

Transcriptional Autorepression of the Stress-inducible Gene *ATF3**

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Previously, we demonstrated that *ATF3* (activating transcription factor-3) is a stress-inducible gene, and the protein it encodes is a transcriptional repressor. In this report, we present evidence suggesting that *ATF3* represses the transcription of its own gene. Interestingly, efficient repression requires a consensus ATF/cAMP-responsive element site in the promoter and a previously unidentified *ATF3*-binding site immediately downstream from the TATA box. Although this new site resembles the known ATF/cAMP-responsive element sequences at the flanking sequence, it differs from them at the center key residues. These observations indicate that *ATF3* can tolerate variations in the center of the binding sites if the flanking sequences are favorable. The repression of the *ATF3* promoter by its own gene product provides a mechanistic explanation, at least in part, for the transient expression pattern of the *ATF3* gene upon stress induction.

All cells exhibit alterations in gene expression in response to extracellular stress signals such as elevated temperature, lack of nutrients, and exposure to toxins (for a review, see Ref. 1). 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 Ref. 2). One such immediate-early gene is *ATF3* (for a review, see Ref. 3). Previously, we demonstrated that *ATF3* is induced by many physiological stress signals in animal models (4, 5). By *in situ* hybridization, we demonstrated that the steady-state mRNA level of *ATF3* greatly increases upon exposure to a variety of stresses: seizure, toxic chemicals, mechanical injury, ischemia, and ischemia coupled with reperfusion (for a review, see Ref. 3). In each case, *ATF3* is induced in the corresponding tissue that is exposed to the stress signal. Therefore, the induction of *ATF3* is a common cellular response to many stress signals: it is neither tissue- nor stress-specific.

In addition to animal model systems, *ATF3* has also been demonstrated by many investigators to be induced in cultured

cells by a variety of treatments. These treatments include the following: (a) growth-stimulating factors such as serum (6–9), fibroblast growth factor (10), and epidermal growth factor (11); (b) cytokines such as interferons and interleukin-4 (12, 13); (c) genotoxic agents such as ionizing radiation, methyl methane-sulfonate, and ultraviolet light (14); and (d) agents known to induce the c-Jun N-terminal kinase/stress-activated protein kinase stress signaling pathway such as anisomycin (15) and cycloheximide (11). Therefore, *ATF3* is induced by a variety of extracellular signals, and many of them are stress signals. In this context, it is interesting to note that, as indicated by Brown and co-workers (8), the temporal program of transcription during the response of human fibroblasts to serum appears to be a paradigm for stress response during wound healing rather than cell proliferation. This is because normal cells do not encounter serum (the soluble fraction of clotted blood), but become exposed to serum in the context of a wound. Therefore, the induction of *ATF3* by serum in cultured cells may reflect a part of the complex cellular stress response to wounding. Epidermal growth factor (in serum) and fibroblast growth factor (in the extracellular matrix) are also factors that the cells will encounter during wounding; thus, their use in cultured cells may also be viewed as a paradigm for wound healing. In summary, in both animal and cell culture models, *ATF3* is induced by many signals that can be classified as stress signals, supporting the idea that *ATF3* is a stress-inducible gene.

In most cases of *ATF3* induction, the expression of *ATF3* is immediate (within 2 h) and transient (Refs. 4 and 16; for a review, see Ref. 3). At present, the mechanism for the transient nature of *ATF3* induction is not clear. Two clues prompted us to hypothesize that *ATF3* autorepresses its own gene. First, the human *ATF3* gene promoter has a consensus ATF¹/CRE site (15). Second, *ATF3* is a transcriptional repressor (7). This autorepression hypothesis provides an explanation, at least in part, for the transient nature of *ATF3* induction (see “Discussion”). In this report, we present evidence supporting this hypothesis. Significantly, we identified an unexpected *ATF3*-binding site that is critical for the autorepression of the *ATF3* promoter. This site differs from the consensus ATF/CRE site in the key central residues and is immediately downstream from the TATA box.

