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Proto-Oncogene FosB: the Amino Terminus Encodes a Regulatory Function Required for Transformation

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Overexpression of some members of the Fos gene family, including FosB, leads to transformation of established rodent fibroblasts. We have previously shown that transformation by FosB requires the presence of a C-terminal transcriptional activation domain. We now report that transformation by FosB also requires an intact DNA-binding domain composed of the functionally bipartite basic region and leucine zipper as well as sequences present in the N terminus that serve a regulatory function. Deletion of the N-terminal sequences results in proteins impaired in transcriptional activation and transformation. This region does not itself function as a transcriptional activation domain but instead regulates the transactivation functions present in the FosB-Jun complex. The requirement for this N-terminal region can be abolished by the presence of a strong constitutive activation domain. The primary sequence of the region that we have defined is highly conserved in the Fos family of proteins, suggesting functional conservation.

Exposure of cells to extracellular peptide growth factors initiates a cascade of biochemical events that result in cell division. These events include an increase in the synthesis and activity of several transcription factors that mediate, at least in part, the cellular response to growth factors. The transcription factor AP1, a sequence-specific DNA-binding factor, is composed of a complex mixture of polypeptides derived from the Fos and Jun gene families (18, 21, 31, 35, 40). AP1 activity is rapidly and transiently induced in response to growth factors and mitogens, and AP1 DNA-binding sites are sufficient to confer transcriptional induction by the growth-promoting phorbol esters (4, 17, 23, 25, 28, 37, 38, 54, 55). That AP1 mediates the cellular response to growth factors is suggested by the observation that deregulated expression of certain members of the Fos and Jun families results in the neoplastic transformation of susceptible cells.

Mutational analysis of the c-Fos protein supports the hypothesis that sequence-specific DNA-binding activity is a prerequisite for transforming activity. The products of all transforming Fos proteins show high-affinity sequence-specific DNA-binding activity as part of a heterodimeric complex with c-Jun, and conversely, all mutant Fos proteins that do not associate with both Jun and DNA do not retain transforming activity (32, 44). Heterodimer formation and DNA-binding activity are specified by small discrete domains known as the leucine zipper and basic region, respectively, together termed the BLZ motif (18, 21, 31, 33, 34, 36, 41, 48). The functional integrity of the BLZ motif is therefore required for transforming activity.

Although the BLZ motif is required for transformation by Fos proteins, expression of the BLZ motif alone is not sufficient to induce transformation (19). We and others have previously described the existence of two functionally distinct proteins, FosB and FosB2, that are generated by alternative splicing of the primary transcript from the FosB gene (12, 29, 30, 54). FosB, a 338-amino-acid protein with transforming activity, was originally identified as the product of the FosB gene, an early-response gene with strong homology to c-Fos (55). FosB2, a 237-amino-acid protein, corresponds to a C-terminal truncation derivative of FosB and lacks transforming activity (12, 22, 29, 30). Both proteins bind Jun proteins and DNA with high affinity, and therefore the difference in transforming activity is not the result of differences in DNA binding (29, 30, 54). We have previously shown that the difference in transforming activity between FosB and FosB2 is due to the presence of a strong transcriptional activation domain in the C-terminal amino acids unique to FosB (53). This conclusion was supported by three lines of evidence: (i) mutations in the C-terminal region unique to FosB impaired transformation and transcriptional activation in a coordinate manner, (ii) the C-terminal amino acids from FosB function as a strong activation domain when linked to heterologous DNA-binding domains, and (iii) fusion of FosB2 to well-characterized transcriptional activation domains results in fusion proteins with transforming activity (53).

The results to date support the hypothesis that transformation by Fos proteins is the result of transcriptional activation of genes containing AP1 sites (43, 44, 53). To further characterize the molecular requirements for transformation, we have extended our mutational analysis of the FosB protein to regions outside the C-terminal transactivation domain. As expected, we find that deletion of either the basic region or the leucine zipper results in proteins without transforming activity. Although large portions of the amino terminus are dispensable, the presence of a small region (amino acids 41 to 73) is required for transforming activity. Deletions of this sequence impair transcriptional activation by FosB, but this region does not function as a transcriptional activation domain when fused to a heterologous DNA-binding domain. Furthermore, the requirement for this region is not apparent in fusion proteins that contain a strong constitutive activation domain. The results demonstrate that transformation by FosB protein requires the presence of a positive regulatory domain in the amino terminus.

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MATERIALS AND METHODS

Cells. 208F cells, azaguanine-resistant derivatives of Rat-1 cells, were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% calf serum and antibiotics. Focus-forming assays were performed by growing cells in DMEM supplemented with 5% calf serum and 2×10^{-6} M dexamethasone as previously described (27, 52). Recombinant retrovirus was generated by transfection of the packaging cell line Am12 by the calcium phosphate method followed by selection with G418 (300 μg/ml). 208F cells were infected with recombinant virus in the presence of Polybrene (4 μg/ml). At 24 h after infection, cells were split into four plates; two were selected in G418 (400 μg/ml), and two were used to count the number of transformed foci 12 days after infection. Cos cells were transfected by the calcium phosphate method. For chloramphenicol acetyltransferase (CAT) assays, 208F cells were transfected by the calcium phosphate method.

