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Interaction of ATF6 and Serum Response Factor

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Serum response factor (SRF) is a transcription factor which binds to the serum response element (SRE) in the c-*fos* promoter. It is required for regulated expression of the c-*fos* gene as well as other immediate-early genes and some tissue-specific genes. To better understand the regulation of SRF, we used a yeast interaction assay to screen a human HeLa cell cDNA library for SRF-interacting proteins. ATF6, a basic-leucine zipper protein, was isolated by binding to SRF and in particular to its transcriptional activation domain. The binding of ATF6 to SRF was also detected in vitro. An ATF6-VP16 chimera activated expression of an SRE reporter gene in HeLa cells, suggesting that ATF6 can interact with endogenous SRF. More strikingly, an antisense ATF6 construct reduced serum induction of a c-*fos* reporter gene, suggesting that ATF6 is involved in activation of transcription by SRF. ATF6 was previously partially cloned as a member of the ATF family. The complete cDNA of ATF6 was isolated, and its expression pattern was described.

Expression of the c-*fos* gene is rapidly transcriptionally activated in serum-starved cells by serum and many other mitogens. Mapping of the c-*fos* promoter suggests that a key sequence element is the serum response element (SRE) (15). SRE-like elements have also been found to be critical for the expression of other immediate-early genes, a number of muscle-specific genes, and interleukin-2 receptor (15). The main nuclear protein found to bind the SRE is serum response factor (SRF). SRF is a 508-amino-acid protein of the MADS box family that binds to DNA as a dimer (21). In addition to central DNA binding and dimerization domains (spanning the conserved MADS box), it contains a C-terminal transcriptional activation domain (13).

One mechanism for serum induction of the SRE is through a family of SRF-associated proteins, ternary complex factors (TCFs) (27). TCFs are encoded by three related genes (*Elk1*, *Sap1*, and *Sap2*/*Net*/*Erp*) with *ets*-related DNA binding domains (22, 27). TCFs contact both SRF and a specific sequence element and bind to SRF only when a DNA binding site is adjacent to the SRF site. TCFs also contain a transcriptional activation domain which is regulated by protein kinases from the mitogen-activated protein kinase family (27, 28). Activation of c-*fos* expression by serum and various growth factors at least partially works by activating mitogen-activated protein kinases. A mutation of the TCF site in the c-*fos* promoter, however, does not have a large effect on serum induction of a reporter gene (6, 11). This and other results suggest that there is a TCF-independent mechanism for serum induction of c-fos (10, 12, 14).

The TCF transcriptional activation domain acts in conjunction with the transcriptional activation domain of SRF to increase c-*fos* transcription (9, 12, 14). The transcriptional activation domain of SRF can also function alone when it is fused to the DNA binding domain of GAL4, but it is not regulated by serum in this context (13). One mechanism for the activation of transcription by the SRF transcriptional activation domain appears to be its binding to the general transcription factor TFIIF. We previously found that SRF bound the RAP74 subunit of TFIIF in vitro and that mutations in either SRF or RAP74 that abrogated this binding also reduced the ability of SRF to activate transcription in vitro (16, 30).

In vivo footprinting suggests that SRF constitutively occupies the c-*fos* SRE in cells (8). However, SRF does not activate transcription in uninduced cells, suggesting that there is negative regulation of SRF prior to activation. In order to better understand how SRF is regulated by serum and how this regulation controls its ability to activate transcription, we utilized a yeast interaction screen to identify additional SRF-interacting proteins. We describe here the identification of one such protein, ATF6, which binds to the SRF transcriptional activation domain and is itself a DNA-binding protein. An antisense ATF6 construct strongly reduced serum induction of a c-*fos* reporter gene, suggesting that ATF6 is a critical component of the SRE-SRF complex.

MATERIALS AND METHODS

Yeast interaction screen. The screening of a human HeLa cell cDNA library was performed essentially as previously described (2). The indicator strain, ActL, contains a high-affinity SRF binding site in front of a *cyc1-lacZ* gene. The SRF expression vector pSD0.7 encodes full-length SRF under the control of a galactose-inducible promoter (2). The HeLa cell cDNA library used was a randomly primed cDNA library fused to the C terminus of the VP16 transcriptional activation domain under the control of a galactose-inducible promoter as previously described (2). To screen the library, transformed yeast cells were plated directly onto nitrocellulose filters and grown for approximately 22 h on selective medium containing 2% glucose. The filters were then transferred to plates that contained galactose instead of glucose and grown for an additional 20 h. Color assays for β-galactosidase activity were performed by submerging the filters in liquid nitrogen to permeabilize cells and placing them on Whatman 3MM paper soaked with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate buffer (2). Positive colonies were retested and purified.

Positive colonies were cured of either the SRF-expressing plasmid (pSD0.7; *TRP1* marker) or cDNA-expressing plasmids (*URA3* marker) by standard procedures and retested by the color assay. VP16-tagged cDNA library plasmids were recovered by preparing DNA from a 10-ml culture of pSD0.7-cured yeast and transforming it into *Escherichia coli* MC1066 by electroporation. The isolated plasmids were transformed into the indicator yeast strain containing SRF (pSD0.7) and tested by the color assay for activation of *lacZ* expression. To test the interaction with SRF deletion mutants, vectors that express these forms of SRF were used instead of pSD0.7. For samples without SRF, the control *TRP1* vector pRS314 was used (25).

For the liquid β-galactosidase assays, yeast cells were grown overnight in selective medium plus 2% glucose, diluted 1:50 in selective medium plus 2% galactose, and grown until the optical density at 600 nm was 0.6 to 0.8. β-Galactosidase activities were determined as previously described (2).

Plasmids. The plasmids constructed for this study are described below. Further details and maps are available upon request.

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(i) Yeast expression plasmids. Mutant SRF expression plasmids were derived from pSD0.7, which expresses full-length SRF with a galactose-inducible promoter and a *TRP1* marker (2). These plasmids included amino acids 114 to 508, 114 to 245, or 1 to 412 of SRF.

(ii) Bacterium expression plasmids. A glutathione *S*-transferase (GST)–c1.12 fusion protein was made by using PCR primers to place the c1.12 region of ATF6 3′ of GST between the *Bam*HI and *Xba*I sites of pGTK, a GST expression vector provided by Hua Zhu. A fusion of maltose-binding protein (MBP) and SRF was made by placing a fragment that encodes amino acids 266 to 508 of SRF 3′ to MBP in plasmid pMalC2 (New England Biolabs). pMBP-c1.12 was obtained by inserting c1.12 into the *Eco*RI-to-*Xba*I sites of pMalC2. pSRF(114-508) was expressed in pET3a and was described previously (23).

(iii) Mammalian reporter and expression plasmids. The reporter gene plasmids pO-Fluc, pSRE-Fluc, and pSRE.M-Fluc were described previously (14). They contain a minimal human c-*fos* reporter gene (−53 to +45) fused to a luciferase gene with no site, a single copy of the c-*fos* SRE, and a mutant SRE, respectively. The reporter gene plasmids Fos-WT and Fos-pm12 contain −356 to +109 of the mouse c-*fos* promoter inserted 5′ to the luciferase gene in the pGL3 vector (Promega). Fos-pm12 contains four base mutations in the SRE that abolish SRF binding (5).