EXPERIMENTAL PROCEDURES

Plasmids—Human cDNAs for ATF1, ATF3, ATF3(1–100), and ATF4 were inserted into the following vectors for expression: pCG (17) for expression in mammalian cells and pTM1 (18) for expression in the vaccinia system or in the reticulocyte lysate translation system. pATF3-CAT, which contains the human *ATF3* promoter (–1850 to +34) linked to the chloramphenicol acetyltransferase (CAT) reporter gene, referred to as wild-type CAT in this study, was described previously (15). Dele-

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¹ The abbreviations used are: ATF, activating transcription factor; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus.

tion constructs of the *ATF3* promoter were generated using exonuclease III (Life Technologies, Inc.). Δ CRE-CAT, which lacks the consensus ATF/CRE site, was constructed by ligating the -1850 to -119 upstream region of the *ATF3* promoter to the -82 to $+34$ downstream region of the *ATF3* promoter. Δ -20-CAT was constructed by polymerase chain reaction-based mutagenesis using the wild-type CAT construct as a template, resulting in the replacement of the -27 to -11 region by the GAL4 DNA-binding sequence (5'-CGGAGGACAG-TACTCGA-3'). Δ Both-CAT, which contains both mutations described above, was constructed by polymerase chain reaction-based mutagenesis as described above, except that the Δ CRE-CAT construct was used as a template to include the Δ CRE mutation.

Cell Culture, Transfection, and CAT Assay—Maintenance of monolayer HeLa or COS-1 cells, calcium phosphate transfection, and CAT assay were as described previously (7).

In Vitro Protein Synthesis and Electrophoretic Mobility Shift Assay (EMSA)—pTM1 derivatives encoding ATF1 and ATF3 proteins were transcribed by T7 polymerase (Life Technologies, Inc.) and translated using the TNT reticulocyte lysate system (Promega) according to the manufacturer's instructions. DNA binding reactions and gel electrophoresis were carried out as described (19).

Expression and Purification of Vaccinia Virus-expressed ATF3—Expression and purification of vaccinia virus-expressed ATF3 were as described previously (20), except that 10 mM dithiothreitol was included in the dialysis step. Coomassie Blue staining of the purified fractions showed only bovine serum albumin, which was added to help stabilize the protein (data not shown), indicating that the concentration of the fusion protein or any contaminating proteins was low. The presence of ATF3 was confirmed by immunoblotting (data not shown).

DNase I Footprinting and Maxam-Gilbert Sequencing—DNase I footprinting was carried out as described previously (21) with histidine-tagged ATF3 (His-ATF3) purified from the vaccinia virus expression system. The amount of His-ATF3 required for each experiment was empirically determined by titration of each batch of protein. The DNA fragment contains the -49 to $+8$ region of the *ATF3* promoter. Maxam-Gilbert sequencing was performed as described (22).

RESULTS

ATF3 Represses Transcription of Its Own Promoter—To test whether ATF3 represses the transcription of its own promoter, we cotransfected into HeLa cells a plasmid expressing ATF3 (pCG-ATF3) with a plasmid containing a CAT reporter driven by a 1.8-kilobase fragment of the human *ATF3* promoter. This region has been demonstrated to confer inducibility of the *ATF3* promoter by stress signals such as anisomycin (15). Fig. 1 shows that ATF3 repressed the transcription of the *ATF3* promoter 10-fold. This repression is dependent on the DNA binding activity of ATF3 because ATF3(1-100), a truncated form of ATF3 that lacks the basic region-leucine zipper DNA-binding domain (7), did not repress this promoter. In fact, it activated the promoter \sim 3-fold. This activation by ATF3(1-100) is consistent with our previous observation that ATF3, when not bound to DNA, can activate transcription presumably by sequestering inhibitory cofactors away from the promoter (7). Furthermore, the repression of the *ATF3* promoter by ATF3 is not a general characteristic of the ATF/CREB family of transcription factors because ATF4, a transcriptional activator (23), activated the promoter. Therefore, we conclude that ATF3 represses the transcription of its own promoter.

A Previously Unidentified ATF3-binding Site in the ATF3 Promoter—To delineate the regions of the *ATF3* promoter that are functionally important for its repression by ATF3, we generated a series of deletion constructs from the 5'-end of the promoter by exonuclease III digestion. For the convenience of discussion, we refer to these constructs by the nucleotide numbers of their 5'-deletion points. All constructs have the 3'-end at nucleotide $+34$. The longest construct, p(-1850), will be referred to below as the wild-type "full-length" construct (wild-type CAT). Surprisingly, ATF3 repressed all of these constructs equally well, irrespective of the presence or absence of the consensus ATF/CRE site at -90 (Fig. 2).

