, which expresses T7 polymerase, and then transfected with the appropriate pTM1 constructs. After a 24-h incubation period, the cells were harvested and sonicated. Cellular extracts were incubated with 10 μl of glutathione-conjugated agarose beads (Pharmacia) and washed as described in reference 31. Immunoprecipitation was performed as described previously (10).

RNA isolation and Northern blot analysis. Poly(A) RNA was isolated from rat liver by using the guanidine thiocyanate method and oligo(dT)-cellulose (57). Three micrograms of poly(A) RNA was loaded on a 1% formaldehyde agarose gel, transferred to a Duralon membrane (Stratagene), UV cross-linked, and hybridized to ^{32}P-labeled rat ATF3, rat gadd153/Chop10, or cyclophilin (14) partial cDNA according to standard techniques (57).

Detection of ATF3 in crude liver extract. Crude rat liver extracts from CCl_4-treated livers were prepared by a previously described method (59). The extract was loaded on a P11 phosphocellulose column (Whatman) and washed with 0.1 M salt. The 0.5 M salt eluate was concentrated by using a Centricon-10 column (Amicon) and phosphorylated by heart muscle kinase (Sigma) in the presence of [γ-^{32}P]ATP. The radiolabeled fraction was then precleared with nonimmune serum and immunoprecipitated with an ATF3 antibody (Santa Cruz).

Animal experiments. All animals were male Sprague-Dawley rats, and the procedures were performed at Zivic-Miller Laboratories, Inc. (Zelienople, Pa.). All rats were anesthetized with ether unless otherwise indicated; tissues were frozen by the isopentane method.

(i) Hepatectomy. Seven-week-old rats were anesthetized, and 70% of the liver (midventral lobes) from each rat was removed and saved as control liver. Two hours after hepatectomy, the rats were sacrificed and the regenerating livers were collected.

(ii) CCl_4 treatment. Eight-week-old rats were anesthetized and intragastrically injected with 0.08 ml of 100% CCl_4, a dose that caused 20% death (52); 3.5 h later, the rats were sacrificed and the livers were collected. Control rats received saline instead of CCl_4. For the time course experiment, the rats were treated as described above and sacrificed at the indicated times.

(iii) Alcohol treatment. Four-week-old rats were fasted overnight, anesthetized, and intragastrically injected with 100% ethanol (1.7 ml/100 g of body weight; 50% lethal dose according to reference 69). Two hours later, the animals were sacrificed and the livers were collected. Control rats received saline instead of alcohol.

(iv) Coronary artery ligation. Seven-week-old rats were anesthetized, and the left anterior descending (LAD) coronary artery of each rat was ligated to induce ischemia. Two hours later, the rats were sacrificed and the pale regions of the left ventricle wall were collected. Sham-operated rats received the same procedures without the coronary artery ligation.

(v) Coronary artery ligation and reperfusion. Seven-week-old rats were anesthetized with pentobarbital, and the LAD coronary artery of each rat was ligated by tying a slipknot, using a silk suture. Two hours later, the left ventricles were reperfused by removing the suture. One hour after reperfusion, the rats were sacrificed and the region of the left ventricle wall adjacent to the LAD coronary artery was collected. Sham-operated rats received the same procedures without the coronary artery ligation and reperfusion.

(vi) PTZ treatment. Seven-week-old rats were intraperitoneally injected with pentylenetetrazole (PTZ; Sigma p6500, 5% solution in saline, 50 mg/kg of body weight). Two hours later, the rats were anesthetized and the brains were collected. Control rats received saline instead of PTZ.

(vii) Light stimulation. The experiment was based on protocols described elsewhere (35, 36). Eight-week-old rats were entrained in a 12-h dark/12-h light cycle for 2 weeks. Three hours into the dark phase, the rats were exposed to light (>30 lx) for 2 h and sacrificed, and the brains were collected. The control rats were subjected to similar handling without light exposure. Two hours later, the rats were sacrificed under dim red light (Kodak 1A filter), and the optic nerves were severed before the lights were turned on to collect the brains.

Preparation of probes for in situ hybridization. Plasmids pBSSK-ATF3 and pBSSK-Jun containing the corresponding rat cDNAs were linearized and used as templates to make RNA. The RNA probes were synthesized in 5 μl of transcription buffer (40 mM Tris-HCl [pH 8.0], 25 mM NaCl, 8 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine) containing 50 ng of template DNA, 125 μCi of [α-^{33}P]UTP (1,000 Ci/mmol; Amersham), 500 μM each ATP, CTP, and GTP, 20 U of RNasin, and 25 U of T7 polymerase (antisense probes) or T3 polymerase (sense probes) at 40°C for 2 h. The reaction mixture was diluted to 25 μl with transcription buffer, 0.5 U of RQ1 DNase (Promega) was added, and the mixture was incubated at 37°C for 15 min. The probes were separated from unincorporated triphosphates by a Sephadex G-50 minicolumn, degraded to an average size of 150 nucleotides by base hydrolysis (58), ethanol precipitated, and stored at −20°C in hybridization solution (10% dextran sulfate, 50% ultrapure formamide [U.S. Biochemical], 0.3 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 10 mM dithiothreitol, 0.5 mg of denatured tRNA per ml).

In situ hybridization. The in situ hybridization procedure was modified from protocols described previously (45, 58). Frozen tissues were sectioned (in 12- to 14-μm thickness) by a Zeiss HM500 cryostat, fixed with 4% paraformaldehyde, and dehydrated with a 30, 50, 70, 85, 95, and 100% ethanol series. All sections were hybridized with the indicated probes (0.5×10^7 to 2×10^7 dpm/ml) at 55°C for 16 to 20 h in a humidified chamber. To remove any nonhybridized single-stranded RNAs, sections were incubated in an RNase A solution (20 μg of RNase A per ml, 10 mM Tris-HCl, 1 mM EDTA, 0.5 M NaCl) at 37°C for 30 min and then subjected to standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) washes including a 0.1× SSC wash at 70°C for 30 min. The sections were dehydrated as described above with addition of 0.3 M ammonium acetate in the ethanol solutions to prevent the dissociation of the RNA-RNA hybrid and then analyzed by liquid autoradiography, using Kodak NTB-2 emulsion diluted in an equal volume of distilled water according to the manufacturer's instructions. The exposure time was predetermined by the intensity of the signal in an overnight X-ray autoradiograph. Sections were counterstained with either cresyl violet or hematoxylin and eosin according to standard histological techniques (41) and then photographed with either a Zeiss Axiophot microscope with bright-field, dark-field, or double-filter exposure or a Zeiss Stemi 2000-C stereoscope with dark-field exposure. The double-filter exposure consists of a dark-field exposure with a 48 79 00 Zeiss filter followed by a bright-field exposure with an 80A Kodak Wratten gelatin filter.

Immunohistochemistry. Immunohistochemical staining was carried out with an Elite ABC kit as instructed by the manufacturer, (Vector), with the following modifications. Tissue sections (12 to 14 μm) were fixed with Histochoice Tissue Fixative MB (Amresco); after incubation with normal goat serum, the sections were blocked by sequential incubations with 100 μg of avidin D (Sigma) per ml and 400 μg of biotin (Sigma) per ml for 15 min each and then extensively washed

in PBS to remove the background due to the endogenous biotin in the liver. gadd153/Chop10 antibody was affinity purified and used at a dilution of 1:500.

RESULTS

ATF3 is induced by a variety of physiological stresses. To determine the expression pattern of ATF3 in regenerating liver, we examined rat livers at 2 h after partial hepatectomy by in situ hybridization. As shown in Fig. 1A and B, the antisense RNA probe detected signals in regenerating liver but did not detect any signals in the normal liver, indicating that the ATF3 mRNA level increases during liver regeneration. The hybridization specificity was demonstrated by the observation that the sense RNA probe did not hybridize to either normal or regenerating liver (data not shown). The signals detected by the antisense RNA probe were patchy and spread throughout the liver and did not correspond to any specific structure of liver.

Because the liver is the major organ that metabolizes toxins, we examined the expression pattern of ATF3 in liver at 3.5 h (see Discussion) after intragastric injection of the hepatoxin CCl_4. As shown in Fig. 1C and D, ATF3 was expressed in cells around central veins; it was, however, not expressed in the rest of the liver. Alcohol, another hepatoxin, also induced ATF3 (Fig. 1E and F). In contrast to CCl_4, alcohol induced the expression of ATF3 in clusters of cells throughout the liver (the black spots in Fig. 1F), not just in cells around the central veins.

