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Structure and mapping of the *fos*B gene. FosB downregulates the activity of the *fos*B promoter

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ABSTRACT

We have determined the genomic structure of the *fos*B gene and shown that it consists of 4 exons and 3 introns at positions also found in the c-*fos* gene. By deletion analysis we have characterized a region upstream of the TATA box which is the promoter region of the gene. Several consensus sequences have been identified, including an SRE and AP-1 binding site whose relative positions are identical to those in the 5′ upstream region of the c-*fos* gene. We have also shown that FosB and c-Fos can downregulate the activity of the *fos*B promoter to a similar extent. The *fos*B gene is located in the [A1-B1] region of mouse chromosome 7.

INTRODUCTION

Growth factors and other mitogens are capable of rapidly inducing the expression of a set of genes in quiescent fibroblasts. These genes which have been named immediate early genes encode for transcription factors, cytoskeletal proteins, cytokines and other proteins of yet unknown functions (for review 1, 3). The *bona fide* or putative transcription factors encoded by the immediate early genes that have been identified so far include two related proteins containing zinc fingers, Krox-20/Egr-2 (4–6) and Krox-24 (7, 8), also named Zif/268 (9–11), NGFIA (12) and Egr-1 (13), a nuclear receptor, N10 (14) also named Nur/77 (15) and NGFIB (16), c-Rel (17), and the different members of the Jun and Fos families, such as c-Jun (18, 19), JunB (20), JunD (21, 22), c-Fos (23, 24), FosB (25), Fra-1 (26) and Fra-2 (27, 28). The initial observation that c-Jun and c-Fos can form heterodimers (29–34) has been extended to the different members of these families. c-Fos (35) as well as FosB (25) and Fra-1 (36, 37) can associate with the three Jun proteins enhancing their DNA binding to AP-1 sites indicating that the transcription factor AP-1 comprises a collection of related inducible protein complexes that interacts with similar sequence motifs. Therefore it would be of interest to understand the mechanisms controlling the expression of the different components of the AP-1 activity especially considering that stimulation of cell proliferation induces the co-expression of these proteins leading to the formation of different Jun:Fos heterodimers in the cell (38). The c-*fos* gene has been the most extensively studied of all the immediate early genes and it has been demonstrated that its activation is in part mediated by upstream cis-elements, some of which have been also found in other immediate early genes (reviewed in 39).

One of the members of the *fos* family, *fos*B (25), presents a rapid and transient kinetics of induction similar to c-*fos* (24), in contrast to *fra*-1 (26) and *fra*-2 (27, 28) which have a more delayed response. Therefore it was of interest to characterize the gene structure of *fos*B in order to determine its possible similarity with c-*fos* and to study the upstream elements involved in the control of its expression. We have shown that the structure of the *fos*B gene is very similar to that of c-*fos* and that they share the cis-elements CRE, SRE, AP-1 and NF-1 in the 5′ upstream region. The expression of the *fos*B gene is transrepressed by FosB and c-Fos proteins.

MATERIALS AND METHODS

Cell culture

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

Cell transfections

NIH 3T3 cells were plated in DMEM supplemented with 10% FCS at 4×10^5 cells per 100 mm diameter dishes 20 h before DNA transfection. Cells were transfected with the different CAT-constructs by the calcium phosphate coprecipitation method (40) and exposed to the precipitate for 12 to 16 h, washed twice in PBS and kept in 0.5% FCS for 36 to 48 h. Cells were stimulated

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for 3 h with 20% FCS before harvesting. In all cases the plasmid pCH110 containing the β-galactosidase gene under the control of the simian virus 40 early promoter (41) was used as internal control of the efficiency of transfection. Typically, 2 μg of reporter plasmid and 3 μg of pCH110 were used. The total amount of DNA used for the transfections was kept at 20 μg by adding pUC18.

For transrepression experiments, cells were exposed to the precipitate for 4 to 6 h, followed by a 2 min 15% glycerol shock and then washed twice with 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 incubating in DMEM containing 0.5% FCS for 36 to 48 h. Three hours before harvesting, cells were stimulated with 20% FCS.

The CAT and β-galactosidase activities were determined as described (42, 43). The results were quantitated by cutting out and counting the spots in a liquid scintillation counter. All results were standardized according to the internal levels of β-galactosidase and/or to protein content.

Genomic library screening and Southern blotting

Two hundred micrograms of mouse liver genomic DNA were partially digested with *Eco*RI. The digested DNA was size fractionated in a linear 15% to 40% sucrose gradient by centrifugation in a SW28 (Beckman) rotor for 20 h at 22,000 rpm at 15°C. Ten micrograms of size-fractioned DNA (25 to 45 kb) were used for ligation into the vector pHC79 (GIBCO-BRL; *Eco*RI digested and phosphatase treated) and the library was packaged using *in vitro* lambda packaging system (Amersham). The bacterial strain used for transformation was DH5α MCR (GIBCO-BRL). A total of 1.5×10^5 colonies were screened as previously described (44). A 3.1 kb *fos*B cDNA random labeled to a specific activity of 1×10^9 cpm/μg was used as a probe (45, 46). For Southern blotting experiments, DNA restriction fragments were separated on 1.2% agarose gels and transferred to a nitrocellulose membrane (47). Hybridization was carried out in 50% formamide, 0.5% SDS, 5×SSC (1×SSC = 150 mM sodium chloride, 15 mM sodium citrate) and 5×Denhardt's solution at 42°C for 12 to 16 h. Filters were extensively washed in 0.1×SSC containing 0.5% SDS at 60°C.