All of the mammalian expression vectors used were derived from pCGN, in which expression is driven by the cytomegalovirus (CMV) promoter and the expressed proteins are fused to the influenza virus hemagglutinin antigen at the amino terminus at an *Xba*I site (26). pCFN is identical to pCGN, except for a simian virus 40 nuclear localization signal added after the hemagglutinin epitope tag and before the inserted gene (provided by Hua Zhu). pCFN-VP16c1.12 (see Fig. 3) contains the *VP16-c1.12* fusion gene (isolated in the yeast screen) inserted in the *Xba*I site of pCFN. The full-length ATF6 expression vector, pCGN-ATF6, contains ATF6 from the first start codon to the 3′ end at the *Xba*I site of pCGN. In pCFN-c1.12, the c1.12 fragment of ATF6 was expressed by placing it at the *Xba*I site of pCFN. The antisense ATF6 vector pAS-ATF6 contains the c1.12 region of ATF6 in the antisense orientation at the *Xba*I site of pCFN.

In vitro binding of SRF and ATF6. GST-c1.12, GST, MBP-c1.12, MBP-SRF (266-508), MBP, and SRF(114-508) proteins were expressed in *E. coli* BL21. Expression was induced in mid-log phase by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Then cells (1 liter) were harvested, resuspended in 20 ml of BC100 buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 0.2 mM EDTA, 100 mM KCl, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.05% Nonidet P-40), sonicated, and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant was recentrifuged, and the subsequent supernatant was used for purification. SRF(114-508) was purified as previously described (23). GST and MBP proteins were bound to glutathione-Sepharose 4B (Pharmacia) or amylose agarose (New England Biolabs) by adding 0.5 ml of resin for every 5 ml of supernatant for 1 h at 4°C. The resins were washed with BC100 buffer four times at 4°C. The binding of GST proteins was confirmed by eluting three times with 10 mM reduced glutathione in 50 mM Tris (pH 8.0) and pooling the eluates. MBP fusion proteins were eluted from the amylose resin with 10 mM maltose in BC100 buffer. The purities and quantities of proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie blue staining.

Aliquots (20 μl) of glutathione-Sepharose beads bearing equal amounts of either GST or the GST-c1.12 fusion protein were incubated with 200 ng of SRF(114-508) or MBP-SRF(266-508) in 100 μl of BC100 buffer. The mixtures were gently rocked on a rotating wheel at room temperature for 1 h. The beads were pelleted at 3,000 × *g* for 2 min, and the supernatant was saved as the unbound fraction. The beads were washed four times with 1 ml of BC100 buffer, and bound proteins were eluted three times by incubation with 20 μl of 10 mM reduced glutathione in 50 mM Tris (pH 8.0) at room temperature for 10 min and pooled. Both unbound and eluted proteins (40 μl [each]) were analyzed by immunoblotting with anti-C-terminal SRF serum (18) and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G.

Transfection and luciferase assays. All cells were grown in Dulbecco's modified Eagle medium containing 10% newborn calf serum. One 60-mm-diameter plate of cells (HeLa or Cos) was transfected by standard calcium phosphate methods with a total of 7 μg of DNA composed of 2 μg of reporter constructs, 3 μg of vector or expression constructs, and 2 μg of pCMV-β-galactosidase as an internal control. Luciferase and β-galactosidase reporter gene activities were assayed 42 h later as previously described (14), except that luciferase samples were counted for 15 s in a Berthold Lumat luminometer. Luciferase levels were normalized for transfection efficiency with β-galactosidase activities. In some experiments at 16 h after the beginning of transfection, HeLa cells were washed and serum starved in Dulbecco's modified Eagle medium with 0.2% newborn calf serum for 36 h. Starved cells were either left alone or induced with 20% newborn calf serum for 4 h. The results given are the means of three independent transfection assays done in duplicate, and variations are shown as the standard errors of the means.

cDNA cloning. ATF6 cDNA was further cloned from the sequence of the c1.12 cDNA clone isolated in the yeast screen. The region 3′ of *c1.12* was cloned by using a 3′ rapid amplification of cDNA ends (RACE) system (Gibco-BRL). As described by the manufacturer, 5 μg of total HeLa RNA was primed with an oligo(dT)-containing adapter primer (5′-GGCCACGCGTCGACTAGTACT TTTTTTTTTTTTTTT-3′) and extended with reverse transcriptase (Super-Script II RT RNase H^-). After first-strand cDNA synthesis, the original mRNA template was digested with RNase H. Then the cDNA was amplified by two stages of PCR with nested ATF6 primers. First, ATF6 primer c1.12-GSP1 (annealing to positions 989 to 1012 of ATF6) was used with an abridged universal amplification primer (AUAP; 5′-GGCCACGCGTCGACTAGTAC-3′) which overlaps part of the adapter sequence used to prime first-strand cDNA synthesis. Second, nested ATF6 primer c1.12-AccI (5′-ATCGAGAATCCGCTTGT CAGTCTC-3′; nucleotides 1033 to 1054) and AUAP were used. The ends were treated with T4 DNA polymerase, and PCR products were subcloned into the *Not*I and *Acc*I sites of pBluescript II SK.

The region 5′ to the c1.12 clone was isolated by using a 5′ RACE system (Gibco-BRL). The first-strand cDNA was primed with ATF6-specific primer GSP-2BT (positions 880 to 856). Terminal transferase (TdT) was used to add poly(dC) tails to the 3′ ends of the cDNA. The tailed cDNA was amplified by two-step PCRs. The first PCR was performed with primer AAP (5′-GGCCACGCGTC GACTAGTACGGGIIGGGIIGGGIIG-3′) and ATF6-specific primer C1.12BT3 (positions 736 to 717). The second amplification was done with this AUAP and nested ATF6 primer GSP-Acc (5′-CGAGGGCAGAACTCCAGGTGCTT-3′; nucleotides 794 to 773). Three PCR products were obtained and were subcloned into pBluescript II SK.

The full-length cDNA sequence was obtained by sequencing both strands with vector and gene-specific primers. Sequence comparisons were performed with a BLAST search of the GenBank database.

Northern blot analysis. Total cellular RNA was isolated from an 80% confluent 10-cm-diameter plate with 7 ml of TRIzol reagent (Gibco-BRL). Hybridization probes were gel purified and labeled with [α-^{32}P]dATP by using a Primer-It RmT random primer labeling kit (Stratagene) as described by the manufacturer. Total RNA (7 μg) was electrophoresed and transferred to nitrocellulose by standard procedures. The filters were prehybridized in QuikHyb hybridization solution (Stratagene) for 30 min at 68°C and hybridized with 2.5×10^6 cpm of hybridization solution per ml for 1.5 h at 68°C. The filters were washed twice for 15 min at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and twice for 30 min at 60°C with 0.1× SSC–0.05% SDS. The mouse multitissue Northern blot (Clontech) contained 2 μg of poly(A)$^+$ RNA from the indicated tissues. The human β-actin probe (Clontech) was used to confirm the amount of RNA in each lane.

Immunoblotting of ATF6. MBP-c1.12 fusion protein was affinity purified on amylose agarose as described above and used to raise antiserum against ATF6. MBP-c1.12 (100 μg) was injected subcutaneously into a rabbit, and 50 μg was subsequently injected at 2, 3, and 7 weeks after the initial injection.

