Both Products of the \textit{fosB} Gene, FosB and Its Short Form, FosB/SF, Are Transcriptional Activators in Fibroblasts

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We demonstrate that a member of the \textit{fos} family, the \textit{fosB} gene, gives rise to two transcripts by alternative splicing of exon 4, generating two proteins, FosB of 338 amino acids and a short form, FosB/SF, which contains the DNA binding and dimerization domains but not the 101 amino acids of the C terminus. FosB/SF activates an AP-1–chloramphenicol acetyltransferase construct in NIH 3T3 cells, as determined by transient and stable transfections, although more weakly than does FosB. In contrast to FosB, FosB/SF has lost its ability to repress the dyad symmetry element of the \textit{c-fos} gene. FosB/SF when expressed in excess to FosB can downmodulate the activity of FosB. Constitutive expression of high levels of FosB/SF in NIH 3T3 cells has no significant inhibitory effect in the induction of cell proliferation or cell cycle progression, indicating that FosB/SF is not a negative regulator of cell growth. This conclusion is further confirmed by the observation that the majority of the Jun molecules are complexed with FosB/SF in the FosB/SF-overexpressing cells.

The transcription factor AP-1, originally discovered in human cells as an activity that binds selectively to enhancer elements in the promoter region of simian virus 40 and of many other vertebrate genes (2, 27), has been demonstrated to be a protein complex composed of different members of the Jun and Fos families (for a review, see reference 54). The first cellular gene identified to encode a transactivator similar to AP-1 was \textit{c-Jun} (1, 4), the cellular homolog of the viral oncogene \textit{v-Jun} (31). Two other members of this family, \textit{JunB} (44) and \textit{junD} (16, 43), have also been identified. A series of elegant studies has demonstrated that c-Jun and c-Fos form a complex in vivo that recognizes the AP-1 target sequence (54). As in the case of c-Jun, genes sharing a significant similarity with \textit{c-fos} have also been described: \textit{fra-1} in rats (8), \textit{fosB} in mice (57), and \textit{fra-2} in humans and chickens (33, 40). The highly conserved common region of these families of proteins is composed of two structures: a basic motif, which is thought to bind directly to DNA, and a leucine zipper required for dimerization (6, 25). The three Jun proteins, c-Jun, JunB, and JunD, can form homodimers and bind to an AP-1 site, but they differ in their binding affinities (46), and it has been demonstrated that c-Jun and JunB have different transcriptional and biological activities (7, 51, 52). The Fos proteins, c-Fos, FosB, Fra-1 and Fra-2, differ from the Jun proteins in that they do not form homodimers and have no intrinsic specific DNA binding activity (9, 13, 38, 57). However, the binding affinity and transcriptional activation by the Jun proteins are dramatically increased upon dimerization with Fos proteins (1, 9, 13, 46, 57). The complexity arising from the possible associations between different Jun and Fos:Jun:Fos proteins has been further increased by the finding that c-Jun can dimerize with other leucine zipper-containing proteins (3, 18, 30).

Interestingly, the members of \textit{jun} (44, 45, 47) and \textit{fos} (8, 12, 24, 36, 40, 57) families belong to the set of genes which are rapidly induced by growth factors in quiescent fibroblasts (5, 15, 26), indicating that the Jun and Fos proteins may play an important role in the control of cell proliferation. It has been demonstrated that following serum stimulation of quiescent fibroblasts, different Jun:Fos heterodimers are formed and that their proportion is governed by their concentration (21). A strong evidence that these proteins play an important role in normal cell growth is the observation that intracellular expression of antisense \textit{c-fos} inhibits the entrance to the cell cycle of serum-stimulated quiescent cells (39) and cell cycle progression in exponentially growing cells (17). This finding has been further supported by the fact that microinjection of anti-Fos (22, 42) and anti-Jun (22) antibodies efficiently inhibits induction of cell proliferation and cell cycle progression.

