ATF3 Gene
GENOMIC ORGANIZATION, PROMOTER, AND REGULATION*

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ATF3 gene, which encodes a member of the activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family of transcription factors, is induced by many physiological stresses. As a step toward understanding the induction mechanisms, we isolated the human ATF3 gene and analyzed its genome organization and 5′-flanking region. We found that the human ATF3 mRNA is derived from four exons distributed over 15 kilobases. Sequence analysis of the 5′-flanking region revealed a consensus TATA box and a number of transcription factor binding sites including the AP-1, ATF/CRE, NF-κB, E2F, and Myc/Max binding sites. As another approach to understanding the mechanisms by which the ATF3 gene is induced by stress signals, we studied the regulation of the ATF3 gene in tissue culture cells by anisomycin, an approach that has been used to study the stress responses in tissue culture cells. We showed that anisomycin at a low concentration activated the ATF3 promoter and stabilizes the ATF3 mRNA. Significantly, co-transfection of DNAs expressing ATF2 and c-jun activates the ATF3 promoter. A possible mechanism implicating the C-jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) stress-inducible signaling pathway in the induction of the ATF3 gene is discussed.

Transcriptional regulation plays an important role in both differentiation and homeostasis (for reviews see Refs. 1 and 2). We have been studying the ATF/CREB family of transcription factors (Refs. 3–6; for reviews see Refs. 7–9). Members of the ATF/CREB family bind to a consensus DNA sequence (TGACGTCA), have a similar DNA binding domain (the basic region/leucine zipper (bZip) domain), and form selective heterodimers with each other via the leucine zipper region. Although all ATF/CREB proteins share similarity in their bZip domains, subgroups of proteins share additional similarity in other regions. For example, ATF1 (5), CREB (4, 6), and CREM (3) are similar in regions that contain the phosphorylation sites. Similarly, ATF2/CRE-BP1 (Ref. 10; also named HB16 in Ref. 11) and ATF2a (12) share similarity in regions outside the bZip domain: the first 100 N-terminal residues and the last 13 C-terminal residues. It is possible that proteins within a given subgroup have closely related functions. Proteins between subgroups, however, are completely different from each other outside the DNA binding domain, indicating that they may interact with different proteins or ligands and have different functions.

Consistent with this idea, ATF1 and CREB have been demonstrated to stimulate transcription in response to cAMP and calcium influx (13–16), whereas ATF2/CRE-BP1 has been demonstrated to stimulate transcription in response to viral induction (17–19). ATF3, on the other hand, is not an activator; it represses transcription when bound to DNA (20). However, ATF3 can heterodimerize with J un proteins, and the ATF3/J un heterodimers have been demonstrated to activate transcription (21, 22). Therefore, depending on the cellular context, ATF3 may repress transcription as homodimers or activate transcription as heterodimers.

Recently, we found that the level of ATF3 mRNA greatly increases both in tissue culture cells after serum stimulation (20) and in whole organisms after physiological stressors. Using rats as a model system, we demonstrated that ATF3 mRNA level increased in mechanically injured liver after partial hepatectomy and in chemically injured liver treated with toxins such as carbon tetrachloride or alcohol. ATF3 was also induced in blood-deprived heart (ischemic heart) after coronary artery ligation and in reperfusion-injured heart after coronary artery ligation coupled with reperfusion. Furthermore, ATF3 was induced in postseizure brain treated with pentylentetrazole. Significantly, ATF3 was not induced in the suprachiasmatic nuclei in entrained rats receiving light stimulation during their subjective night. One difference between light stimulation and the rest of the treatments is that light stimulation does not elicit cellular injuries, whereas the others do. Therefore, these results suggest a correlation between ATF3 gene expression and cellular injuries.