RNA and protein analysis. RNA isolation and Northern (RNA) blotting were carried out as previously described (51). For transin mRNA analysis, cells were grown in DMEM with 0.5% calf serum for 24 h prior to isolation of RNA. For protein analysis, 6-cm-diameter dishes of Cos cells were transfected with 6 μg of DNA corresponding to different FosB mutants. At 48 h after transfection, cells were labeled with 500 μCi of ^{35}S-amino acids per ml for 2 h. After lysing of cells in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) plus 0.5% sodium dodecyl sulfate (SDS), the lysates were boiled for 5 min and then diluted in RIPA buffer to a final concentration of 0.1% SDS. Samples were incubated successively with a FosB-specific antiserum (raised against a peptide corresponding to amino acids 80 to 96) and protein A-Sepharose. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.

Immunofluorescence. Transiently transfected Cos cells grown on glass coverslips were fixed in 50% methanol–50% acetone for 8 min at −20°C. The coverslips were then incubated in a 1:100 dilution of affinity-purified FosB antisera followed by fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody. The coverslips were mounted and visualized by immunofluorescence microscopy.

In vitro analysis of FosB proteins. c-Jun and different mutant FosB plasmids were transcribed in vitro and translated in rabbit reticulocyte lysate. For assays involving FosB–c-Jun heterodimer formation, unlabeled FosB proteins were mixed with ^{35}S-labeled c-Jun protein and incubated for 30 min at room temperature before dilution in RIPA buffer and immunoprecipitation with the FosB antiserum. The immunoprecipitates were then separated by SDS-PAGE. FosB proteins were not labeled in this set of experiments because it is difficult to separate FosB and c-Jun proteins by SDS-PAGE. The epitope against which the FosB antiserum was raised (amino acids 80 to 96) is not present in the deletion mutant FosB D74-141. An antiserum against the conserved c-Fos M peptide (amino acids 128 to 152), which also recognizes FosB, was used for the analysis of this mutant.

For gel shift analysis, equal volumes of reticulocyte lysate containing unlabeled proteins were incubated with a ^{32}P-labeled oligonucleotide corresponding to the collagenase AP1 site as previously described (54). The complexes were then separated by electrophoresis through 5% polyacrylamide gels in 0.5% Tris-borate-EDTA.

CAT assays. 208F cells transiently transfected with 1 μg of the GAL4 reporter construct pG5B-CAT, 4 μg of different GAL4-FosB expression plasmids, and 1 μg of the β-galactosidase expression plasmid BAG were lysed 48 h after transfection. Cell lysates were then prepared and analyzed for CAT activity as previously described (16). All samples were normalized for transfection efficiency by using equal amounts of β-galactosidase activity. To verify expression of the GAL4-FosB fusion proteins, nuclear extracts from transiently transfected cells were prepared according to the method of Andrews and Faller (3). Extracts were analyzed for GAL4 DNA-binding activity by electrophoretic mobility shift assay. The GAL4 DNA-binding site corresponded to the consensus site defined by Giniger et al. (13); the sequence of the top strand of the 21-bp DNA fragment was 5′-GCC GGA AGA CTC TCC TCC GCC-3′.

Plasmids. The FosB and FosB2 cDNAs cloned from mouse 3T3 cells have been previously described (54). FosB deletion mutants contain precise deletions of the amino acids specified. The mutants were generated by using oligonucleotides corresponding to the desired mutations to prime DNA synthesis from a uracil-containing single-stranded DNA template produced by *dut ung* bacteria (24). The FosB2-VP16 fusion cDNA has been previously described (53). Deletion mutants of FosB2-VP16 were generated by the polymerase chain reaction. The sequences of all mutants were verified by nucleotide sequencing. The various mutants were subcloned into the retroviral vector SLX-CMV (42) to generate recombinant retrovirus and into the simian virus 40-based expression vector CMX (49) for expression in Cos cells.

The reporter plasmid pG5B-CAT contains five tandem repeats of the GAL4 DNA-binding site and the basal promoter from the adenovirus E1b gene fused to CAT sequences. This plasmid has previously been described (20).

To generate GAL4-FosB fusion genes, the polymerase chain reaction was used to amplify various segments of the FosB gene encoding the specified amino acids. Amplified fragments contained a 5′ *Eco*RI site and a 3′ *Xba*I site; polymerase chain reaction-amplified fragments were cloned into the vector pSG424, which encodes the DNA-binding domain of GAL4 (amino acids 1 to 147) in a simian virus 40-based expression construct (39). The nucleotide sequences of the FosB inserts were verified by sequencing. Plasmid GAL4-FosB (226-338) has previously been described (53).