Ribonuclease protection assay

A *Bam*HI-*Hin*dIII fragment comprising nucleotides −380 to +309 of *fos*B genomic DNA was cloned into Bluescript KS (+) plasmid. 0.5 μg of cloned DNA was *in vitro* transcribed by T3 RNA polymerase in the presence of 100 μCi of [^{32}P]-UTP (800 Ci/m mol) for 60 min at 37°C. Then the DNA was digested with 50 μg of RNase-free DNase I for 15 min at 37°C and subsequently the RNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol using 20 μg of tRNA as a carrier. The specific activity of the RNA obtained was approximately 2×10^8 cpm/μg. The probe RNA (2×10^6 cpm total) was then hybridized with 10 μg of total RNA from serum stimulated NIH 3T3 cells or 10 μg of tRNA as a control at 45°C overnight after being denatured at 90°C for 5 min. The hybridized RNAs were then digested with 2 μg of DNase-free Ribonuclease A and 1.2 μg of Ribonuclease T1 for 60 min at 30°C. The reaction was terminated by addition of SDS (0.5% final) and digestion with 0.1 μg of proteinase K for 15 min at 37°C. After extraction and precipitation the ribonuclease digested products were analyzed on a denaturating 6% polyacrylamide sequencing gel.

Construction of *fos*B-CAT plasmids

To construct the −962-*fos*B-CAT plasmid, a *Pst*I-*Hin*dIII fragment of approximately 1.3 kb comprising nucleotides −962 to 308 of the *fos*B genomic DNA was cloned into the vector pBLCAT3 (48). The other *fos*B-CAT plasmids were constructed by PCR amplifying the desired fragments and then cloning in *Pst*I-*Kpn*I sites of the pBLCAT vector. The oligonucleotides corresponding to the promoter region of the *fos*B gene used for PCR amplification were: 5'AGCACATTTTCAAGCTCACCG-AG 3' (nucleotides −884 to −862); 5'AGCGGCGGGCGGG-TTGCCACAG 3' (nucleotides −535 to −514); 5'AGGATACCAAACAAACACTGGG 3' (nucleotides −465 to −444); 5'ATATGGCTAATTGCGTCACAGG 3' (nucleotides −424 to −403); 5'CAGGAACTCCGGGGAGGGCGGGG 3' (nucleotides −406 to −384); 5'CAGAACGCAGCCTTGGG-GACCCCCG 3' (nucleotides −358 to −334); 5'AGTTGCGG-GTGACCGAAGCGCGGG 3' (nucleotides −225 to −202); 5'GCAGCTCCGTTTCATTCATAAGACTG 3' (nucleotides −170 to −145); 3'GAGGCTTGGCCTCTGATT 5' (nucleotides 292 to 309).

All these oligonucleotides contain in their 5' end an extra *Pst*I site and in their 3' end an extra *Kpn*I site. The PCR products were ligated into *Pst*I-*Kpn*I sites of the vector pBLCAT2. All constructs were confirmed by sequencing (49).

The c-*fos* DSE- and *fos*B SRE-CAT constructs were obtained by cloning into *Sal*I sites of the pBLCAT2 vector (48) the following oligonucleotides:

5'TCGACAGGATGTCCATATTAGGACATCTGC3'
3'GTCCTACAGGTATAATCCTGTAGACGCAGCT5'
and
5'TCGACTGGCGAGCTCCTTATATGGCTAATTGCG3'
3'GACCGCTCGAGGAATATACCGATTAACGCAGCT5'
respectively.

Chromosome spread preparation

In situ hybridization experiments were carried out using metaphase spreads from a WMP male mouse in which all the autosomes except 19 were in the form of metacentric robertsonian translocations. Concanavalin A-stimulated lymphocytes were cultivated at 37°C for 72 h with 5-bromodeoxyuridine added for the final 6 h of culture (60 μg/ml of medium), to ensure a chromosomal R-banding of good quality. The 2.6 kb *fos*B clone in pGEM1 was tritium labeled by nick-translation (50) to a specific activity of 1.3×10^8 dpm/μg. The radiolabeled probe was hybridized to metaphase spreads at a final concentration of 100 μg/ml of hybridization solution as previously described (51).

After coating with nuclear track emulsion (NTB_2, KODAK), the slides were exposed for 21 days at 4°C, then developed. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first stained with buffered Giemsa solution and metaphases photographed. R-banding was then performed by the fluorochrome-photolysis-Giemsa method and metaphases rephotographed before analysis.

Gel retardation assay

The annealing of the two strands of a corresponding double-stranded oligonucleotide was performed in 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 50 mM NaCl, starting at 95°C for 10 min and cooling down slowly to room temperature. The sequence of the DSE and SRE oligonucleotides used for the gel retardation assays, have been described above under construction of *fos*B-CAT plasmids.