We next investigated whether ATF3 is induced by heart injury. Since ischemia (deficiency of blood) is the most common cause of heart injury, we used an established animal model, left coronary artery ligation, to mimic myocardial ischemia. As shown in Fig. 1G and H, ATF3 was locally induced in the endocardium side of the ventricle wall (indicated by an arrow; see Discussion). In the control heart from a sham-operated rat, ATF3 was not induced. In ischemic myocardium, reintroduction (reperfusion) of blood into the ischemic tissue causes further cellular injuries. We therefore examined the expression of ATF3 mRNA in rats receiving left coronary artery ligation followed by reperfusion. As shown in Fig. 1I and J, ATF3 was also induced in reperfusion-injured heart: it was induced in a loop-shaped area that was much larger than the patchy region observed in ischemia. We conclude that both myocardial ischemia and ischemia-reperfusion induce ATF3.

Prolonged seizure causes brain injury. To investigate whether ATF3 is induced in brain by seizure, we examined the expression of ATF3 in rats treated with PTZ, which causes seizure (61). As shown in Fig. 1K and L, ATF3 was induced in the dentate gyrus, a region implicated in seizures, but not in other regions of the brain (data not shown).

The above-described experiments (mechanical injury, chemical intoxication, heart injuries, and seizure) all involve cellular injury. To find out whether ATF3 is also induced by treatments that do not elicit cellular injury, we examined the expression of ATF3 after light stimulation during the dark phase of the circadian cycle. As shown in Fig. 1M and N, ATF3 was not induced in the suprachiasmatic nucleus (SCN), where the circadian pacemaker resides. As a positive control, we demonstrated that the treatment did induce in SCN the c-*fos* gene (data not shown), a gene known to be induced by light stimulation (35).

In summary, we demonstrate that ATF3 is induced in a variety of stressed tissues: mechanically injured liver, toxin-injured liver, ischemic heart, ischemic reperfused heart, and postseizure brain. Significantly, these conditions do not increase the level of CREB mRNA, which encodes a bZip protein (data not shown), indicating that an increased mRNA level in stressed tissues is not a general response of all genes encoding bZip proteins.

ATF3 heterodimerizes with gadd153/Chop10. The foregoing observations prompted us to consider the possibility that ATF3 mediates responses to physiological stresses. This in turn raises an important question: What are the target promoters of ATF3? Since ATF3 is induced in a variety of tissues by different injuries, we propose that the specificity of the target is determined by the physiological context and by the ATF3-interacting proteins in the cell. As a first step toward testing this possibility, we screened a cDNA expression library with radiolabeled ATF3 to isolate interacting proteins (see Materials and Methods). We isolated a cDNA clone encoding the bZip protein gadd153/Chop10. As demonstrated by a protein-protein blot analysis, radiolabeled ATF3(bZip), which only contains the bZip region, bound to gadd153/Chop10 and to itself but not to ATF1; the binding of ATF3 to gadd153/Chop10 was consistently greater than that to itself (Fig. 2B, lanes 1 to 3). Furthermore, radiolabeled ATF1 did not bind to gadd153/Chop10 (Fig. 2B, lane 5). These results indicate that the interaction between ATF3 and gadd153/Chop10 was not nonspecific. The leucine zipper region was, as expected, necessary for the interaction between ATF3 and gadd153/Chop10, because ATF3(1-100), a truncated ATF3 lacking the leucine zipper, did not bind to gadd153/Chop10 (Fig. 2C).

To examine the in vivo interaction between ATF3 and gadd153/Chop10, we coexpressed in HeLa cells ATF3 and GST-gadd153, a fusion protein containing GST and gadd153/Chop10. We isolated whole cell extracts, purified GST-gadd153 by agarose beads conjugated with glutathione, and examined the presence of ATF3 by Western blotting (immunoblotting). As shown in Fig. 3A, ATF3 was copurified with GST-gadd153 by the glutathione beads (lane 2), indicating an interaction between ATF3 and the fusion protein. The specificity of the interaction was confirmed by the lack of interaction between ATF3 and GST alone (Fig. 3A, right panel). Another indication that ATF3 and gadd153/Chop10 interact with each other in vivo was that ATF3 specifically coimmunoprecipitated with gadd153/Chop10 when coexpressed in HeLa cells (Fig. 3B). Furthermore, immunofluorescence analysis indicates that, as expected, ATF3 is a nuclear protein (Fig. 3C). As demonstrated previously (54), gadd153/Chop10 is also a nuclear protein. This colocalization, taken together with coimmunoprecipitation and copurification, strongly suggests that when expressed in the same cells, ATF3 and gadd153/Chop10 can interact with each other.

gadd153/Chop10 negatively regulates ATF3. gadd153/Chop10 is a C/EBP-homologous protein, but it contains several amino acid substitutions at conserved residues in its basic region, rendering it unable to bind to the C/EBP site (54). Consequently, it acts as a negative regulator of C/EBP proteins, such as C/EBPα and C/EBPβ (54), by forming nonfunctional heterodimers with them. In light of these observations, we tested whether dimerization between gadd153/Chop10 and ATF3 results in a heterodimer unable to bind to the ATF/CRE consensus site. As shown in Fig. 4A, gadd153/Chop10 inhibited the binding of ATF3 to the ATF/CRE consensus site. We then asked whether gadd153/Chop10 also affects the binding of ATF3 to ATF/CRE-related DNA sequences. We examined AP-1, ELAM-1, E4F, and Enk-2 sites. In addition, we examined C/EBP site and a chimeric site composed of the ATF/CRE and C/EBP half sites. Figure 4B shows the sequences of these sites. As shown in Fig. 4C, the ATF3 homodimer bound to the AP-1, ELAM-1, E4F, and chimeric sites specifically; addition of gadd153/Chop10 abolished the binding. ATF3 did not bind to the Enk-2 or C/EBP site; importantly, addition of