To detect ATF6 in mammalian cells, 2×10^7 Cos or HeLa cells were lysed with 300 μl of 1× SDS loading buffer (50 mM Tris [pH 6.8], 0.2% SDS, 0.1% bromophenol blue, 10% glycerol, 0.5 mM dithiothreitol). Lysates (60 μl) were analyzed by standard immunoblotting procedures with preimmune or anti-ATF6 serum (1:800 dilution) and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (20 ng/ml).

Cos cells were transfected with 7 μg of full-length ATF6 expression plasmid pCGN-ATF6 or control vector pCGN. Transfected-cell lysates (20 μl) were analyzed with anti-ATF6 antiserum as described above or with monoclonal antibody 12CA5 against influenza virus hemagglutinin antigen (1:700) (26).

RESULTS

Screen for SRF-interacting proteins in yeast. To identify proteins which interact with SRF and which either regulate or execute its activity, we screened a human HeLa cell cDNA library in the yeast *Saccharomyces cerevisiae*. We utilized the system of Dalton and Treisman (2) that includes a *lacZ* reporter gene with an SRF binding site upstream of a *cyc1* promoter. The SRF binding site used, ActL, binds SRF with high affinity but does not bind Mcm1, a yeast protein homologous to SRF. SRF and a HeLa cell cDNA library, fused to the C terminus of the herpesvirus VP16 transcriptional activation domain, were expressed with galactose-inducible promoters on plasmids with *TRP1* and *URA3* markers, respectively.

SRF activates transcription only weakly in yeast; therefore, activation of the *lacZ* gene was dependent upon recruitment of a VP16 fusion protein to the promoter. This system has an advantage over the standard two-hybrid method in that it allows SRF to bind directly to the promoter and attain its proper conformation and potentially allows an interacting protein to bind to both SRF and DNA, thus stabilizing their interaction. This is especially critical since GAL4-SRF chimeras are not serum regulated in HeLa or NIH 3T3 cells (13). This system was successfully used to clone one of the TCF family members, Sap1 (2).

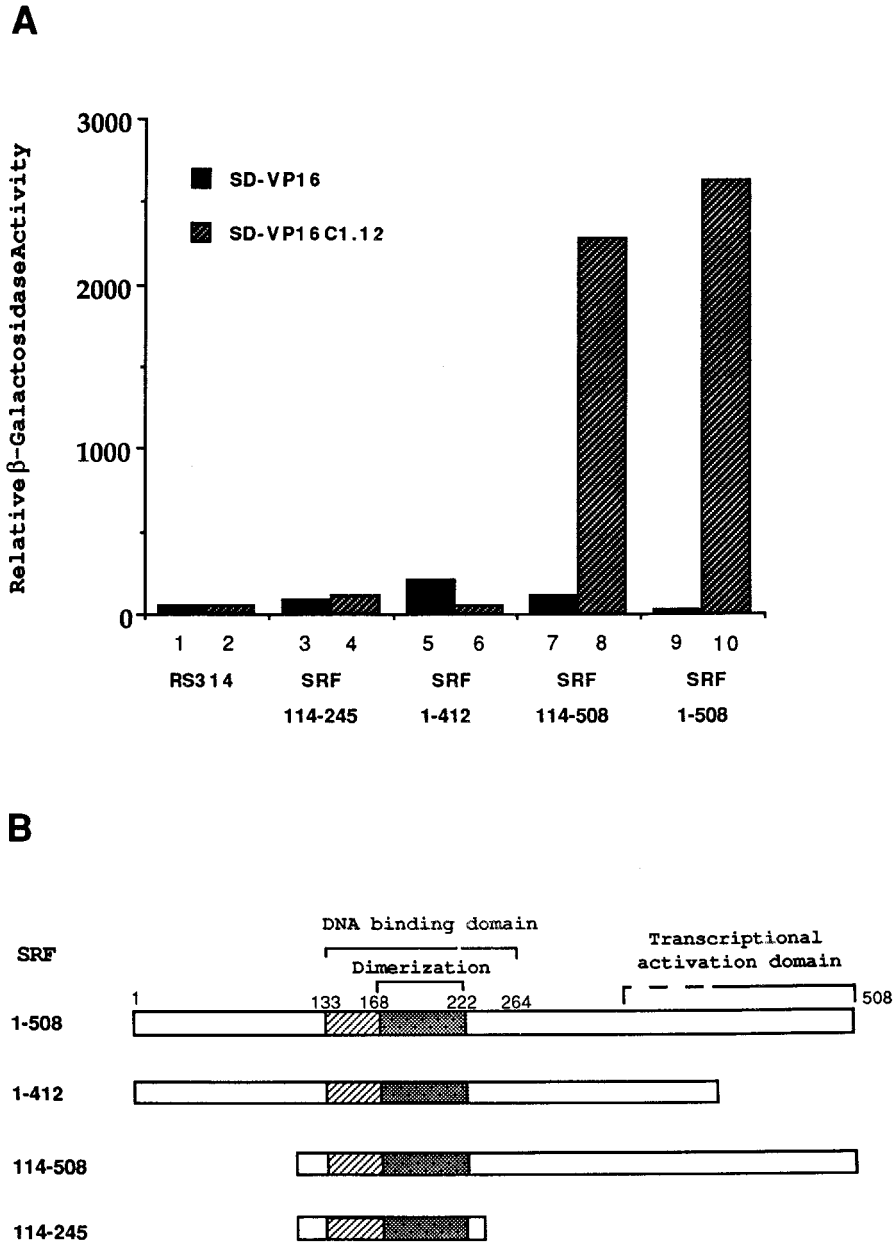

FIG. 1. Interaction of clone c1.12 with SRF in yeast. (A) Clone c1.12 fused to VP16 (SD-VP16-c1.12) or vector (SD-VP16) were expressed in yeast strain ActL with a control *TRP1* plasmid, pRS314, or the indicated SRF variants. Yeast strain ActL contains an integrated copy of an SRF binding site next to a *cyc1-lacZ* reporter gene. After the induction of protein expression with galactose, a liquid β-galactosidase assay was used to measure the interaction of SRF expression plasmids with VP16-c1.12. Each point was repeated at least two times, and the results of a representative experiment are shown. (B) Schematic diagrams of SRF deletion mutants and functional domains of SRF. Full-length SRF consists of 508 amino acids. The region from amino acids 222 to 264 enhances DNA binding but is not absolutely required.

In a screen of approximately 400,000 yeast colonies, 22 positive colonies were identified. Of these, 13 were found to be dependent upon both the SRF and VP16 cDNA vectors by curing of individual plasmids and retransformation of the VP16 cDNA plasmids into the reporter strain. Restriction enzyme analysis and DNA sequencing of the isolated plasmids showed that nine had a longer form Sap1 and two had a shorter-form Sap1 (both forms have been previously described [2]). Sequencing of clone c4.6 showed that it was identical to a lysosomal membrane glycoprotein, CD63 antigen. Due to its presumed cellular localization, this clone was not studied further. Sequencing of clone c1.12 showed that it was identical to a region of ATF6, a member of the ATF basic leucine zipper family of DNA-binding proteins.