We have found that one of the members of the \textit{fos} family, the \textit{fosB} gene (57), gives rise to two mRNA products by alternative splicing of exon 4, generating two FosB proteins, FosB and a shorter form, FosB/SF, which are simultaneously induced by serum in resting fibroblasts. While our work was in progress, Nakabeppu and Nathans (38) also demonstrated that the \textit{fosB} gene gives rise to two mRNA products by alternative splicing. They showed that in transfection assays of F9 cells, the short form of FosB lost its capacity to activate a promoter with an AP-1 site and behaved like a dominant negative form of Fos in these cells. That finding led them to postulate that the cellular function of FosB/SF is to limit the activity of Jun and Fos proteins following mitogen induction. Since the \textit{fosB} gene was identified as an immediate-early gene in fibroblasts, we found it of interest to investigate the activity of FosB/SF in these cells and its possible function during their growth response. We have demonstrated that FosB/SF activates transcription in fibroblasts and that its constitutive overexpression does not inhibit induction of cell proliferation or cell cycle progression, despite the fact that more than 80% of the Jun proteins are complexed with FosB/SF. We conclude that in mouse fibroblasts, which are representative of mitogen-stimulated cells, FosB/SF is not a dominant negative regulator but functions as a transcriptional activator.

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MATERIALS AND METHODS

Cell culture. NIH 3T3 and Swiss 3T3 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U of penicillin per ml and 50 μg of streptomycin per ml). Confluent cells were made quiescent by incubation for 48 h in 0.5% FCS (NIH 3T3 cells) or 2.5% FCS (Swiss 3T3 cells). For stimulation, both cell lines were incubated in 20% FCS for the indicated periods of time.

To detect DNA synthesis following 24 h of serum stimulation, 5-bromodeoxyuridine (Sigma) at a final concentration of 100 μM was added to the medium 14 h before the cells were fixed. For indirect immunofluorescence, cells were fixed by using cold methanol (4°C) for 10 min, rehydrated in phosphate-buffered saline (PBS), and incubated for 30 min in 1.5 M HCl to denature the DNA. After the coverslips were washed several times with PBS, the cells were incubated with a mouse antibromodeoxyuridine monoclonal antibody (1:50; Becton Dickinson) for 30 min at room temperature, washed with PBS, and then incubated for another 30 min (room temperature) with donkey polyclonal anti-mouse immunoglobulins Texas red conjugated to detect DNA synthesis (1:50; Jackson Immunoresearch). After being washed in PBS, the coverslips were mounted on slides, using Fluoromount G (Southern Biotechnic).

Growth curves for a total of 6 days were obtained by seeding 3 × 10⁴ cells into 35-mm wells (six-well plates; Corning) in DMEM containing different concentrations of FCS. Medium was renewed every other day. Duplicate cultures were trypsinized daily and resuspended in PBS containing 3.5% formaldehyde for fixing the cells and counted with a Coulter Counter.

Cell labeling and immunoprecipitation. (i) Cell labeling. For pulse-labeling, quiescent cultures grown in 24-well plates stimulated with 20% FCS were labeled for 30 min with [35S]-methionine (800 μCi/ml) in methionine-free medium at the indicated times. Prior to labeling, cells were washed with DMEM without methionine. Labeling during the first 30 min following stimulation was done in the presence of dialyzed FCS. For continuous labeling, exponentially growing cells in 24-well plates were washed twice with DMEM without methionine before incubation for 6 h with [35S]-methionine (800 μCi/ml) in methionine-free medium in the absence of serum. In all cases, two wells per point were used.

(ii) Immunoprecipitation. Cellular lysates were prepared under non-denaturing or denaturing conditions. For non-denaturing conditions, cells were lysed on ice by adding RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1% Na2O4C, 2,5-diphenyloxazole (PPO) in N,N-dimethylformamide, 0.25 mM phenylmethylsulfonyl fluoride). For denatured cell extracts, cells were lysed in denaturing buffer (50 mM Tris-HCl [pH 7.5], 0.5 M SDS, 70 mM β-mercaptoethanol), boiled for 10 min, and then diluted by adding 4 volumes of RIPA buffer without SDS.

Cell lysates (final volume, 2 ml) were incubated with 6 μl of antiserum for 1 h on ice and then with 15 μl of protein A-Sepharose beads (PharMacia) for 2 to 3 h on a roller system at 4°C. The complex with the protein A-Sepharose beads was washed twice with buffer A (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), once with buffer B (10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), and once with buffer C (10 mM Tris-HCl [pH 7.5]). The Laemmli sample buffer and then run overnight in a 12.5% acrylamide–bisacrylamide gel (200:1) at 12 mA per gel. Fixed gels were incubated twice for 1 h in dimethyl sulfoxide, incubated for 3 h in 20% 2,5-diphenyloxazole (PPO) in dimethyl sulfoxide, and then washed for 30 min with water. Gels were dried and exposed to Kodak X-Omat AR film at −70°C. All lysates were first treated with preimmune serum as described above. Characterization of the anti-Fos and anti-Jun antibodies has been described elsewhere (21).