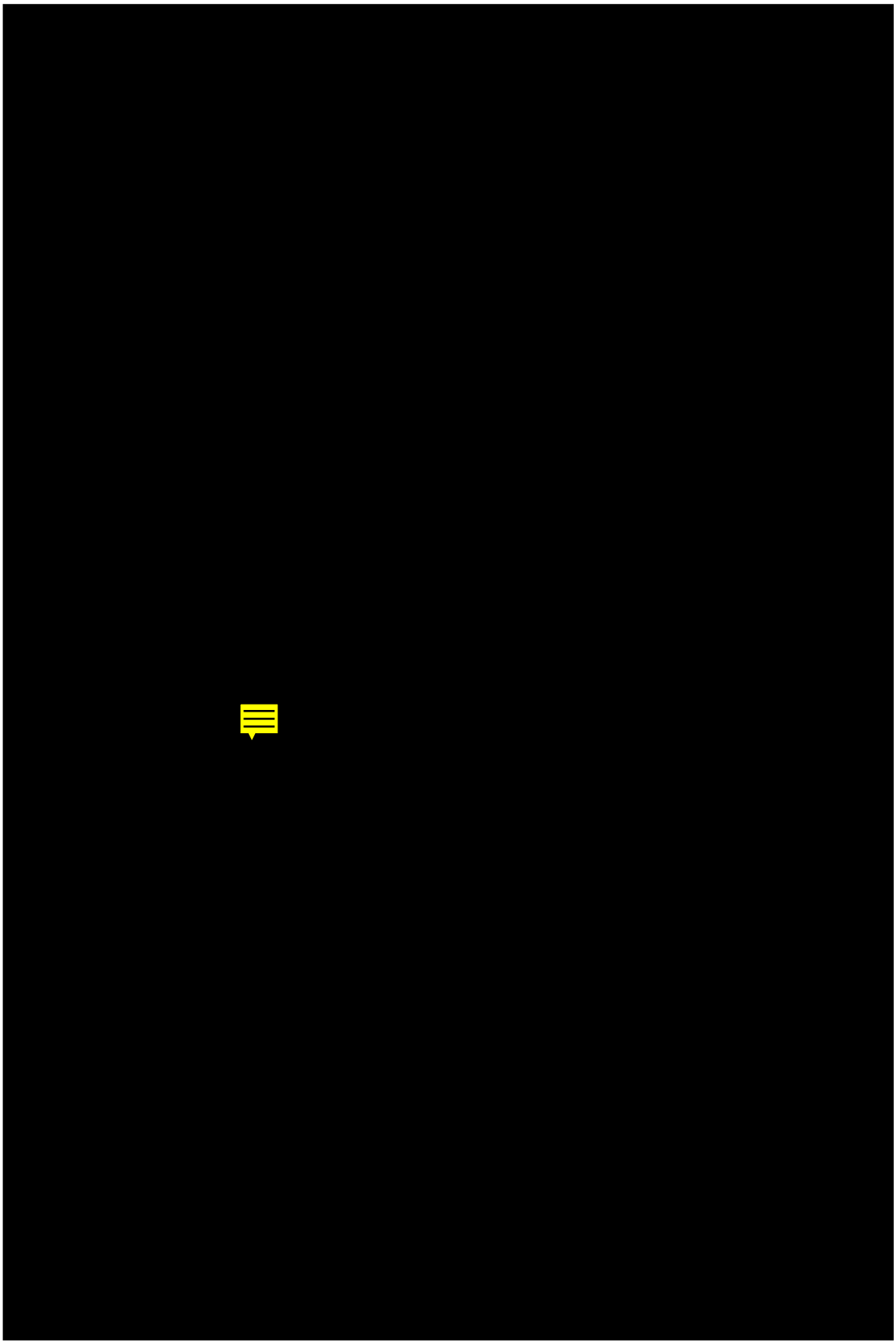

FIG. 1. Rat ATF3 mRNA was induced in a variety of tissues by different physiological stresses. Tissue sections from rats subjected to the indicated treatments were analyzed by in situ hybridization using ATF3 antisense RNA as the probe. The signals were detected by autoradiographic emulsion for various exposure times. The sections were counterstained by hematoxylin and eosin (A to J) or cresyl violet (K to N). Depending on the intensity of the signals and the type of counterstains, we generated the pictures by using different photographic techniques: bright-field photography giving black signals on stained sections, dark-field photography giving pinkish white signals on a green background, and double-filter photography giving pink signals on a blue background. (A and B) Normal versus regenerating liver, 21-day exposure, dark field, bar = 50 μm. (C and D) Saline- versus CCl_4-treated liver, 10-day exposure, bright field, bar = 50 μm. CV, central vein; PT, portal triad which consists of the portal vein, artery, and bile duct. (E and F) Saline- versus alcohol-treated liver, 21-day exposure, bright field, bar = 30 μm. (G and H) Normal versus ischemic heart, 10-day exposure, dark field, bar = 30 μm. En and Ep indicate the endocardium and epicardium side of the ventricle wall, respectively. (I and J) Normal versus ischemic reperfused heart, 5-day exposure, dark field, bars = 200 μm (I) and 250 μm (J). (K and L) Saline- versus PTZ-treated brain, 14-day exposure, double filter, bar = 100 μm. DG, dentate gyrus. (M and N) Unstimulated versus light-stimulated brain, 21-day exposure, double filter, bar = 125 μm. The pictures show only the base of the anterior hypothalamus where the paired SCNs locate. The dotted lines indicate SCNs; they are meant to help explain the figures, not to indicate the precise locations or sizes of SCNs. The line that vertically divides the two SCNs is the third ventricle (3V).

gadd153/Chop10 did not result in any appreciable binding to these sites (see Discussion). Taken together, these results indicate that gadd153/Chop10 inhibited ATF3 from binding to ATF/CRE and several related sites.

These results predict that dimerization of gadd153/Chop10 with ATF3 should also block the transcriptional function of ATF3 on promoters containing ATF/CRE or related sites. Since the ATF3 homodimer acts as a transcriptional repressor

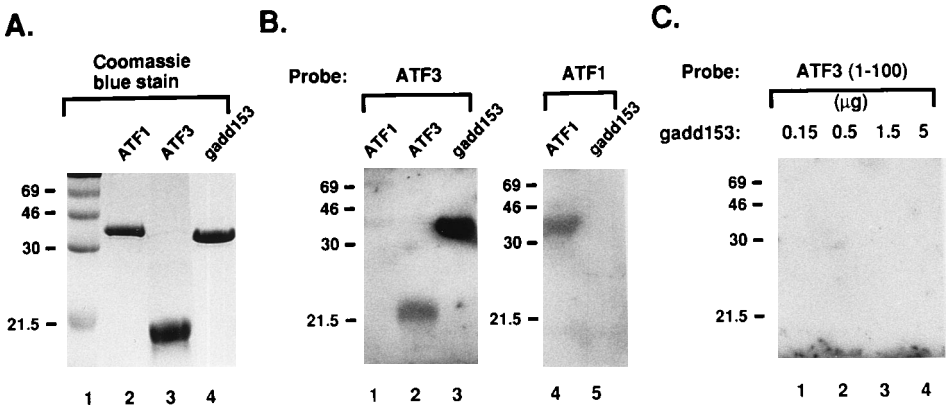

FIG. 2. The interaction between ATF3 and gadd153/Chop10 was not nonspecific and was dependent on the leucine zipper. (A) Coomassie blue stain to ensure that similar amounts of protein were used. ATF1 (lane 2), ATF3(bZip), which contains the bZip region of ATF3 (lane 3), and gadd153/Chop10 (lane 4) expressed from *E. coli* were analyzed by electrophoresis on an SDS-polyacrylamide gel and stained. Lane 1 shows rainbow size marker (Amersham). Size markers in kilodaltons are indicated on the left. (B) Interaction between ATF3 and gadd153/Chop10 was not nonspecific. ATF1, ATF3(bZip), and gadd153/Chop10 were analyzed by protein-protein blotting using radiolabeled ATF3(bZip) (lanes 1 to 3) or ATF1 (lanes 4 and 5) as the probe. Size markers in kilodaltons are indicated on the left. (C) The leucine zipper was necessary for the interaction between ATF3 and gadd153/Chop10. Increasing amounts of gadd153/Chop10 were analyzed by protein-protein blotting using radiolabeled ATF3(1-100), which lacks the leucine zipper, as the probe. Size markers in kilodaltons are indicated on the left.

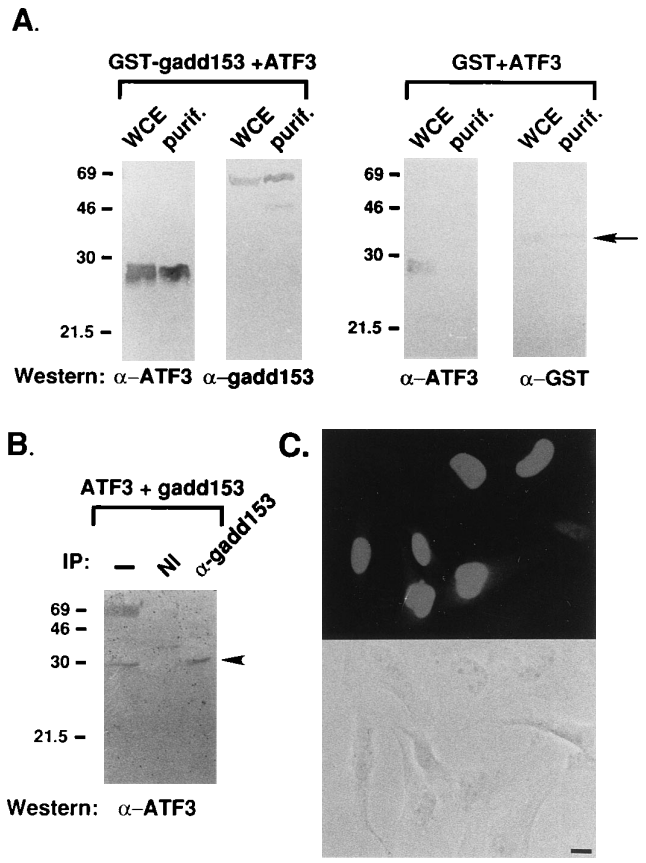

FIG. 3. ATF3 and gadd153/Chop10 interacted with each other in vivo. (A) ATF3 was copurified with GST-gadd153 by glutathione-agarose beads. HeLa cells were cotransfected with DNA expressing ATF3 and DNA expressing the GST-gadd153 fusion protein or GST alone. Whole cell extract (WCE) or the glutathione beads-purified fraction (purif.) was loaded on an SDS-polyacrylamide gel and then immunoblotted with anti (α)-ATF3, anti-gadd153, and anti-GST antibodies as indicated. The arrow indicates GST. Size markers in kilodaltons are indicated on the left. (B) ATF3 coimmunoprecipitated with gadd153/Chop10. HeLa cells were cotransfected with DNA expressing ATF3 and DNA expressing gadd153/Chop10. Whole cell extract (WCE) or the pellet from immunoprecipitation (IP) using a nonimmune serum (NI) or anti-gadd153 antibody was loaded on an SDS-polyacrylamide gel and immunoblotted with an anti-ATF3 antibody. The arrowhead indicates ATF3. Size markers in kilodaltons are indicated on the left. (C) ATF3 localized in the nucleus. HeLa cells were transfected with DNA expressing ATF3 and analyzed by immunofluorescence using the anti-ATF3 antibody. Top, fluorescent picture; bottom, phase-contrast picture. Bar = 12 μm.

when bound to DNA (10), we examined whether gadd153/Chop10 could relieve the repression by ATF3 in a cotransfection assay. Previously, we demonstrated that the E-selectin promoter, which contains an ELAM-1 site and several NF-κB sites (67, 68) was activated by NF-κB and that the activated promoter activity was effectively repressed by ATF3 (10). Figure 5 shows that this repression was completely relieved by gadd153/Chop10. Importantly, gadd153/Chop10 by itself did not activate transcription, indicating that the apparent relief of repression was not due to the masking of repression by transcriptional activation. In fact, transfection of gadd153/Chop10 alone resulted in a slight repression of the E-selectin promoter. This was probably because gadd153/Chop10 heterodimerized with some endogenous bZip proteins important for the activation of the E-selectin promoter. Consistent with previous observations (67), mutation of the ELAM-1 site completely abolished the promoter activity even in the presence of NF-κB (Fig. 5B). The observation that gadd153/Chop10 relieved the transcriptional repression by ATF3 in a transient transfection assay also supports the notion that these two proteins can interact with each other in vivo.