A liquid β-galactosidase assay was used to demonstrate the interaction of SRF with VP16-c1.12 in yeast (Fig. 1A). Full-length SRF (1 to 508) did not activate expression when it was expressed with a VP16 only control vector (SD-VP16) but activated strongly when it was expressed with SD-VP16-c1.12 (Fig. 1A, lanes 9 and 10). VP16-c1.12 also did not activate without SRF (Fig. 1A, lane 2).

Various deletion mutants of SRF were used to map the region of SRF required for interaction with c1.12. Amino acids 133 to 222 of SRF are required for DNA binding and dimer-

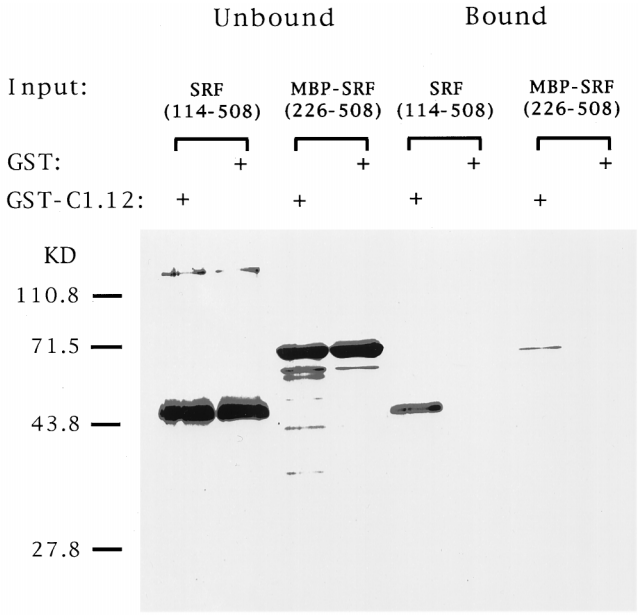

FIG. 2. Physical association between ATF6 and SRF in vitro. The c1.12 ATF6 fragment was bacterially expressed as a GST fusion protein, GST-c1.12. Normalized amounts of GST-c1.12 and GST were immobilized on a glutathione-agarose resin and incubated with 200 ng of either SRF(114-508) or a chimera of MBP fused to SRF [MBP-SRF(266-508)]. Bound proteins were eluted with glutathione, and bound and unbound proteins were analyzed by immunoblotting with anti-SRF serum. Molecular mass standards (in kilodaltons [KD]) are given on the left. +, presence.

ization as well as interaction with TCFs (21, 27). The C-terminal region of SRF contains a transcriptional activation domain (13). Only deletions that retained the DNA binding domain were used since this assay requires the binding of SRF to the SRE-containing reporter gene. As shown in Fig. 1A, deletion of the SRF amino terminus [SRF(114-508)] did not abolish this interaction (lane 8). However, deletion of the SRF C terminus [SRF(1-412)] abolished binding to c1.12 (Fig. 1A, lane 6). In addition, the SRF DNA binding region alone [SRF(114-245)] was not sufficient for binding to c1.12 (Fig. 1A, lane 4). Similar results were obtained by β-galactosidase filter assay (data not shown). Both SRF(1-412) and SRF(114-245) were expressed since they interacted well with VP16-Sap1 (16) (data not shown). Besides affecting the binding of SRF to c1.12, a deletion to amino acid 412 of SRF abolishes transcriptional activation by a GAL4-SRF chimera (13). These results suggest that the c1.12 protein product interacts with the SRF transcriptional activation domain.

As described below (see Fig. 5), the predicted amino acid sequence from the 570-bp sequence of c1.12 shows that it is identical to the ATF6 sequence in a region that spans the basic region but includes only half of the leucine zipper dimerization domain. As such, it would not be predicted to bind DNA directly. This suggests that c1.12 (and therefore ATF6) can bind SRF without making additional DNA contacts.

Binding of ATF6 and SRF in vitro. To further demonstrate the binding of ATF6 and SRF, we assayed for the binding of recombinant SRF to c1.12. The partial ATF6 fragment isolated in the yeast screen was bacterially expressed as a GST fusion protein. GST-c1.12 or GST alone was immobilized on glutathione-Sepharose beads. The amounts of GST-c1.12 and GST immobilized on beads were normalized, as judged by the amount of protein eluted by glutathione (data not shown). Immobilized GST-c1.12 and GST were incubated with either SRF(114-508) or a chimera of MBP fused to SRF, MBP-SRF(266-508). Bound and unbound proteins were analyzed by immunoblotting with anti-SRF serum. As shown in Fig. 2, both forms of SRF bound to GST-c1.12 beads but not to GST beads. About 5% of the total input SRF(114-508) bound, but significantly less of MBP-SRF(226-508) was eluted. These results further suggest that SRF binds ATF6 and that a domain of SRF larger than the minimal transcriptional activation domain is required for optimal ATF6 binding.

Interaction of ATF6 with SRF in HeLa cells. To test whether ATF6 can interact with SRF in mammalian cells, we expressed VP16-c1.12 in HeLa cells with an SRE reporter gene. Endogenous SRF binds the SRE in serum-starved cells but does not activate transcription (15). If ATF6 binds SRF in cells, we would predict that VP16-c1.12 would activate an SRE reporter gene by bringing the VP16 transcriptional activation domain to the promoter. For this experiment, we used a reporter gene in which the luciferase reporter gene is controlled by a minimal c-*fos* promoter (−53 to +45) without an SRE (O-Fluc), with a c-*fos* SRE (SRE-Fluc), or with a mutated SRE that cannot bind SRF (SRE.M-Fluc) (14). Transfection of VP16-c1.12 activated the SRE reporter gene but did not activate the reporter gene without an SRE or with a mutated SRE (Fig. 3). VP16-c1.12 also superinduced the SRE reporter gene in serum-starved cells, suggesting that SRF and ATF6 can interact before activation of expression (data not shown). Serum in-

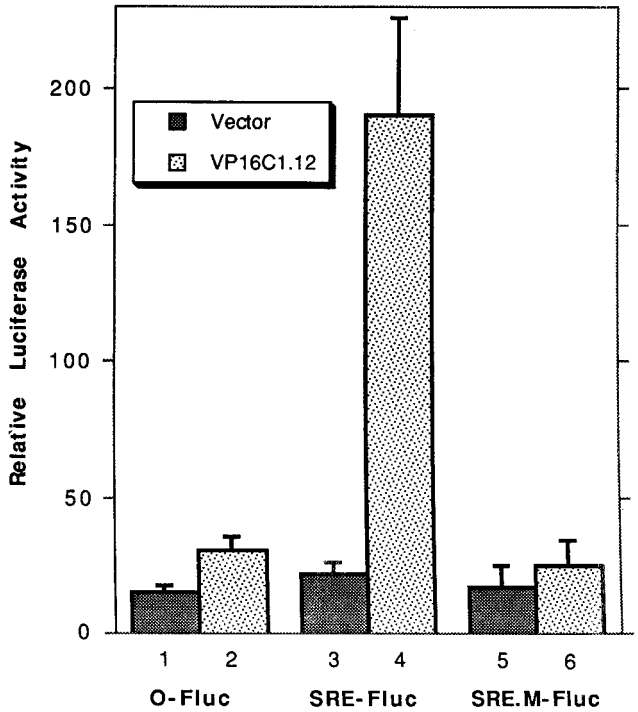

FIG. 3. Interaction of ATF6 with SRF in HeLa cells. The c1.12 fragment of ATF6 was expressed in a CMV expression vector fused to the VP16 transcriptional activation domain. Vector VP16-c1.12 or a vector control with no insert was transfected into HeLa cells with a luciferase reporter gene containing a minimal c-*fos* promoter (−53 to +45) without an SRE (O-Fluc), with a wild-type c-*fos* SRE (SRE-Fluc), or with a mutant SRE (SRE.M-Fluc). An internal control plasmid of pCMV-β-galactosidase was also included. After transfection, cells were grown in 10% serum for 42 h. Cell lysates were assayed for luciferase and β-galactosidase activities. Luciferase activities were normalized to the β-galactosidase activity in each sample. Data are the means ± standard errors of the means of at least six repetitions.

