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Regulation of the *cfos* Serum Response Element by C/EBPβ

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Serum response element binding protein (SRE BP) is a novel binding factor present in nuclear extracts of avian and NIH 3T3 fibroblasts which specifically binds to the *cfos* SRE within a region overlapping and immediately 3′ to the CArG box. Site-directed mutagenesis combined with transfection experiments in NIH 3T3 cells showed that binding of both serum response factor (SRF) and SRE BP is necessary for maximal serum induction of the SRE. In this study, we have combined size fractionation of the SRE BP DNA binding activity with C/EBPβ antibodies to demonstrate that homodimers and heterodimers of p35C/EBPβ (a transactivator) and p20C/EBPβ (a repressor) contribute to the SRE BP complex in NIH 3T3 cells. Transactivation of the SRE by p35C/EBPβ is dependent on SRF binding but not ternary complex factor (TCF) formation. Both p35C/EBPβ and p20C/EBPβ bind to SRF in vitro via a carboxy-terminal domain that probably does not include the leucine zipper. Moreover, SRE mutants which retain responsiveness to the TCF-independent signaling pathway bind SRE BP in vitro with affinities that are nearly identical to that of the wild-type SRE, whereas mutant SRE.M, which is not responsive to the TCF-independent pathway, has a nearly 10-fold lower affinity for SRE BP. We propose that C/EBPβ may play a role in conjunction with SRF in the TCF-independent signaling pathway for SRE activation.

The serum response element (SRE) is a regulatory sequence in the *cfos* promoter that is necessary and sufficient for the rapid induction of the *cfos* gene by serum, growth factors, and phorbol esters (9, 14, 51; reviewed in reference 48). The SRE binds a ubiquitous and constitutively expressed (in most cell types) transcription factor, serum response factor (SRF) (36, 52, 53). The binding of SRF to the SRE is essential for serum and growth factor activation of the *cfos* promoter (48). SRF binding sites are also constitutive promoter elements in many muscle-specific promoters (3, 46, 54). SRF is a 62-kDa protein that binds to DNA as a dimer. The DNA binding and dimerization domains of SRF are both located within a central core of the 508-amino-acid protein (36). This core domain (also termed the MADS box) has substantial homology (70%) with the yeast regulatory protein MCM1 (36), which is also involved in extracellular signal-regulated and cell-type-specific transcription (30, 44). However, owing to differences between the basic N-terminal regions of these domains, the two proteins have related but distinct DNA binding specificities (58).

The essential role which SRF plays in serum- and growth factor-responsive transcription now appears to be due to the convergence of at least two independent signaling pathways upon the SRF (13, 17, 19, 24). Growth factors which activate the Ras–Raf–mitogen-activated protein (MAP) kinase cascade and phorbol esters target a family of transcription factors containing N-terminal *ets* domains and forming ternary complexes with SRF and SRE DNA (20, 25; reviewed in reference 49). The family of ternary complex factors (TCFs) includes SAP-1 (5), Elk-1 (21), and SAP-2/ERP/NET (11, 28). They cannot bind the SRE autonomously. Contacts with the SRF protein and a purine-rich *ets* motif, CAGGAT, 2 bp 5′ of the SRF site are required for recruitment to the SRE (23, 38, 42, 43, 50). TCFs form protein-protein interactions with SRF in a domain within SRF's dimerization domain (33, 42). Activation of p42 and p44 MAP kinases by growth factor stimulation or phorbol ester treatment results in the reversible phosphorylation of TCFs within a conserved C-terminal region (18, 20, 22, 25, 29, 59). This modification regulates transcriptional activation by the ternary complex, since multiple phosphorylation of the carboxy-terminal region is necessary for efficient transactivation by this domain in TCF proteins (22, 29). Growth factor-induced phosphorylation of TCFs has also been reported to stimulate ternary complex formation in some cases (10); however, the carboxy-terminal region of Elk-1 acts as a growth factor-regulated transcriptional activation domain even when brought to DNA by fusion with heterologous DNA binding domains from Gal4 or LexA (22, 29).

cfos promoter mutants that cannot bind TCF are not responsive to activation of the Ras-Raf-MAP kinase pathway (13, 17, 19, 24). However, they remain responsive to serum induction through a second, TCF-independent pathway that still requires SRF (17, 19, 24). In the absence of TCF, SRF can also mediate transcriptional activation by the serum mitogen lysophosphatidic acid (LPA), as well as by intracellular activation of heterotrimeric G proteins by the aluminum fluoride ion (AlF_4^-) (19). Recently, Hill et al. (19) demonstrated that TCF-independent regulation of the SRE is mediated by activated forms of the Rho family GTPases. More specifically, functional RhoA is required for serum-, LPA-, and AlF_4-induced transcriptional activation by SRF, and two other Rho family members, Racl and CDC42Hs, also potentiate SRF activity (19). Thus, two types of signaling pathways, one mediated by Ras and the other by Rho family GTPases, converge on the SRE and require SRF. While the Ras pathway targets TCF, it is not yet clear whether accessory proteins are also involved in the SRF-linked, TCF-independent signaling pathway. Both Hill et al. (17) and Johansen and Prywes (24) have found that TCF-independent transcriptional regulation by SRF requires that it be bound to DNA via its own DNA binding domain. However, mutations in the SRF DNA binding domain that do not block DNA binding nonetheless block activation via the TCF-independent pathway (17). Hill et al. (17, 19) have proposed that these mutations disrupt recognition of DNA-bound SRF by a second accessory factor, distinct from TCF, that is activated by

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serum-induced signals. This putative recognition factor could be a critical target for Rho family-mediated signaling to the nucleus, although such an accessory protein has not yet been identified.

We have previously reported on the presence of a factor, SRE binding protein (SRE BP), in avian and mouse NIH 3T3 fibroblasts that specifically binds to the *cfos* SRE. We have shown by site-directed mutagenesis that the binding of this factor to a region of the SRE overlapping and just 3′ to the SRF is required for maximal serum responsiveness of the SRE (2). We now report that the SRE BP complex is composed in part of homo- and heterodimers of two forms of transcription factor C/EBPβ. C/EBPβ is a member of the basic leucine zipper family of transcription factors (1, 4, 6, 32, 56). The C/EBPβ gene encodes three in-frame methionines which can potentially give rise to three translation products of 38, 35, and 20 kDa (values are for the rat or murine gene). p20C/EBPβ, which is probably initiated by leaky ribosome scanning to position +152 (7), lacks a transactivation domain and therefore acts as an inhibitor of transcription. We have shown that many fibroblast cell types, such as NIH 3T3 cells, express both the long (38- and 35-kDa) and short (20-kDa) forms of C/EBPβ at approximately equal levels (41). Both p35 and p20 homodimers, as well as p35-p20 heterodimers, contribute to the SRE BP complex in nuclear extracts.

In transient transfection experiments, expression of p20C/EBPβ completely blocks serum induction of the *cfos* SRE, while p35C/EBPβ potentiates activity approximately 20- to 30-fold, even in serum-deprived cells. We have further shown that transactivation of the SRE by p35C/EBPβ is independent of TCF but does require DNA-bound SRF. Interestingly, both p35C/EBPβ and p20C/EBPβ can bind to a chimeric glutathione *S*-transferase (GST)-SRF fusion protein in vitro. Moreover, SRE BP binds well to SRE mutants that retain responsiveness to the TCF-independent serum-induced signaling pathway, but binding is impaired by mutations which block this pathway. We suggest that C/EBPβ may be a recognition factor targeted by the SRF-linked TCF-independent signaling pathway, although we cannot rule out the possibility that other factors may be involved as well.

MATERIALS AND METHODS

Nuclear extracts and gel shift assays. Nuclear extracts were prepared from NIH 3T3 or BALB/c 3T3 fibroblasts by 0.53 M NaCl extraction as previously described by Sears and Sealy (41), except that in some cases (Fig. 3 and 9) final dialysis of the 0.5 M NaCl extract was replaced by the addition of an equal volume of 10 mM HEPES (pH 8)–25% glycerol containing phosphatase and protease inhibitors prior to storage at −70°C. Protein concentrations were determined by the method of Schaffner and Weissman (39) with bovine serum albumin (BSA) as the standard.

DNA binding assays were performed with nuclear extract, sodium dodecyl sulfate (SDS) size-fractionated samples, or recombinant C/EBPβ protein, ^{32}P-labeled SRE DNA, and poly(dI-dC):poly(dI-dC) at the amounts indicated in the figure legends in a final volume of 20 μl containing 10 mM HEPES (pH 8), 1 mM $MgCl_2$, 2.5 mM dithiothreitol (DTT), 2 mM spermidine, 20 mM KCl, and 10% glycerol. As shown in Fig. 6 and 9, DNA binding assays were carried out in 20-μl volumes containing 7.5 mM HEPES (pH 8), 2.5 mM Tris (pH 7.5), 1 mM EDTA, 5 mM β-mercaptoethanol, 5 mM DTT, 5 mM spermidine, 100 mM NaCl, and 10% glycerol (buffer I conditions). The DNA binding assays shown in Fig. 3 were also carried out using buffer I conditions except that 200 mM NaCl was used. Binding reactions were carried out at room temperature for 30 min. Where noted in the figure legends, antisera, preimmune serum, or BSA was added to the protein samples prior to DNA addition. Following binding reactions, samples were analyzed on native 5 or 6% polyacrylamide gels containing 25 mM Tris base, 190 mM glycine, and 1 mM EDTA (pH 8.5). Gels were subsequently dried for autoradiography.