In all types of induction, ATF3 mRNA level greatly increased within 2 h after stimulation. This quick induction of the ATF3 gene by many physiological stressors suggests that ATF3 may play an important role in stress responses. It is not clear, however, how stress signals induce ATF3 gene. As a first step toward understanding the induction mechanisms, we isolated the human ATF3 gene and analyzed its 5′-flanking region. We also studied the regulation of the ATF3 gene in tissue culture cells by anisomycin, an approach that has been used to study...
the stress responses in tissue culture cells (24–27). In this report, we present the genome organization of the ATF3 gene and the sequence analysis of its 5'-flanking region. We also describe the effects of anisomycin on the stability of ATF3 mRNA and the activity of ATF3 promoter. The involvement of the stress-inducible JNK/SAPK signal transduction pathway (for a review see Ref. 28) in the induction of ATF3 mRNA and the activity of ATF3 gene is discussed.

MATERIALS AND METHODS

Plasmids—pH2.8k, a pGEM3 derivative, contains the HindIII 2.8-kilobase fragment as shown in Fig. 1. This fragment contains the following regions from the ATF3 gene: the 5'-flanking region (−1850 to +1), the first exon (+1 to +167), and 680 base pairs from the first intron. pATF3-CAT contains the −1850 to +34 region of the ATF3 gene. pE45M-CAT contains the −330 to +17 region of the adenovirus E4 promoter with an internal deletion from −138 to −65 (29). pCG-ATF2 was described previously (20).

Screening of the Genomic Library—Genomic DNA isolated from a human placenta was partially digested with MboI. DNA fragments ranging from 16 to 20 kilobases were isolated by sucrose density gradient and cloned into the λ-FixI vector (Stratagene). Approximately 100 plaques were screened with 32P-labeled ATF3 cDNA.

Cell Culture, Transfection, and Chloramphenicol Acetyltransferase (CAT) Assay—Cell culture, transfection, and CAT assays were performed as described previously (20) with the following modifications. For comparison of promoter activities, HeLa cells were transfected with 2 μg of the indicated reporter construct in addition to 5 μg of pGEM4 (Promega) carrier DNA by the calcium phosphate method. CAT assays were performed 36 h after transfection. For induction experiments, NIH 3T6 cells were transfected with 2 μg of pATF3-CAT or pG6TI-CAT by Lipofectamine (Life Technologies, Inc.), followed by starvation for 72 h in medium containing 0% serum and induction for 24 h in medium containing 20% fetal bovine serum or in medium containing 0.5% fetal bovine serum and one of the following reagents: 50 ng/ml anisomycin or 50 ng/ml of actinomycin D. CAT assays were performed after induction. For co-transfection experiments, HeLa cells were transfected with 2 μg of pATF3-CAT in addition to 5 μg of pCG-ATF2 expressing ATF2, pCMV-5-3 expressing c-jun, or 2.5 μg of each DNA. In the reporter alone control, 5 μg of pGCG was added.

Cell Labeling and Immunoprecipitation—Cell labeling and immunoprecipitation were described previously (20).

In Vitro Transcription—In vitro transcription by crude nuclear extract was carried out as described previously (30). The CAT-primer, 5'-GCCATGCTATGTAACAGCG-3', is complementary to the region from +29 to +49 of the CAT mRNA. Nuclear extracts were made from uninduced or induced HeLa cells according to Dignam et al. (31). Induction was carried out for 2 h in medium containing 50 ng/ml anisomycin.

Northern Blot and Primer Extension—Northern blot and primer extension were carried out as described previously (20). The primer (5'-CTCCGGGCGCAGGG-3') is complementary to the region from +141 to +156 of the ATF3 mRNA. Extended products were resolved on an 8% polyacrylamide sequencing gel, and the sequence ladder was generated from the same primer using pH2.8k as a template.

In Situ Hybridization—5 x 10⁶ HeLa cells were grown on Superfrost Plus glass slides (VWR Scientific) and stained in medium containing 0.25% serum for 48 h. The cells were then incubated with growing medium in the absence or presence of 50 ng/ml anisomycin for 2 h. In situ hybridization was carried out as described previously.²

Analysis of mRNA Half-life—HeLa cells were induced with 20% fetal bovine serum and 50 ng/ml anisomycin for 2 h, washed with phosphate-buffered saline, and incubated with medium containing 10% fetal bovine serum and 25 μg/ml 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Sigma). In one set of plates, 50 ng/ml of anisomycin was also included. Total RNA was isolated at the indicated time points and analyzed by Northern blot using ATF3 or GAPDH cDNA as probe.