ATF3 and gadd153/Chop10 are expressed in CCl_4-treated liver in inverse but overlapping manners. Having demonstrated that gadd153/Chop10 is a negative regulator of ATF3, we then asked whether this inhibition is physiologically relevant. As a first step toward answering this question, we examined the expression of rat gadd153/Chop10 by using the animal models described above. We examined the liver for the following reasons. First, C/EBP proteins, such as C/EBPα, C/EBPβ (also named LAP and NF-IL-6), and DBP, have been shown to regulate liver cell-specific genes (3, 18, 50; for reviews, see references 38 and 44). It is possible that gadd153/Chop10 is also involved in regulating gene expression in liver. Second, gadd153/Chop10 is induced in liver by amino acid starvation and inflammation (43, 64), indicating that it may also be regulated by some of the treatments used in the present study. Using Northern blot analysis, we examined the expression of gadd153/Chop10 after CCl_4 treatment. As shown in Fig. 6A and B, gadd153/Chop10 mRNA levels were high in the saline-treated liver, low at 1.5 h after CCl_4 treatment, and high again at 7.5 h. Significantly, these expression kinetics were inversely correlated to that of ATF3: ATF3 mRNA was nondetectable in the saline-treated liver, increased to high levels at 1.5 h after CCl_4 treatment, and returned to nondetectable levels at 17.5 h.

To better characterize the expression of ATF3, we examined the level of ATF3 mRNA by RT-PCR, a method that is more sensitive than Northern blot analysis. As shown in Fig. 6C, ATF3 mRNA was clearly detectable in the saline-treated liver (lane 9) and in the livers collected at 7.5 and 17.5 h after CCl_4 treatment (lanes 6 and 7). Because under these conditions the levels of gadd153/Chop10 mRNA were high (Fig. 6A and B), we conclude that ATF3 and gadd153/Chop10 are expressed in inverse but overlapping manners.

To confirm that ATF3 and gadd153/Chop10 proteins, not just mRNAs, are present in the same cells, we examined the liver sections by immunohistochemistry. As shown in the left panel of Fig. 7A, gadd153/Chop10 protein was present throughout the liver section at 17.5 h after CCl_4 treatment. We note that it was more abundant around the portal triads than in the rest of the liver; this result is consistent with the in situ hybridization result which showed higher mRNA levels around the portal triads (70). The specificity of the assay was indicated by the complete lack of signals when an anti-gadd153 antibody was omitted in immunohistochemistry (right panel). In addition, incubation of the antibody with excess amounts of gadd153/Chop10 also abolished the signals (data not shown). Immunohistochemistry of the liver section at 17.5 h after CCl_4 treatment failed to detect any ATF3 (data not shown), probably because the level of ATF3 was too low. To circumvent this problem, we took advantage of the observation that ATF3 can be phosphorylated by heart muscle kinase in vitro (9). We made liver extract, partially purified the extract by a phosphocellulose column, radiolabeled ATF3 in vitro, immunoprecipitated it, and then analyzed it on a protein gel. As shown in Fig. 7B, a band of the expected size was precipitated by the ATF3 antibody but not by the nonimmune serum or by the ATF3 antibody in the presence of an excess amount of ATF3. We conclude that ATF3 protein is present in the liver at this time point. Since gadd153/Chop10 is present throughout the liver, it must overlap with ATF3 in some liver cells, irrespective of the subtissue localization of ATF3.

We next asked whether the inverse correlation of ATF3 and gadd153/Chop10 expression is specific to CCl_4 treatment. We

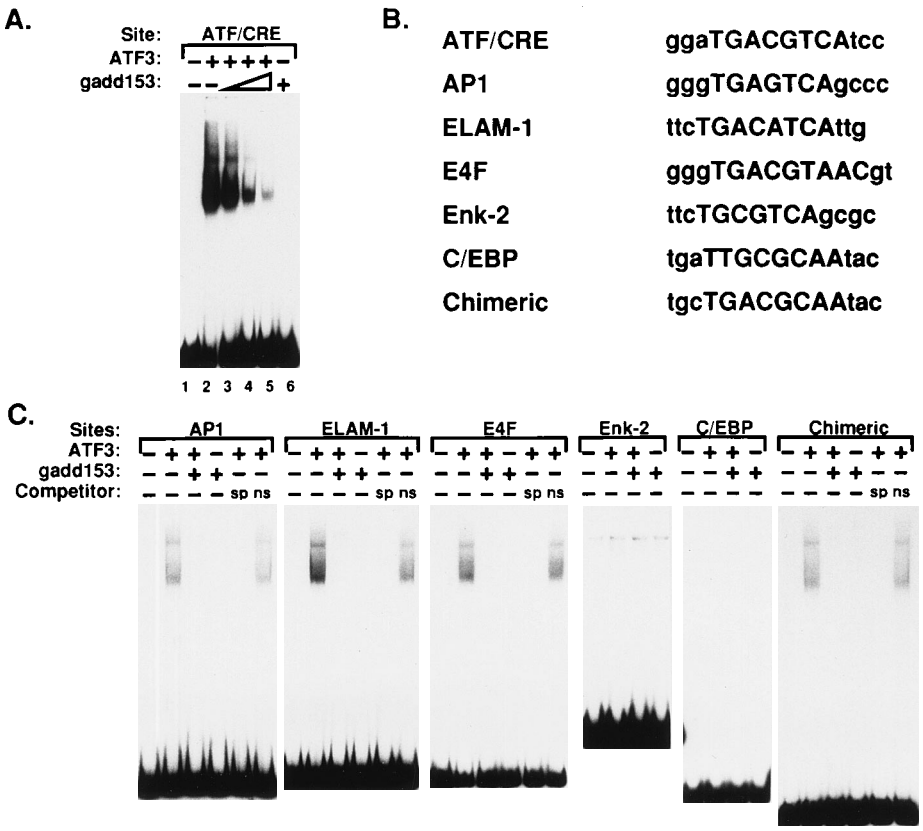

FIG. 4. gadd153/Chop10 inhibited ATF3 from binding to the ATF/CRE consensus sequence and several related sites. (A) gadd153/Chop10 inhibited ATF3 from binding to the ATF/CRE consensus site. One microliter of ATF3 generated by reticulocyte lysate was mixed with a radiolabeled DNA fragment containing the consensus ATF/CRE site in the absence (lane 2) or presence of gadd153/Chop10 generated by reticulocyte lysate (lanes 3 to 5; 1, 3, and 9 μl, respectively). Lane 6 shows the result of mixing 9 μl of gadd153/Chop10 and DNA together, indicating that gadd153/Chop10 by itself did not bind to the ATF site. Reticulocyte lysate not programmed to generate any specific protein was included to ensure that each reaction mixture contained the same amount of reticulocyte lysate. (B) Sequences of the ATF/CRE consensus and related sites. (C) gadd153/Chop10 inhibited ATF3 from binding to several ATF/CRE related sites. Radiolabeled DNA fragments containing the indicated sites were incubated with 1 μl of ATF3, 9 μl of gadd153/Chop10, or both. Reticulocyte lysate not programmed to generate any specific protein was included so that each reaction contained the same amount of reticulocyte lysate. Specific (sp) or nonspecific (ns) competitor DNA (200-fold) was included as indicated to demonstrate the specificity of binding.

examined the levels of ATF3 and gadd153/Chop10 mRNA after alcohol and partial hepatectomy. The level of gadd153/Chop10 mRNA decreased at 2 h after partial hepatectomy but did not decrease significantly at 3.5 h after alcohol treatment (Fig. 8B); the level of ATF3 mRNA increased after these treatments (Fig. 8A), consistent with the in situ hybridization results shown in Fig. 1. These results indicate that the inverse correlation between ATF3 and gadd153/Chop10 expression is not a specific response of the liver to CCl_4; they also indicate that the inverse correlation is not a general response of the liver to all stresses.

c-Jun is induced in the cells surrounding the central veins by CCl_4 treatment. As described earlier, we hypothesize that the target promoters of ATF3 are determined by the physiological context and by the ATF3-interacting proteins in the cells. To find out whether any bZip proteins are induced in the same cells that express ATF3, we examined the expression of c-Jun after CCl_4 treatment for the following reasons. First, c-Jun has been demonstrated to heterodimerize with ATF3 in vitro (22). Second, c-*jun* is an immediate-early gene and has been shown to be induced by many stimuli. Third, the specific subtissue localization of ATF3 after CCl_4 treatment provides a convenient landmark against which to compare the distribution of expression. As shown in Fig. 9D, at 3.5 h after CCl_4 treatment, c-Jun was induced around the central veins, the same cell population that expressed ATF3. Examination of c-Jun at other time points after CCl_4 treatment also indicated coexpression of c-Jun with ATF3 (71).