One pmol of the oligonucleotide was end-labeled with [^{32}P]-dCTP by filling in the overlapping ends using the Klenow fragment of DNA polymerase I. Six pmoles of the radioactive deoxynucleotide were used to assure complete labeling of the double-stranded oligonucleotide. *In vitro* translated proteins (0.5 to 1 μl) were added to Dignam's buffer D (18 μl) containing 5 mM spermidine and 1 mg/ml BSA and if not otherwise indicated, 1 μg of pUC19. The DNA-protein complexes were resolved on a 7% polyacrylamide gel (39:1; acrylamide: bisacrylamide) in 0.25×TBE (20 mM Tris-boric acid pH 8.3, 0.25 mM EDTA) run at room temperature for 16 h at 100V.

RESULTS

Genomic structure of *fos*B

To isolate the *fos*B gene, a 2 kb *fos*B cDNA was used to screen a mouse genomic cosmid library. Two clones were isolated from 1.5×10^5 colonies screened. One of them, cosmid B2, which cross-hybridized to probes from the 5′ and 3′ region of *fos*B cDNA, was further characterized. The region containing the *fos*B gene was defined by restriction mapping analysis and Southern blotting. The complete *fos*B cDNA clone was demonstrated to be included in a 8 kb *Eco*RI fragment. This was subcloned and

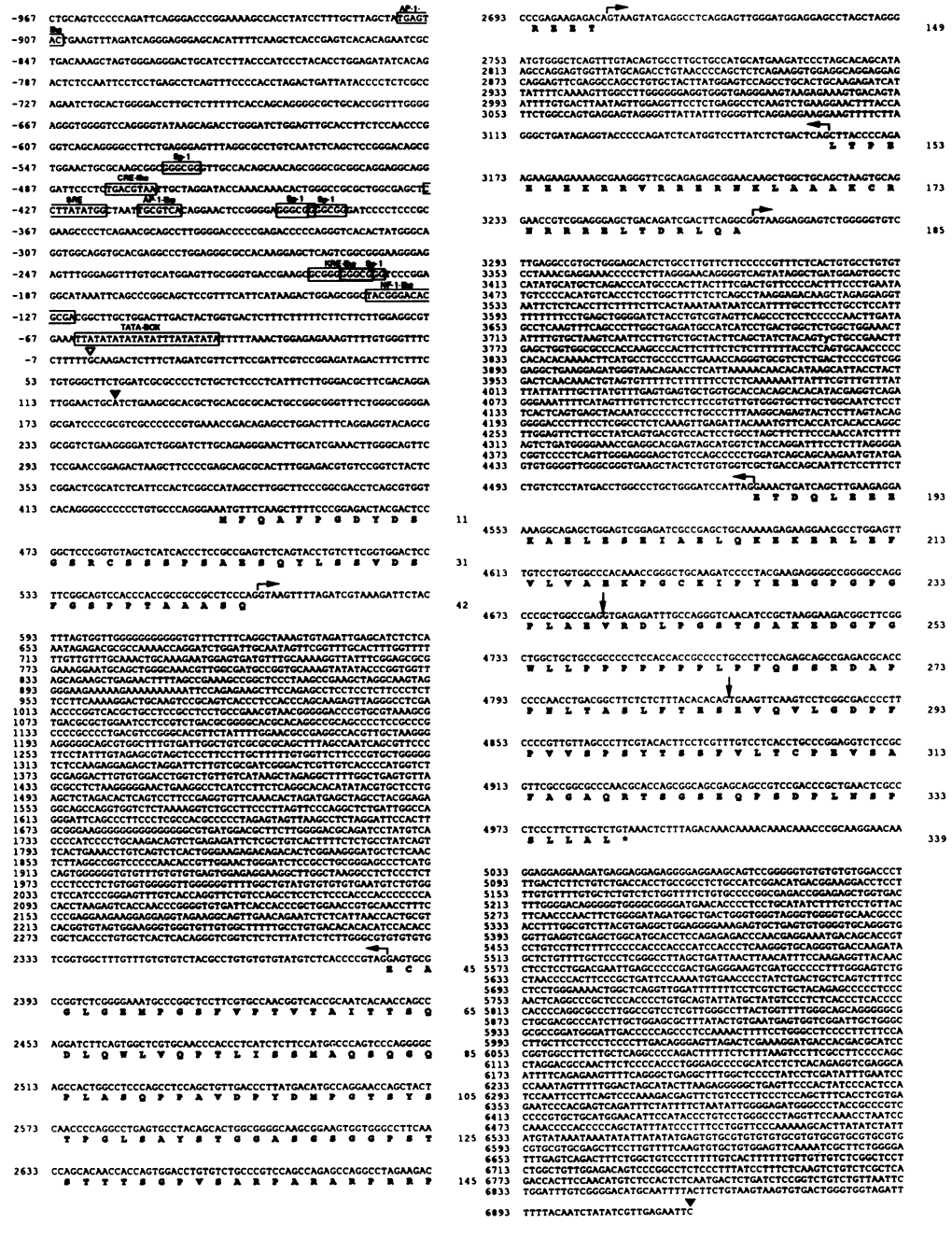

Figure 1. Nucleotide sequence of the *fos*B gene and of the 5′ flanking region. The site of transcriptional initiation is indicated by an open triangle. The first and last nucleotides corresponding to the *fos*B cDNA are indicated by closed triangles. The 3′ untranslated region of exon 4 is not complete and ends in a natural *Eco*RI site. The intron-exon boundaries are indicated by broken arrows. The TATA box and other elements are boxed. The arrowheads indicate the site for alternative splicing which would generate *fos*B/sf.

by detailed restriction mapping the presence of several introns was detected. Then the 8 kb fragment was completely sequenced confirming the presence of the introns (Figure 1). The precise location of the introns was established by comparison of the genomic sequence to *fos*B cDNA. The *fos*B gene encloses 4 exons and 3 introns. The complete coding sequence is distributed in the 4 exons in a genomic fragment of approximately 4.6 kb.