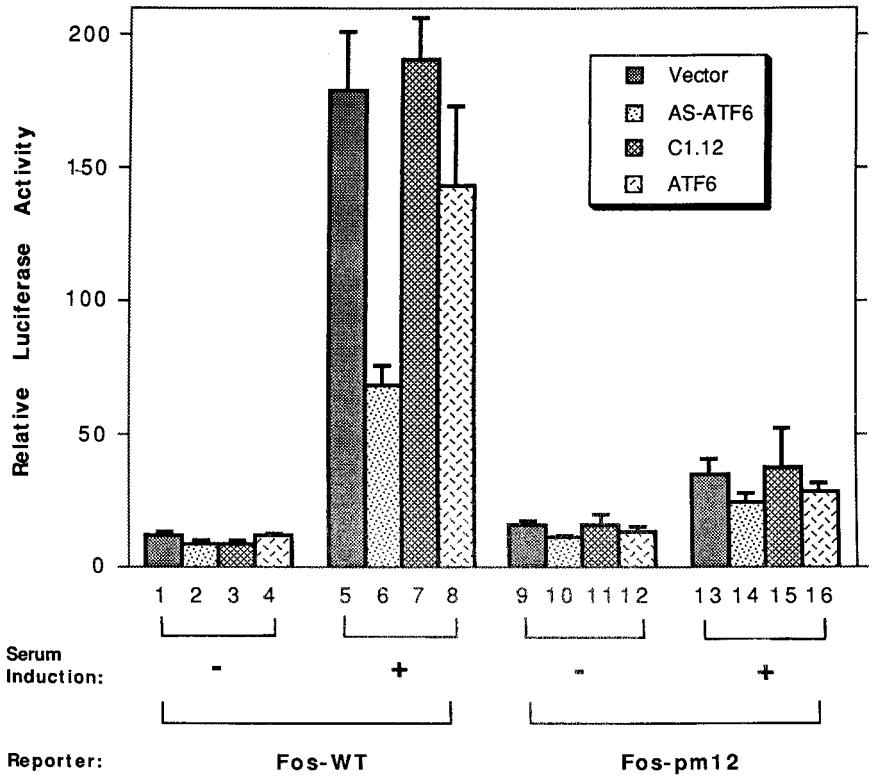

FIG. 4. Antisense ATF6 reduces serum-induced expression of a c-*fos* reporter gene. c-*fos*–luciferase reporter genes (Fos-WT and Fos-pm12) were transfected into HeLa cells with CMV expression plasmids. Fos-pm12 contains four point mutations in the c-*fos* SRE that abolish SRF binding. Either vector alone (pCGN), an antisense ATF6 vector (AS-ATF6), the c1.12 fragment of ATF6, or full-length ATF6 was transfected as indicated. An internal control plasmid, pCMV-β-galactosidase, was also included. After transfection (16 h), HeLa cells were serum starved (−) for 36 h and induced (+) with 20% newborn calf serum for 4 h as indicated. Cell lysates were assayed for luciferase and β-galactosidase activities. Luciferase activities were normalized to the β-galactosidase activity in each sample. Data are the means ± standard errors of the means of at least six repetitions.

duction of SRE-Fluc increased the level of expression about eightfold. This serum-induced level was further activated about twofold by VP16-c1.12 (data not shown). VP16-c1.12 had no significant effect on the expression of an internal control plasmid, pCMV-β-galactosidase (data not shown). The results of these experiments suggest that ATF6 can bind to SRF in HeLa cells in addition to binding to SRF in vitro and in yeast.

Requirement of ATF6 for c-*fos* expression. We tested whether ATF6 is required for serum-induced c-*fos* expression by transfecting an antisense ATF6 construct into HeLa cells with a mouse c-*fos* promoter (−356 to +109) reporter gene. The antisense construct contained the c1.12 region of ATF6 in the antisense orientation under the control of a CMV promoter. We also tested the effects of expression of full-length ATF6 (described below) and c1.12 (fused to an epitope tag and a nuclear localization signal). The latter may be expected to function as a dominant negative by interfering with endogenous ATF6 binding to SRF.

Expression from the c-*fos* promoter (Fos-WT) was induced by serum induction of serum-starved HeLa cells (Fig. 4, lanes 1 and 5). A mutant c-*fos* promoter (Fos-pm12) showed that serum induction was sensitive to mutations in the SRE that abolish SRF binding (Fig. 4, lanes 9 and 13). Expression of the antisense ATF6 construct reduced serum-induced expression from Fos-WT by over 60% (Fig. 4; compare lanes 5 and 6). The antisense construct did not significantly reduce the expression of Fos-pm12 or Fos-WT in uninduced cells (Fig. 4). The internal control plasmid, pCMV-β-galactosidase, was also not detectably affected by the antisense ATF6 vector (data not shown). These results suggest that ATF6 is specifically required in HeLa cells for SRF-mediated activation of the c-*fos* promoter.

In contrast to the antisense ATF6 construct, expression of c1.12 or full-length ATF6 did not significantly affect expression of the c-*fos* reporter gene (Fig. 4). There may be sufficient endogenous ATF6 that overexpression has no effect. The c1.12 product may not have inhibited for a number of reasons; e.g., it may not have been expressed highly enough to inhibit the endogenous ATF6-SRF interaction.

Full-length ATF6 cDNA cloning. ATF6 was originally cloned from a cDNA expression library probed with a double-stranded oligonucleotide containing ATF binding sites (7). This original clone spanned the basic region and leucine zipper domain, but the full-length cDNA was not isolated. In addition, although ATF6 was isolated with a multimerized ATF site probe, it did not bind to a single ATF site; therefore, it was not characterized further (7). To obtain a cDNA containing the entire ATF6 coding region, we performed 5′ and 3′ RACE PCRs with HeLa RNAs. On the 3′ side of the c1.12 clone, 3′ RACE PCR gave a single band of 1.5 kb.

Three 5′ cDNA clones which differed at the very 5′ end were isolated by 5′ RACE PCR. These results suggest that there may be three forms of *ATF6* which differ in their 5′ regions but are identical through the rest of the gene. One of these, however, was found to be the predominant form in HeLa cells by Northern and quantitative reverse transcription-PCR analyses. The abundances of the other forms were at least 20-fold lower.