The observation that ATF3 repressed constructs without the

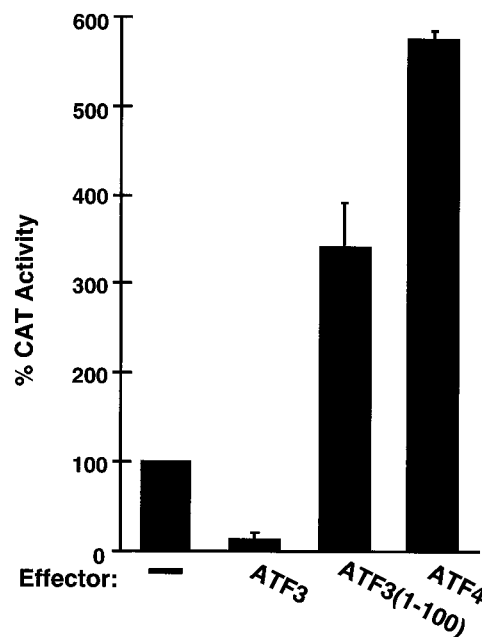


FIG. 1. ATF3 represses transcription of the *ATF3* promoter. HeLa cells were cotransfected with a CAT reporter driven by the *ATF3* promoter and pCG derivatives that express ATF3, ATF3(1-100), or ATF4. The vector (pCG) carrying the cytomegalovirus (CMV) promoter was included in some experiments to ensure that each transfection mixture contained the same amount of CMV promoter. CAT activity from the reporter transfected with the pCG vector (first bar) was arbitrarily defined as 100%. The average of three experiments is shown.

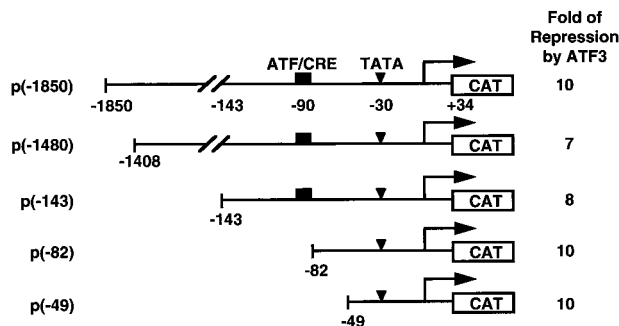


FIG. 2. Determination of the minimal region of the *ATF3* promoter necessary for repression by ATF3. CAT reporters driven by the indicated promoter regions were transfected into HeLa cells in the presence of pCG-ATF3. The vector (pCG) carrying the CMV promoter was included in some experiments to ensure that each transfection mixture contained the same amount of CMV promoter. The -fold repression by ATF3 was calculated by dividing the CAT activity in the absence of ATF3 by that in the presence of ATF3. A representative of three experiments is shown.

consensus ATF/CRE site (the p(-82) and p(-49) constructs) was quite unexpected because there is no previously identified ATF-binding site in the region downstream from -49 . These results imply that the -49 to $+34$ region contains novel binding site(s) that can mediate the repression by ATF3 (either directly or indirectly). To test this idea, we performed EMSA using DNA fragments containing different regions of the *ATF3* promoter. ATF3 was synthesized *in vitro* using the reticulocyte lysate-coupled transcription and translation system; this *in vitro* synthesized ATF3 bound specifically to a variety of previously defined ATF3-binding sites as demonstrated by competition and supershift experiments (see Fig. 7; see figures in Refs. 4, 7, and 20; data not shown). Fig. 3A shows a schematic of different *ATF3* promoter regions, and Fig. 3B shows representative results. Consistent with its ability to repress the p(-82) and p(-49) reporters, ATF3 bound to the -82 to $+34$