The putative 5′-cap-nucleotide is located 40 nucleotides downstream from a complex TATA box. As shown in Figure 1, within the first 500 bases 5′ upstream of the starting site of the *fos*B gene a few cis-acting elements can be identified by their similarity with consensus DNA motifs. There are one KRE-like sequence (Krox responsive element, 8), one AP-1-like sequence, one SRE, one CRE-like sequence, four Sp-1 binding sites and one NF-1-like site. There is another AP-1 like sequence at position −909. It is interesting to note that the relative locations of the SRE (−428 to −419) and the AP-1-like sequences (−413 to −407) resemble very much that found in the 5′ upstream region of the c-*fos*, *Krox-20* and *Krox-24* genes (11, 52, 53). The strict conservation of these elements possibly reflects their importance in the regulation of these genes.

Comparison of the 5′ untranslated regions of the *fos*B cDNA clone AC113−1 (61) and the homologous genomic region revealed a difference in the sequence. The first 881 nucleotides of the AC113−1 cDNA are not present in the corresponding 5′ genomic sequence, likely due to a cloning artifact. The identity between the 5′ untranslated region of AC113−1 cDNA and the corresponding genomic region ends in nucleotide 123 of the genomic sequence. In light of this discrepancy we determined the starting site of *fos*B by ribonuclease protection assay. For this, a single-stranded RNA probe comprising nucleotides −380 to +309 of the genomic *fos*B generated by *in vitro* transcription in the presence of [^{32}P]-UTP (Figure 2, lane 1) was hybridized against total RNA of serum-stimulated NIH 3T3 cells and then digested with ribonuclease as described in Materials and Methods. As shown in Figure 2, lane 2, ribonuclease treatment of the hybridized labeled RNA generates a major protected fragment of approximately 320 nucleotides, which is consistent with the length expected considering the genomic *fos*B sequence. This confirms that the major 5′ cap-nucleotide is located 40 nucleotides downstream from a complex TATA box and demonstrates that the discrepancy found between the size of the 5′ untranslated region of the AC113−1 cDNA clone (61) and that predicted from the genomic *fos*B sequence is the result of an artifact produced during the construction of the cDNA library. These results also indicate that, although weaker, there are other possible transcription initiation sites further upstream of the complex TATA box, as illustrated by the presence of protected fragments of approximately 420 and 470 nucleotides. Indeed, there are two sequences in the *fos*B genomic sequence located at positions −184 to −175 and −157 to −150, approximately 120 and 90 nucleotides upstream of the complex TATA box (−63 to −41) which resemble a TATA box. The protected fragments smaller 320 nucleotides are possibly generated by the presence of incomplete labeled transcripts.

The first exon of the *fos*B gene, which is 565 nucleotides long, contains the complete 5′ untranslated region (439 nucleotides long) and the region encoding the first 42 amino acids. This is separated by the largest intron of the gene (1,819 nucleotides long) from the second exon encoding 107 amino acids. The third exon which encodes 36 amino acids is the smallest exon of the gene and is of identical size to the corresponding exon of the c-*fos* gene (54). This exon contains the basic region of the DNA

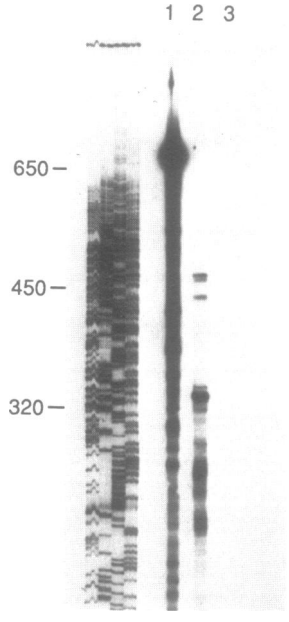

Figure 2. Mapping of *fos*B mRNA starting site. A fragment comprising nucleotide −380 to +309 of *fos*B genomic DNA cloned into Bluescript KS (+) was *in vitro* transcribed in the presence of [^{32}P] UTP. The single-stranded RNA product was hybridized against 10 μg of total RNA from serum stimulated NIH 3T3 cells or 10 μg of tRNA as control. The hybridized RNAs were digested with ribonuclease and analyzed in a 6% polyacrylamide sequencing gel as described in Material and Methods. Lane 1: Transcribed RNA without RNase treatment. Lane 2: Product of the RNase digestion of the transcribed RNA hybridized to total RNA from serum stimulated NIH 3T3 cells. Lane 3: Product of the RNase digestion of the transcribed RNA hybridized to tRNA. The number on the left refers to the size of a known DNA sequence.