RESULTS

Dimerization and DNA binding by mutant FosB proteins. To determine the functions of different regions of the FosB protein, a series of deletion mutants was generated. The schematic diagram in Fig. 1 displays the general features of the proteins derived from the FosB gene. The basic region consists of amino acids 142 to 182, and the leucine zipper extends from amino acids 183 to 211. In the first set of experiments, the N terminus of the protein was divided into two regions. FosB and c-Fos show strong homology between amino acids 2 and 73 and much less homology between amino acids 74 and 141. We have previously shown that amino acids 226 to 338 constitute a strong transactivation domain of the proline-rich type and that this domain is required for transforming activity (53).

To assess the effect of these mutations on the ability of FosB proteins to interact with c-Jun, in vitro translation of the mutant proteins was carried out in reticulocyte lysate. After mixing of unlabeled FosB proteins with ^{35}S-labeled

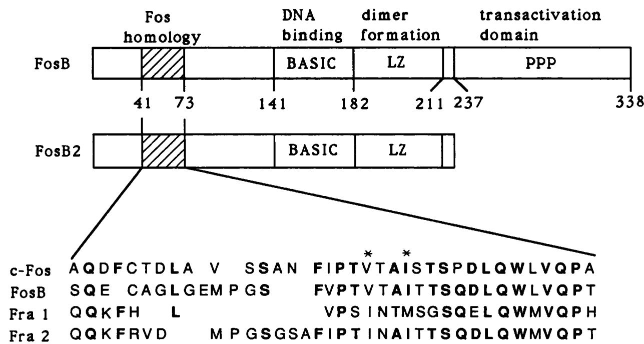


FIG. 1. Schematic diagram of FosB and FosB2 proteins. The major landmarks include the basic region (amino acids 142 to 182), the leucine zipper (LZ; 183 to 211), and the C-terminal transcriptional activation domain (ppp; 226 to 338). N-terminal sequences required for transforming activity by the FosB protein are identified. The sequence conservation of this region in the Fos family of proteins is shown by an amino acid sequence alignment of the proteins in the region corresponding to FosB amino acids 41 to 73. The sequences are from human c-Fos, Fra-1, and Fra-2, and mouse FosB. Residues conserved in three or more of the family members are shown in bold type; asterisks denote sites of mutations in the proteins encoded by murine sarcoma virus strains FBJ and FBR.

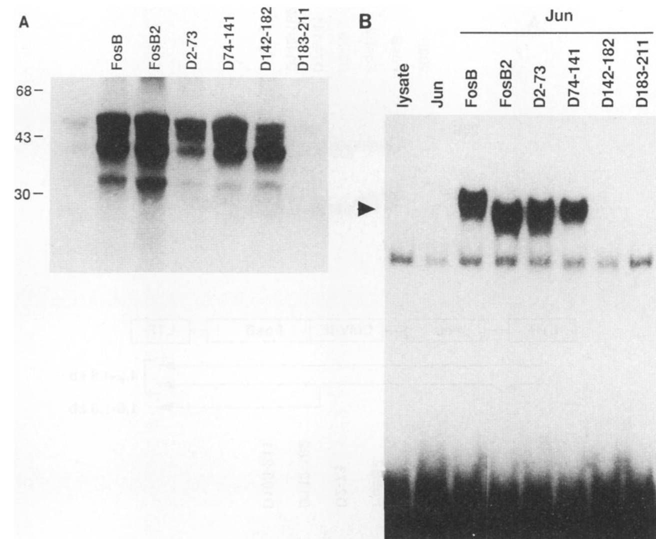


FIG. 2. (A) Association of mutant FosB proteins with c-Jun. ^{35}S-labeled c-Jun protein produced in rabbit reticulocyte lysate was mixed with different unlabeled FosB proteins (the numbers designate the amino acids deleted). After mixing, the proteins were immunoprecipitated with an antiserum specific for FosB except for mutant FosB D74-141, which was immunoprecipitated with an antiserum against the conserved basic region of c-Fos. The immunoprecipitates were then resolved by SDS-PAGE and visualized by autoradiography. Jun protein expressed in reticulocyte lysate migrates as a series of bands because of phosphorylation on multiple sites. The migration of molecular mass standards is shown in kilodaltons. (B) DNA-binding activity of mutant FosB proteins. Different FosB proteins and c-Jun protein produced in rabbit reticulocyte lysate were incubated with a ^{32}P-labeled probe corresponding to the collagenase AP1 site. The resulting DNA-protein complexes were separated on native polyacrylamide gels and visualized by autoradiography. In the first lane is reticulocyte lysate without added RNA; in the second lane is reticulocyte lysate programmed with c-Jun RNA; the remaining lanes contain reticulocyte lysate programmed with c-Jun RNA plus the designated FosB RNA. The arrowhead designates the migration of FosB-Jun complexes. On longer exposures, a complex corresponding to homodimeric c-Jun is observed.

c-Jun protein, dimer formation was measured by the ability of an antiserum specific for FosB to coimmunoprecipitate c-Jun protein. In this assay, all of the FosB mutant proteins except FosB D183-211, corresponding to a deletion of the leucine zipper domain, formed heterodimers with c-Jun (Fig. 2A). This result demonstrates that the leucine zipper is required for efficient dimer formation with c-Jun, a result consistent with the mutational analysis of c-Fos protein (18, 21, 33, 36, 41, 44, 48).