SDS size fractionation of DNA binding activity. Nuclear extract was freshly prepared from two 100-mm-diameter dishes of NIH 3T3 cells. All steps and solutions were at 4°C. Cells were washed three times with phosphate-buffered saline (PBS) containing 0.1 mM Na vanadate and were scraped into the same buffer and collected by centrifugation. Cells were lysed by vortexing in 300 μl of buffer A containing phosphate and protease inhibitors (41). Crude nuclei were collected by centrifugation at 5,000 × *g* for 10 min in an Eppendorf centrifuge. Nuclei were resuspended in 300 μl of buffer A, and an equal volume of 2× SDS loading buffer (100 mM Tris [pH 6.8], 2% SDS, 20% glycerol, 2% β-mercaptoethanol, 0.2% bromophenol blue) was added. The sample was sonicated with a microtip (setting, 1.5; 30% duty cycle; 30 s) to reduce viscosity but was not boiled prior to electrophoresis to maximize recovery of DNA binding activity. Size fractionation was performed by separating up to 70 μl of nuclear extract on an SDS–12% polyacrylamide gel at 160 V for 3.5 h. The gel was incubated in transfer buffer (33 mM Tris base, 192 mM glycine, 20% methanol) at 4°C for 15 min prior to transfer of fractionated proteins to Immobilon-P membranes (Millipore Corp.) as previously described (41). Following transfer, the membrane was briefly stained in 0.5% Ponceau S in water to visualize protein molecular mass standards. Lanes containing nuclear extract proteins were cut into 3-mm slices, and proteins in each slice were eluted and renatured at 4°C by vigorous shaking for 2 to 3 h in a solution containing 50 mM Tris (pH 7.5), 0.1 mM EDTA, 100 mM NaCl, 20% glycerol, 100 μg of BSA per ml, 1% Triton X-100, and 5 to 10 mM DTT.

Immunoblots. Nuclear extract proteins were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane as described above for SDS size fractionation of DNA binding activity. Following transfer, the membrane was blocked in TBS-T buffer (0.1 M Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature with gentle rocking. The membrane was briefly washed in TBS-T buffer containing 0.5% nonfat dry milk prior to incubation with a 1:5,000 dilution of anti-C/EBPβ C-terminal peptide antibody (Santa Cruz Biotechnology) in 0.5% nonfat dry milk–TBS-T buffer for 1 h at room temperature. The membrane was washed for 1 h at room temperature in 0.5% nonfat dry milk–TBS-T buffer with three buffer changes. The membrane was then incubated with a 1:2,000 dilution of goat anti-rabbit immunoglobulin G (Boehringer Mannheim) in 0.5% nonfat dry milk–TBS-T buffer for 1 h at room temperature. Following extensive washing, the secondary antibody was detected with the Renaissance chemiluminescence detection kit (Dupont NEN) following the manufacturer's instructions.

Antibodies. All antibodies were purchased from Santa Cruz Biotechnology except for the guinea pig polyclonal anti-LAP antibody which was previously described by Sears and Sealy (41). This polyclonal antibody does not cross-react with C/EBPα or C/EBPδ. It was affinity purified by incubating the antiserum with recombinant His-tagged LAP immobilized on Immobilon-P filter strips. Bound antibody was eluted from the filter strips in 0.2 M glycine-HCl, pH 2.2, and immediately neutralized with 1.5 M Tris, pH 9.

Radiolabeled and competitor DNAs. Oligonucleotides were prepared by automated DNA synthesis in the Diabetes Research and Training Center DNA Core (Vanderbilt University) and were purified over desalting columns as previously described (2). Radiolabeled probe for gel shift assays was labeled with polynucleotide kinase and was gel purified as previously described (2). Nonradiolabeled competitor DNAs were gel purified and quantitated by A_{260} measurements as previously described (2). Sequences of the oligonucleotides used are as follows. Linker sequences are in lowercase. Wild-type and mutant CArG boxes are in boldface.

c-fos SRE tcgggCAGGATGT**CCATATTAGG**ACATCTGCc
cGTCCTACA**GGTATAATCC**TGTAGACGgagcc

*fos** tcgggCAGGATGT**CGATATTACG**ACATCTGCc
cGTCCTACA**GCTATAATGC**TGTAGACGgagcc

FSS CTCGAGGATGTC**CCTATTAGG**TAATTAAGATCT

SRE.M CTCGAGGATGTC**CCAATCGGG**ACATCTAGATCT

SRE.LP ATGTACTGTATGT**ACATATTAGT**ACATCTGC

Cell culture, transfections, and CAT assays. NIH 3T3 fibroblasts were obtained from the American Type Culture Collection and maintained in Dulbecco modified Eagle medium (DMEM) (high glucose) supplemented with 0.22% sodium bicarbonate, 10% calf serum, 25 U of penicillin G sodium per ml, and 25 mg of streptomycin sulfate per ml. Transfections were performed by the calcium phosphate coprecipitation technique (12). Cells were plated in 60-mm-diameter dishes 1 day prior to transfection at a density between 0.5×10^6 and 1×10^6 cells/dish. Transfections were performed with 15 μg of plasmid DNA per 5 ml of medium. Cells were exposed to the $CaPO_4$-DNA precipitate for 8 h. The medium was then removed, and after the monolayer was washed with Tris-glucose buffer, cells were placed in complete medium unless stated otherwise in the figure legends. Twenty-four hours after transfection, cells were placed in DMEM containing 0.5% calf serum for 36 h. Cells were then either harvested without further treatment or stimulated with 15% fetal calf serum (Hyclone) and harvested at the times indicated in the figure legends. Cell extracts were prepared as previously described (41), and chloramphenicol acetyltransferase (CAT) assays were performed on extract samples containing equivalent protein as previously described (41).

Reporter genes and expression vectors. The e-CAT reporter gene plasmid has been previously described (2) and contains the long terminal repeat (LTR)

minimal promoter (sequences from −54 to +103) linked to the CAT gene. Wild-type or mutant SRE oligonucleotides were cloned into e-CAT at a unique *Acc*I site at −235 relative to the start site of transcription. The sequences of the inserts were as follows:

cfos SRE tcgggCAGGATGT**CCATATTAGG**ACATCTGCc
cGTCCTACA**GGTATAATCC**TGTAGACGgagcc

*fos** tcgggCAGGATGT**CGATATTACG**ACATCTGCc
cGTCCTACA**GCTATAATGC**TGTAGACGgagcc

TCF_m gggCGATCGTG**CCATATTAGG**ACATCTGCccc
cccGCTAGCAC**GGTATAATCC**TGTAGACGggg

Linker sequences are in lowercase. The CArG box is in boldface, and the locations of the TCF binding sites are underlined. The nonbinding SRF mutant sequence designated fos* is from the paper by Treisman (53). The nonbinding TCF mutant sequence, TCF_m, is a chimeric site in which sequences 5′ of the CArG box in the *cfos* SRE have been replaced with the corresponding sequences 5′ of the CArG box in the Rous sarcoma virus LTR SRF (EFIII) site. The block of five transversions (TCGTG) introduced 5′ to the CArG box has been shown to prevent TCF binding by Shaw et al. (43). Where necessary, oligonucleotides were treated with DNA polymerase I at 4°C to generate blunt ends prior to cloning. Plasmids with a single insert in the correct orientation were selected, and the absence of mutations was confirmed by DNA sequencing.

Cytomegalovirus (CMV)-LAP and CMV-LIP were generous gifts from U. Schibler (University of Geneva, Geneva, Switzerland). CMV–NF-IL6 was a kind gift from S. Akira (Osaka University, Osaka, Japan). CMV-C/EBPβ(lz−) was constructed in this laboratory by inserting a 937-bp *Pst*I fragment from pRsetB-LAP (41) into the *Pst*I site of expression vector CMV-5. The 937-bp fragment encodes amino acids 1 to 265 of rat C/EBPβ. pRsetA-LIP(lz−) was constructed from pRsetA-LIP (41) by digestion with *Pst*I and religation to delete the *Pst* fragment encoding the C-terminal 32 amino acids of LIP. Because of the removal of the normal termination codon, translation proceeds for 28 vector-encoded amino acids before terminating. pRsetB-LAP(1–139) was constructed from pRsetB-LAP by *Bst*BI digestion to delete a *Bst*BI fragment encoding amino acids 140 to 297. Although the normal termination codon is removed, translation proceeds only one amino acid (alanine) into the vector before terminating.

Recombinant proteins. Histidine-tagged LAP was produced in *Escherichia coli* and purified by nickel affinity chromatography as described by Sears and Sealy (41). To obtain GST-SRF, a 1,563-bp fragment encoding amino acids 1 to 508 of human SRF was generated by PCR amplification of pGEM3.5 (36) (a kind gift from R. Treisman) with Amplitaq DNA polymerase and the GeneAmp PCR kit (Perkin-Elmer) according to the manufacturer's instructions. The primers used for PCR contained *Eco*RI recognition sites so that upon digestion of the PCR product with *Eco*RI, the resultant fragment was inserted into the *Eco*RI site of pGEX-4T-1 (Pharmacia). Chimeric GST-SRF was produced from pGEX-4T-SRF in BL21 host cells by induction with IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C. Bacterial cells were lysed by sonication at 4°C in PBS containing 0.1 mM phenylmethylsulfonyl fluoride and 1 μg of aprotinin per ml. Triton X-100 was added to a final concentration of 0.1%. After 30 min of gentle mixing, the lysate was clarified at 12,000 × *g* for 10 min. The supernatant was incubated with glutathione-Sepharose beads at 4°C for 30 min. The beads containing GST proteins were collected by low-speed centrifugation and were washed extensively with PBS containing 0.1% Triton X-100 and the protease inhibitors listed above before use.

Analysis of SRF and C/EBPβ interaction in vitro. C/EBPβ proteins in pRset vectors as described under "Reporter genes and expression vectors" were transcribed and translated in vitro by using the TNT T7 coupled reticulocyte lysate system from Promega according to the manufacturer's instructions. Translated proteins were radiolabeled with the EXPRE^{35}S protein labeling mix (Dupont NEN). Four microliters of each translation mixture was incubated with an aliquot of glutathione-Sepharose beads containing approximately equivalent amounts of either GST-SRF or GST in buffer containing 8 mM Tris (pH 7.5), 0.8 mM EDTA, 80 mM NaCl, 10% glycerol, 10 mM DTT, 0.05% Nonidet P-40, 1 μg of aprotinin per ml, and 0.1 mM sodium vanadate (binding buffer). The amount of GST protein bound to the beads was estimated by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis of GST or GST-SRF proteins that had been released by boiling an aliquot of beads in Laemmli sample buffer (Bio-Rad). To reduce background binding, beads were preincubated with unprogrammed reticulocyte lysate (10 μl/20 μl of packed beads) for 1 h at 4°C in binding buffer. After collection by centrifugation, blocked beads were washed once with binding buffer before incubation with radiolabeled protein for 1 h at room temperature. After three rapid washes in binding buffer at 4°C, proteins bound to the beads were released by boiling for 5 min in Laemmli sample buffer (Bio-Rad) and were analyzed by SDS-polyacrylamide gel electrophoresis.