DNA Sequencing—Dideoxy sequencing was carried out using the regular method with Taq polymerase (Promega) or the cycle sequencing method (Life Technologies, Inc.).

RESULTS

Isolation and Characterization of the Human ATF3 Gene—An unamplified λ-FixI genomic library prepared from size-fractionated human placenta DNA was screened using ATF3 cDNA as probe. Two overlapping clones, λ16kb-1 and λ20kb-2, were further characterized using the N-terminal or C-terminal cDNA as probe; only λ16kb-1 contained the 5' end of the gene. The λ16kb-1 clone was further mapped, subcloned, and sequenced to identify the mRNA coding region, exon/intron junctions, and the 5'-flanking sequences. Fig. 1 shows the restriction maps for EcoRI and HindIII. The positions of the exons were determined by Southern blot analysis using cDNA or oligonucleotides as probes. DNA fragments containing the exons were further characterized by detailed restriction mapping and sequencing. Comparison of the genomic and cDNA sequences with the restriction map suggested that the human ATF3 mRNA is derived from four exons distributed over 15 kilobases, designated as exons A, B, C, and E in Fig. 1. Exon A encodes the 5'-untranslated region. Exon B contains the AUG initiation codon and encodes the N-terminal 80 amino acids. Exon C encodes 36 amino acids, which is mostly the basic region. Exon E encodes 65 amino acids, which is mostly the leucine zipper (ZIP) domain; it also encodes the 3'-untranslated region. This organization indicates that exons B, C, and E each encode a functional domain, consistent with the modular nature of many exons. Fig. 2 summarizes the exon organization, and Table I shows the sequences around the exon/intron junctions.

As reported previously (20), there is an alternatively spliced isoform of ATF3, ATF3AZip. ATF3AZip contains an additional exon between exons C and E; this additional exon introduces an in-frame termination codon, resulting in a truncated protein lacking the leucine zipper region at the C terminus. In addition, the last three nucleotides (AAA) in exon C were spliced out in this isoform, resulting in a glutamine (Q) residue instead of a lysine (K) residue at the end of the corresponding domain. The splicing event resulting in ATF3AZip was shown previously (20); the exon organization of ATF3AZip is shown in Fig. 2.

Identification of the Transcriptional Start Site and Analysis of the 5'-Flanking Sequences—To identify the transcriptional

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**Fig. 1. Structure of the human ATF3 gene.** Exons are indicated by boxes and designated as A, B, C, D, and E. EcoRI (R) and HindIII (H) restriction sites are shown. pH2.8k contains the indicated 2.8-kilobase fragment.
To analyze the promoter, we sequenced the 5'-flanking region. Fig. 4 shows the sequence of the 5'-flanking 1850 nucleotides. Inspection of the 5'-flanking sequence revealed a consensus TATA element around -30 and, interestingly, a consensus ATF/CRE site around -90. We also noticed several other transcription factor binding sites. Among them, two classes of binding sites are especially interesting. One is the inducible site such as the ATF/CRE, AP1, and NF-kB sites; the other is the site implicated in cell cycle regulation, such as the Myc/Max and E2F binding sites. It is not clear, however, whether any of these binding sites are functionally important for the promoter activity. The promoter region also contains other transcription factor binding sites, such as the SP1, AP2, AP3, and octamer binding sites, although they are not indicated in Fig. 4.