The effect of these mutations on the ability of FosB protein to bind DNA with high affinity as part of a heterodimeric complex with c-Jun was assessed by performing electrophoretic mobility shift assays using a ^{32}P-labeled AP1 probe (Fig. 2B). Once again, this assay yielded results consistent with the previous mutational analysis of c-Fos protein (32, 34, 48). Specifically, deletions of either the basic region (FosB D142-182) or the leucine zipper (FosB D183-211) resulted in proteins that did not bind DNA as part of a heterodimeric complex with c-Jun protein. However, FosB proteins with deletions in the N terminus showed DNA-binding activity comparable to that of the wild type (compare D2-73 and D74-141 with FosB in Fig. 2B).

Transformation by mutant FosB proteins. To assess the biologic activities of the various FosB mutant proteins, cDNAs corresponding to the different mutants were cloned into the retroviral vector SLX-CMV (42). We have previously shown that expression of FosB protein from this vector can induce neoplastic transformation of 208F fibroblasts and that expression of the transin gene, a known AP1 target gene, is increased in the infected cells (53). 208F cells infected with recombinant retroviruses capable of directing the expression of the different mutants were analyzed for expression of viral RNA by Northern blotting. Figure 3A shows that RNA from each of the viruses was expressed to approximately the same level and that expression of FosB RNA from the viruses constitutes marked overexpression compared with expression of the endogenous FosB gene. Immunoprecipitation of FosB proteins from metabolically labeled Cos cells transfected with the mutant cDNAs showed that all of the proteins were expressed in approximately the same amount (Fig. 3B). It should be noted that expression of FosB D74-141 could not be measured because the peptide that the antiserum was raised against is deleted in this mutant. The subcellular distribution of the mutant proteins was assayed by immunofluorescence of transiently transfected Cos cells (Fig. 4). FosB, FosB2, and the mutant FosB proteins all showed a nuclear pattern of immunofluorescence. This result is consistent with previous data on c-Fos showing that no single mutation is sufficient to result in the production of protein localized predominantly to the cytoplasm (46). Taken together with the in vitro data, these results demonstrate that any difference in the biologic properties of the mutant proteins is not the result of decreased expression or altered subcellular distribution. Furthermore, with the exceptions of FosB D142-182 and FosB D183-211, which contain deletions of the basic region and leucine zipper, respectively, all of the proteins possess normal DNA-binding activity.

To assess transforming activity, virus stocks corresponding to each of the FosB mutants were used to infect 208F cells. After infection, the cells were split into duplicate

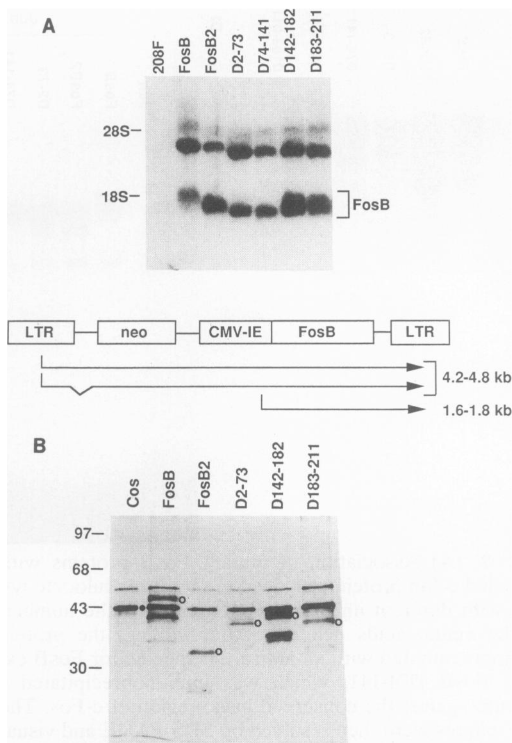


FIG. 3. (A) Analysis of FosB RNA from virus-infected cells. Mutant FosB cDNAs were cloned into the retroviral vector SLX-CMV. Recombinant virus produced by transfection into the retroviral packaging cell line Am12 was used to infect 208F cells. RNA from infected 208F cells (isolated by selection in G418) was separated on formaldehyde agarose gels and analyzed by Northern blotting using a FosB probe. Positions of the rRNAs are indicated. Three transcripts are detected in infected cells. A schematic diagram of transcripts expected from the SLX-CMV FosB virus is shown. The 1.6- to 1.8-kb transcript corresponds to mRNA initiated at the cytomegalovirus immediate-early promoter (CMV-IE) that is translated into FosB protein, while the 4.2- to 4.8-kb transcripts correspond to unspliced and spliced transcripts originating from the long terminal repeat (LTR). (B) Immunoprecipitation of mutant FosB proteins. Cos cells transiently transfected with plasmids expressing the different FosB mutants were metabolically labeled with ^{35}S-amino acids, and immunoprecipitated FosB proteins were separated by SDS-PAGE. Samples were boiled prior to immunoprecipitation in order to dissociate Jun proteins, which migrate very close to FosB on SDS-PAGE. The band present at 45 kDa in all samples probably represents the endogenous FosB protein of Cos cells. Open circles denote the proteins produced from the transfected genes.