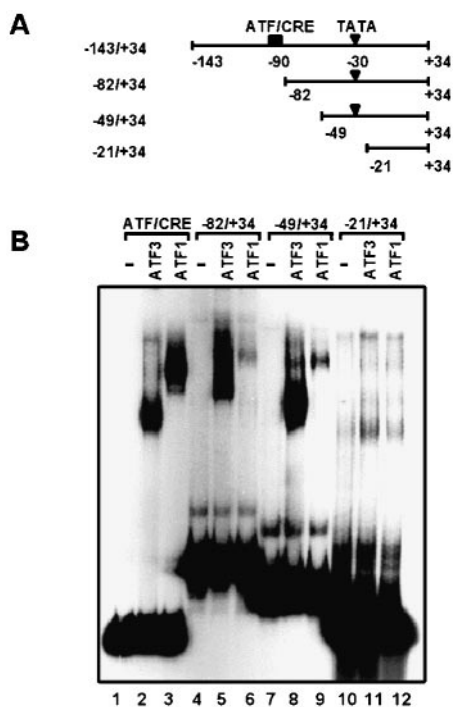


FIG. 3. A previously unidentified ATF3-binding site on the ATF3 promoter. *A*, shown is a schematic representation of various regions of the ATF3 promoter used for EMSA in *B*. Regions of the promoter are indicated by numbers relative to the transcriptional start site (+1). *B*, radiolabeled DNA fragments containing the consensus ATF/CRE site or the indicated regions of the ATF3 promoter were incubated with non-programmed reticulocyte lysates (lanes 1, 4, 7, and 10), lysates programmed to produce ATF3 (lanes 2, 5, 8, and 11), or lysates programmed to produce ATF1 (lanes 3, 6, 9, and 12) for EMSA analysis.

and -49 to +34 fragments efficiently (Fig. 3*B*, lanes 5 and 8). ATF1, another member of the ATF/CREB family of proteins, bound to these fragments only weakly (Fig. 3*B*, lanes 6 and 9), indicating that the -82 to +34 and -49 to +34 regions have a higher affinity for ATF3 than for ATF1. Control experiments showed that the same amount of ATF1 bound to the consensus ATF/CRE site efficiently (Fig. 3*B*, lane 3), excluding the possibility that the inefficient binding is due to the lack of active protein. More important, ATF3 did not bind to the -21 to +34 region of the ATF3 promoter (Fig. 3*B*, lane 11), indicating that the -49 to -22 region is necessary for ATF3 binding.

Analyses of the Novel Binding Site—To delineate the precise binding site for ATF3, we first determined the minimal region required for ATF3 binding. We performed exonuclease III deletion from the 3'-end of the -49 to +34 fragment and assayed the binding by EMSA. As shown in Fig. 4, fragment E (-49 to -9) was the smallest fragment in this deletion series that still bound to ATF3. The next fragment, fragment F, did not bind to ATF3. Because the only difference between fragments E and F is that fragment F lacks residues -16 to -9 at the 3'-end, this result indicates that residues -16 to -9 are important for the binding. To confirm this result, we performed DNase I footprint analysis using ATF3 derived from the vaccinia virus expression system. As shown in Fig. 5*A*, ATF3 protected a 17-base pair region from -26 to -10, supporting the EMSA results described above. Fig. 5*B* indicates the binding site defined by DNase I footprinting and the end points of fragments E and F for comparison. Because the residues at both the 5'- and 3'-boundaries of the protected region (-26 to -10) were not efficiently cleaved by DNase I in naked DNA, it is not possible to conclude whether they are protected by ATF3 or not. Therefore, residues -26 to -10 define the minimal region protected by