RESULTS

SRE BP is a heterodimer of p35C/EBPβ and p20C/EBPβ. To further characterize the SRE BP DNA binding activity, we initially wanted to determine an approximate molecular mass for this factor. A nuclear extract from BALB/c 3T3 fibroblasts was fractionated by SDS-polyacrylamide gel electrophoresis, and proteins from the gel were transferred to an Immobilon-P membrane. The membrane was cut into 3-mm-long slices, and proteins eluted and renatured from each slice were tested for DNA binding activity with radiolabeled SRE DNA. The results are shown in Fig. 1A. SRE DNA binding activity was recovered in fractions with approximate molecular masses of 38 kDa (lane 9) and 20 kDa (lane 14). These DNA binding activities were specific, as shown by experiments with competitor DNAs (data not shown). However, compared with the mobility of SRE BP in the unfractionated nuclear extract (lane 17), the 38-kDa complex migrated more slowly and the 20-kDa complex migrated more rapidly. This suggested that the 38- and 20-kDa DNA binding activities, when mixed, might give rise to a complex with the same mobility as that of SRE BP. This is indeed the case as shown in Fig. 1B. When proteins in the 38- and 20-kDa fractions were mixed at 37°C prior to DNA addition, a complex of intermediate mobility which matched that of SRE BP was formed. These results indicate that SRE BP is composed in part of a heterodimer of 38- and 20-kDa proteins. We cannot exclude the possibility that other proteins, whose binding activities are not recovered after renaturation, contribute independently to SRE BP binding activity. We note that SRF binding activity is not recovered after this procedure.

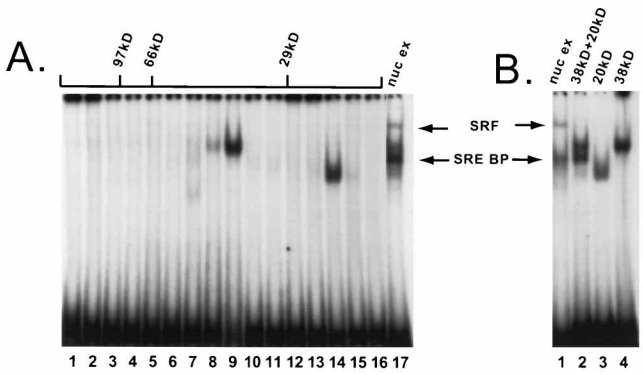

FIG. 1. Size fractionation of SRE BP DNA binding activity. (A) Nuclear extract (nuc ex) from NIH 3T3 cells was fractionated on an SDS–10% polyacrylamide minigel and transferred to an Immobilon-P membrane, and the separated proteins were renatured in an elution solution as described in Materials and Methods. Gel shift assays were performed with 10 μl of each size fraction and 0.5 ng of ^{32}P-labeled SRE DNA in the presence of 100 ng of poly(dI-dC):poly(dI-dC), as described in Materials and Methods. A scale of apparent molecular masses in kilodaltons, based on protein standards run in parallel on the SDS gel, is shown above lanes 1 to 16. In lane 17, approximately 5 μg of unfractionated nuclear extract from BALB/c 3T3 cells was mixed with 0.5 ng of ^{32}P-labeled SRE DNA in the presence of 500 ng of poly(dI-dC):poly(dI-dC) as described in Materials and Methods. SRF and SRE BP complexes are indicated. (B) Gel shift assays were performed with 5 μg of unfractionated nuclear extract as in panel A, lane 17 (lane 1), 5 μl each of the 38- and 20-kDa SDS-sized fractions mixed at 37°C for 20 min prior to DNA addition (lane 2), 5 μl of the 20-kDa SDS-sized fraction (lane 3), or 5 μl of the 38-kDa SDS-sized fraction (lane 4). Protein samples were mixed with 0.5 ng of ^{32}P-labeled SRE DNA in the presence of 100 ng of poly(dI-dC):poly(dI-dC) as described in Materials and Methods except for lane 1, which contained 500 ng of poly(dI-dC):poly(dI-dC). Ten nanograms of aprotinin was added to the protein sample shown in lane 2 prior to incubation at 37°C. This figure was prepared with a Mirror 800 color scanner and a Power Macintosh 8100/80 with Adobe Photoshop 2.5.1 and Adobe Illustrator 5.5.

Metz and Ziff (34) have shown that rat p35C/EBPβ binds to the *cfos* SRE in vitro at a site coincident with the binding site for SRE BP. The C/EBPβ gene encodes three in-frame methionines which can potentially give rise to three translation products of 38, 35, and 20 kDa. We have shown that many

fibroblast cell types, such as NIH 3T3 cells, express both the long (38- and 35-kDa) and short (20-kDa) forms of C/EBPβ at approximately equal levels as determined by Western blotting (41). Because the sizes of the long and short forms of C/EBPβ are remarkably similar to the sizes of the two components of the SRE BP heterodimer identified in Fig. 1, we wanted to determine whether the 38- and 20-kDa SRE binding activities did in fact comigrate with p35C/EBPβ, p38C/EBPβ, and p20C/EBPβ. We repeated the SDS gel electrophoresis, except that this time duplicate adjacent lanes of an NIH 3T3 nuclear extract were transferred to an Immobilon-P membrane. One lane was processed for immunoblotting with a C-terminal peptide antibody specific for C/EBPβ (Santa Cruz Biotechnology), and the other was cut into slices for recovery of DNA binding activities. It is clear that the fractions containing p35C/EBPβ (Fig. 2A, lane 7) and p20C/EBPβ (Fig. 2A, lane 16) also contain the SRE binding activities. Both p38C/EBPβ and p35C/EBPβ can be resolved on the larger gel used for Fig. 2, whereas these species were not separated on the minigel employed for Fig. 1. It has not been established whether p38C/EBPβ and p35C/EBPβ are different translation products initiating at the first (+1) and second (+24) methionines or whether p38 is a more highly phosphorylated form of p35. In any case it appears that only p35 is capable of binding the SRE in this analysis. The additional band in the Western blot of approximately 45 kDa is probably due to nonspecific cross-reactivity of this antibody with actin. The protein-DNA complexes in lanes 3 to 6 of Fig. 2 are also nonspecific; they can be eliminated by using more poly(dI-dC):poly(dI-dC) in the DNA binding assay, as was the case for the assays shown in Fig. 1.

To confirm that the SRE binding activities in lanes 7 and 16 of Fig. 2A are indeed C/EBPβ, these fractions were treated with C/EBPβ C-terminal peptide antibody (Santa Cruz Biotechnology) prior to the addition of radiolabeled SRE DNA. As shown in Fig. 2B, the binding of p35 was blocked by this antibody, whereas p20 binding activity was partially supershifted. p20 DNA binding activity can be completely eliminated as well by using more C/EBPβ antibody (data not shown). In contrast, a control peptide antibody of the same concentration (also from Santa Cruz Biotechnology) raised against the C terminus of a family member, C/EBPδ, had no effect on the p35 and p20 SRE binding activities. The nonspecific DNA binding proteins shown in lanes 4 to 6 were also not affected by either antibody and served as useful controls. We conclude that SRE BP is composed at least in part of a heterodimer of p35C/EBPβ and p20C/EBPβ.

p35C/EBPβ and p20C/EBPβ homodimers also contribute to SRE BP. These results initially appeared to be at odds with earlier results from our laboratory in which we found in gel shift assays that the SRE BP complex present in NIH 3T3 nuclear extracts was not affected by antibodies to rat NF-IL6 (p35C/EBPβ) or a C-terminal C/EBPβ peptide antibody (kindly provided by Steve McKnight) (2). We repeated these experiments using the same C/EBPβ peptide antibody from Santa Cruz Biotechnology used for Fig. 2. Because of the addition of high levels of DTT in the DNA binding reactions, we can now resolve the SRE BP present in NIH 3T3 nuclear extract into three distinct complexes. On the basis of the mobilities of complexes identified in the size-fractionated experiments of Fig. 1, the slowest-migrating complex corresponds to a p35C/EBPβ homodimer and the fastest-moving complex migrates as the p20C/EBPβ homodimer. The complex of intermediate mobility comigrates with a p35-p20 heterodimer. When NIH 3T3 nuclear extract is treated with C/EBPβ peptide antibody prior to DNA addition (Fig. 3, lane 4), some, but not all, of the SRE BP complex is eliminated. The results are also presented graphically in Fig. 3. Only about 50% of the SRE BP complex can be eliminated by the C-terminal C/EBPβ peptide antibody, even if increasing amounts are added. This is not due to lack of availability of the C-terminal epitope in a portion of the SRE BP complexes because a similar result was obtained with a polyclonal antibody generated to the entire p35C/EBPβ protein (αLAP) in our laboratory. Most of the decrease in the SRE BP complex comes from the elimination of p35 homodimers and p20 homodimers; the putative heterodimer complex is decreased by only 25%. However, these effects are specific for the C/EBPβ antibodies, as a C-terminal peptide