plates and monitored for the appearance of foci of transformed cells or G418-resistant colonies. The results of the focus assays are shown in Table 1. As expected, both FosB D142-182 (deletion of the basic region) and FosB D183-211 (deletion of the leucine zipper) did not possess transforming activity. Analysis of the transforming activity of the N-terminal deletion mutants showed a striking contrast. FosB D74-141 transformed with activity approximately equal to that of wild-type FosB, while FosB D2-73 was severely impaired in transforming activity, generating only 2% the number of foci of the wild-type protein.

The transin gene encodes an extracellular metalloproteinase and is a known target of AP1-mediated transcriptional activation. We and others have previously shown that transcriptional induction of the endogenous transin gene correlates well with the transforming activity of Fos proteins (26, 43, 53). As a measure of transcriptional activation by FosB proteins, we measured the steady-state level of transin mRNA in infected cells (Fig. 5). Quantification of the data by scanning densitometry is shown in Table 1. The results show that those mutants that are not transforming (D2-73, D142-183, and D184-211) are also impaired in their ability to induce expression of transin mRNA, while FosB D74-141 retains transforming and transactivating properties similar to those of full-length FosB. We draw two inferences from the analysis of transcriptional activation and transforming activity by the FosB mutants. First, as expected, mutants with impaired DNA-binding activity are coordinately impaired in transforming activity. Second, there is an N-terminal region of the protein (amino acids 2 to 73) that is required for both transcriptional activation and neoplastic transformation, despite the fact that DNA-binding activity is unaffected.

Delineation of N-terminal sequences required for transformation. To better characterize the effect of mutations in the N-terminal region of the protein, this region was subdivided for further mutagenesis. Previous analysis of the c-Fos protein had demonstrated that the first 40 amino acids were not required for transforming activity, but a larger deletion of the first 110 amino acids resulted in a protein severely impaired in its ability to induce focus formation (19). On the basis of this result, we generated mutations corresponding to deletions of amino acids 2 to 40 and 41 to 73. In vitro translation of these mutant proteins showed that neither of the mutations affected the ability of the proteins to bind DNA (data not shown; see D2-73 in Fig. 2A). Viruses capable of directing expression of the mutant FosB proteins were generated and used to infect 208F cells as before. RNA analysis of infected cells showed that the mutant viral RNAs (D2-40, D41-73, and D2-73) were expressed to approximately the same level as was wild-type FosB (Fig. 6A). Focus assays were performed with the different viruses, and the results are displayed in Table 1. FosB D2-40 displayed a nearly wild-type level of transforming activity, while mutant FosB D41-73 was severely impaired in transforming activity. These results were correlated with the expression of mRNA from the endogenous transin gene in infected cells (Fig. 6B and Table 1). Again, a correlation was found between transcriptional activation of the endogenous transin gene and transforming activity. FosB D2-40 induced expression of transin mRNA, while FosB D41-73 did not. The results support our previous contention that N-terminal sequences are required for transformation by FosB and further delimit the sequences required for transformation to amino acids 41 to 73.

The N-terminal region does not function as a transactivation domain. The results of the mutational analysis define an N-terminal region (amino acids 41 to 73) that is not required for high-affinity DNA binding or correct subcellular distribution but is required for transcriptional activation and transformation to neoplastic growth. We have previously demonstrated the presence of a strong activation domain in the C terminus of FosB. Furthermore, we have shown that the presence of such a domain is required for transforming activity. We tested the hypothesis that the N-terminal region including amino acids 2 to 73 encodes a second transactivation domain by examining the ability of this region to function as a transcriptional activator when fused to the heterologous DNA-binding domain of the yeast transcription factor GAL4. These fusion genes were then tested for the ability to direct transcription from reporter constructs containing GAL4 DNA-binding sites after transient transfection