To determine whether ATF3 and c-Jun can form a heterodimer in vivo, we used the GST and coimmunoprecipitation approaches described above. As shown in Fig. 9E and F, ATF3 and c-Jun can interact with each other when coexpressed in HeLa cells. This result, in conjunction with the coinduction of ATF3 and c-Jun around the central veins by CCl_4, suggests that ATF3 and c-Jun may form heterodimer in the liver after CCl_4 treatment. However, because ATF3 can also heterodimerize with gadd153/Chop10, we compared the affinity between ATF3 and c-Jun with that between ATF3 and gadd153/Chop10. We incubated radiolabeled ATF3 with different amounts of c-Jun or gadd153/Chop10 and found that ATF3 had similar affinities to c-Jun and gadd153/Chop10 (Fig. 10). Given that the affinities are similar, it is unlikely that gadd153/Chop10 can compete with c-Jun to heterodimerize with ATF3, because the level of gadd153/Chop10 is much lower than that of c-Jun when ATF3 is induced. We note that, in addition to c-Jun, other bZip proteins may heterodimerize with ATF3, depending on their concentrations and on their affinities to ATF3.

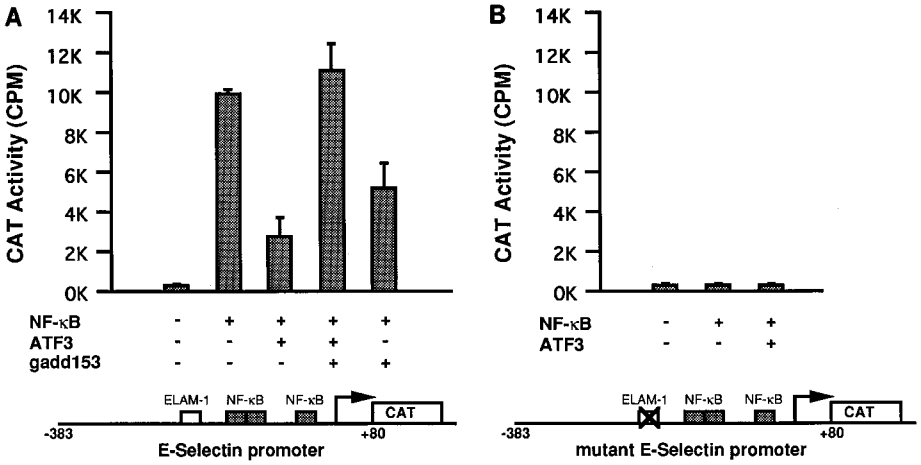

FIG. 5. gadd153/Chop10 relieved the repression of E-selectin promoter by ATF3. (A) E-selectin–CAT reporter was transfected into COS-1 cells with plasmids pCGN-p50 and pCGN-p65 (NF-κB), pCG-ATF3, and pCG-gadd153. The plasmid DNA carrying only the cytomegalovirus promoter (pCG) was included in some calcium phosphate-DNA mixes to ensure that each transfection mix contained the same amount of promoter. (B) Mutant E-selectin–CAT reporter containing a mutated ELAM-1 site (TCACGACGGT) was cotransfected with DNA expressing ATF3 or NF-κB as indicated. The average CAT activities from three experiments are shown.

DISCUSSION

Induction of ATF3 by stress signals is an early event. In this report, we show that ATF3 mRNA increases upon stimulation by a variety of physiological stresses. Since in situ hybridization detects steady-state mRNA levels, it is not clear whether the induction is due to an increase of mRNA synthesis, a decrease of degradation, or both. Nevertheless, it is clear from the CCl_4 time course experiment that the ATF3 mRNA level increases shortly after stimulation and returns to a low level after several hours. In this context, it should be noted that all animals described in this report except those from the CCl_4 and alcohol experiments (see below) were sacrificed at 2 h after stimulation.

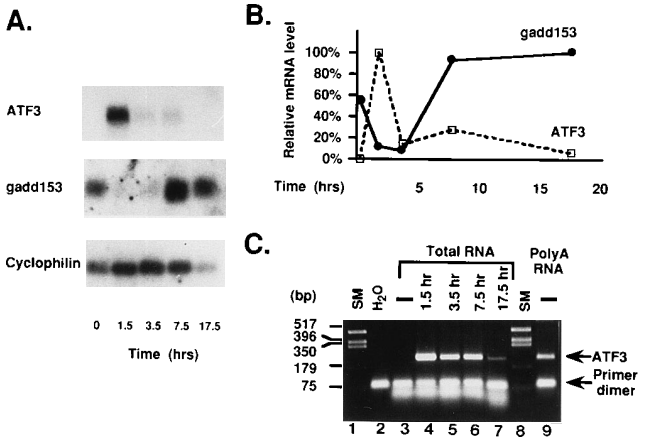

FIG. 6. ATF3 and gadd153/Chop10 were expressed in CCl_4-treated liver in inverse but overlapping manners. (A) Northern blot analysis indicated inverse expression of ATF3 and gadd153/Chop10. Rats intragastrically injected with CCl_4 were sacrificed at the indicated times; poly(A) RNA was prepared from liver and analyzed (3 μg per lane) by Northern blotting using radiolabeled ATF3, gadd153/Chop10, or cyclophilin cDNA as the probe. Cyclophilin is abundant and ubiquitous in tissue and phylogenetic distribution (14). (B) Quantitation of the Northern blot. The signals in panel A were quantitated by a PhosphorImager (Molecular Dynamics). ATF3 and gadd153/Chop10 signals were normalized against cyclophilin, and the highest expression point was arbitrarily defined as 100%. (C) RT-PCR analysis indicated overlapping expression of ATF3 and gadd153/Chop10. ATF3 mRNA was present in the normal liver and in the livers collected at 7.5 and 17.5 h after CCl_4 treatment. Total RNA (10 μg) or poly(A) RNA (1 μg) from saline-treated liver (−) or CCl_4-treated livers collected at the indicated times was analyzed by RT-PCR using a primer set to amplify ATF3. An aliquot of the product was loaded on the gel. As a control, water was used to replace RNA (H_2O). Size markers (SM) (derived from a pGEM3 *Hin*fI digest) are indicated on the left.

Induction of ATF3 in a variety of stressed tissues. (i) Mechanically injured liver. The liver is one of the few organs that can regenerate. During liver regeneration, expression of the albumin gene decreases while expression of the α-fetoprotein gene increases (51). This expression pattern is similar to that during the early fetal stage, leading to the hypothesis that liver regeneration mimics the dedifferentiated state of liver. In this report, we demonstrate that ATF3 is induced during liver regeneration. Because the induction of ATF3 precedes the decrease of albumin gene expression and because the ATF3 homodimer is a transcriptional repressor (10), it would be interesting to determine whether the albumin promoter is a target for ATF3. We hypothesize that in addition to repressing transcription, upon induction by stress, ATF3 heterodimerizes with other proteins and activates transcription. c-Jun is a potential candidate for such a protein (see below for an explanation). However, the target promoters for the ATF3-Jun heterodimer remain to be identified.