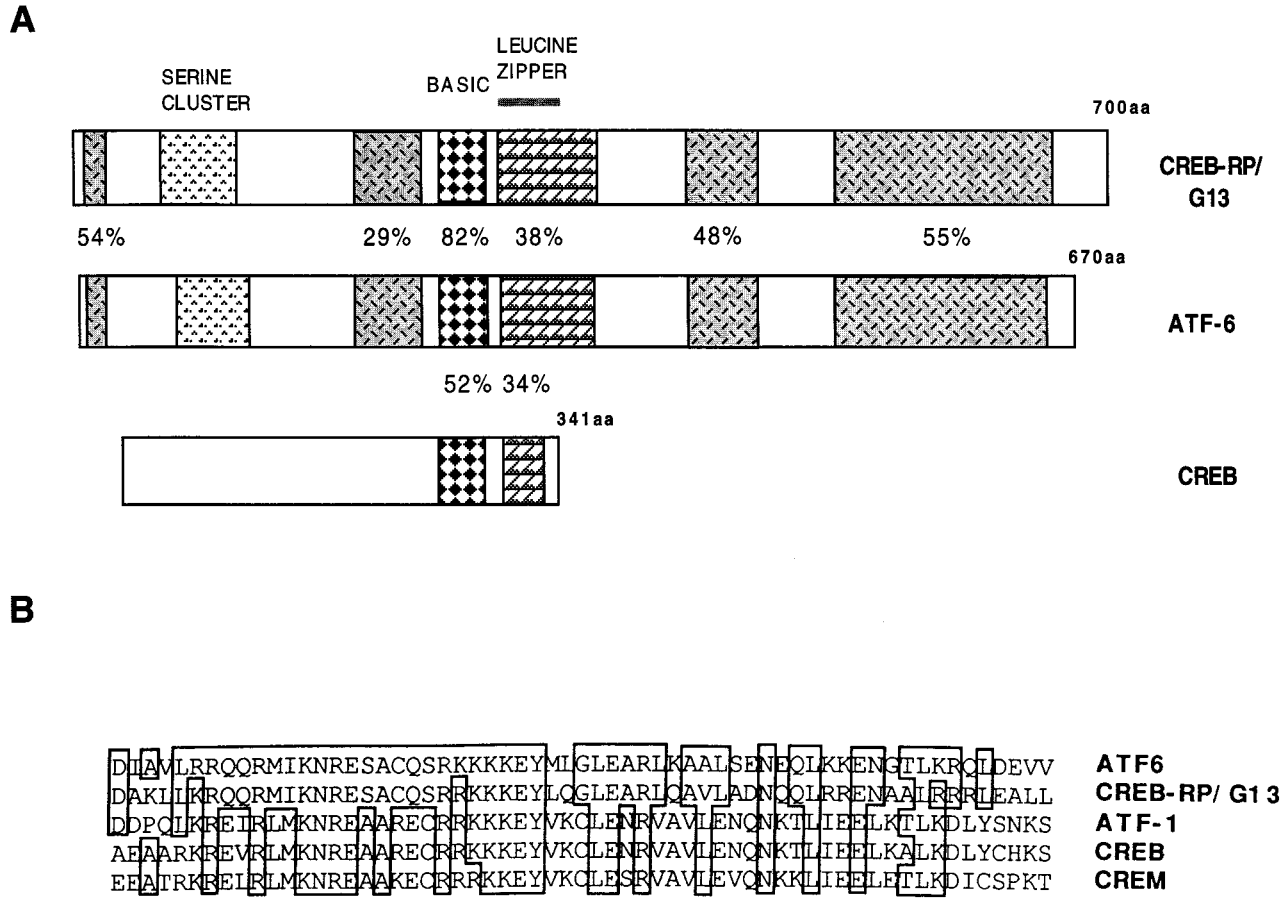

FIG. 5. Similarity of ATF6 to CREB-RP/G13. (A) Diagrams of each region in *ATF6* and *CREB-RP* (also called *G13*) which shows significant similarity. For comparison, similarity to *CREB* is confined to the basic-leucine zipper domain. The percentages of identity for domains are shown between the genes compared. Besides these regions, a serine-rich section of *CREB-RP* was noted (56% serine) (20). As shown, there is also a serine-rich region in *ATF6* (41% serine), but there is no significant direct amino acid sequence similarity in this region. aa, amino acids. (B) Amino acid similarity in the basic-leucine zipper domain. A sequence alignment of the mammalian proteins most similar to ATF6 in the basic-leucine zipper domain in a BLAST search of the GenBank database is shown. ATF6 is most similar to CREB-RP (also called G13) (17, 20), followed by human ATF-1 (29), human CREB (19), and human CREM (4). In this region, ATF6 has 61% identity and 84% similarity to human CREB-RP, 44% identity and 69% similarity to ATF-1, 42% identity and 72% similarity to CREM, and 40% identity and 65% similarity to CREB.

The predominant form extended 507 bases 5′ of the c1.12 clone.

cDNA sequence. Sequencing of the major ATF6 cDNA form revealed that the length of the complete ATF6 cDNA was 2,474 bp. A diagram of the structure of the predicted amino acid sequence is shown in Fig. 5A. The length of the cDNA is consistent with the size of the major mRNA band detected on Northern blots of HeLa RNA (Fig. 6A). The cDNA sequence predicts an open reading frame from nucleotides 43 to 2052 for a protein product of 670 amino acids with a calculated molecular mass of 74,576 Da. The 3′ end of the cDNA contains 422 nucleotides of untranslated sequence which contains two potential polyadenylation signals (AAATAA) followed by a poly(A) tail.

The putative DNA binding domain of ATF6 is located in the center of the protein, with the basic region from amino acids 303 to 330 followed by a leucine zipper sequence of six hydrophobic amino acids (LALLVL) every seven amino acids. This basic-leucine zipper region is similar to those of more than 30 members of the leucine zipper superfamily currently in the GenBank database. The sequence is most similar to that of *CREB-RP* (also called *G13*), a *CREB*-related gene cloned in the HLA locus (17, 20). Otherwise, the highest similarities are to *ATF-1*, *CREB*, and *CREM* (4, 19, 29) (Fig. 5B).

Outside the basic-leucine zipper region, *ATF6* is similar only to *CREB-RP*. The extents of identity for various domains are shown in Fig. 5A. A short conserved domain near the amino terminus is 41% acidic in *CREB-RP*, which is suggestive of a transcriptional activation domain, but only two of the acidic amino acids are conserved in *ATF6*. Besides the three other conserved domains shown in Fig. 5A, both *ATF6* and *CREB-RP* contain a serine-rich domain (41% and 56% serine, respectively) in the N-terminal region, but there is no significant direct amino acid sequence similarity in this region.