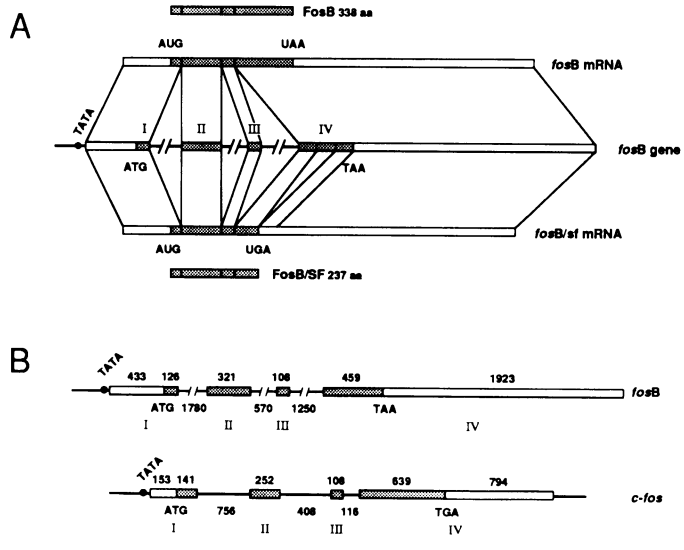

Figure 3. (A) Generation of the two *fos*B transcripts. The *fos*B and *fos*B/sf mRNAs generated by alternative splicing are presented with the position of the sites for translation initiation and termination. The FosB and FosB/SF protein products are shown. (B) Comparison of the genomic structures of *fos*B and c-*fos*. The exons are indicated by boxes and the coding regions are shadowed. The exons are numbered with roman numerals. The size of each exon, intron and non-coding region is indicated. c-*fos* gene structure was taken from (54).

binding domain and presents an 89% identity with c-*fos*. The fourth exon is the longest of all. It encodes the last 153 amino acids of *fos*B protein and contains a fragment 1,923 nucleotides long of the 3′ untranslated region which is incomplete in our genomic subclone. Considering that the size of the *fos*B mRNA is approximately 5 kb, the genomic clone is missing nearly 1.7 kb of the 3′ region. Therefore the complete exon 4 would be approximately 4 kb long, of which 3.6 kb would correspond to the 3′ untranslated region. This exon can be alternatively spliced at positions 4,685 and 4,825, giving rise to a truncated form of *fos*B, *fos*B/sf, which generates a protein of 237 amino acids (FosB/SF) and includes the basic and leucine zipper domain (55, 56). The 5′ and 3′ deletion endpoints contain a typical acceptor and donor splice site respectively (57). Although the *fos*B/sf transcript is only missing 140 nucleotides, it encodes a protein 101 amino acids shorter due to an in frame stop codon generated in the alternative spliced site (Figure 1 and Figure 3A). This alternative splicing in exon 4 has not been observed in the other members of the *fos* family. That the overall structure of the *fos*B gene is very similar to that of c-*fos* strengthens the notion that they have evolved from a common ancestor gene. A similar structure has been also reported for *fra*-2 (28). Both contain 4 exons and 3 introns and their relative size has been conserved (Figure 3B). It is interesting to mention that the first 40 amino acids encoded in exon 4 comprise the leucine zipper of FosB which has a 67% identity with the corresponding leucine zipper of c-Fos, also encoded in exon 4. In contrast, the remaining 113 amino acids of FosB encoded in this exon present no significant homology with c-Fos with the exception of the last 12 amino acids that are highly conserved in the Fos protein family.

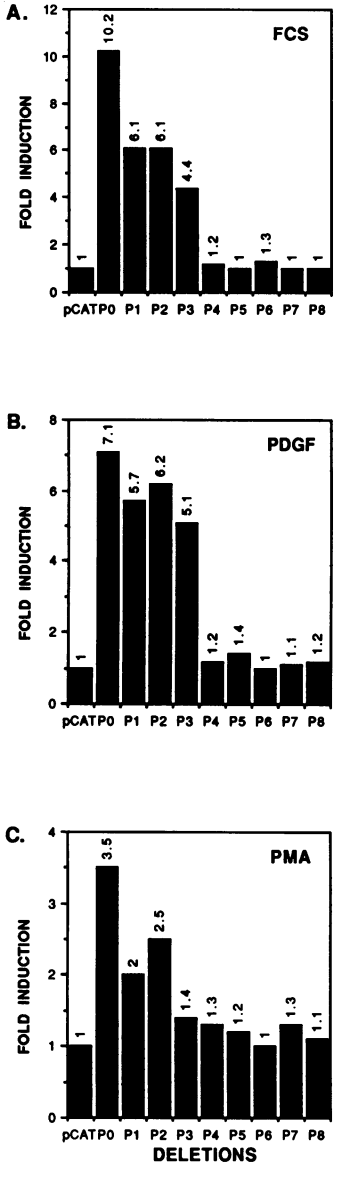

Figure 4. Induction of CAT activity in cells transfected with different *fos*B-CAT plasmids after treatment with (A) 20% FCS, (B) 10 ng/ml of PDGF, and (C) 100 ng/ml of PMA. NIH 3T3 cells were cotransfected with the indicated *fos*B-CAT plasmids and the β-galactosidase plasmid pCH110 and were incubated for 36 to 48 h in 0.5% FCS before stimulation for 3 h with the indicated agents. The CAT activity of each construct was determined using equivalent amounts of nuclear extracts and was normalized to the β-galactosidase activity of each extract. The graphs indicate the fold induction of CAT activity respect to the non-stimulated cells for each construct. The results represent the average of 6 independent transfections.