(ii) Chemically injured liver. CCl_4 is a hepatoxin that has been used to induce liver regeneration and liver cirrhosis. Induction of liver regeneration is indicated by the increase of [^{3}H]thymidine incorporation at 24 h after a single injection of CCl_4 (51). Induction of liver cirrhosis, however, requires repeated injection of CCl_4 (52). We examined the level of ATF3 at 3.5 h after a single injection of CCl_4 without repeated treatment, because we reasoned that ATF3 is encoded by an early gene and its expression probably increases shortly after the initial injection. Since CCl_4 administered intragastrically reached a maximum level in the liver after 1.5 h (52), we examined the level of ATF3 at 3.5 h after injection to allow 2 h of induction after CCl_4 reached maximum levels in the liver. Although both CCl_4 and partial hepatectomy can induce liver regeneration, they induced different patterns of ATF3 expres-

FIG. 7. Both ATF3 and gadd153/Chop10 proteins were present in the liver at 17.5 h after CCl_4 treatment. (A) gadd153/Chop10 was present throughout the liver section as indicated by immunohistochemistry. (Left) The liver section collected at 17.5 h after CCl_4 treatment was pretreated with avidin-biotin blocking steps, incubated with affinity-purified gadd153/Chop10 antibody, incubated with biotin-conjugated secondary antibody, and detection by using an Elite ABC kit (Vector). CV, central veins; PT, portal triad. (Right) The section was analyzed as described above except for omission of the first antibody. Bar = 20 μm. (B) ATF3 was present in the liver at 17.5 h after CCl_4 treatment. Liver extract was partially purified by a phosphocellulose column, and the 0.5 M salt eluate which contained ATF3 was dialyzed, radiolabeled with heart muscle kinase in the presence of [γ-^{32}P]ATP, and then immunoprecipited (IP) with a nonimmune antibody (NI; lane 1) or affinity-purified ATF3 antibody in the presence (lane 2) or absence (lane 3) of an excess amount of ATF3. Pellets from immunoprecipitation were analyzed by electrophoresis on an SDS-polyacrylamide gel and autoradiography. ATF3 is indicated by an arrowhead; size markers in kilodaltons are indicated on the left.

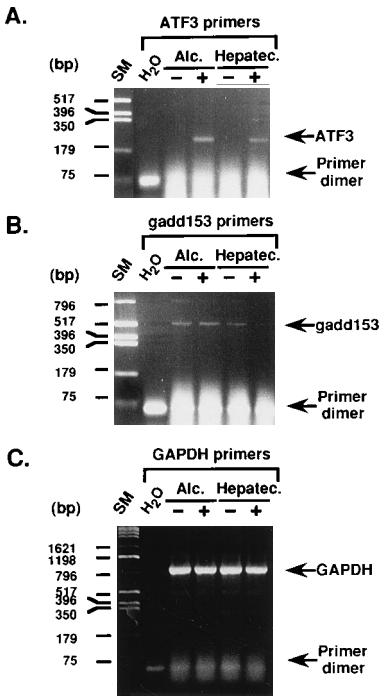

FIG. 8. Partial hepatectomy but not alcohol treatment decreased the level of gadd153/Chop10 mRNA. Total RNA (10 μg) from control livers, from livers collected at 3.5 h after alcohol treatment (Alc.), and from livers collected at 2 h after partial hepatectomy (Hepatec.) was analyzed by RT-PCR, using primer sets to amplify ATF3 (A), gadd153/Chop10 (B), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; C) cDNAs as indicated. An aliquot of the product was loaded on the gel. H_2O, water control for each primer set. Size markers are indicated on the left.

sion: CCl_4 induced ATF3 around the central veins, whereas partial hepatectomy induced ATF3 in random clusters. This finding indicates that the regenerative process induced by CCl_4, where the liver architecture is preserved, may be different from that induced by partial hepatectomy, where the liver architecture is destroyed. It is intriguing that CCl_4 induces necrosis (cell death) around the central veins (53), where ATF3 is induced. It would be interesting to determine whether the expression of ATF3 has any causal relationship with necrosis.

(iii) Stressed heart. Most heart attacks are due to lack of blood flow to the heart wall (myocardial ischemia). In the animal model used to study myocardial ischemia, the LAD coronary artery is surgically occluded because it is the predominant artery that supplies blood to the major portions of the left ventricle wall. Because the arterial flow enters the ventricle wall from the epicardial side to the endocardial side, oxygen is extracted from the blood by the epicardial cells before it reaches the endocardium. Consequently, the endocardium is usually more severely ischemic than the epicardium in a patient suffering a heart attack (30). This explains our observation that ATF3 was induced in the endocardium but not in the epicardium. However, as shown in Fig. 1H, the expression of ATF3 was not uniformly distributed in the endocardium; instead, it was patchy. This was probably because ischemia is characteristically heterogeneous, a result of heterogeneity in the local blood flow (24).

Reintroduction of blood (reperfusion) is necessary to reverse the ischemic damage before it becomes irreversible. However, reperfusion itself also causes injury (40). It is generally accepted that oxygen free radicals and inflammatory responses contribute to reperfusion injury (40). As shown in Fig. 1J, after myocardial ischemia-reperfusion, ATF3 was induced in a broad, loop-shaped region, a pattern distinct from the patchy expression observed in myocardial ischemia. The

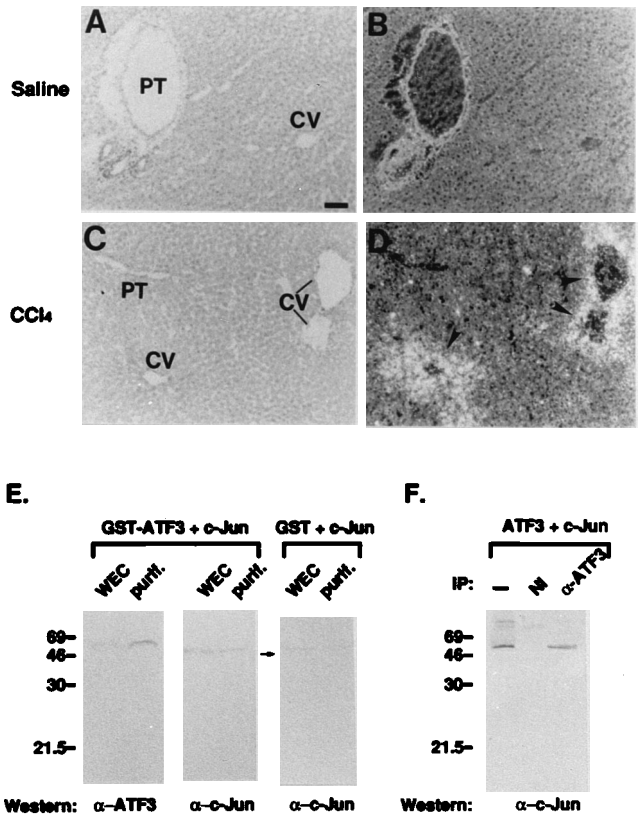

FIG. 9. c-Jun may form a heterodimer with ATF3 after CCl_4 treatment. (A to D) c-Jun was induced around the central veins, where ATF3 was induced. Saline-treated (A and B) or CCl_4-treated (C and D) liver sections collected at 3.5 h after treatment were analyzed by in situ hybridization using c-Jun antisense RNA as the probe. Bright-field (A and C) and dark-field (B and D) pictures are shown. Central veins (CV) and portal triad (PT) are indicated. The signals are indicated by arrowheads. Bar = 50 μm. (E) c-Jun was copurified with GST-ATF3 by glutathione-agarose beads. HeLa cells were cotransfected with DNA expressing c-Jun and DNA expressing the GST-ATF3 fusion protein or GST alone. Whole cell extract (WCE) or the glutathione bead-purified fraction (purif.) was loaded on an SDS-polyacrylamide gel and immunoblotted with an anti (α)-ATF3 or anti-c-Jun antibody as indicated. The arrow indicates c-Jun. (F) c-Jun coimmunoprecipitated with ATF3. HeLa cells were cotransfected with DNA expressing c-Jun and DNA expressing ATF3. Whole cell extract or pellet from immunoprecipitation (IP) with a nonimmune serum (NI) or anti-ATF3 antibody (α-ATF3) was loaded on an SDS-polyacrylamide gel and immunoblotted with an anti-c-Jun antibody. Size markers in kilodaltons are indicated on the left in panels E and F.