Plasmids. (i) Reporter vectors. The AP-1 (OLIGO1 [46])-containing plasmids were constructed by inserting OLGIO1 (5'-TTCAGAATCTATGACATCCGTGCAG-3') into the SalI site of pBLCAT 2 (29). Constructs 1×TRE- and 3×TRE-tk-CAT have been described previously (2). DSE-tk-CAT was constructed by cloning an oligonucleotide containing the dyad symmetry element (DSE) of the c-fos promoter (−322 to −297 [56]), 5'-CAGGATGCATATTT AGGACATCTGC-3', into the SalI site of pBLCAT 2. pCH110, a plasmid containing the β-galactosidase coding sequences under the control of the simian virus 40 early promoter (14), was purchased from Pharmacia.

(ii) Expression vectors. The complete coding regions of c-fos, fosB, fosB/sf and c-jun were cloned into the mammalian expression vector pMexNeo, in which the polyclinmer sequence is flanked by a Moloney murine sarcoma virus long terminal repeat and a polyadenylation signal of simian virus 40 (32). The identity of all constructs was confirmed by sequencing.

Cell transfections. (i) Transient transfection. Transient transfections were performed by using a standard calcium phosphate coprecipitation technique (11), with minor modifications. NIH 3T3 cells maintained in DMEM supplemented with 10% FCS were trypsinized and plated 24 h before transfection onto 100-mm dishes, so that at the time of transfection they were about 50% confluent. Two to three hours before transfection, cells were refed with fresh medium. Cells were exposed to the precipitate for 12 to 16 h, washed twice in PBS, and incubated for 36 to 48 h in 0.5% FCS before harvesting.

Typically, 2 μg of reporter plasmid and 3 μg of pCH110 (pSV2-β-galactosidase as an internal control [11]) were used. The amount of the expression vector pMexNeo (32) containing either c-fos, fosB, or fosB/sf is indicated in each case. The total amount of transfected DNA was always adjusted to 10 μg with pUC18.

For transpression experiments, cells were exposed to the precipitate for 4 to 6 h, subjected to a 2-min glycerol shock (15%), and then washed twice in PBS. In this case, the total amount of transfected DNA was always adjusted to 15 μg with pUC18.

After transfection, cells were made quiescent by incubation in DMEM containing 0.5% FCS for 36 to 48 h. Three hours before harvesting, cells were stimulated by addition of 20% FCS.

Chloromphenicol acetyltransferase (CAT) and β-galactosidase activities were determined as described previously (11, 34). The results were quantitated by cutting out and counting the spots in a scintillation counter. All results were standardized according to the internal levels of β-galactosidase and/or to protein content.

(ii) Stable transfection. NIH 3T3 cells were plated in DMEM supplemented with 10% FCS at 4 × 10⁴ cells per 100-mm-diameter dish 24 h before DNA transfection. Cells were transfected with 10 μg of pMexNeo-fosB or pMexNeo-fosB/sf by calcium phosphate-mediated precipitation and exposed to the precipitate for 14 h. Twenty-four hours later, cultures were split and seeded in DMEM supplemented with 10% FCS containing G418 (800 μg/ml). The expressing
clones were screened by immunofluorescent staining using a specific anti-FosB antibody.

PCR amplification and cloning of fosB/sf. One microgram of total RNA from NIH 3T3 cells stimulated with 20% FCS for 1 h was used for the reverse transcription reaction. The RNA was incubated at 42°C for 1 h in a mixture containing 25 U of avian myeloblastosis virus reverse transcriptase (Boehringer), 1 mM of each dNTP, 50 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl2, 0.1% [wt/vol] gelatin. The final volume was 10 µl.