Expression pattern of ATF6. To determine the expression pattern of ATF6, we performed multitissue Northern blotting of mouse mRNA. First, we confirmed that the human ATF6 probe (using the c1.12 region) could detect the mouse message by comparing the signal with HeLa RNA to that from a mouse myelomonocytic leukemia cell line, WEHI-3 (Fig. 6A). A 2.5-kb band was present in both cell lines, but an 8-kb band was much stronger in WEHI-3 cells. On the multitissue Northern blot, ATF6 mRNA could be detected in all the tissues tested, with the notable exception of spleen tissue (Fig. 6B). Three

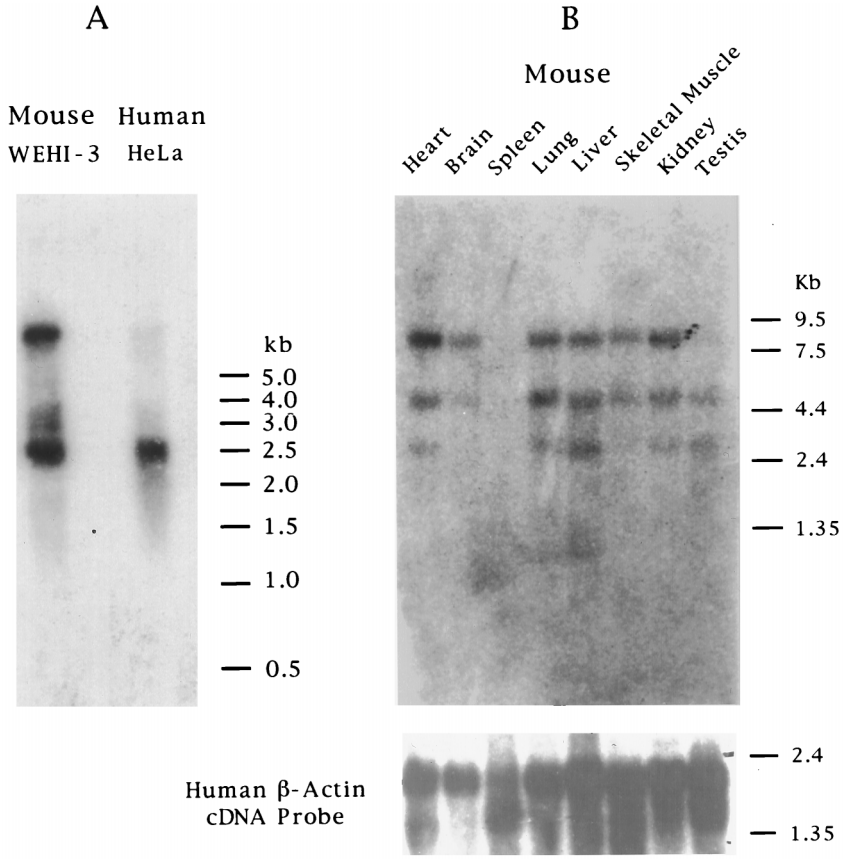

FIG. 6. Tissue distribution of ATF6 expression. (A) HeLa (human cervical carcinoma) and WEHI-3 (mouse promyelocytic leukemia) cell RNAs were probed with the ATF6-specific c1.12 probe. (B) Northern blot of mRNAs from the indicated mouse tissues hybridized with the ATF6-specific c1.12 probe. To control for mRNA amounts, the blot was reprobed with a human β-actin cDNA probe. An alternatively spliced form of β-actin is detected in spleen mRNA.

bands, of 2.5, 4.5, and 8 kb, were detected. The distribution of these bands varied. Each of these bands was also detected by probes from the 5′ and 3′ ends of ATF6 (data not shown). We cannot account for the different-sized messages at this time. Since the three ATF6 probes detected the same bands and we used fairly stringent hybridization conditions, it does not appear that we detected cross-reactive genes.

ATF6 protein product. We raised antiserum to *ATF6* to show that its protein product was made in cells. The c1.12 region of *ATF6* was expressed in *E. coli* as an MBP fusion protein, purified, and injected into rabbits. First, the antiserum was found to bind a GST-c1.12 fusion protein on an immunoblot, showing that it is reactive to ATF6 (data not shown). It also detected in vitro-translated ATF6 but not in vitro-translated ATF-1 or ATF-2 (data not shown). To determine the molecular weight of ATF6 in mammalian cells, we used the antiserum to probe an immunoblot of HeLa and Cos cell lysates. In both lysates, a 90-kDa band was detected. This was not detected with preimmune serum, although a 65-kDa band was detected by both sera (Fig. 7, lanes 1 to 4). Transfection into Cos cells of ATF6 in a CMV expression vector, fused to an influenza virus epitope tag, showed overexpression of a comigrating 90-kDa band. This band was detected by anti-ATF6 and anti-epitope (HA) sera (Fig. 7, lanes 6 and 8). The migration of the ATF6 band was somewhat higher than the expected molecular mass (75 kDa). This may be due to conformational effects on ATF6 migration or to posttranslational modifications. In argument somewhat against posttranslational modifications, in vitro-translated ATF6 comigrated with HeLa cell ATF6 at 90 kDa (data not shown). The 90-kDa ATF6 band was also detected in nuclear extracts of HeLa cells, suggesting that it is at least partially localized to the nucleus (data not shown).

Two pieces of evidence suggest that we have cloned the entire ATF6 coding region. First, the ATF6 cDNA is similar in size to the RNA band detected on Northern blots (Fig. 6). Second, the transfected ATF6 protein product (90 kDa) comigrated with an endogenous band reactive with the anti-ATF6 serum (Fig. 7). Nevertheless, although we did not obtain any cDNA clones with further 5′ sequence by 5′ RACE, it remains possible that the mRNA 5′ sequence extends further.

DISCUSSION

We have identified ATF6 as a protein which interacts with SRF in yeast and in vitro. The results of transfection experiments showed that ATF6 can also interact with SRF in HeLa cells and is required for serum-induced expression of c-fos.

Interaction with SRF. Deletion mutants of SRF showed that the SRF transcriptional activation domain is required for its interaction with ATF6 in yeast. Deletion of the C terminus of SRF (amino acids 413 to 508) abolished transcriptional activation by SRF in HeLa cells (13) and the interaction of SRF with ATF6 in yeast. The C-terminal half of SRF was sufficient for binding to ATF6 in vitro, but a larger fragment bound more effectively. This may be because there are additional contacts