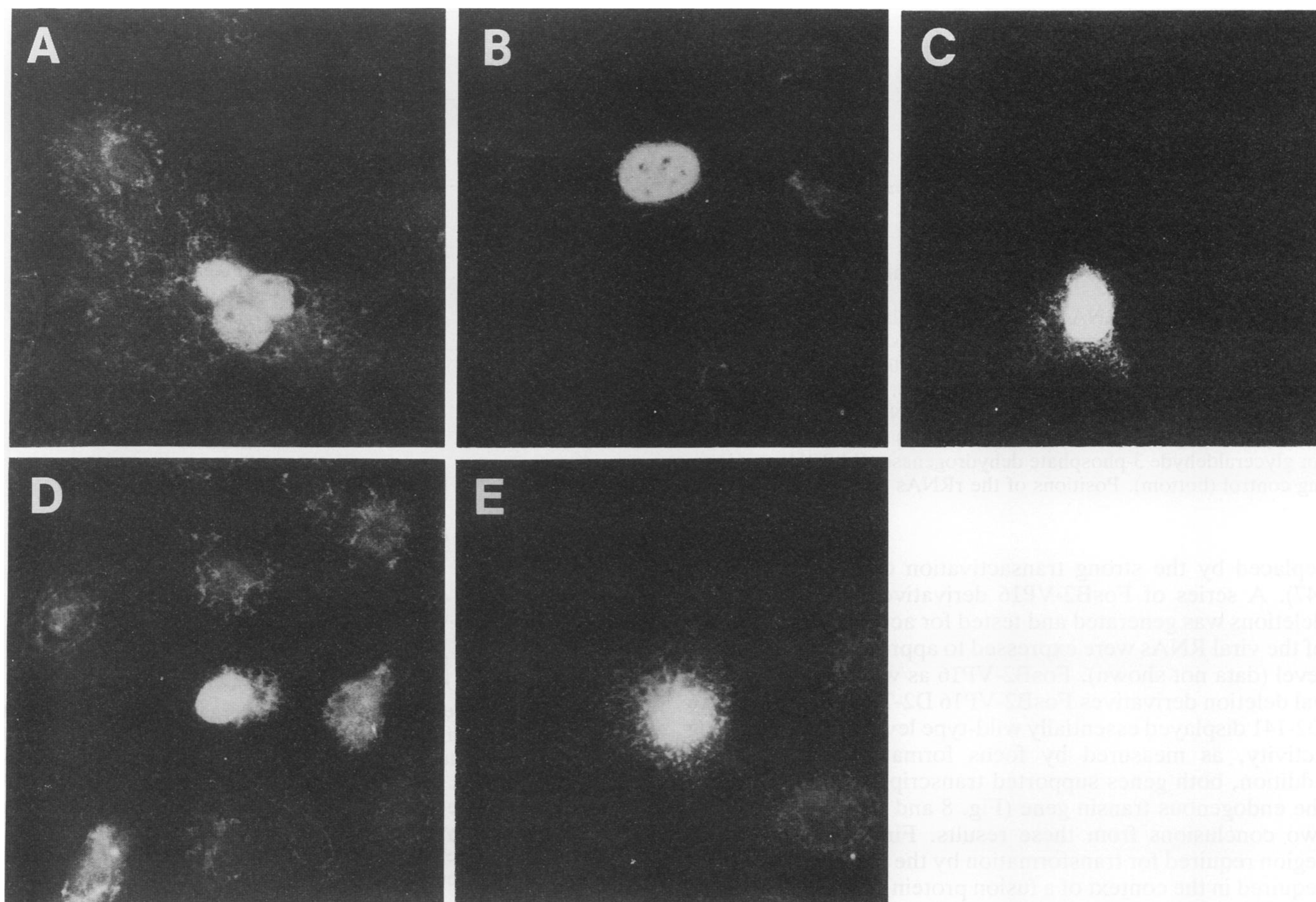


FIG. 4. Nuclear localization of mutant FosB proteins. Plasmids encoding different FosB proteins were transfected into Cos cells grown on glass coverslips. At 48 h after transfection, cells were fixed and then incubated successively in affinity-purified FosB antiserum and fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin. FosB proteins were visualized by immunofluorescence microscopy. The panels show nuclear staining of FosB (A), FosB2 (B), FosB D2-73 (C), FosB D142-182 (D), and FosB D183-211 (E). The faint nonspecific staining of untransfected cells can also be seen.

into 208F cells. The fusion proteins GAL4-FosB (2-73) and GAL4-FosB (2-100) did not function as transcriptional activators in this assay, although the fusion protein GAL4-FosB (226-338), which contains the previously described C-terminal activation domain, did (Fig. 7). Lack of transcriptional activation by GAL4-FosB (2-73) and GAL4-FosB (2-100) is not the result of decreased expression of the fusion proteins, as nuclear extracts prepared from cells transfected with each of the GAL4 derivatives expressed approximately equal amounts of DNA-binding activity, as measured by electrophoretic mobility shift assays using a GAL4 DNA-binding site as the probe (data not shown). These results suggest that although the N-terminal region consisting of amino acids 41 to 73 is required for transcriptional activation in the intact FosB molecule, this region by itself does not function as a transactivation domain in a heterologous context.