For PCR amplification, 2 µl of the reverse transcriptase products was mixed with 1 U of Taq DNA polymerase (Cetus Corp.) and 50 pmol of primer. Each primer contained clamp and EcoRI sites (indicated in bold). The primers used were fosB 5'-GGGCCCGAATTCGGCCCAGGGGTTTCGCAG-3', fosB 5'-GGGCCCGAATTCGGCCCAGGGAAATGGTTTACAGTTTT-3', and fosB 3'-ACACCGTGGAACCTTGGGAAACTGAAAGACAGCTTAAAGGCCC-5'.

Amplification was performed in sequential cycles of denaturation of the DNA at 95°C for 30 s, primer annealing at 52°C for 30 s, and elongation at 72°C for 90 s, using the Perkin-Elmer Cetus Gene Amp DNA amplification reagent kit. Forty cycles were used. Ten microliters of each PCR product was analyzed in a 1.5% agarose gel.

To clone the PCR products, 50 µl (one-half) of the PCR sample was digested with 10 µg of protease K (Boehringer) in 200 µl of a 0.5% SDS solution at 50°C for 1 h. The sample was then extracted once with phenol and twice with chloroform-isooamyl alcohol (24:1) and ethanol precipitated. The pellet was resuspended in 50 µl of distilled water and digested with EcoRI. The digested DNA was run on a 4% NuSieve GTG agarose gel (FMC) in TAE (40 mM Tris-acetate [pH 8.0], 0.25 mM EDTA). The EcoRI fragments were cloned into Bluescript KS' (Stratagene) according to the cloning protocol for NuSieve GTG agarose. The PCR fragments were sequenced as described previously (49).

To obtain the complete fosB/sf, the PCR fragment in Bluescript KS' (plasmid 21R-A12) was digested with PstI and EcoRI, and the 630-bp PstI-EcoRI fragment containing the leucine zipper and 3' coding region of the short form was isolated. This fragment was ligated with a Sall-PstI fragment from the fosB cDNA clone. The ligated product was cloned into Bluescript KS' and pMExNeo vectors.

In vitro transcription and translation. c-fos, fosB, and c-jun vectors for in vitro transcription have been described previously (46). fosB/sf was cloned into Bluescript KS'. In vitro transcription was carried out with the Boehringer transcription kit, using the T7 and T3 promoters and 7-methyllguanosine capping structure according to the manufacturer's instructions. The c-fos, fosB, c-jun, and fosB/sf vectors were linearized with XbaI, EcoRV, SphI, and XbaI, respectively. In vitro translation in a rabbit reticulocyte lysate system was done with the Promega translation kit, using [35S]methionine as the label.

RESULTS

Existence of two forms of FosB. During the course of our studies on the induction of FosB following serum stimulation of Swiss 3T3 cells, we observed that in all cases two major bands that followed identical kinetics were immunoprecipitated with anti-FosB antibodies (Fig. 1A). Of these, only the higher-molecular-size form (52 kDa) showed the expected size for FosB, leaving the smaller form of 37 kDa unidentified. Two important observations suggested to us that the 37-kDa protein was directly related to FosB. First, immunopurified anti-FosB antibodies preabsorbed against c-Fos and Fra-1, in order to diminish cross-reactivity with related molecules, recognized the 37-kDa protein, and second, the protein displayed a kinetic of induction identical to that of FosB. To further investigate the nature of the 37-kDa protein, we determined whether it was associated in vivo with some of the Jun proteins. For this purpose, serum-stimulated Swiss 3T3 cells were labeled for 2 h with [35S]methionine. Lane Q, immunoprecipitates of quiescent cells labeled for 30 min with [35S]methionine. (B) Quiescent Swiss 3T3 cells stimulated with 20% FCS were continuously labeled with [35S]methionine for 2 h, lysed under nonnondenaturing conditions, and incubated with anti-JunB antibody. The immunoprecipitate was denatured and reprecipitated with anti-JunB and anti-FosB antibodies as described in Materials and Methods. (C) In vitro translation products of fosB/sf and fosB mRNAs using a rabbit reticulocyte lysate system. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