Effect of progressive deletions of *fos*B 5′ upstream cis-elements in gene induction

To determine if the 1 kb 5′ upstream sequence of the *fos*B gene is sufficient for gene activation, a recombinant *fos*B-CAT fusion gene was used. To construct this plasmid (PO) the genomic *fos*B sequence (nucleotides −962 to 308) was ligated into the *Pst*I-*Hin*dIII sites of the vector pBLCAT3 (48). NIH 3T3 cells were transfected and tested for inducibility as described in Materials and Methods. As shown in Figure 4 the −962-*fos*B-CAT (PO) presents marked stimulation of CAT expression by serum and platelet-derived growth factor (PDGF) and to a lesser extent by phorbol 12-myristate 13-acetate (PMA). To investigate which is the minimal 5′ upstream fragment of *fos*B that confers inducibility to these different agents, several progressive deletion *fos*B-CAT fusion genes were constructed (see Figure 5). The −884-*fos*B-CAT plasmid (P1), which lacks the AP-1-like sequence in position −912 to −906, presents an approximately 6-fold stimulation of CAT expression by serum and PDGF compared to only 2-fold stimulation with PMA. The induction of P1 is significantly lower than that of the −962-*fos*B-CAT plasmid (PO), specially with serum, indicating that under these conditions the AP-1-like sequence plays a role in the induction of the gene. The stimulation of CAT expression in the −535-*fos*B-CAT plasmid (P2) by the three agents is comparable to that of P1. This indicates that the region between nucleotides −883 and −536, of the 5′ upstream region of the *fos*B gene, containing no recognizable consensus element, is not essential for the

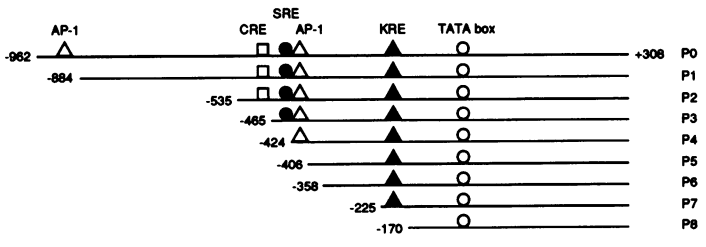

Figure 5. Schematic representation of the upstream elements of the *fos*B gene and the various *fos*B deletion mutants used. The *fos*B-CAT constructs were generated by standard recombinant DNA procedures as described in Materials and Methods.

induction of the gene under these conditions. However, the construct −465-*fos*B-CAT (P3) which lacks the CRE-like sequence located in position −479 to −470 presents a modest but reproducible reduction in its stimulation, indicating that this element contributes to the induction of the *fos*B gene. The most dramatic decrease in stimulation is shown by the construct −424-*fos*B-CAT (P4), where the SRE has been eliminated, it shows a slight or no stimulation of CAT expression by the different stimulants suggesting that this element plays an important role in the induction of the gene. This also demonstrates that the elements such as AP-1, Sp-1 and KRE-like which still remain in −424-*fos*B-CAT are not sufficient to confer inducibility to the gene. Further deletion mutants show no induction of CAT expression.

Activity and binding of *fos*B SRE

The results presented above suggested that the SRE located in position −428 to −419 may play an important role in the induction of the *fos*B gene, therefore we determined whether the SRE was functional, and compared its activity with that of the SRE present in the c-*fos* gene (39). The oligonucleotide containing the c-*fos* SRE also included the flanking symmetrical sequences, therefore we will refer to it as dyad symmetry element (DSE). Cells were transfected with the corresponding constructs and induction of CAT activity by serum was determined. The *fos*B-SRECAT shows a 5-fold induction in CAT activity by serum (Figure 6A). This induction is similar to that observed with the construct −465-*fos*B-CAT which contains the SRE confirming the above observation that deletion of the SRE, as in the −424-*fos*B-CAT construct, eliminates the serum response. Comparison of the CAT activity to serum between the *fos*B-SRE-CAT and −962-*fos*B-CAT construct (PO) indicates that the SRE contributes to nearly half of the total induction by serum. The response to serum confered by the c-*fos* DSE to CAT is consistently higher than the *fos*B SRE, with 8-fold activation by the DSE compared to a 5-fold by the SRE. This correlates with the higher level of induction of the c-*fos* gene compared to the *fos*B gene *in vivo*.