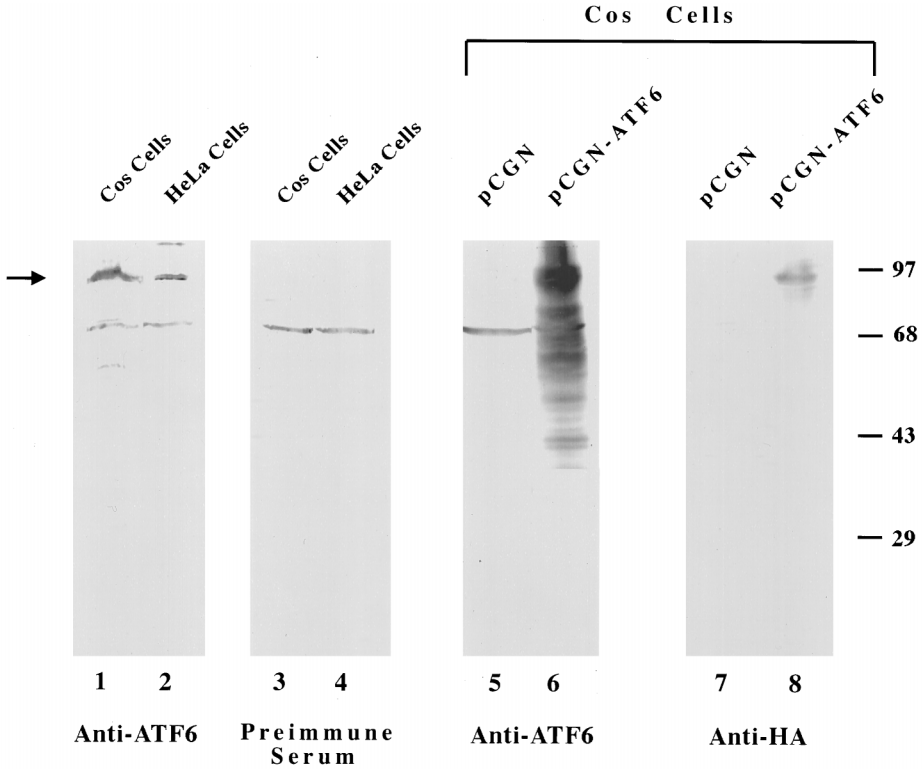

FIG. 7. Identification of an ATF6 protein product. Antiserum to the c1.12 region of *ATF6* was raised in rabbits. Immunoblots of HeLa and Cos cell lysates were probed with either anti-ATF6 or preimmune serum (lanes 1 to 4). The arrow indicates a 90-kDa band detected with anti-ATF6 serum but not with preimmune serum. A CMV expression vector with ATF6 fused to an influenza virus epitope tag (pCGN-ATF6) and a control vector (pCGN) were transfected into Cos cells (lanes 5 to 8). Cell lysates were probed on immunoblots with either anti-ATF6 or anti-epitope (anti-HA) serum as indicated. An endogenous ATF6 band was detected in lane 5 but cannot be seen in the exposure shown.

with ATF6 or because the larger SRF protein attains a more stable conformation to interact with ATF6.

The SRF transcriptional activation domain is required for SRF function in HeLa and NIH 3T3 cells (9, 10, 14). This has been shown by overexpression of SRF with low-affinity SRE reporter genes. Serum-induced expression of these low-affinity SRE reporter genes are dependent upon overexpression of SRF but bind endogenous TCFs. We previously found a complete loss of activity with deletion of the SRF transcriptional activation domain (14), but others have found only a partial loss, presumably due to the contribution of the TCF transcriptional activation domain (9, 10). The finding that the SRF transcriptional activation domain is required for activation with TCF suggests that ATF6 is involved in transcriptional activation along with TCF. Antisense ATF6 expression reduced serum-induced c-*fos*–luciferase expression by 60%. The effect may not have been larger for several reasons. There may be residual activation by TCF without SRF's contribution; proteins similar to ATF6, such as CREB-RP (G13), may have similar activities; or the antisense ATF6 construct may not have completely reduced cellular ATF6 levels. This last possibility is difficult to check in transient-transfection experiments since only a fraction of cells are transfected.

The SRF transcriptional activation domain is not specifically required for serum regulation since its deletion and replacement with a heterologous activation domain (from VP16) resulted in an SRF-VP16 fusion protein that mediated serum induction of a reporter gene (14). These results suggest that ATF6 is not involved in regulating serum induction but participates in the activation of transcription once other components of an SRF complex are activated. Nevertheless, there could be an additional level of regulation that involves ATF6. It will be interesting to determine whether ATF6 is altered in serum-induced cells.

Interaction with ATF6. The region of ATF6 that was isolated in yeast by binding to SRF (clone c1.12) starts before the basic region of the DNA binding domain and extends halfway into the leucine zipper (amino acids 155 to 345 of ATF6). This suggests that it should not be able to dimerize or bind DNA and that these properties are not required for its interaction with SRF. Our preliminary evidence is that deletions of N- or C-terminal parts of c1.12 abolish its interaction with SRF in yeast, suggesting that much of this region is required (unpublished results). Since in these experiments the basic-leucine zipper region of ATF6 was not sufficient for SRF binding, this result also suggests that the basic-leucine zipper domains of other such proteins are not sufficient to bind SRF.

Since ATF6 is a DNA-binding protein, it could bind to the c-*fos* promoter and then contact SRF. It is particularly notable that there are at least two ATF-like sites in the c-*fos* promoter (1, 3). One (a CRE at −60) has strong homologies with consensus ATF sites and binds CREB and ATF-1 in HeLa cells (unpublished results). The second (at −290 immediately 3′ to the SRE) has been called the c-*fos* AP1 site or FAP. This site is more similar to an AP1 site than an ATF site, but it is not clear which factors bind this site in cells. Mutagenesis of this site has also given variable results. It is not required for serum induction of transiently transfected reporter genes in tissue culture cell lines (1, 3) but is required for c-*fos*–*lacZ* expression in transgenic mice (24).

ATF6 was originally isolated in a cDNA expression library probed with a multimerized ATF site but was not able to bind a single ATF site (7). We have not yet been able to demonstrate specific DNA binding with ATF6. This may require identifying a preferred binding site for ATF6 and/or finding a heterodimerization partner for ATF6 that is required for it to bind DNA.

Models for ATF6's role in c-fos regulation. Two attractive models are suggested by our results. One is that ATF6 functions as a coactivator for SRF. Since ATF6 binds to the SRF transcriptional activation domain, it may bridge an interaction to the general transcription machinery. We previously found that SRF binds the RAP74 subunit of the general transcription factor TFIIF in vitro and suggested that this is the mechanism for transcriptional activation by SRF (16, 30). There may be alternative means for activation by SRF; it is also possible that SRF binds both ATF6 and TFIIF at the same time, even though their binding domains overlap.

A second model, not necessarily exclusive to the first, is that ATF6 binds a sequence element in the c-*fos* promoter and then binds SRF. The binding of ATF6 to the promoter could enhance and stabilize its interaction with SRF. This would also allow the formation of a larger promoter complex that could be important in cells for the activation of transcription. Such a complex was proposed from the results with mutations of the c-*fos* promoter in transgenic mice. Mutations in multiple elements had a deleterious effect in transgenic mice, even though they did not affect the expression of transiently transfected reporter genes in tissue culture (24).

***ATF6* sequence.** *ATF6* was originally partially cloned (7), but we have cloned the complete cDNA sequence here. The full sequence reveals that *ATF6* is most similar to *CREB-RP* (*G13*) both in the basic-leucine zipper domain and in a number of other domains (Fig. 5A). While *ATF6* is slightly less similar to *ATF-1*, *CREB*, and *CREM* in the basic-leucine zipper domain (Fig. 5B), it contains no similarity to those genes outside of this region. The *CREB-RP* (*G13*) gene was identified during extensive cloning and sequencing of the human HLA locus (17, 20). Its expression was detected in all the cell lines and tissues tested (20). The extended similarity of CREB-RP (G13) and ATF6 suggests that they have similar functions, such as activating transcription with SRF.

It will be interesting to determine the DNA binding and dimerization properties of ATF6 and CREB-RP (G13) and whether CREB-RP (G13) can also bind SRF. This will help to characterize the SRF transcriptional activation complex and determine whether ATF6 or CREB-RP (G13) binds to a specific site in the c-*fos* promoter. Since these leucine zipper proteins must dimerize to bind DNA, it will be critical to determine with which proteins they dimerize. They may homodimerize, but they may also require a distinct partner to heterodimerize and bind DNA.

The functional domains of ATF6 also need to be determined. It may have a transcriptional activation domain as well as regulatory domains. Since the SRF complex does not activate transcription in uninduced cells, an important mystery to be solved is what changes upon serum induction. One answer is phosphorylation of TCF, but TCF is not required for serum induction (15). One piece of evidence, the transfection of VP16-c1.12 (Fig. 3), suggests that ATF6 can bind SRF in uninduced cells. This suggests that there must be a change in the SRF complex and/or ATF6 that leads to transcriptional activation in serum-induced cells.

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