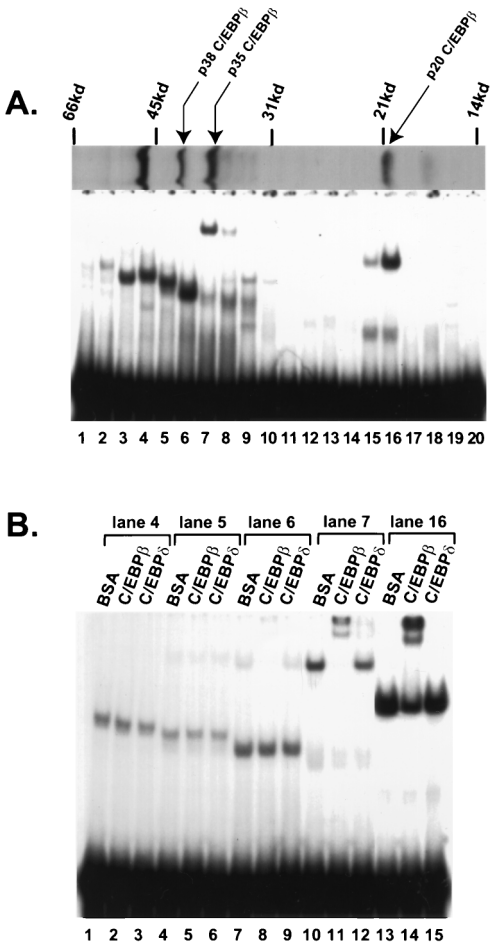

FIG. 2. p35C/EBPβ and p20C/EBPβ contribute to SRE BP DNA binding activity. (A) Nuclear extract from NIH 3T3 cells was fractionated on an SDS–12% polyacrylamide gel and transferred to an Immobilon-P membrane. One lane was processed for Western blotting with anti-C/EBPβ peptide antibody, while the separated proteins with apparent molecular masses between 66 and 14 kDa were renatured in elution solution from a parallel lane as described in Materials and Methods. Gel shift assays were performed with 10 μl of each size fraction and 0.5 ng of ^{32}P-labeled SRE DNA in the presence of 500 ng of poly(dI-dC):poly(dI-dC) as described in Materials and Methods. A scale of apparent molecular masses in kilodaltons, based on protein standards run in parallel on the SDS gel and detected on the Immobilon-P filter by Ponceau S staining, is shown above the lanes. (B) Ten microliters of the SDS-sized fractions from the lanes indicated in panel A was preincubated for 10 min at 37°C with 1 μl of the indicated peptide antibodies (Santa Cruz Biotechnology) or 1 μl of 1-mg/ml BSA in the presence of 20 ng of aprotinin. Protein samples were then mixed with 0.5 ng of ^{32}P-labeled SRE DNA in the presence of 500 ng of poly(dI-dC):poly(dI-dC) as described in Materials and Methods. This figure was prepared with a Mirror 800 color scanner and a Power Macintosh 8100/80 with Adobe Photoshop 2.5.1 and Adobe Illustrator 5.5.

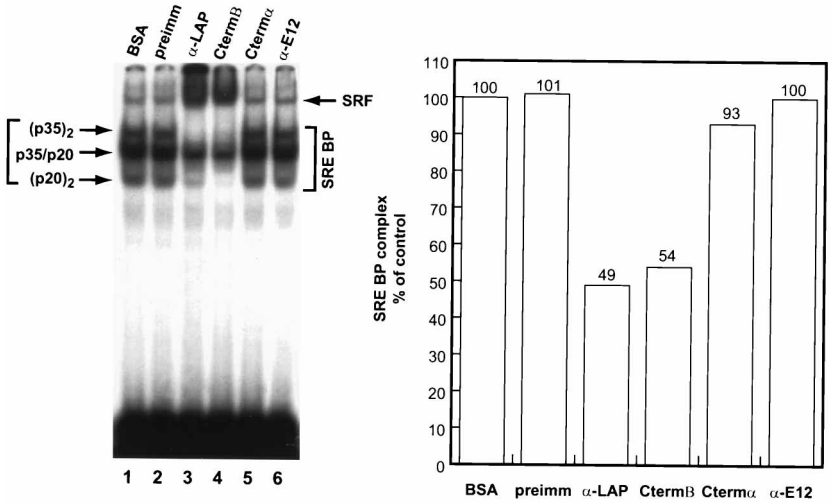

FIG. 3. Effect of C/EBPβ antibodies on SRE BP DNA binding activity in nuclear extract. Approximately 8 μg of NIH 3T3 cell nuclear extract was mixed with 1 μg of BSA (lane 1), 1 μl of guinea pig preimmune serum (lane 2), 1 μl of guinea pig affinity-purified anti-LAP antibody (lane 3), or 1 μl of C/EBPβ (lane 4), C/EBPα (lane 5), or E12 (lane 6) peptide antibodies from Santa Cruz Biotechnology in the presence of 20 ng of aprotinin at 4°C for 2 h. Protein samples were then mixed with 1 ng of ^{32}P-labeled SRE DNA in the presence of 2 μg of poly(dI-dC):poly(dI-dC) as described in Materials and Methods for buffer I conditions. Specific SRF-DNA or C/EBPβ protein-DNA complexes are indicated. The radioactivity levels in specific protein-DNA complexes on the dried gel were quantified with a Bio-Rad molecular imager. The graph represents the decrease in SRE BP complex formation with antibody or preimmune serum incubation. The amount of SRE BP complex formed when nuclear extract was incubated with BSA was assigned a value of 100%. Data are the averages of two separate experiments for the E12 antibody, three separate experiments for the guinea pig antibody and preimmune serum, and five separate experiments for the Santa Cruz Biotechnology C/EBP antibodies and BSA. The average error in the determinations was 14%. This figure was prepared with a Mirror 800 color scanner and a Power Macintosh 8100/80 with Adobe Photoshop 2.5.1 and Adobe Illustrator 5.5.

antibody against C/EBPα or the E12 protein had no effect on SRE BP.

We suspect that our earlier results in which C/EBPβ antibodies had no effect on SRE BP were due in part to the lack of resolution of the complex. We were primarily detecting the putative heterodimer complex, which continues to be minimally affected by C/EBPβ antibodies. It is likely that at least one other factor in addition to the p35C/EBPβ and p20C/EBPβ proteins contributes to the SRE BP complex detected in unfractionated nuclear extracts. However, we have been unable to identify this additional factor. Work from several laboratories has suggested that proteins in the Rel family can form a complex with C/EBPβ (8, 27, 45). Binding sites within the SRE have also been reported for the helix-loop-helix protein E12 (32) and for YY1 (15, 35). However, in addition to the E12 antibodies shown in Fig. 3, antibodies to NF-κBp65 and p50 and to YY1 also have no effect on SRE BP (data not shown).

Expression of p20C/EBPβ blocks serum stimulation of the *cfos* SRE. Since at least 50% of SRE BP is composed of p35C/EBPβ and p20C/EBPβ homodimers and heterodimers, we wished to determine the influence of these proteins on *cfos* SRE transcriptional activation. p20C/EBPβ lacks the transactivation domain present in its longer cousin and thus can act as a repressor of transcription (7, 41). A CMV expression vector encoding p20 was cotransfected into NIH 3T3 cells with a CAT reporter gene construct driven by a single copy of the *cfos* SRE linked to a minimal promoter from the Rous sarcoma virus LTR. The LTR minimal promoter consists of a TATA box and initiation site (2, 40) but no other promoter *cis* elements to influence transcription or SRE function. We could not use the *cfos* SRE linked to the *tk* promoter for these experiments because the *tk* promoter contains a potential C/EBPβ binding site that could complicate the interpretation of the results. After transfection, the cells were rendered quiescent by 40 h of serum deprivation prior to serum stimulation. As shown in Fig. 4, transcription from the *cfos* SRE-CAT construct was increased 10-fold by serum treatment. However, coexpression of p20C/EBPβ completely blocked this serum induction. Transcription mediated by the *cfos* SRE in serum-deprived cells was also decreased slightly (about 30%) by p20C/EBPβ expression. These results suggest that the binding of p20C/EBPβ to the *cfos* SRE may prevent interaction with a factor normally responsible for transactivation of the SRE in response to mitogenic stimulation.

A likely candidate for this factor might be p35C/EBPβ. Indeed, when we cotransfected a CMV expression vector for p35C/EBPβ along with the *cfos* SRE-CAT reporter gene we found that overexpression of p35C/EBPβ stimulated transcription from the *cfos* SRE nearly 30-fold in serum-deprived cells (Fig. 4). Only a small further increase in transcription was observed upon serum stimulation (up to 35-fold), which is not significant given the error associated with the data. Overexpression of p35C/EBPβ alone (which probably results in the formation of primarily p35 homodimers) resulted in an increase in transcription greater than that normally achieved by serum stimulation. We therefore investigated the effects of cotransfection of p35C/EBPβ and p20C/EBPβ to attempt to analyze the effect of heterodimers on *cfos* SRE transcription. Unfortunately, given the low transfection efficiency of NIH 3T3 cells, it is not possible to directly measure the level of transfected protein expression by Western blotting. Because we have shown that p20C/EBPβ is more stable than p35C/EBPβ in NIH 3T3 cells (41), we transfected with a 2:1 ratio of CMV p35C/EBPβ expression vector to CMV p20C/EBPβ expression vector. However, we cannot be certain that this resulted in equal amounts of both p35 and p20 being expressed in the transfected cells. Nonetheless, this ratio of the two expression vectors resulted in a 10-fold increase in *cfos* SRE-driven transcription in quiescent cells, with no further increase upon serum stimulation. These results raise the possibility that the increase in transcription upon mitogenic stimulation could