As the activity of the *fos*B SRE is lower than that of the c-*fos* DSE, and as they share the core sequence CC(A or T)$_6$GG, but the *fos*B SRE lacks the flanking symmetrical sequences present in the c-*fos* DSE (reviewed in 39), we found it of interest to determine whether the *fos*B SRE binds the serum responsive factor (SRF) with a similar affinity to that of the c-*fos* DSE. To perform these studies, *in vitro* translated SRF was incubated with a [^{32}P]-labeled SRE- or DSE-containing oligonucleotide and the protein/DNA complex was analyzed at room temperature using the gel retardation assay. The results show that *fos*B SRE binds SRF very efficiently and competition studies using an unlabeled oligonucleotide containing SRE unrelated sequences demonstrate that the complex formed is specific (Figure 5B). Competition experiments using different concentrations of unlabeled SRE or DSE show that they similarly compete the binding of the labeled SRE indicating that the binding affinity of SRF for both, DSE

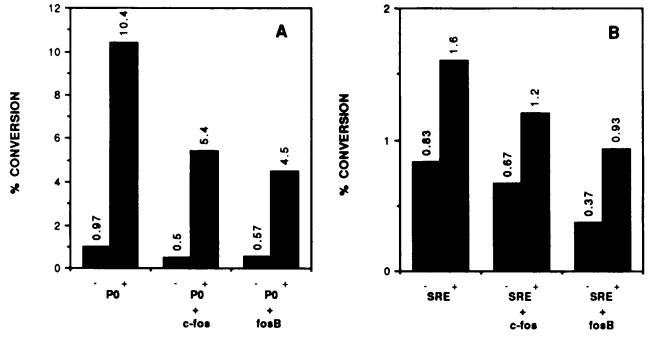

Figure 7. (A) Transrepression of *fos*B promoter by FosB and c-Fos. The −962-*fos*B-CAT construct was transfected into NIH 3T3 cells alone or cotransfected with the expression vector pMexNeo containing either *fos*B or c-*fos*. After 36 to 48 h in 0.5% FCS cells were stimulated for 3 h with 20% FCS. (B) Transrepression of *fos*B SRE by FosB and c-Fos. The *fos*B-SRE-CAT plasmid was transfected alone or cotransfected with either a *fos*B or c-*fos* expressing vector. Cells were kept for 36 to 48 h in 0.5% FCS before stimulation with 20% FCS. An equivalent amount of nuclear extract was used and the percentage of conversion was nominalized to that of galactosidase activity in each case.

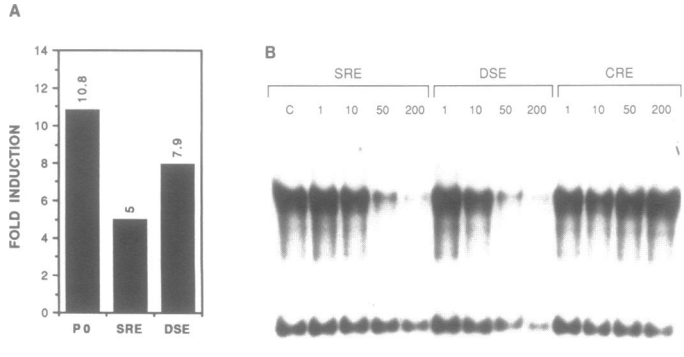

Figure 6. (A) Induction of CAT activity in cells transfected with −962-*fos*B-CAT (PO), *fos*B-SRE or c-*fos*-DSE. NIH 3T3 cells were cotransfected with the indicated constructs and β-galactosidase plasmid pCH110 and were kept in 0.5% FCS for 36 h before stimulation for 3 h with 20% FCS. Equivalent amounts of nuclear extracts were used. The CAT activity was normalized to the β-galactosidase activity of each extract. (B) Competition between *fos*B-SRE and c-*fos*-DSE for SRF. Binding reactions were carried out as described in Materials and Methods using 1 Pl of *in vitro* translated SRF and 0.01 pmol of labeled *fos*B-SRE oligonucleotide per reaction. For competition studies the competitor oligonucleotide was premixed with the labeled oligonucleotide before adding SRF. C = binding of SRE in the absence of competitor oligonucleotide.

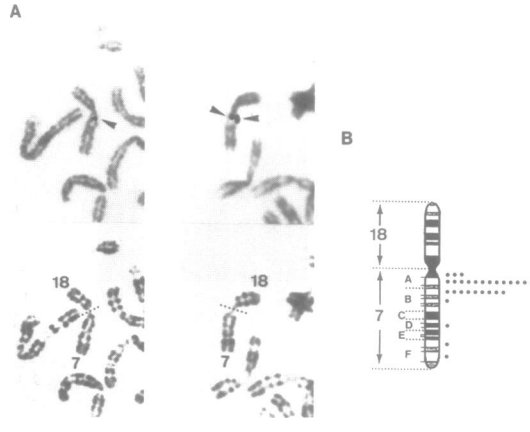

Figure 8. Localization of the *fos*B gene to mouse chromosome 7 by *in situ* hybridization. (A) Two partial WMP mouse metaphases, showing the specific site of hybridization to chromosome 7. Top, arrowheads indicate silver grains on Giemsa-stained chromosomes after autoradiography. Bottom, chromosomes with silver grains were subsequently identified by R-banding. (B) Diagram of WMP mouse Rb (7:18) chromosome indicating the distribution of labeled sites.

and SRE is comparable. Therefore, the differences in the activity of these elements is most likely due to their capacity to bind other of the SRE binding proteins.