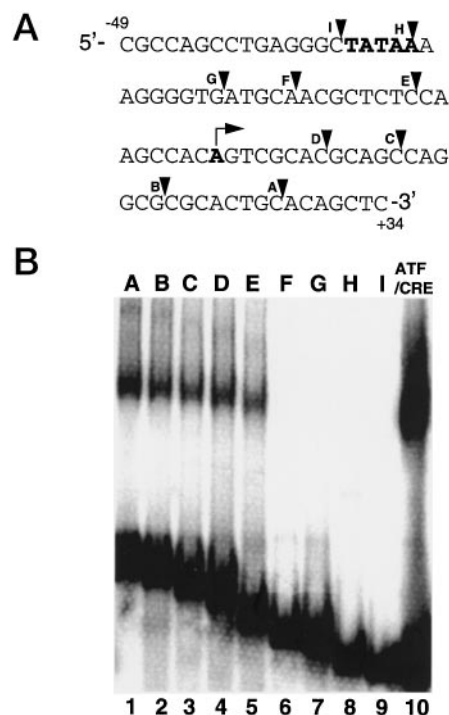


FIG. 4. Deletion analysis of the -49 to +34 region of the ATF3 promoter. *A*, shown is a schematic representation of the ATF3 promoter fragments used in *B*. Individual deletions are represented by a letter (A-I). Each deletion fragment starts at -49 of the ATF3 promoter and ends at the respective arrowhead. The TATA box is indicated in boldface, and the transcription start site (+1) is indicated by an arrow. *B*, radiolabeled fragments A-I (see above) and a fragment containing the consensus ATF/CRE site (TGACGTCA) were analyzed by EMSA in the presence of ATF3 generated by reticulocyte lysate.

ATF3. For the convenience of discussion, we refer to this region as the -20 site.

We compared the sequence at the -20 site to several binding sites that have been demonstrated previously to bind to ATF3 (19, 4, 20): the consensus ATF/CRE, AP-1, ELAM-1, E4F, and CAAT/enhancer-binding protein/ATF chimeric sites. Although it lacks a clear ATF/CRE consensus sequence (TGACGTCA), close inspection of the sequence revealed some interesting features. First, it resembles the consensus ATF/CRE and AP-1 sites at the border nucleotides, although the center key residues are different (Fig. 6*A*). Second, when including the flanking sequence, the -20 site is similar to an ATF/CRE site in the adenoviral *E4* promoter (Fig. 6*B*). Again, the -20 site differs from the *E4* promoter site in the key center residues. These observations indicate that ATF3 can tolerate variations in the center of the binding sites if the flanking sequences are favorable. Interestingly, this site is immediately downstream from the TATA box, raising the possibility that ATF3 may compete with basal transcription factors for binding to DNA.

To compare the affinity of ATF3 for this site versus the consensus ATF/CRE site, we performed a competition experiment by EMSA using the radiolabeled consensus ATF/CRE site as a probe and the unlabeled -20 site or consensus ATF/CRE site as a competitor. As shown in Fig. 7*B*, the unlabeled -20 site (lanes 8-14) was less efficient than the consensus ATF/CRE site (lanes 2-7) as a competitor, suggesting that it is a weaker binding site than the consensus site for ATF3. The specificity of the DNA-protein complex in this assay is demonstrated by the following criteria: (a) only reticulocyte lysate programmed to produce ATF3 gave rise to a strong band, whereas non-programmed or vector-programmed lysate did not (Fig. 7*A*); (b) the complex was shifted by an antiserum against

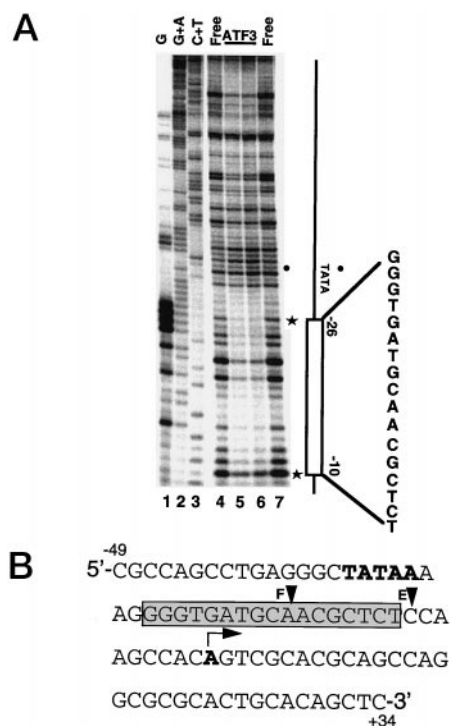


FIG. 5. Determination of the novel ATF3-binding site on the ATF3 promoter. A, shown are the results from DNase I footprint analysis of the ATF3 promoter. The end-labeled promoter fragment (-49 to +8) was analyzed in the absence (lanes 4 and 7) or presence (lanes 5 and 6) of His-ATF3 (5 μ l) purified from the vaccinia virus expression system. Lanes 4 and 7 and lanes 5 and 6 are duplicates. Maxam-Gilbert sequencing of the free probe is shown in lanes 1-3. The closed circle indicates the first T residue of the TATA box, and the rectangle flanked by stars indicates the protected region. B, a portion of the ATF3 promoter sequence is shown, and the region protected by ATF3 is boxed. The arrow indicates the transcription start site (+1). The end points of fragments E and F are indicated by arrowheads.