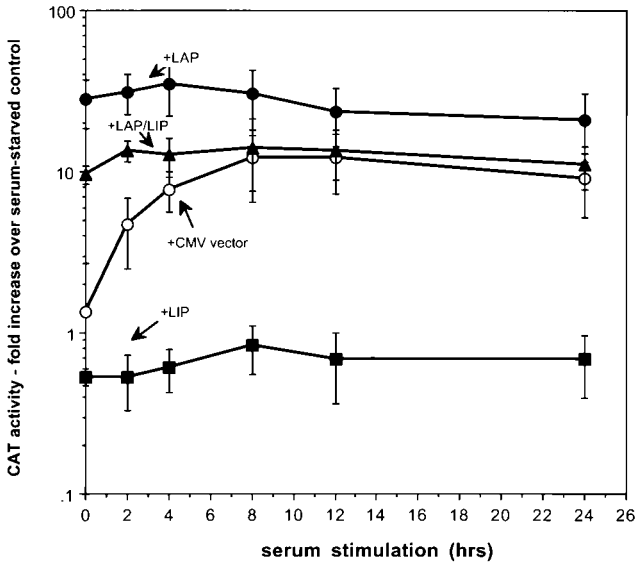

FIG. 4. Regulation of the *cfos* SRE by p20C/EBPβ and p35C/EBPβ. NIH 3T3 cells were transfected as described in Materials and Methods with 10 μg of *cfos* SRE CAT reporter gene and either 2.5 μg of CMV-4 vector (+CMV vector), 2.5 μg of CMV-LAP (p35) (+LAP), 0.5 μg of CMV-LIP(p20) (+LIP), or 1 μg of CMV-LAP and 0.5 μg of CMV-LIP (+LAP/LIP). Total DNA in each transfection was adjusted to 15 μg with pUC19 DNA. Twenty-four hours after transfection, cells were serum deprived for 36 h and all but the plates harvested at time zero were stimulated with 15% fetal calf serum as described in Materials and Methods. Cells were harvested at the indicated times, and CAT activity was measured as described in Materials and Methods. Fold induction was calculated as the increase in CAT activity relative to that obtained with the reporter gene plus pUC19 DNA in serum-deprived cells at time 0. Values are the averages of three independent experiments, and error bars show standard deviations.

be achieved simply by exchanging one p20C/EBPβ subunit for a p35C/EBPβ subunit on the SRE DNA. Alternatively, a p20 homodimer could be replaced after dissociation from the DNA by a p35-p20 heterodimer. In any case, it appears that binding of p35C/EBPβ to the *cfos* SRE could be a limiting factor in quiescent cells.

p35C/EBPβ can activate the *cfos* SRE in the absence of p62^{TCF} binding. TCF was initially identified as a 62-kDa protein that forms a ternary complex with SRF and SRE DNA through a purine-rich sequence (AGGA) at the 5′ end of the SRE (42, 43). TCF, which comprises a small family of Ets domain proteins, including Elk-1 and SAP-1, is phosphorylated by MAP kinases (ERKs) in response to serum, and this modification activates the transcriptional activation function of TCF (reviewed in reference 48). Thus, TCFs regulate SRE activity in response to activation of the Ras-Raf-ERK pathway. Although *cfos* promoter mutants that cannot bind TCF are no longer responsive to the Ras-Raf-ERK signaling pathway, they remain responsive to serum by a second, TCF-independent pathway (17, 19, 24). To determine if C/EBPβ regulation of the SRE is dependent on TCF, we repeated the transfections with a mutant SRE CAT reporter gene (TCF_m-CAT) that does not bind TCF. As seen in Fig. 5, transcriptional activation by p35C/EBPβ was nearly identical regardless of whether the SRE was capable of binding TCF. Similarly, p20C/EBPβ strongly repressed serum induction of both wild-type SRE and the TCF-deficient mutant. In the absence of exogenous C/EBPβ protein expression, the level of serum induction of the TCF mutant SRE reporter gene was consistently lower than that of wild-type SRE at each time point, presumably reflecting the loss of activation through the Ras-Raf-ERK pathway. In this series of experiments we also tested the human C/EBPβ protein, p42 (NF-IL6). Coexpression of human C/EBPβ from a CMV vector resulted in strong stimulation of both the wild-type and TCF mutant SREs in both serum-deprived and -stimulated NIH 3T3 cells. Activation by the 42-kDa human protein was consistently higher (60- to 80-fold) than that by the rat and mouse 35-kDa homologs (20- to 30-fold). The significance of this difference (if any) is not known.

Transactivation of the SRE by p35C/EBPβ is dependent upon SRF binding. TCF-independent serum-induced activation of the SRE has been shown to be dependent on SRF (17, 19, 24). Serum-regulated transcriptional activation by SRF requires that it be bound to the SRE via its own DNA binding domain and can be blocked by mutations in this domain that do not impair DNA binding (17, 24). On the basis of these findings, Hill et al. (17) proposed that DNA-bound SRF undergoes a conformational change, allowing it to interact with a non-TCF "recognition factor" that is the target of serum-induced signals. We asked if transactivation by C/EBPβ is dependent upon SRF binding to the *cfos* SRE. A set of transfections were performed with either the wild-type SRE reporter gene or a CAT reporter gene construct harboring a mutant SRE which no longer binds SRF (fos*CAT). The mutant SRE contains G-C transversions at positions +4 and −4 in the dyad symmetry element of the SRE which disrupt the CArG box and SRF binding (53). However, we previously showed that SRE BP binding in nuclear extracts is unimpaired in this mutant (2). We confirmed this finding using 6× histidine-tagged C/EBPβ (His-LAP) protein purified from *E. coli*. As shown in Fig. 6B, the fos* mutant SRE oligonucleotide competed equally with wild-type SRE DNA for binding purified recombinant p35C/EBPβ protein. We then compared the ability of increasing amounts of the CMV vector encoding the human protein (NF-IL6) to transactivate the wild-type and fos*CAT reporter

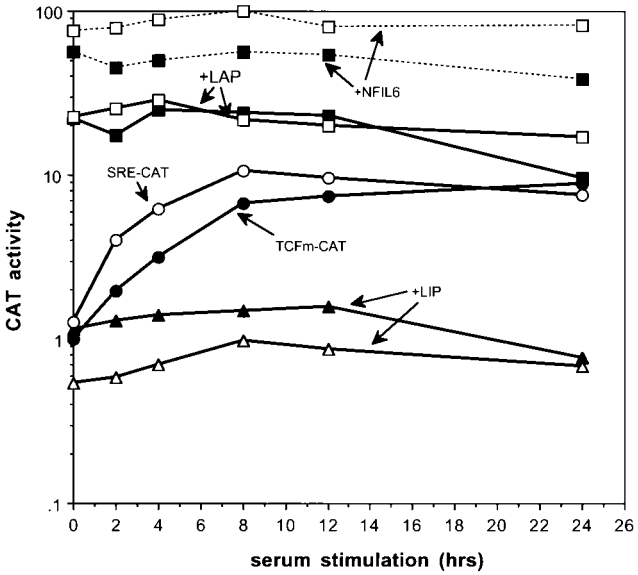

FIG. 5. Regulation of the *cfos* SRE by C/EBPβ is independent of TCF binding. NIH 3T3 cells were transfected as described in Materials and Methods with 10 μg of a TCF mutant SRE CAT reporter gene (solid symbols) and either 2.5 μg of CMV-4 vector (circles), 2.5 μg of CMV-LAP (p35) (squares, solid lines), 0.5 μg of CMV-LIP(p20) (triangles), or 2.5 μg of CMV–NF-IL6 (squares, broken line). Parallel experiments with the wild-type SRE CAT reporter gene are denoted by open symbols. CAT activity is expressed as fold increase, calculated as described in the legend to Fig. 4. Error bars have been omitted for clarity; however, the average error was ±1.6-fold for the CMV-4 and CMV-LIP transfections, ±11-fold for the CMV-LAP transfections, and ±17-fold for the CMV–NF-IL6 transfections.

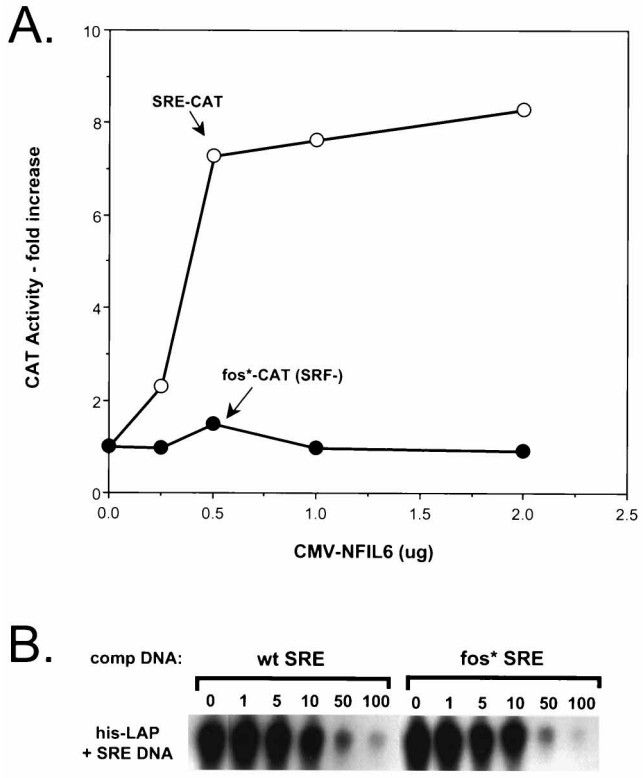

FIG. 6. Regulation of the *cfos* SRE by C/EBPβ requires SRF binding. (A) NIH 3T3 cells were transfected as described in Materials and Methods with either 10 μg of the reporter gene fos*CAT or wild-type SRE CAT along with increasing amounts of CMV–NF-IL6 expression vector as shown. Total DNA in each transfection was adjusted to 15 μg with CMV-4 DNA. Twenty-four hours after transfection, cells were serum deprived for 36 h prior to harvesting. CAT activity was measured as described in Materials and Methods. Fold induction was calculated as the increase in CAT activity relative to that obtained with the appropriate reporter gene in the absence of CMV–NF-IL6. Values are the averages of four determinations; the average error in the data was 40%. (B) Twenty nanograms of His-LAP protein purified from *E. coli* (41) was mixed with 1 ng of ^{32}P-labeled SRE DNA and 1 μg of poly(dI-dC):poly(dI-dC) in the absence or presence of a 1- to 100-fold molar excess of the competitor DNA (comp DNA) indicated in the figure under buffer I conditions as described in Materials and Methods. The His-LAP-SRE DNA complex is shown. This figure was prepared with a Mirror 800 color scanner and a Power Macintosh 8100/80 with Adobe Photoshop 3.0.4 and Adobe Illustrator 5.5.

genes (Fig. 6A). Similar results were obtained with a CMV expression vector for the rat p35C/EBPβ protein (data not shown). Transcriptional activity from the wild-type SRE-driven promoter was increased approximately 10-fold by the greatest amount of CMV–NF-IL6 expression vector used in these experiments, whereas transcription from the fos*-driven promoter was unaffected by NF-IL6 expression. These results indicate that SRF binding to the SRE is necessary for transactivation by C/EBPβ.