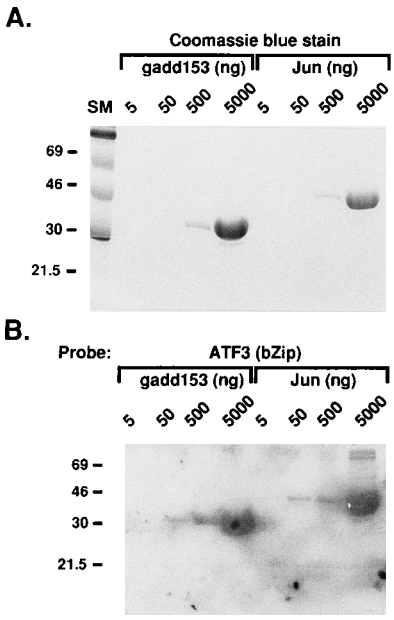

FIG. 10. ATF3 interacted with gadd153/Chop10 and c-Jun with similar affinities. (A) Coomassie blue stain to ensure that similar amounts of protein were used. (B) ATF3 showed similar affinities toward gadd153/Chop10 and c-Jun. Increasing amounts of gadd153/Chop10 or c-Jun were analyzed by protein-protein blot analysis using radio-labeled ATF3(bZip) as the probe. Rainbow size markers (SM) (Amersham) in kilodaltons are indicated on the left.

loop shape is reminiscent of the border zone reported previously (25). Hearse et al. described concentric zones of different degrees of ischemia and postulated that gradients of metabolism and electrophysiology may exist in these zones (25). It is not clear, however, whether the loop-shaped expression of ATF3 is related to the border zone phenomenon.

(iv) Postseizure brain. PTZ inhibits the γ-aminobutyric acid–benzodiazepine–chloride ionophore receptor complex, resulting in increases in neuronal actions and thereby inducing seizures (61). Previously, it has been demonstrated that seizure induces c-*fos* in the dentate gyrus, a region implicated in seizures (47, 56). Here, we demonstrate that, similar to c-*fos*, ATF3 is induced by seizure. Our result is also consistent with the observation that the ATF3 mRNA level increases upon depolarization of neuroblastoma cells in culture (1).

(v) Light-stimulated SCN. Gene expression is important for both the generation and the control of daily light/dark rhythms (references 36 and 62 and references therein). Because the immediate-early genes c-*fos*, c-*jun*, and *jun*B are induced by light stimulation during the subjective night of the animals (36, 55) and because ATF3 is induced by many stimuli (described above), we examined the expression of ATF3 by light stimulation. As shown in Fig. 1N, ATF3 was not induced in the SCN. The failure to induce ATF3 was not due to the procedure failure, because adjacent tissue sections hybridized with the c-*fos* antisense RNA probe (data not shown). Perhaps one difference between light stimulation and all the other treatments described above is the lack of tissue injury during light stimulation. It is tempting to speculate that this difference contributes to the lack of ATF3 induction by light stimulation. One caveat in interpreting our result is that we examined the expression of ATF3 only after light stimulation for 2 h. It remains to be determined whether ATF3 is induced at different time points after light stimulation.

A working hypothesis for the role of ATF3 in the liver's response to CCl_4. In this report, we show that ATF3 heterodimerizes with gadd153/Chop10. gadd153/Chop10 cDNA was first isolated from a hamster library by using subtractive hybridization based on its ability to be induced by UV irradiation (19). It was subsequently shown to be induced by many growth arrest and DNA-damaging agents (7, 20). By a zipper blot method, Ron and Habener isolated a cDNA encoding the C/EBP-homologous protein, which is more than 85% identical to gadd153 (54). They further demonstrated that gadd153/Chop10 acts as a negative regulator of C/EBP proteins by forming nonfunctional heterodimers with them (54). Here, we show that gadd153/Chop10 also acts as a negative regulator of ATF3. In addition, we show that the level of gadd153/Chop10 mRNA was high in the normal liver and greatly decreased upon CCl_4 treatment. Our observation that gadd153/Chop10 was readily detectable in the normal liver is consistent with a previous report demonstrating the presence of gadd153/

Chop10 mRNA in many tissues, including the liver (54). This result, however, is in contrast to that of two reports showing low, basal levels of gadd153/Chop10 mRNA in the normal liver (33, 64). This discrepancy is probably due to different sensitivities of the assays, because different amounts of total RNA or poly(A) RNA were analyzed, and human gadd153/Chop10 cDNA was used to probe rat RNAs in one report.

In this report, we also demonstrate an inverse correlation between the expression of ATF3 and gadd153/Chop10. It is reasonable that the expression of gadd153/Chop10 is low when the expression of ATF3 is high, because gadd153/Chop10 is a negative regulator of ATF3. It would not be reasonable for the cells to induce ATF3 when the concentration of its inhibitor, gadd153/Chop10, is high. However, we emphasize that although the expressions of ATF3 and gadd153/Chop10 were inversely correlated, they did overlap. It is important that under certain conditions, ATF3 and gadd153/Chop10 are coexpressed; otherwise, their ability to interact with each other would not be biologically relevant. As shown in Fig. 6 and 7, under conditions such that the expression of gadd153/Chop10 was high, the expression of ATF3 was low but not completely absent.

As an explanation for this inverse but overlapping expression of ATF3 and gadd153/Chop10 and for the ability of gadd153/Chop10 to negatively regulate ATF3, we suggest the following hypothesis. At the prestress stage, the level of ATF3 is low and the level of gadd153/Chop10 is high; this ensures the functional inhibition of the trace amount of ATF3. Upon stress induction, cells make ATF3 to carry out certain functions and at the same time repress the synthesis of its inhibitor, gadd153/Chop10. ATF3 homodimer represses transcription, turning off certain target promoters; at the same time, ATF3 may form heterodimers with various proteins, such as protein X, which may or may not be induced by the signal. The ATF3-protein X heterodimer may act as an activator and turn on a set of target promoters. One possible candidate for protein X is c-Jun, because it is induced in the same cell population as ATF3 by CCl_4 and because the ATF3-Jun heterodimer has been demonstrated to activate transcription in transient transfection assays (28, 29). At the poststress stage, cells not only downregulate the level of ATF3 but also up-regulate the level of gadd153/Chop10 to functionally inhibit the trace amount of ATF3.

We emphasize that our results do not rule out the possibility that the ATF3-gadd153 heterodimer binds to DNA sequences unrelated to the ATF/CRE consensus. This binding may result in activation or repression of transcription. The concentration of this heterodimer, however, is low in the liver system that we described. It is not clear whether the heterodimer at low concentrations would bind to the target DNA. Clearly, to understand the roles of ATF3 in stress response, it is important to elucidate the heterodimeric partners of ATF3 and to elucidate the target promoters of the ATF3 homodimer and various ATF3 heterodimers.

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The first two authors contributed equally to this work.