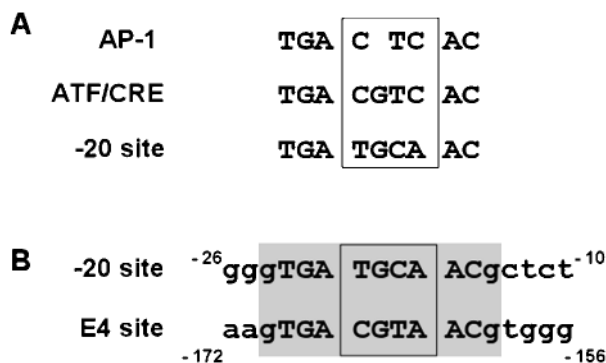


FIG. 6. Sequence comparison of the -20 site with other known ATF-binding sites. A, alignment of the consensus AP-1, consensus ATF/CRE, and -20 sites. The region where the novel site differs from the consensus sequences is boxed. B, sequence comparison of the -20 site with an ATF-binding site on the adenoviral E4 promoter. Uppercase letters indicate the binding sites, and lowercase letters indicate the flanking sequences. The region of similarity (8 out of 11 nucleotides are the same) is shaded.

ATF3, but not by a nonspecific antiserum in the supershift experiment (Fig. 7A); and (c) the complex was competed away by specific competitors (the ATF/CRE and -20 sites), but not by a nonspecific competitor (Fig. 7B, lanes 15-20).

Functional Significance of the -20 and Consensus ATF/CRE Sites—The results from the deletion mapping experiments (Fig. 2) indicated that the -49 to +34 region, which contains the -20 site only, was sufficient to confer repression by ATF3. However, those analyses were carried out using de-