Zipperless C/EBPβ antagonizes SRE-activated transcription. It is possible that the requirement for SRF binding in p35C/EBPβ transactivation of the SRE reflects some physical interaction between these two transcription factors that is essential for transactivation (discussed in more detail below). However, a more trivial explanation is that p35C/EBPβ indirectly leads to higher levels of SRF protein in the transfected cells through its effects on the SRF promoter, on SRF mRNA, or on protein stability. To rule out possible indirect influences of p35C/EBPβ on the SRE, we initially sought to mutate C/EBPβ binding in the SRE to demonstrate that p35C/EBPβ transactivation required direct binding to the SRE DNA. However, although we have down mutations in p35C/EBPβ binding, none of the mutants completely eliminate C/EBPβ binding to the SRE. Due to the overlap of their binding sites, we have not been able to effectively eliminate C/EBPβ binding without also altering SRF recognition. Therefore, we chose an alternative approach to determine if p35C/EBPβ transactivation requires SRE DNA binding. We generated a mutant C/EBPβ protein which lacks the C-terminal 32 amino acids, including 4 of the 5 leucine repeats in the zipper. This leucine zipperless (lz−) C/EBPβ protein is unable to dimerize, and expression of the mutant protein in vitro confirmed that, as expected, it is also unable to bind DNA (data not shown).

We constructed a CMV expression vector for the C/EBPβ (lz−) mutant and performed cotransfection assays with an SRE-driven reporter gene in NIH 3T3 cells. We expected that the truncated C/EBPβ protein, which is incapable of binding SRE DNA, would have no effect on SRE-activated transcription. However, as shown in Fig. 7, expression of the C/EBPβ (lz−) mutant completely blocked serum induction of the SRE. Transcription mediated by the *cfos* SRE in serum-deprived cells was also decreased 80% by C/EBPβ(lz−) expression. We initially suspected that overexpression of the intact N-terminal transactivation domain in C/EBPβ(lz−) in the absence of DNA binding was leading to "squelching" or sequestering of some component of the transcription initiation machinery away from the promoter. We reasoned that if this were the case, then transactivation by wild-type p35C/EBPβ should be antagonized by coexpression of C/EBPβ(lz−) as well.

Surprisingly, when expression vectors for p35C/EBPβ and

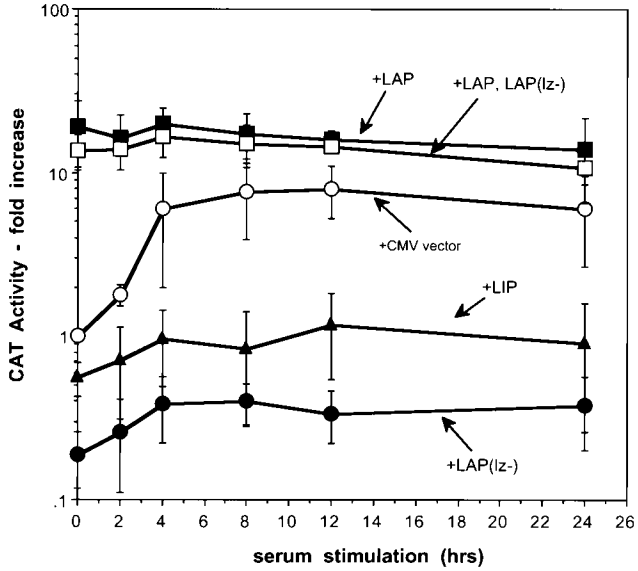

FIG. 7. C/EBPβ(lz−) inhibits SRE transcription. NIH 3T3 cells were transfected as described in Materials and Methods with 10 μg of *cfos* SRE CAT reporter gene and either 2.5 μg of CMV-4 vector (+CMV vector), 2.5 μg of CMV-C/EBPβ(lz−) [+LAP(lz−)], 0.5 μg of CMV-LIP(p20) (+LIP), 2.5 μg of CMV-LAP (p35) (+LAP), or 2.5 μg each of CMV-C/EBPβ(lz−) and CMV-LAP [+LAP, LAP(lz−)]. Total DNA in each transfection was adjusted to 15 μg with pUC19 DNA. Twenty-four hours after transfection, cells were serum deprived for 36 h and all but the plates harvested at time zero were stimulated with 15% fetal calf serum as described in Materials and Methods. Cells were harvested at the indicated times, and CAT activity was measured as described in Materials and Methods. Fold induction was calculated as the increase in CAT activity relative to that obtained with the reporter gene plus pUC19 DNA in serum-deprived cells at time 0. Values are the averages of three independent experiments, and error bars show standard deviations.

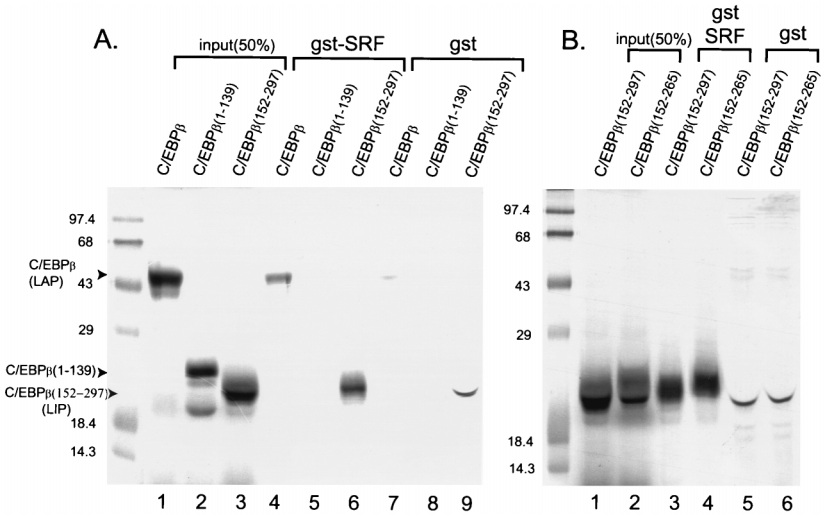

FIG. 8. C/EBPβ binds to GST-SRF in vitro. (A) Radiolabeled C/EBPβ (lanes 1, 4, and 7) or derivatives consisting of N-terminal aa 1 to 139 (lanes 2, 5, and 8) or C-terminal aa 152 to 297 (lanes 3, 6, and 9) were prepared by in vitro transcription and translation of appropriate pRset expression constructs and then incubated with approximately 4 μg of GST or GST-SRF protein immobilized on glutathione-Sepharose. Proteins eluted from the GST-SRF beads (lanes 4 to 6) or GST beads (lanes 7 to 9) by boiling in Laemmli sample buffer were resolved on an SDS–12% polyacrylamide gel and detected by autoradiography. Lanes 1 to 3 contain one-half the amount of the radiolabeled proteins (positions indicated by arrowheads) initially mixed with the beads. The leftmost lane represents ^{14}C-labeled molecular mass markers. (B) Radiolabeled C/EBPβ (aa 152 to 297) (lanes 1, 3, and 5) or a deletion mutant lacking the leucine zipper (aa 152 to 265) (lanes 2, 4, and 6) were prepared by in vitro transcription and translation of appropriate pRset expression constructs and then were incubated with GST or GST-SRF protein immobilized on glutathione-Sepharose as described in the legend for panel A. Proteins eluted from the GST-SRF beads (lanes 3 and 4) or the GST beads (lanes 5 and 6) by boiling in Laemmli sample buffer were resolved on an SDS–12% polyacrylamide gel and detected by autoradiography. Lanes 1 and 2 contain one-half the amount of the radiolabeled proteins initially mixed with the beads. The leftmost lane represents ^{14}C-labeled molecular mass markers. This figure was prepared with a Mirror 800 color scanner and a Power Macintosh 8100/80 with Adobe Photoshop 3.0.4 and Adobe Illustrator 5.5.

C/EBPβ(lz−) were cotransfected in a 1:1 ratio along with the SRE-driven reporter gene into NIH 3T3 cells, no effect of C/EBPβ(lz−) expression on transactivation of the SRE by wild-type p35C/EBPβ was observed (Fig. 7). Although other explanations for this result are possible, the simplest one is that C/EBPβ(lz−) inhibits basal and serum-induced transcription of the SRE by sequestering SRF away from the DNA through protein-protein interactions with SRF that are not dependent on the leucine zipper of C/EBPβ. Wild-type p35C/EBPβ transactivation of the SRE is not antagonized by C/EBPβ(lz−), perhaps because when both are overexpressed, the wild-type p35C/EBPβ dimer competes more effectively for SRF interaction than the C/EBPβ(lz−) monomer.

C/EBPβ binds to GST-SRF in vitro. Since the data in Fig. 7 suggested that C/EBPβ may be able to bind SRF through protein-protein interactions, we proceeded to test this possibility directly in vitro. SRF was expressed as a GST fusion protein and immobilized on glutathione-agarose beads. Beads containing GST-SRF or GST alone were incubated with in vitro-translated C/EBPβ labeled with [^{35}S]methionine. As shown in Fig. 8A, lane 4, C/EBPβ bound to the GST-SRF-containing beads. We also prepared in vitro-translated proteins representing either the N terminus of C/EBPβ (amino acids [aa] 1 to 139) or the C-terminal 145 aa of C/EBPβ comprising p20C/EBPβ (aa 152 to 297). The N terminus of C/EBPβ containing the transactivation domain did not bind GST-SRF in vitro (lane 5), whereas the C-terminal 145 aa common to p35C/EBPβ and p20C/EBPβ were retained on the GST-SRF beads (lane 6). A small amount of p20C/EBPβ bound to beads containing GST alone (Fig. 8A, lane 9; see also Fig. 8B, lane 5), which we have been unable to eliminate even if the beads are blocked with BSA or unprogrammed translation lysate. However, it is clear that the binding of p20C/EBPβ is substantially increased when the chimeric GST-SRF protein is present on the beads.