Promoter Activity of the 5'-Flanking Two-kilobase Region—To find out whether the 5'-flanking region contains promoter activity, we constructed a CAT reporter driven by a fragment containing the 5'-flanking two kilobases (pATF3-CAT), and compared its activity with several that of CAT reporters driven by different sequences: SP1 sites (pG6TI-CAT), adenovirus E4 promoter (pE4SM-CAT), the Rous sarcoma virus long terminal repeat (pRSV-CAT), and the E1B
TATA box (pEC). As shown in Fig. 5A, when transfected into HeLa cells, pATF3-CAT was much more active than pEC; it was similar to pE4SM-CAT and 50% active compared with pG6TI-CAT but only 5% active compared with pRSV-CAT. Consistent with this result, pATF3-CAT displayed similar activity as pE4SM-CAT in an in vitro transcription assay but was less active than pG6TI-CAT (Fig. 5B). pRSV-CAT was not included in this assay, because it was not active in vitro.

As reported previously (20), ATF3 gene is induced by serum stimulation in tissue culture cells. We then examined whether pATF3-CAT responds to serum stimulation in tissue culture cells. We transiently transfected pATF3-CAT into NIH 3T6 cells and assayed for CAT activity. A representative result of three experiments is shown. C, induction assay. NIH 3T6 cells were transfected with either pATF3-CAT or pG6TI-CAT, starved for 72 h, and then induced as indicated for 24 h. CAT activity was assayed, and an average of four results is shown.

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cells, starved the cells in medium containing 0% serum to arrest the cells in G0 phase, and induced the cells with 20% serum. We used NIH 3T6 cells instead of HeLa cells, because serum starvation arrests NIH 3T6 cells better than HeLa cells. As shown in Fig. 5C, pATF3-CAT was induced by serum stimulation, whereas pG6TI-CAT was not. In addition, we examined the inducibility of pATF3-CAT by the phorbol ester tetradecanoyl-phorbol acetate, which also induced the endogenous ATF3 gene. Fig. 5C shows that it slightly induced pATF3-CAT, although the induction was not as high as that of the endogenous ATF3 gene. Taken together, we conclude that the 5'-flanking two-kilobase region contains promoter activity and can confer, as least partly, the responsiveness to several inducing agents.

The Effects of Anisomycin on ATF3 Gene Expression—As described earlier, the ATF3 gene is induced by many physiological stresses. This observation prompted us to ask whether the stress-inducible JNK/SAPK signal transduction pathway (for a review see Ref. 2) is involved in the induction of ATF3 gene by stress signals. As a first step toward answering this question, we examined the induction of ATF3 gene by anisomycin, because anisomycin at subinhibitory concentrations, concentrations that fail to inhibit protein synthesis, can activate the JNK/SAPK signal transduction pathway (32, 33). We examined the expression of the endogenous ATF3 gene in tissue culture cells by both Northern blot analysis and in situ hybridization. As shown in Fig. 6, A and B, anisomycin at 50 ng/ml greatly increased the level of ATF3 mRNA. That anisomycin at this concentration did not significantly inhibit protein synthesis was confirmed as follows. We incubated the cells with [35S]methionine to radiolabel the newly synthesized proteins in the absence or presence of anisomycin and examined the incorporation of [35S]methionine into cellular proteins by an SDS-polyacrylamide gel. As shown in Fig. 6C, at 50 ng/ml (low dose), anisomycin had little effect on the synthesis of total cellular proteins; at 10 μg/ml (high dose), however, anisomycin completely abolished the synthesis. Furthermore, immunoprecipitation using ATF3 antibody indicated that the newly induced ATF3 mRNA is indeed translated to produce ATF3 protein in the presence of 50 ng/ml anisomycin (lanes 5-7). Taken together, this information leads us to conclude that anisomycin at a subinhibitory concentration induces ATF3.