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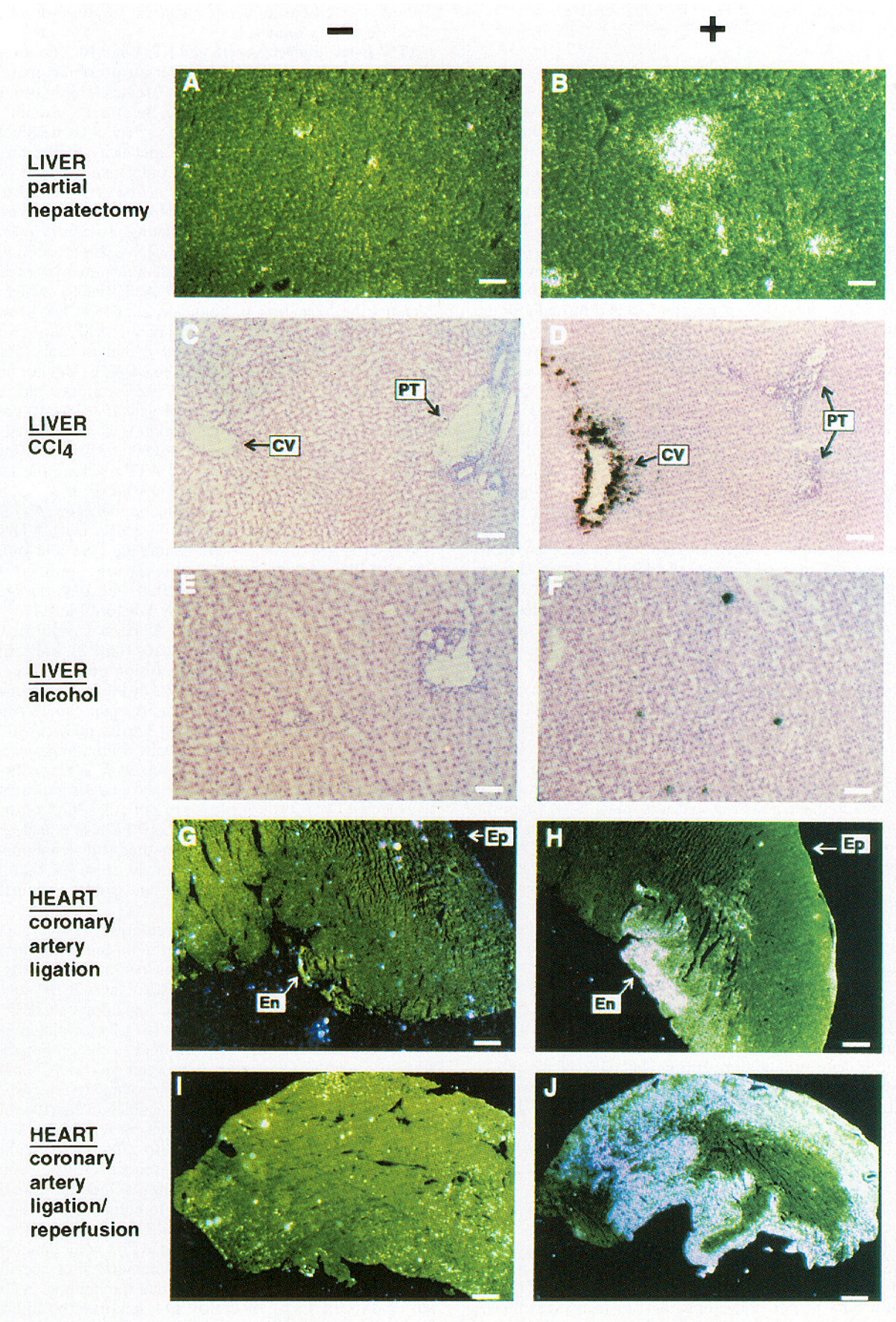
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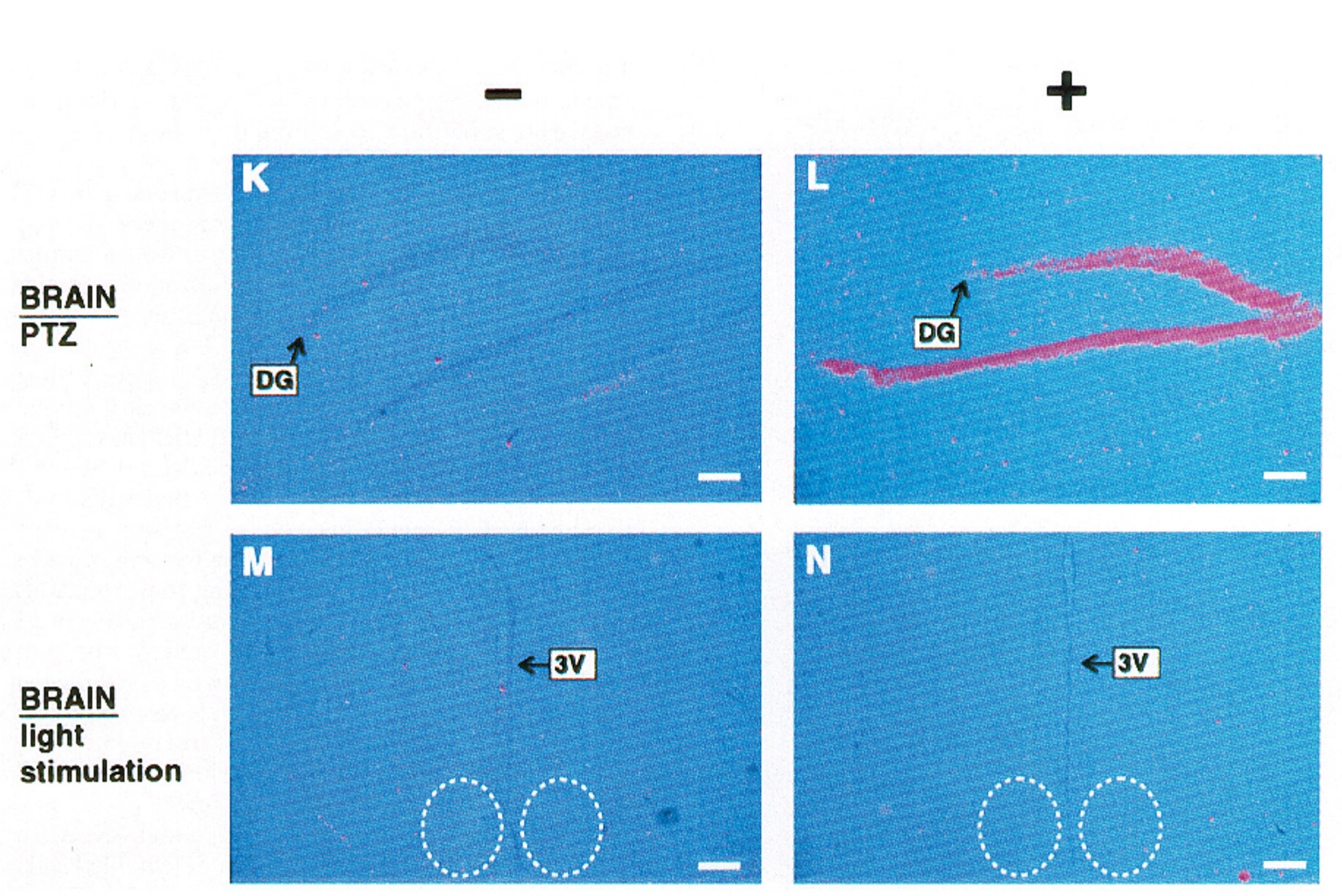
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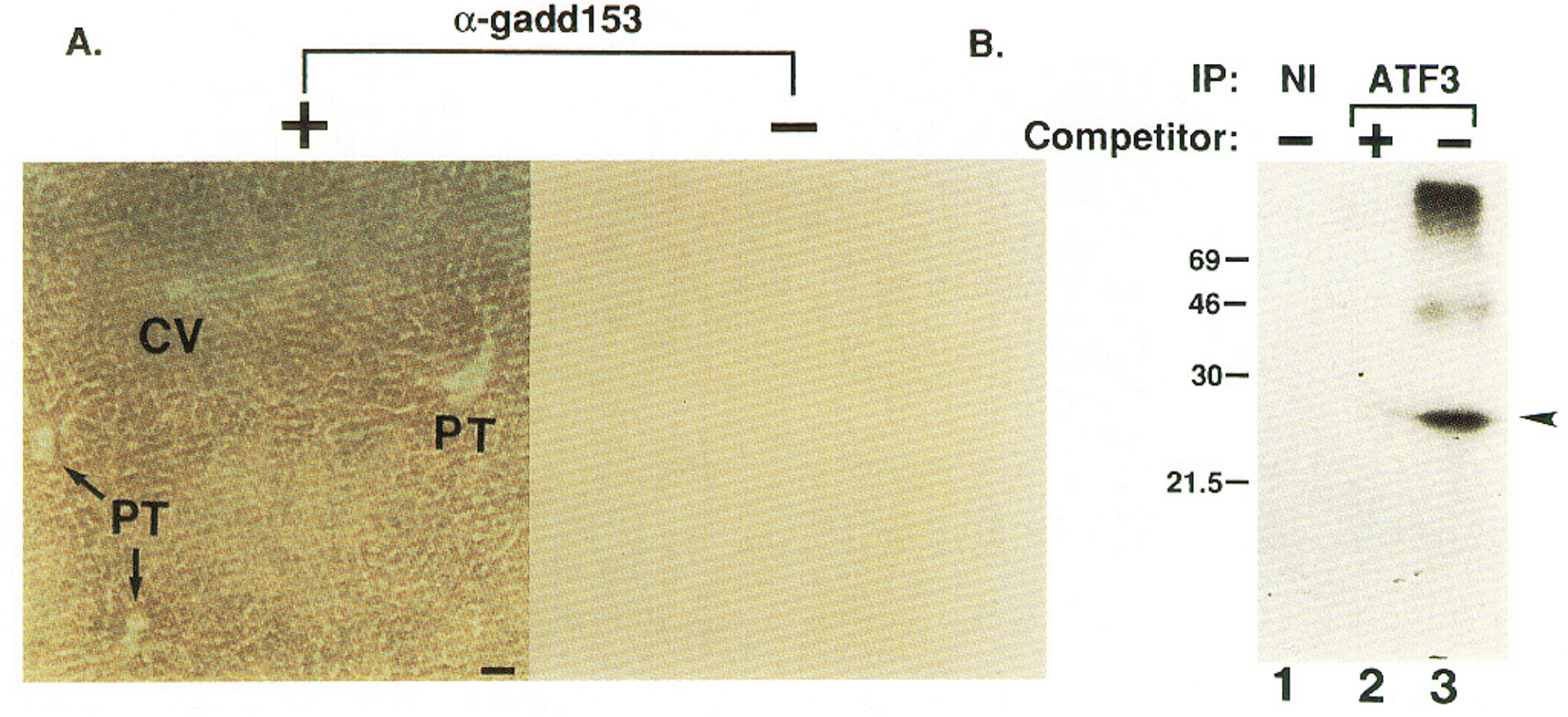
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