FIG. 1. (A) Time course of induction of FosB in serum-stimulated fibroblasts. Quiescent Swiss 3T3 cells (48 h in 2.5% FCS-DMEM) were stimulated with 20% FCS for the indicated periods and pulse-labeled with [35S]methionine for 30 min (30') as described in Materials and Methods. After labeling, cells were lysed under denaturing conditions and incubated with anti-FosB antibody. Lane Q, immunoprecipitates of quiescent cells labeled for 30 min with [35S]methionine. (B) Quiescent Swiss 3T3 cells stimulated with 20% FCS were continuously labeled with [35S]methionine for 2 h, lysed under nonnondenaturing conditions, and incubated with anti-JunB antibody. The immunoprecipitate was denatured and reprecipitated with anti-JunB and anti-FosB antibodies as described in Materials and Methods. (C) In vitro translation products of fosB/sf and fosB mRNAs using a rabbit reticulocyte lysate system. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

PCR analyses of total RNA from serum-stimulated fibroblasts using appropriate fosB primers show that indeed the fosB gene gives rise to two products, one of them lacking 140 nucleotides of the coding region. By comparing the sequences of the PCR products with that of the fosB gene, we determined that the short form of fosB, fosB/sf, lacks 140 nucleotides of exon 4 between nucleotides 4685 and 4825 of the fosB gene. The 5' and 3' deletion endpoints contain typical donor and acceptor splice sites, respectively (35), demonstrating that the fosB gene can be alternatively spliced in exon 4. Although the short transcript is missing only 140
nucleotides, it codes for a protein of 237 amino acids, in contrast to the 338 amino acids of the complete form, as the result of an in-frame stop codon generated in the alternative splicing site. The size of the in vitro-translated FosB/SF protein is identical to that of the low-molecular-weight form immunoprecipitated with the anti-FosB antibody (Fig. 1C). We also demonstrated that in vitro-translated FosB/SF not only can complex with Jun but also increases its DNA binding activity similarly to FosB (not shown). These results convinced us that the small-size band detected with the anti-FosB antibody in vivo is in fact FosB/SF. Similar results have been recently reported by Nakabeyashi and Nathans (37).

Transactivation by FosB and FosB/SF. To investigate whether FosB and FosB/SF differ in their capability to transactivate, we cotransfected NIH 3T3 cells with pMexNeo-fosB or pMexNeo-fosB/sf. NIH 3T3 cells were chosen because they represent a model for studying the role of AP-1 proteins following mitogen activation. Reporter constructs containing different AP-1 binding sites, OLG1O1- and TRE-tk-CAT, were used since we had previously shown that the nucleotides flanking the AP-1 consensus sequence have a strong effect in the binding of Jun and Jun:Fos complexes (46). Our initial experiments demonstrated that pMexNeo-fosB did not require the cotransfection of pMexNeo-c-jun to activate the transcription of an AP-1-CAT construct in NIH 3T3 cells, possibly because of the high levels of Jun proteins present; therefore, we performed all transfections in the absence of a c-Jun-expressing vector. Transfection of fosB and fosB/sf showed that both products can activate transcription of OLG1O1- and TRE-tk-CAT constructs (Fig. 2). Activation was observed for a single copy of AP-1 as well as when AP-1 was cloned in an oligomerized form. Single-copy OLG1O1 and TRE are weaker in their activation, and both forms of FosB similarly increased their transcription by 1.5- to 3-fold. However, when OLG1O1 and TRE were used in an oligomerized form, a strong activation of CAT transcription was observed with the two FosB forms, although FosB/SF was a weaker activator. FosB induced CAT expression on average 15- to 20-fold, while FosB/SF increased it only 5- to 8-fold. The highest activation was obtained with the 5×OLG1O1-tk-CAT construct; therefore, it was selected for further studies.

To better compare the activities of FosB and FosB/SF, different concentrations of the corresponding expression vectors were used for transfections. As shown in Fig. 3, the activation of CAT expression correlates with the amount of FosB-, FosB/SF-, and c-Fos-expressing vector transfected. A significant induction of 5×OLG1O1-tk-CAT could be detected when only 0.05 μg of fosB or fosB/sf expression vector was used. Transfection of fosB consistently gave the strongest activation, enhancing transcription two- to threefold more than did fosB/sf and c-fos. These differences in activity could partly be due to differences in the expression of the Fos proteins. We feel, however, that this is unlikely because all proteins were expressed from the same expression vector and similar results were obtained in many independent experiments. Transfection with pMexNeo-c-fos activated CAT expression similarly to what was observed with pMexNeo-fosB/sf, further supporting the notion that FosB/SF has a significant transactivating activity.