Having localized the interaction domain of SRF and C/EBPβ to the C terminus of C/EBPβ common to p35 and p20, we then asked if binding to GST-SRF in vitro was dependent on the leucine zipper domain. In vitro-translated, [^{35}S]methionine-labeled p20C/EBPβ (aa 152 to 297) and a truncated version of p20C/EBPβ (aa 152 to 265) containing the same leucine zipper deletion assayed in transfection experiments shown in Fig. 7 were tested for binding to GST-SRF beads. (Because translation of C/EBPβ [aa 152 to 265] terminates in vector sequences, the zipperless protein is very close in size to the wild-type p20C/EBPβ protein.) Deletion of the leucine zipper had no effect on the ability of the C terminus of C/EBPβ to bind SRF in vitro (compare lanes 3 and 4 of Fig. 8B). These data are consistent with the possibility that C/EBPβ(lz−) inhibits SRE-driven transcription by sequestering SRF from the DNA via protein-protein interactions in vivo. However, we cannot exclude the possibility that the leucine zipper is required for interaction with SRF and that the 28 vector-encoded amino acids included at the C terminus of C/EBPβ(lz−) in Fig. 8B (lanes 2 and 4) compensate for this loss, although there is no obvious amino acid sequence homology. We are currently expressing C/EBPβ(lz−) without the additional vector-encoded amino acids to address this concern.

C/EBPβ binding to mutant SREs correlates with the SRF-linked, TCF-independent signaling pathway. The contribution of SRF to serum-mediated activation of the SRE has been extensively studied with mutated SREs that bind chimeric SRF proteins with altered DNA binding specificity. Johansen and Prywes (24) generated a mutant SRE, termed FSS, that binds endogenous SRF poorly but can bind a chimeric SRF molecule having the DNA binding domain of the related yeast protein, MCM1. They showed that the FSS site in reporter gene constructs can be induced by serum independently of TCF. In addition, Hill et al. (18) generated an altered SRE, SRE.M, which also could not bind endogenous SRF but could bind a

similar chimeric SRF molecule with the DNA binding domain of MCM1. The SRE.M site, however, is not appreciably induced by serum in the absence of TCF binding. The differences between the FSS and SRE.M sites are in the core A/T region of the SRE, which is more extensively mutated in SRE.M (Fig. 9). FSS and SRE.M also differ in 3′ flanking sequences, which are wild type in SRE.M but derived from the yeast STE6 gene in FSS. These differences presumably lead to the inactivation of the TCF-independent signaling pathway in SRE.M, but this effect is probably not due to differential affinities for the chimeric SRF protein, since Johansen and Prywes (24) found that their chimeric SRF molecule bound better to the SRE.M site in vitro.

We examined the ability of p35C/EBPβ to bind the FSS and SRE.M sites in vitro. If C/EBPβ is involved in the TCF-independent pathway for serum regulation, we would expect this protein to still bind the FSS mutant but perhaps be impaired in binding SRE.M. We found this to be the case, as shown in Fig. 9. Radiolabeled wild-type SRE DNA was incubated with nuclear extract from NIH 3T3 cells in the presence of increasing amounts of competitor FSS or SRE.M DNA or in the absence of such DNA. Compared with the wild-type SRE as competitor, the C/EBPβ complex of proteins in nuclear extract showed identical binding affinity for the FSS mutant (Fig. 9A). However, the binding affinity for SRE.M DNA was approximately 10-fold lower (Fig. 9B; representative data shown in Fig. 9D). Thus, recognition of these altered SREs by C/EBPβ proteins correlates directly with the responsiveness of the mutants to the TCF-independent signaling pathway. The lower affinity of C/EBPβ for the nonresponsive SRE.M mutant is likely due to the CG change in the AT-rich core of this mutant, which alters the first two nucleotides of the C/EBPβ binding site. This change is not present in FSS. Moreover, although the 3′ flanking sequences in FSS are derived from the yeast STE6 gene (24), they happen to contain a slightly better match to the C/EBPβ consensus sequence than do the wild-type SRE and SRE.M. Taken together, these data suggest that C/EBPβ is a potential target for the TCF-independent signaling pathway(s) to the nucleus.

Hill et al. (17) characterized an SRE mutant, SRE.LP, which contains a low-affinity SRF-binding site and thus is not serum responsive. However, this mutant still retained significant activity in the presence of activated Rho family proteins, which was further potentiated by overexpression of SRF (19). Hill et al. (19) suggested that the response of SRE.LP to activated Rho proteins could reflect the continued ability of this mutant to bind the active recognition factor, which was then able to facilitate binding of SRF to the low-affinity site in this mutant. Interestingly, as shown in Fig. 9, the binding affinity of C/EBPβ for SRE.LP is nearly identical to that of the wild-type SRE. It is thus possible that unaltered binding of C/EBPβ to the SRE.LP mutant may contribute to the continued response of this mutant to Rho-mediated signaling.

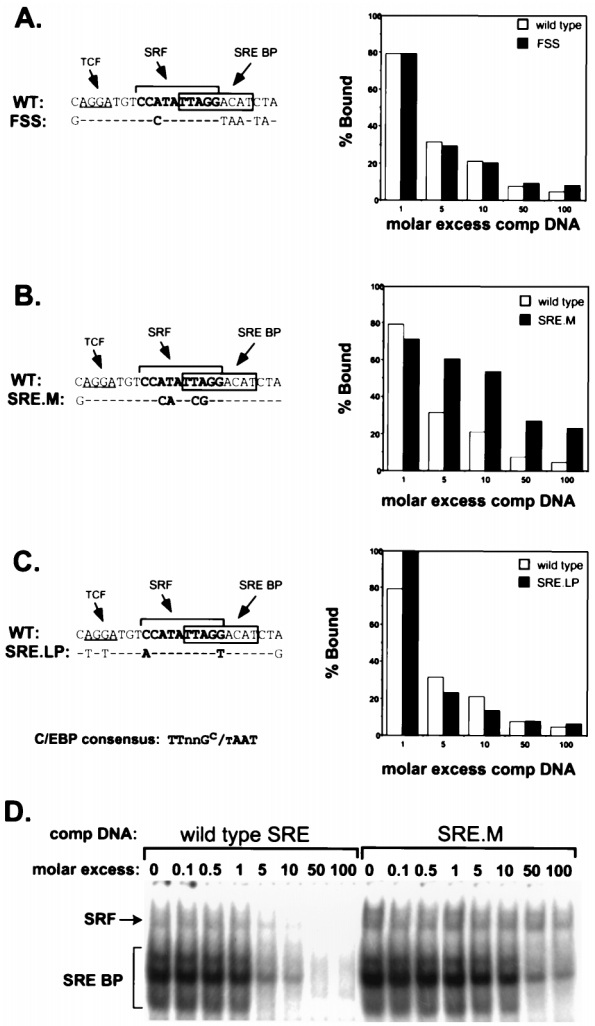

FIG. 9. SRE BP binding to SRE mutant DNAs. Gel shift assays were performed as described in Materials and Methods using buffer I conditions with approximately 8 μg of nuclear extract from NIH 3T3 cells and 1 ng of ^{32}P-labeled SRE DNA in the presence of 2 μg of poly(dI-dC):poly(dI-dC) and the indicated amount of competitor SRE mutant oligonucleotide (solid bars) or wild-type SRE DNA (open bars). Radioactivity levels in the SRE BP-DNA complexes on the dried gel were quantified with a Bio-Rad molecular imager. The ratio of DNA bound in the presence versus the absence of competitor DNA for each concentration of competitor DNA added to the binding reaction mixtures was calculated and plotted as shown. Values are the averages of duplicate determinations. Sequence changes between the wild type and FSS (A), SRE.M (B), and SRE.LP (C) mutants are shown at the left. Complete sequences of the wild-type and mutant SRE oligonucleotides used in the competitions are given in Materials and Methods. (D) A representative electrophoretic mobility shift assay used to obtain the graphical data presented in panel B is shown. Only the SRF-DNA and SRE BP-DNA complexes are shown. The amount of SRE BP complex plotted in panels A to C represents the summation of the radioactivity levels in each of the three discernible bands within the SRE BP complex, although similar results were obtained by considering each of the three SRE BP bands individually. This figure was prepared with a Mirror 800 color scanner and a Power Macintosh 8100/80 with Adobe Photoshop 2.5.1 and Adobe Illustrator 5.5.