Because both Northern blot and in situ hybridization detect steady-state mRNA levels, the increase could be due to the increase of mRNA synthesis or the increase of mRNA stability. It is possible that anisomycin treatment stabilizes ATF3 mRNA because the 3'-untranslated region of ATF3 mRNA contains several AUUUA sequences, which have been demonstrated to destabilize mRNA (for reviews see Refs. 34 and 35). To find out whether anisomycin increases the stability of ATF3 mRNA, we compared the stability of ATF3 mRNA in the absence and presence of anisomycin as follows. We treated HeLa cells with 20% serum and 50 ng/ml of anisomycin for 2 h to increase the steady-state level of ATF3 mRNA. After removal of serum and anisomycin, we added DRB to inhibit further RNA synthesis, allowing the existing RNA to turn over. We then analyzed ATF3 mRNA by Northern blot at various time points to assay for its stability. In one set of plates, we added anisomycin back to determine whether it affects the stability of ATF3 mRNA. As shown in Fig. 7, anisomycin at 50 ng/ml moderately increased the stability of ATF3 mRNA.

To find out whether anisomycin increases the activity of ATF3 promoter, we examined pATF3-CAT in the absence and presence of anisomycin. We transiently transfected pATF3-CAT into NIH 3T6 cells, starved the cells for 72 h, and induced them for 24 h in medium containing 50 ng/ml anisomycin. As shown in Fig. 5C, pATF3-CAT was more active in the presence of anisomycin than in the absence of anisomycin. A control reporter, pG6TI-CAT, was not activated by anisomycin. We note that pG6TI-CAT was less active than pATF3-CAT in NIH 3T6 cells (Fig. 5C) but was more active than pATF3-CAT in HeLa cells (Fig. 5A). This discrepancy was probably due to the differences between these two cell lines. The observation that pATF3-CAT can be induced by anisomycin was recapitulated by an in vitro transcription assay; nuclear extracts made from anisomycin-treated HeLa cells transcribed the ATF3 promoter at a higher activity than nuclear extracts from untreated HeLa cells (Fig. 8). These two extracts, however, showed no difference in transcribing the control promoter composed of SP1 sites (Fig. 8). These results suggest that the increase of steady-state ATF3 mRNA level in the presence of anisomycin was, at least partly, due to an increase of the ATF3 promoter activity.

As described earlier, anisomycin activates the JNK/SAPK signal transduction pathway (32, 33). Two transcription factors, ATF2 and c-Jun, have been demonstrated to be phospho-

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3 G. S. Liang, C. D. Wolfgang, B. P. C. Chen, T.-H. Chen, and T. Hai, unpublished results.
ATF3 gene. We also describe the exon/intron junctions of two alternatively spliced isoforms: ATF3 and ATF3a2ip. However, the physiological relevance of these isoforms is not clear at present. Other ATF/CREB family transcription factors have also been demonstrated to have alternatively spliced isoforms. Some examples are CREM (for a review, see Ref. 40), CREB (Refs. 41–43; for a review see Ref. 9), ATF2 (44), and ATFα (12, 45). In light of the large and complex genome organization of both CREM and CREB genes, it is possible that the small, 15-kilobase genome region of the human ATF3 gene described in this report represents only a portion of the gene. We are currently further characterizing the ATF3 genome to clarify this point.

The 5′-Flanking Region of the ATF3 Gene—Analysis of the 5′-flanking 1.8-kilobase region revealed various transcription factor binding sites. Two groups of binding sites are of special interest: the inducible sites and the sites implicated in cell cycle regulation. The inducible sites include the ATF/CRE, AP1, and NF-κB sites. Because these sites have been demonstrated to be induced by signals such as cAMP, calcium influx, UV irradiation, and cytokines (for reviews see Refs. 9 and 46–52), their presence is consistent with our observation that ATF3 gene is induced by many stimulations. Their presence also supports the observation described in this report that the induction is, at least partly, due to the activation of the ATF3 promoter. In addition, the presence of the ATF/CRE consensus sequence in the ATF3 promoter suggests that the ATF3 gene may be regulated by the ATF/CREB family of transcription factors. The observation that co-expression of ATF2 and Jun activates the ATF3 promoter is consistent with this notion. The presence in the ATF3 promoter of the Myc/Max and E2F sites, sites implicated in cell cycle regulation, implies that the expression of ATF3 gene may also be regulated in an cell cycle-dependent manner. We are currently pursuing this possibility.