FosB/SF can downmodulate FosB activity. Since the transactivation of 5×OLG1O1-tk-CAT by FosB/SF was normally two- to threefold lower than that obtained with FosB, we decided to determine whether a high level of expression of FosB/SF could interfere with the activity of FosB. Cotransfection of equal amounts of pMexNeo-fosB/sf with pMex Neo-fosB led to a small but reproducible inhibition of approximately 20 to 30% in the induction of CAT expression compared with the activation observed with pMexNeo-fosB.
alone (Fig. 4). However, when the amount of fosB/sf transfected was fourfold higher than that of fosB, the final induction of CAT expression was similar to that observed upon transfection of fosB/sf alone, suggesting that FosB is subject to strong competition by FosB/SF under these conditions. This result was expected in part because both FosB and FosB/SF can efficiently interact with Jun proteins; therefore, when FosB/SF is much more abundant than FosB, the majority of the Jun molecules in the cell will be complexed with FosB/SF. Surprisingly, cotransfection of fosB/sf with c-fos seemed to have no effect on c-Fos activity; the final induction of CAT expression was similar to that observed when c-fos was transfected alone.

**Transactivation by FosB/SF in stable transfectants.** To confirm that FosB/SF could activate transcription, we decided to generate NIH 3T3 cell lines constitutively expressing FosB and FosB/SF and compare their capacities to induce expression of plasmid 5×OLIG01-tk-CAT. The transfected clones selected by neomycin resistance were screened for FosB and FosB/SF expression by indirect immunofluorescence using a specific anti-FosB antibody. Clones presenting a homogeneous population of cells expressing high levels of the proteins were chosen for further studies. Transfection of these clones with different concentrations of plasmid 5×OLIG01-tk-CAT showed that both FosB- and FosB/SF-expressing cells induced CAT expression 2- to 10-fold more than did control NIH 3T3 cells, depending on the concentration of reporter plasmid used, confirming our results for transient expression assays (Fig. 5).

We further demonstrated that the increase in transcription observed in FosB- and FosB/SF-expressing cells correlates with an increase in the AP-1 binding activity present in the cells as determined by gel retardation assays. The AP-1 binding activity detected in nuclear extracts from FosB- and FosB/SF-expressing cells was three to five times higher than in control cells (not shown).

**FosB/SF does not repress DSE induction.** Several lines of evidence indicate that cFos can repress its own expression through the DSE (20, 28, 41, 50, 53) and the expression of other immediate-early genes via CARG elements (10). It also has been demonstrated that the 27 C-terminal amino acids of c-Fos which are conserved in the Fos family are essential for this repression (10, 55). Therefore, as FosB/SF is missing this region, we found it of interest to determine whether this protein was able to repress the expression of a reporter gene driven by the c-fos DSE. NIH 3T3 cells were cotransfected with plasmid DSE-tk-CAT and a pMexNeo plasmid containing c-fos, fosB, or fosB/sf and then incubated for 36 to 48 h in 0.5% FCS before stimulation with 20% FCS for 3 to 4 h. Expression of c-Fos reduced the activation of the DSE by serum approximately threefold (Fig. 6). Similarly to c-Fos, expression of FosB inhibited induction of the DSE threefold. However, FosB/SF not only did not repress activation of the DSE but consistently enhanced its activation, possibly by competing with the intracellular Fos proteins induced by serum. These results confirm and extend the previous observations that the conserved C-terminal amino acids of Fos proteins are essential for repression.

**Effect of FosB/SF on cell growth.** The finding that FosB/SF stimulates transcription to a lesser extent than does FosB and that it can downmodulate the activity of the latter prompted us to investigate whether FosB/SF could act as a negative modulator in cell proliferation. For this purpose, three stable transfectants of NIH 3T3 cells constitutively expressing FosB/SF were used. As the fosB gene is ex-
pressed mainly during the G₁-to-G₂ transition, we determined the capacity of quiescent FosB/SF-expressing clones to enter the cell cycle following serum stimulation. Confluent cultures of different FosB/SF-expressing clones were rendered quiescent by incubation in 0.5% FCS for 48 h before stimulation with different serum concentrations. The capacity to enter the cell cycle was determined by monitoring the percentage of cells in DNA synthesis after 24 h of stimulation. The independent FosB/SF-expressing clones compared with control NIH 3T3 cells show a similar capacity to enter the S phase at all concentrations of serum used (not shown). It is important to note that in these clones the expression of FosB/SF compared with FosB is at least 5- to 10-fold higher than the maximum transient expression observed 2 h following serum stimulation of NIH 3T3 cells.