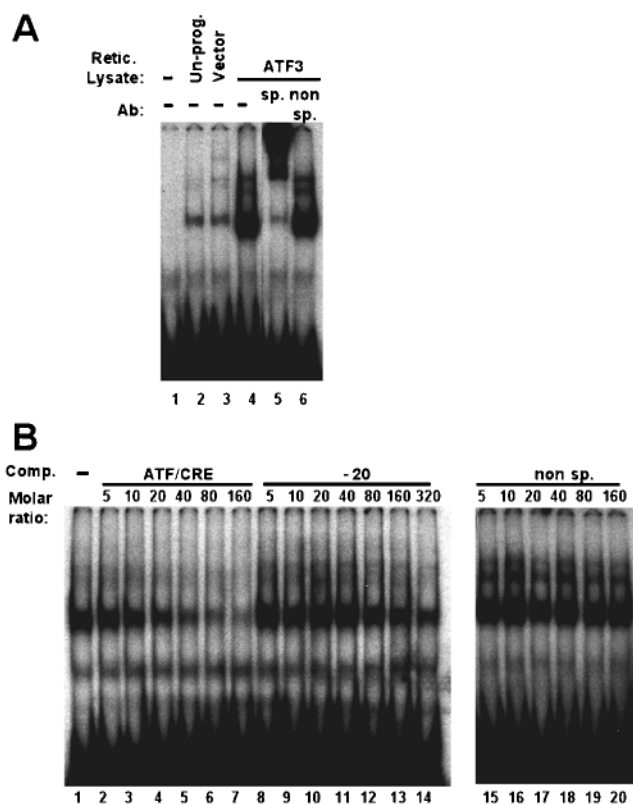


FIG. 7. ATF3 has a higher affinity for the ATF/CRE site than for the -20 site. A, a radiolabeled DNA fragment containing the consensus ATF/CRE sequence (TGACGTCA) was used for EMSA in the absence (lane 1) or presence (lanes 2-6) of various reticulocyte (Retic.) lysates: non-programmed (lane 2), programmed with vector (lane 3), and programmed to produce ATF3 (lanes 4-6). Specific antiserum against ATF3 (sp.; lane 5) or nonspecific antiserum (non sp.; lane 6) was added in the supershift experiment. B, competition (Comp.) experiments were carried out with increasing amounts of DNA fragments containing the consensus ATF/CRE sequence (lanes 2-7), the -49 to +34 region of the ATF3 promoter indicated as the -20 site (lanes 8-14), or a nonspecific sequence derived from the multicloning region of pGEM3 (non sp.; lanes 15-20). The molar ratios of the competitor DNA to the probe are indicated above the lanes. Ab, antibody.

letion constructs, not in the context of the full-length promoter (-1850 to +34). To better determine the importance of the -20 and consensus ATF/CRE sites, we generated three mutant promoter constructs in the full-length context: 1) Δ CRE, with the consensus ATF/CRE sequence (-118 to -83) deleted; 2) Δ -20, with the novel site replaced by the GAL4 site; and 3) Δ Both, with both sites mutated as described above. Fig. 8A shows a schematic of the constructs. The replacement of the -20 site by the GAL4 site completely abolished the ability of ATF3 to bind to this region as shown by EMSA (data not shown).

We first determined whether each of the ATF3-binding sites is important for the "basal" promoter activity. As shown in Fig. 8B, both sites were important for the promoter activity because loss of either site led to a significant decrease in the promoter activity. This result suggests that each site is bound by some factors that contribute to the basal activity of the "wild-type" promoter. Deletion of both sites almost completely abolished the promoter activity. To test the importance of these sites for the repression by ATF3, we transfected the mutant promoters with expression constructs encoding ATF3. As shown in Fig. 8C, ATF3 repressed the wild-type promoter 10-fold, but did not repress the mutant promoters that lack either site. We note that Fig. 2 shows that ATF3 efficiently repressed the p(-82) and p(-49) constructs, which lack the consensus ATF/CRE site

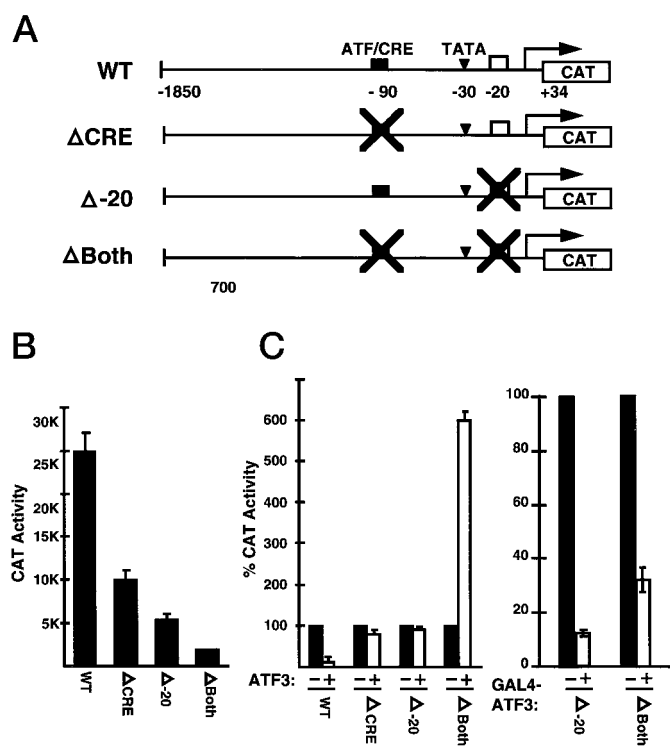


FIG. 8. Both the ATF/CRE and -20 sites are important for the basal activity of the ATF3 promoter and the repression of the promoter by ATF3. A, schematic representation of the CAT reporters used in B and C. The positions of the elements are indicated by numbers relative to the transcriptional start site (+1) as indicated by arrows. B, the ATF/CRE and novel ATF3-binding sites are important for the basal activity of the ATF3 promoter. HeLa cells were transfected with the indicated CAT reporters, and CAT activities were determined. The average of three experiments is shown. C, the ATF/CRE and novel ATF3-binding sites are required for the repression of the ATF3 promoter by ATF3. Cells were transfected with the indicated CAT reporters in the absence (-) or presence (+) of pCG-ATF3 or pGAL4-ATF3 as indicated. The vector (pCG) carrying the CMV promoter was included in some experiments to ensure that each transfection mixture contained the same amount of CMV promoter. The percent CAT activity was calculated by arbitrarily defining each individual reporter activity in the absence of ATF3 as 100%. The average of three experiments is shown. WT, wild-type.