We hypothesized that regulation of FosB activity by the N-terminal region occurs by regulation of a transactivation domain located either in the C terminus of FosB or on the Jun partner. Such a hypothesis predicts that the N-terminal region may not be required in the presence of a constitutive transcriptional activation domain. To test this prediction, we exploited a fusion gene that we have previously described, FosB2-VP16 (53). This fusion gene is essentially a derivative of FosB in which the C-terminal activation domain has been

TABLE 1. Transin mRNA expression and focus-forming activity

Virus	Transin mRNA expression (%)[a]	Focus-forming activity (%)[b]
None	0	0
FosB	100	100
FosB2	14	0
FosB D2-73	12	2
FosB D74-141	90	83
FosB D142-182	11	0
FosB D183-211	7	0
FosB D2-40	72	92
FosB D41-73	14	3
FosB2-VP16	120	96
FosB2-VP16 D2-73	127	116
FosB2-VP16 D2-141	112	105

[a] Determined by densitometric scanning of the autoradiograms shown in Fig. 5, 6, and 8. Values are normalized to those for the glyceraldehyde 3-phosphate dehydrogenase loading control and have been adjusted to a value of 0% for 208F cells and 100% for cells infected with virus SLX-CMV FosB.

[b] Measured by infecting 208F cells with the different viruses. At 24 h after infection, the cells were split into duplicate plates that were monitored for the appearance of either transformed foci or G418-resistant colonies. The focus-forming activity was calculated by dividing the number of transformed foci by the number of G418-resistant colonies; the activity of FosB virus was assigned a value of 100%. The values are derived from at least two separate experiments with each virus.

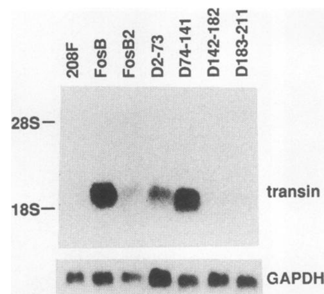


FIG. 5. Transin mRNA expression in cells expressing different FosB mutant proteins. Expression of the transin gene was measured by Northern analysis of RNA isolated from 208F cells infected with recombinant retroviruses expressing different FosB mutants. Cells were grown in 0.5% serum for 24 h before RNA isolation. The filter was hybridized first with a transin probe (top) and then with a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control (bottom). Positions of the rRNAs are indicated.

replaced by the strong transactivation domain from VP16 (47). A series of FosB2-VP16 derivatives with N-terminal deletions was generated and tested for activity as before. All of the viral RNAs were expressed to approximately the same level (data not shown). FosB2-VP16 as well as the N-terminal deletion derivatives FosB2-VP16 D2-73 and FosB2-VP16 D2-141 displayed essentially wild-type levels of transforming activity, as measured by focus formation (Table 1). In addition, both genes supported transcriptional activation of the endogenous transin gene (Fig. 8 and Table 1). We draw two conclusions from these results. First, the N-terminal region required for transformation by the FosB protein is not required in the context of a fusion protein containing a strong constitutive activation domain. This finding suggests that either the FosB C-terminal activation domain or the Jun transcriptional activation domain is subject to regulation by the FosB N terminus. Second, the protein FosB2-VP16 D2-141 consists entirely of three small functional domains (the FosB basic region and leucine zipper and the VP16 activation domain). Transformation by this fusion protein

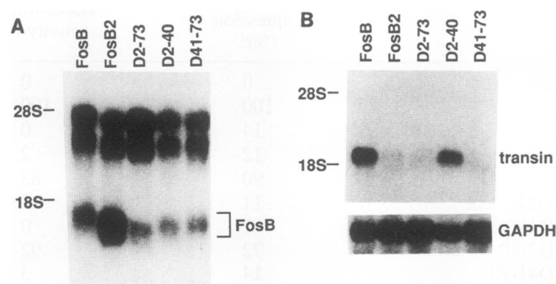
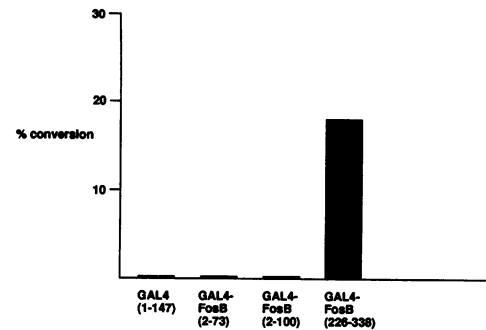


FIG. 6. (A) FosB RNA analysis from cells expressing FosB mutants with N-terminal deletions. RNA was isolated from 208F cells infected with different FosB mutant viruses and separated on formaldehyde agarose gels. After blotting to nylon membranes, the filter was probed with a FosB cDNA. A schematic diagram of the expected transcripts is shown in Fig. 3A. (B) Transin mRNA expression in cells expressing FosB proteins with N-terminal deletions. Cells infected with retroviruses expressing derivatives of FosB were grown in 0.5% serum for 24 h, RNA was isolated, and transin mRNA expression was analyzed as described above. Hybridization with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was used to demonstrate equal loading of the RNA samples.