Since high levels of FosB/SF expression did not have a significant inhibitory effect on the induction of cell proliferation, we decided to investigate whether the protein could affect cell growth in asynchronous cultures. FosB/SF-expressing clones were plated at similar cell densities and grown in media with different serum concentrations for 6 days. Their growth rates were determined by daily counting of the cells. No significant differences in the rates of proliferation of the FosB/SF-expressing clones and control NIH 3T3 cells were found at any serum concentration used (Fig. 7). These results indicate that constitutive expression of high levels of FosB/SF in NIH 3T3 cells does not inhibit quiescent cells to enter the cell cycle or diminish cell growth.

**FosB/SF is complexed with Jun proteins.** The observation that high expression of FosB/SF in NIH 3T3 cells did not significantly diminish their growth properties could be due to an inefficient interaction between FosB/SF and the different Jun proteins, especially in exponentially growing cells in which the FosB proteins are normally expressed at very low or undetectable levels. Although after serum stimulation FosB/SF efficiently associates with Jun proteins, it is possible that differences in posttranslational modifications in exponentially growing cells can inhibit the complex formation of FosB/SF and Jun proteins. This prompted us to investigate the existence of complexes between FosB/SF and c-Jun, JunB, and JunD in some of the clones during exponential growth. Cells were labeled with [35S]methionine for 6 h, and native (nondenatured) cellular lysates were prepared in RIPA buffer and immunoprecipitated with anti-FosB antibody. The complex formation between Fos and Jun proteins is completely inhibited in RIPA buffer; however, preexistent complexes remain stable. The immunocomplexes were dissociated by boiling for 10 min in the presence of 0.5% SDS and then diluted fivefold with RIPA buffer without SDS. This treatment avoids the reassociation of the different Fos and Jun proteins. The samples were reprecipitated with anti-FosB antibody and then sequentially immunoprecipitated with anti-JunD, c-Jun, and -JunB antibodies. As shown in Fig. 8A, exponentially growing cultures of the different stable transfectant cell lines express high levels of FosB/SF, in contrast to the NIH 3T3 parental cells. Complexes between FosB/SF and the different Jun proteins can be readily detected in nondenatured cellular lysates of transfected cells (Fig. 8B). Furthermore, considering the amount of c-Jun, JunB, and JunD that is coprecipitated with FosB/SF and their total cellular amount, it is possible to infer that more than 80% of the c-Jun, JunB, and JunD molecules are associated with FosB/SF in these cells under growing conditions. This view was further confirmed by the observation that in the supernatant of the cellular lysates immunoprecipitated with the anti-FosB antibody under nondenaturing conditions, only a small fraction of c-Jun, JunB, and...
FIG. 8. (A) FosB/SF expression in three independent stable transformants resulting from transfection of NIH 3T3 cells with pMexNeo-fosB/SF. The cell lines used are indicated above the lanes. (B) Fraction of the Jun proteins (c-Jun, JunB, and JunD) associated with FosB/SF in the transfected cell line 10A8. Exponentially growing cells were labeled for 6 h with [35S]methionine and lysed either under denaturing conditions to immunoprecipitate FosB/SF (A) and the total amount of each Jun protein (B, Total) or under nondenaturing conditions to detect the Jun proteins associated with FosB/SF (B, Complexed) as described in Materials and Methods. The fraction of noncomplexed c-Jun, JunB, and JunD (B) corresponds to the amount of Jun proteins remaining in the supernatants of the cellular lysates after immunoprecipitation with anti-FosB antibody under nondenaturing conditions.

JunD was present (Fig. 8B). The amount of the FosB/SF complexed with a particular Jun protein correlates well with the relative amount of each Jun protein present in the cell, being most abundant with c-Jun. These results demonstrate that although most of the Jun molecules are complexed with FosB/SF in the transfected NIH 3T3 cells, FosB/SF does not act as a negative regulator of cell proliferation.