DISCUSSION

We have previously reported on a novel binding factor, SRE BP, present in nuclear extracts of avian and NIH 3T3 fibroblasts which specifically binds to the *cfos* SRE within a region overlapping and immediately 3′ to the CArG box. Site-directed mutagenesis combined with transfection experiments in NIH 3T3 cells showed that binding of both SRF and SRE BP is necessary for maximal serum induction of the SRE (2). In this study, we have combined size fractionation of the SRE BP DNA binding activity with C/EBPβ antibodies to demonstrate that homodimers and heterodimers of p35C/EBPβ (a transactivator) and p20C/EBPβ (a repressor) contribute to the SRE BP complex in NIH 3T3 cells. Other investigators have previously found that C/EBPβ can bind to the *cfos* SRE. Metz and Ziff used the *cfos* SRE DNA motif to screen a cDNA expression library and isolated rat C/EBPβ (32). They showed that in PC12 cells cyclic AMP stimulates phosphorylation and translocation of C/EBPβ to the nucleus to bind the SRE and activate *cfos* expression (31). However, nuclear translocation was

not observed in other cell types, such as HeLa cells. Stein et al. (45) have also shown that rat C/EBPβ transactivates a multimerized *cfos* SRE in F9 embryonal carcinoma cells. In these cells, synergistic stimulation was observed when NF-κB was coexpressed with C/EBPβ, reflecting a cross-coupling between these two transcription factors as a result of a physical association between the bZIP domain of C/EBPβ and the Rel homology domain of NF-κB. Our results extend these previous observations by showing that in fact multiple forms of C/EBPβ contribute to the SRE BP complex and can influence SRE function.

p35C/EBPβ and p20C/EBPβ can regulate SRE activity. Both p35C/EBPβ and p20C/EBPβ are expressed at approximately equal levels in a variety of cell types (41). Quantitation of gel mobility shift assays with nuclear extract from NIH 3T3 cells and SRE DNA indicates that C/EBPβ binding activity is roughly equally composed of one-third each p35-p20 heterodimers, p35 homodimers, and p20 homodimers. We do not know if this represents their relative amounts in the cell, since the nuclear extraction procedure could disrupt the balance of heterodimers and homodimers. We have shown that p35C/EBPβ and p20C/EBPβ have the potential to regulate SRE activity in a directly opposing fashion. In transfection assays, overexpression of p20C/EBPβ, which lacks the N-terminal transactivation domain, completely prevented serum induction of the SRE. Overexpression of p35C/EBPβ resulted in transactivation of the SRE, consistent with the results of previous investigators. The fact that transactivation was observed even in serum-deprived cells suggests that a mitogen-induced modification of p35C/EBPβ is not absolutely required to "unmask" the transactivation potential of this transcription factor when it is bound to the SRE as is the case, for example, with TCF. However, we cannot rule out the possibility that such a mitogen-induced modification would normally promote the binding of p35C/EBPβ to the SRE in preference to p20C/EBPβ or enhance the transactivation potential of this transcription factor (see below), and we are eliminating the need for such a modification by selective overexpression of p35C/EBPβ.

Not all of the SRE BP binding complex can be supershifted by inclusion of C/EBPβ antibodies in the gel shift assay. Approximately 50% of the SRE BP complex remains unaffected and is likely to represent the binding of another factor(s) distinct from C/EBPβ. We have tested a variety of other transcription factor antibodies, singly and in combination with C/EBPβ antibodies, and have shown that they have no effect on the SRE BP complex in gel shift assays. These include, C/EBPα, C/EBPδ, E12, NF-κBp65, p50, and YY1. Binding sites within the SRE have been reported for the helix-loop-helix protein E12 (32) and for YY1 (15, 35). As previously mentioned, NF-κB has been shown to physically interact with C/EBPβ (8, 27, 45). However, none of these factors appear to contribute to the SRE BP complex. Further studies will be necessary to define the as yet unidentified protein(s) contributing to SRE BP binding activity and to evaluate their functional significance.

Is C/EBPβ an accessory factor involved in the TCF-independent pathway of SRE activation? Although we cannot rule out the participation of other factors, our data suggest that C/EBPβ is an attractive candidate for an accessory protein proposed to play a role in conjunction with SRF in the TCF-independent signaling pathway for SRE activation. This suggestion is based on the following observations. First, transactivation of the SRE by p35C/EBPβ is dependent on SRF binding but not TCF binding. A mutant SRE that cannot bind SRF was not transactivated by p35C/EBPβ. Second, SRE mutants which retain responsiveness to the TCF-independent signaling pathway bind p35C/EBPβ in vitro with an affinity nearly identical to that of the wild-type SRE. The SRE.M mutant, which is not responsive to the TCF-independent pathway, has a nearly 10-fold lower affinity for p35C/EBPβ. Third, we have demonstrated that C/EBPβ binds to SRF in vitro via protein-protein interactions that likely involve a 113-amino-acid domain within the C terminus of p20C/EBPβ that is exclusive of the leucine zipper. We have provided indirect evidence that such protein-protein interactions may also occur in vivo, given the ability of non-DNA-binding mutant C/EBPβ(lz−) to inhibit SRE-driven reporter gene transcription but not p35C/EBPβ-activated SRE transcription. Further studies will be needed to establish whether p35C/EBPβ is a recognition factor for SRF and/or a target of RhoA-mediated signaling. In particular, we are currently investigating the domain(s) of SRF required for binding C/EBPβ in vitro and whether such a protein-protein association occurs in vivo by using epitope-tagged proteins in coprecipitation assays.

Possible mechanisms for SRE transactivation by SRF and C/EBPβ. If C/EBPβ is an accessory factor in serum activation of the SRE through the TCF-independent pathway, then how might this transcription factor function along with SRF in regulating SRE transcription in response to mitogens? Since both p35C/EBPβ and p20C/EBPβ are capable of binding the SRE in serum-deprived or -stimulated cells, the balance of these two forms of C/EBPβ bound to the SRE in vivo could be regulated by mitogenic signaling. However, we do not have any evidence that nuclear translocation is involved. Both p35 and p20 are located in the nucleus in similar amounts in serum-deprived or -stimulated NIH 3T3 cells (39a). Also, the distribution of homodimers and heterodimers that make up SRE BP does not appear to change, as indicated by gel shift assays performed with nuclear extracts from serum-deprived or -stimulated cells, suggesting that direct regulation of p20C/EBPβ or p35C/EBPβ DNA binding activity is unlikely. Given that SRF is required for p35C/EBPβ transactivation and that C/EBPβ interacts with SRF in vitro, one possibility is that occupancy of the SRE by p35 homodimers, p35-p20 heterodimers, or p20 homodimers is governed by SRF. SRF is thought to occupy the SRE constitutively. In serum-deprived cells, p20 homodimer binding to the SRE in the presence of SRF may be favored. Upon activation of the TCF-independent signaling pathway, a conformational change in SRF could facilitate interaction and stabilization of p35 homodimers or p35-p20 heterodimers at the SRE, ultimately resulting in efficient transactivation. In vivo footprinting experiments have not found detectable changes in protein occupancy of the SRE upon serum stimulation (17). However, the exchange of p35C/EBPβ for p20C/EBPβ would be compatible with this observation in that both proteins have the same DNA binding domain and thus would be expected to exhibit extremely similar, if not identical, footprints on SRE DNA.

This scenario does not require, but is not incompatible with, the direct modification of C/EBPβ proteins by a nuclear kinase activated by the TCF-independent signaling pathway. For example, phosphorylation of p35C/EBPβ and/or p20C/EBPβ could enhance or diminish, respectively, the interaction of these factors with SRF and thereby shift the balance of C/EBPβ bound to the SRE toward the transactivator (p35C/EBPβ). Another not necessarily mutually exclusive role for phosphorylation could be to "activate" a dormant transactivation domain within p35C/EBPβ. Both Williams et al. (57) and Kowenz-Leutz et al. (26) have argued that C/EBPβ may assume a tightly folded conformation in which the activation domain is masked by intramolecular interaction with a bipartite negative regulatory domain, at least in certain nonpermis-

sive cell types. C/EBPβ has been shown to be a substrate for multiple protein kinases, including calcium-dependent kinases (55), MAP kinases (34), cyclic AMP-dependent protein kinase (31, 47), and kinases activated by stimulation of protein kinase C (47). Phosphorylation at specific sites (near and within the bZIP region) by these kinases can result in increased transactivation (34, 47) or can derepress the transactivation domain (26). We have not yet mapped any specific sites of modification of C/EBPβ upon serum treatment or identified the kinase(s) involved. Such studies may provide important details on a possible mechanism that could regulate activation of the *cfos* SRE by C/EBPβ and SRF.

Although the transfection experiments suggest that altering the balance of p35 homodimers, p35-20 heterodimers, and p20 homodimers bound to the SRE could serve to regulate transcription, it is important to emphasize that we do not yet know whether this strategy is employed in the cell. Thus, it is possible that only the transactivator p35C/EBPβ is involved in SRE activation through a mechanism that leads to unmasking of its transactivation domain and/or promotes binding to SRF; p20C/EBPβ may not function to repress transcription from this element in quiescent cells. However, if this is the case, then it becomes necessary to explain why p20C/EBPβ does not compete with p35C/EBPβ for binding the SRE, since both proteins are capable of binding this element with approximately equivalent affinities in vitro and since both proteins are present in NIH 3T3 cells and many other fibroblasts at approximately equal levels. The reverse situation is also possible, in which p20C/EBPβ acts as a brake on SRE activity in serum-deprived cells; but p35C/EBPβ, although capable of transactivating the SRE, does not actually perform such a function in serum-stimulated cells. However, mutation of the SRE BP binding site in the *cfos* SRE constitutes a loss of function mutation, contrary to what would be expected if the SRE BP binding site were only a negative-acting element. It is possible that a positive-acting factor other than p35C/EBPβ recognizes the SRE BP binding site in the SRE. If so, this unidentified factor could either heterodimerize with p20C/EBPβ or displace the inhibitor protein. The SRE BP binding activity that is not recognized by C/EBPβ antibodies is a possible candidate for such a factor.

Further insight into the potential roles of p35C/EBPβ and p20C/EBPβ in SRE function could likely be gained by examining the formation and regulation of a ternary complex between either of these two forms of C/EBPβ, SRF, and SRE DNA in vitro. However, we have been unable at this time to demonstrate the formation of such a complex. This may be due to technical difficulties such as inappropriate assay conditions, proteins which lack a key modification, or instability of the complex. It is also possible that C/EBPβ and SRF complex formation on SRE DNA requires other factors (such as YY1, for instance) to obtain the required DNA conformation. There may even be coactivator proteins that bind to C/EBPβ and/or SRF to facilitate assembly of a multifactorial complex. We anticipate that the further analysis of the interplay between SRF and C/EBP proteins, both in vivo and in vitro, will at some point converge to provide a better understanding of the mechanism by which transient serum activation of *cfos* gene transcription is accomplished.

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