(at -90). Those results may appear to contradict the results shown here (Fig. 8). One explanation for this apparent discrepancy is that Fig. 8 is derived from full-length mutant promoters, whereas Fig. 2 is derived from truncated mutant promoters that lack not only the binding sites of interest, but also other regions that may contribute to the proper controls of the promoter. Significantly, ATF3 activated the promoter that lacks both sites. This is consistent with our previous observations that ATF3 activates transcription of promoters without its binding sites, presumably by sequestering inhibitory cofactors away from the promoter (7). Taken together, these data suggest that, in the full-length promoter context, both binding sites are necessary for efficient repression of the ATF3 promoter by ATF3; neither site alone works efficiently.

Because the mutation at the -20 site is a replacement of the site with the GAL4-binding site, we asked whether the GAL4-ATF3 fusion protein can repress transcription of the mutant promoters. As shown in Fig. 8C, GAL4-ATF3 repressed the Δ-20 promoter, which contains two binding sites for this fusion protein: 1) the GAL4 site at -20 for the GAL4 portion of the fusion protein to bind and 2) the consensus ATF/CRE site at -90 for the ATF3 portion of the fusion protein to bind. The GAL4-ATF3 fusion protein also repressed the ΔBoth promoter, which contains only one binding site (the GAL4 site at -20) for

the fusion protein to bind. Consistently, the repression was not as efficient as that on the Δ-20 promoter, which contains two binding sites. These results are consistent with the notion that ATF3 is a transcriptional repressor and that a strong binding site at -20 is sufficient for GAL4-ATF3 to repress the promoter.

DISCUSSION

Transient Nature of ATF3 Induction—Previously, we (Ref. 4; for a review, see Ref. 3) and others (16) demonstrated that the induction of ATF3 by stress signals is transient in nature: the mRNA level of ATF3 increases shortly after exposure of cells to stress signals, peaks at around 2 h in most cases, and declines to the basal level after a few hours. In this report, we present evidence indicating that ATF3 can repress its own promoter. This autorepression plays an important role in the prevention of sustained gene expression. However, we note that it by itself cannot explain the transient nature of induction. The induced mRNA must be relatively unstable; otherwise, the mRNA level will stay high even if the synthesis is turned off shortly after induction. Consistently, ATF3 mRNA is unstable, with a half-life of 30 min in HeLa cells (15). The 3'-untranslated region of ATF3 mRNA contains several AUUUA sequences (7, 16), a motif that has been demonstrated to destabilize mRNA (for reviews, see Refs. 24 and 25). In this context, it is interesting to note that autorepression of promoter activity has been described for other genes such as *c-fos* (26), *ICER* (27), *c-myc* (28), and *c-rel* (29). Significantly, many of these genes are also inducible genes. Therefore, autorepression appears to be a common strategy that nature utilizes to achieve a transient expression of many inducible genes.

Novel Binding Site for ATF3—As described under "Results," we identified an unexpected ATF3-binding site in its own promoter. This site is necessary for efficient autorepression by ATF3. Two features of this binding site are interesting. First, it differs from the consensus ATF/CRE-binding site in the central key residues. Second, it is immediately downstream from the TATA box. The variation in the binding site indicates the flexibility of ATF3 in binding to DNA; it also underscores the importance of elucidating the spectrum of binding sites for ATF3. At present, it is not clear whether this novel site is a general binding site for all ATF/CREB proteins. Our results suggest that it prefers ATF3 over ATF1. However, a systematic and comprehensive study using other ATF/CREB proteins is needed to address this question. It is intriguing that this binding site is immediately downstream from the TATA box, raising the possibility that ATF3 may repress transcription by competing with basal transcription factors for binding to the promoter. Clearly, this is only a speculation at this point, and much work is required to test this supposition.

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