DISCUSSION

In this study, we have demonstrated that the immediate-early gene fos shows rise to two protein products, FosB and FosB/SF, by an alternative splicing of exon 4. The FosB/SF protein retains the dimerization and basic domains and efficiently interacts with Jun proteins, enhancing their binding activities, as determined by gel retardation assays. Both FosB proteins are rapidly and transiently induced in about equal amounts following serum stimulation and form complexes in vivo with the different Jun proteins.

Transient and stable transfection of NIH 3T3 cells with FosB- and FosB/SF-expressing plasmids showed that both forms can activate the transcription of a reporter gene with upstream AP-1 sites. FosB is the stronger transactivator, particularly when its action is mediated by multiple AP-1 sites. The stronger transactivation by FosB has been observed for different AP-1-containing sequences, not only when compared with FosB/SF but also when compared with c-Fos, suggesting that FosB is one of the most potent activators of the Fos family. This view is supported by the observation that FosB has a stronger effect than c-Fos and Fra-1 in the binding of Jun proteins to different AP-1-containing sequences. Since the two forms of FosB have similar activities in fibroblasts, the biological significance of FosB/SF in these cells is poorly understood.

We have shown that FosB/SF when expressed in excess to FosB can downmodulate the activity of FosB and that the activation level obtained is similar to that of FosB/SF alone. This finding indicates that the two FosB molecules are part of the same activating pathway and compete for common effectors. In contrast to the competition observed between both forms of FosB, c-Fos seems to have no effect, suggesting that although in NIH 3T3 cells FosB and c-Fos share common effectors, their activation may proceed via slightly different pathways.

The observation that FosB/SF is less active than FosB, and the fact that it has lost the capacity to transrepress, indicates that the 101 amino acids of the C terminus contain an activation domain that is necessary but not essential for FosB transcriptional activity and agrees with the previous observations that the last 27 C-terminal amino acids which are conserved in the Fos family are required for transrepression (10, 55). The clear identification of the putative activation domain present in the C-terminal region will require further studies.

Although FosB/SF is a weaker transactivator than FosB and can compete for common effectors, the experiments with the stable transfectants demonstrate that a 5- to 10-fold increase in FosB/SF over the normal cellular level expressed following serum stimulation does not diminish the capacity of resting cells to enter the cell cycle. Furthermore, the growth rate of exponentially growing cells constitutively expressing high levels of FosB/SF is similar to that of their control counterpart. This finding, together with our finding that FosB/SF is complexed with the Jun proteins in growing cells, strongly suggests that FosB/SF does not have a negative effect on normal cell growth.

It has been previously demonstrated by using antisense c-fos expression (39) and microinjection of anti-Fos antibodies (42) that the activity of c-Fos is required for resting cells to enter the cell cycle and for cell cycle progression of exponentially growing cells. Furthermore, we have shown that microinjection of antibodies against any single member of the Jun family significantly inhibits the entrance to the cell cycle of resting cells and that Jun and Fos protein activity is required for cell cycle progression (22). These results, together with the findings presented in this report, led us to conclude that FosB/SF in NIH 3T3 cells displays a significant transcriptional activity; otherwise, its overexpression would have a detrimental effect on cell proliferation.

Previous studies concerning the structure and function of v-fos protein demonstrated that truncation of more than 160 amino acids corresponding to the C terminus of c-Fos caused only a small reduction in focus formation (10, 56). Furthermore, a region of the v-Fos protein, corresponding to c-Fos amino acids Met-111 to Ile-206, conserves the ability to transform and to stimulate cell proliferation (56). This region, which is highly conserved among the different Fos proteins, is present in FosB/SF. The identity of FosB and c-Fos in this 85-amino-acid segment is 78% and, if conservative changes are considered, is over 90%; therefore, it would be expected that FosB/SF when associated with Jun proteins will still generate an active transcription complex. Accordingly, we have recently demonstrated that overexpression of FosB/SF in Rat-1A cells increases the ability of the cells to grow in semisolid medium and to produce tumors in BALB/c (nu/nu) mice, although to a lesser extent than does FosB (23).

In conclusion, the different lines of evidence presented in this report demonstrate that FosB/SF functions as a transactivator and that it is not a negative regulator of normal cell proliferation in fibroblasts.

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