The Effects of Anisomycin on the Stability of ATF3 mRNA. HeLa cells were treated with 20% serum and 50 ng/ml anisomycin for 2 h to increase the steady-state level of ATF3 mRNA. After removal of serum and anisomycin, DRB was included in the medium at a concentration of 25 μg/ml to inhibit RNA synthesis, allowing the existing RNA to decay. No anisomycin (− Anisomycin, lanes 2–6) or 50 ng/ml of anisomycin (+ Anisomycin, lanes 8–12) was included in addition to DRB to examine the effects of anisomycin on ATF3 mRNA stability. Total RNA was isolated at the indicated times and analyzed (30 μg/lane) by Northern blot using ATF3 or GAPDH cDNA as probe. RNAs from the uninduced cells were also analyzed (lanes 1 and 7).

DISCUSSION

Genome Organization of the Human ATF3 Gene—In this report, we describe the genome organization of the human gene. We are currently further characterizing the ATF3 genome to clarify this point.

The Effects of Anisomycin on the Stability of ATF3
mRNA—As shown in Fig. 7, 50 ng/ml of anisomycin moderately increased the stability of ATF3 mRNA. In this context, it is important to point out that a previous report demonstrated that, in C3H 10T1/2 cells, the half-life of c-fos and c-jun mRNA was 15 and 30 min, respectively, in the presence of subinhibitory concentrations of anisomycin, whereas their half-lives were more than 6 h in the presence of inhibitory concentrations of anisomycin (23). However, not available were the half-lives of these mRNAs in the absence of anisomycin in C3H 10T1/2 cells. Consequently, it is not clear whether these mRNAs are more stable in the presence of subinhibitory concentrations of anisomycin than in the absence of anisomycin. Therefore, our observation that a subinhibitory concentration of anisomycin stabilizes the ATF3 mRNA does not necessarily contradict their results.

Regulation of the ATF3 Promoter by Anisomycin—In this report, we demonstrate that anisomycin activates the ATF3 gene in tissue culture cells. Although anisomycin is a protein synthesis inhibitor, this activation is independent of its ability to inhibit protein synthesis, because anisomycin at 50 ng/ml, a concentration that does not significantly affect protein synthesis, can still activate the ATF3 gene. Therefore, anisomycin activates the ATF3 gene, not by its ability to generally inhibit protein synthesis, but by its intrinsic ability to activate certain intracellular machinery. One candidate for such a machinery is the JNK/SAPK signal transduction pathway, because anisomycin has been demonstrated to activate this pathway independent of its ability to block protein synthesis (23, 32, 33). Therefore, our results indicate a correlation between the activation of the JNK/SAPK pathway and the activation of the ATF3 gene.

The activation of the ATF3 gene by anisomycin is, at least in part, due to the stimulation of the ATF3 promoter, because a CAT reporter driven by the ATF3 promoter can be activated by anisomycin (Fig. 5C). Significantly, the ATF3 promoter can also be activated by the coexpression of ATF2 and c-jun (Fig. 9). Because these two transcription factors have been demonstrated to be phosphorylated and activated by the JNK/SAPK pathway (24–26, 28, 36–39), our preliminary evidence is consistent with the notion that the JNK/SAPK pathway may be involved in the activation of the ATF3 promoter by anisomycin. This notion is reminiscent of the observation that this pathway mediates the induction of the c-jun gene by genotoxic agents (39).

We emphasize that although our results are consistent with the notion that the JNK/SAPK signaling pathway may be involved in the induction of ATF3 gene by anisomycin, they do not prove it. We also note that although the anisomycin approach has been used successfully as a model system to study stress responses in tissue culture cells (24–27), it is not clear whether the mechanisms by which anisomycin induces ATF3 gene in tissue culture cells are the same as that by which physiological stresses induce ATF3 gene in the whole organisms. Clearly, many more experiments are required to clarify these points.

In summary, we analyzed the genome organization and promoter sequences of the human ATF3 gene. We also studied the regulation of ATF3 gene by anisomycin. These results should aid future studies of the induction of ATF3 gene by stress signals.

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