Repression of *fos*B by FosB and c-Fos

It has been previously demonstrated that c-Fos can transrepress its own expression and that this effect is mediated by the SRE (58−61). The finding that the SRE of *fos*B plays a role in the induction of the gene and the fact that *fos*B, like c-*fos*, is transiently expressed prompted us to determine if FosB could transrepress its own expression. For these studies the −962-*fos*B-CAT plasmid (PO) was transfected alone or cotransfected with the mammalian expression vector pMexNeo containing *fos*B into NIH 3T3 cells. As shown in Figure 7A the CAT activity of the PO construct when cotransfected with the *fos*B expressing vector is inhibited nearly 60% indicating that FosB protein can transrepress the expression of its own gene. Parallel experiments cotransfecting the PO plasmid with pMexNeo-c-*fos* demonstrate that c-Fos protein can also transrepress the activity of the *fos*B promoter (Figure 7A). Cotransfection of PO with a pMexNeo construct expressing another transcription factor, such as Krox-24, had no effect in the activity of the *fos*B promoter (not shown). These observations were further extended by the cotransfection of SRE-CAT with pMexNeo containing either *fos*B or c-*fos* (Figure 7B). The results show that indeed the SRE of *fos*B can be inhibited by either FosB or c-Fos expressing vectors. Similar studies using a DSE-CAT construct demonstrate that FosB also inhibits the activation of c-*fos* DSE, suggesting that both c-Fos and FosB not only autoregulate their own expression but also can regulate each others expression.

Chromosomal localization of *fos*B

The high degree of amino acid conservation and similarity on the overall structure of *fos*B and c-*fos* strongly argues that they evolved from a single ancestral gene by gene duplication. We found of interest to determine if the duplicated genes were maintained in the same chromosomal locus or if they were dispersed. To determine the chromosomal localization of the *fos*B gene, *in situ* hybridization experiments were carried out using mouse metaphase spreads. A recombinant pGEM1 plasmid containing a 2.6 kb *fos*B cDNA was used as a probe. In the 100 metaphase cells examined after *in situ* hybridization there were 159 silver grains associated with chromosomes and 29 of these (19%) were located on chromosome 7; the distribution of grains on this chromosome was not random: 86.2% of them mapped to the [A1-B1] region of chromosome 7 with a maximum in the A band. (Figure 8) These results demonstrate that the localization of the *fos*B gene is on the 7A band of the murine genome, most probably in the distal part of this band. Therefore, *fos*B gene is located in a different chromosome than c-*fos* that is located in the [E−D] region of chromosome 12 in the mouse genome (62).

DISCUSSION

In this paper we describe the genomic structure of *fos*B, an immediate early gene whose product is a component of the AP-1 complexes. The general exon-intron distribution of *fos*B is similar to c-*fos* demonstrating that these genes have been originated from a common ancestor gene. However, *fos*B and c-*fos* are not closely located in the mouse genome.

The characterization of the *fos*B promoter demonstrates that its activation by serum, PDGF and PMA is in part mediated via its SRE. Deletion of the SRE produces the most dramatic decrease in activation of the *fos*B promoter. The results obtained with the different promoter deletion mutants indicate some contribution from other upstream elements such as the AP-1- and CRE-like sequences in the induction of the gene. The SRE when tested separate from the rest of the 5′ upstream region is functional and its activity is nearly half of that of the complete *fos*B promoter. The *fos*B SRE activity is weaker than the DSE present in the c-*fos* gene, however by competition experiments a similar binding affinity of these elements was found for SRF, suggesting that their differences in activity is most likely due to their capacity to bind other of the SRE binding proteins. A striking observation is that in the *fos*B promoter 6 nucleotides from the 3′ end of the SRE there is an AP-1-like sequence identical and at the same distance from the SRE to that in the c-*fos* promoter. This strong conservation suggests a regulatory role for this sequence. At least in two other genes, *Krox*-20/*zif*268 (11, 52) and *Krox*-24 (53) the presence of an AP-1-like sequence at the 3′ end of the SRE has been demonstrated, but they differ from that of *fos*B and c-*fos*. In all these genes the SRE plays an important role in their activation, but the differences in their level of expression and kinetics of induction cannot be explained by the differences in the activities of their SREs, as some contain four or five functional SREs and they are induced at lower levels or present slower kinetics of induction compared to c-*fos* (11, 52, 53). This indicates that the other elements present in the promoters of these genes have a strong influence in their responses. There are two other important similarities between the *fos*B and c-*fos* promoter that need to be mentioned. One is the presence of a CRE sequence in *fos*B which is situated 50 nucleotides to the 5′ end of the SRE which resembles the c-*fos* CRE, also located at the 5′ end of the SRE, but at a distance of 28 nucleotides. The other element that is conserved between these promoters is an NF-1-like site which is situated in position −180 to −165 in the c-*fos* promoter and in position −136 to −121 in *fos*B. Therefore, at least four elements are common to the *fos*B and c-*fos* promoter, and most important, their relative location is well conserved. The similarity of these promoters explains in part the fact that the *fos*B and c-*fos* genes present the most alike kinetics of induction in fibroblasts.

We have also shown that the activity of the *fos*B promoter can be downregulated by FosB and c-Fos to a similar extent. Promoter constructs carrying only *fos*B SRE are still repressible by both Fos proteins. Moreover, the activity of a c-*fos* DSE promoter construct is also downregulated by FosB and c-Fos indicating that both proteins have the capacity to transrepress the transcription of each other. It is important to mention that high expression of either FosB or c-Fos in fibroblasts is sufficient to induce abnormal or malignant growth. Therefore, transrepression is a very effective mechanism to tightly control the levels of expression of these proteins.

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