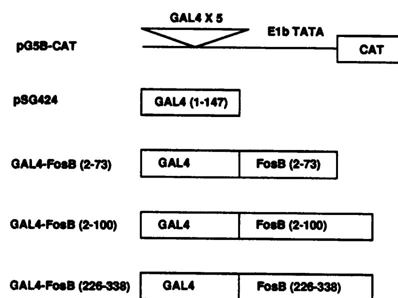


FIG. 7. Evidence that the N-terminal region of FosB does not function as a transcriptional activation domain. Different regions of FosB were fused to the GAL4 DNA-binding domain. Plasmids encoding the fusion proteins were transiently transfected into 208F cells along with the reporter construct pG5B-CAT. At 48 h after transfection, cell lysates were prepared and analyzed for CAT activity. The numbers specify the FosB amino acids present in the fusion protein. Schematic diagrams of the plasmids are shown.

suggests that there are only three requirements for transformation by Fos proteins: dimerization with Jun, high-affinity DNA binding, and a transcriptional activation domain. It seems likely that these functions represent the minimal requirements for transformation by Fos proteins.

DISCUSSION

We have analyzed various properties of the FosB gene by systematic mutagenesis. Together with our previous analysis, these studies extend our understanding of the molecular details of transformation by FosB. One conclusion is that heterodimer formation and DNA binding are required for both transformation to neoplastic growth and transcriptional activation. As is the case with c-Fos, complex formation and DNA binding are specified by discrete functional domains. The leucine zipper of FosB is required for heterodimer formation with c-Jun, and the integrity of the entire BLZ motif is required for sequence-specific DNA binding as part of a FosB-Jun complex. Mutations that abolish high-affinity DNA binding result in proteins that do not activate transcription or transform cells in vitro. This conclusion is in agreement with the results of previous mutational analysis of the c-Fos protein (32, 44).

We have previously shown that DNA-binding activity is not sufficient for transformation to neoplastic growth by FosB; a C-terminal transactivation domain present in FosB, but not FosB2, is also required (53). Here we provide evidence that mutations in the N-terminal region (amino acids 41 to 73) of FosB abolish both transcriptional activa-

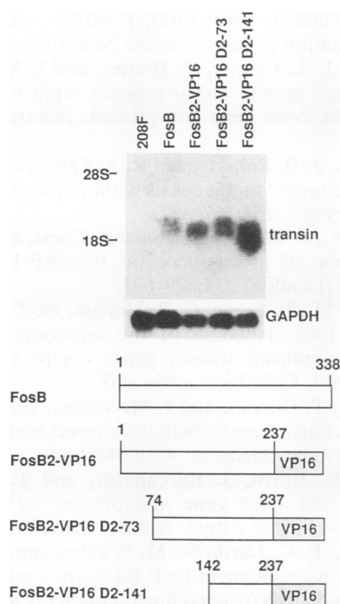


FIG. 8. Transin mRNA analysis in cells expressing FosB2-VP16 derivatives. 208F cells infected with viruses expressing derivatives of FosB2-VP16 were starved of serum for 48 h and analyzed for expression of transin mRNA as described above. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control is also shown. Schematic diagrams of the FosB2-VP16 fusion genes are shown.

tion and transforming activity. The region defined by the mutational analysis does not itself function as an activation domain when fused to a heterologous DNA-binding domain (Fig. 7), suggesting that it may regulate an activation domain present in the FosB-Jun complex. This hypothesis is supported by experiments demonstrating that the presence of a constitutive activation in the FosB2-VP16 fusion protein removes the requirement for N-terminal sequences.

The biochemical mechanisms underlying the requirement for N-terminal sequences remain obscure. One possibility is that deletions in this region of the protein severely perturb the tertiary structure of the molecule. However, as these mutant proteins participate in high-affinity DNA-binding complexes with c-Jun, complete unfolding of the protein seems unlikely. Furthermore, whatever conformational changes are induced by N-terminal deletion do not affect the activity of the FosB2-VP16 fusion protein.

A second possibility is that the N-terminal region either directly or indirectly regulates the transcriptional activation domains present in the FosB-Jun complex. There are precedents for this type of interaction. For example, the transcriptional activation domain of c-Jun is subject to regulation by interaction with the δ domain through a biochemical mechanism that remains obscure (5–7, 9, 10). A second example is the demonstration that the region surrounding the protein kinase A phosphorylation site on the cyclic AMP response element-binding protein does not function as a transactivation domain, but its presence (in a phosphorylated form) is required for the function of a separate activation domain (14, 15). With regard to this possibility, we note that the FosB C-terminal activation domain (amino acids 226 to 338) functions when fused to the GAL4 DNA-binding domain in the absence of FosB N-terminal sequences (53), suggesting it may be the Jun activation domain that is regulated. We are currently testing the possibility that the N-terminal regulatory region of FosB interacts with the c-Jun activation domain.

Comparison of our results with previous mutational analysis of the v-Fos protein is illustrative. First, previous data have demonstrated that although the first 40 amino acids of v-Fos are not required for transformation, an N-terminal deletion of the first 110 amino acids severely impairs transforming activity (19). This truncated v-Fos protein binds DNA and is expressed at levels equivalent to those of the full-length protein (19). The N-terminal region between amino acids 41 and 73 is one of the most conserved regions of the Fos family of proteins outside the BLZ motif, demonstrating that there is conservation of sequence (Fig. 1). Whether this sequence conservation underlies functional conservation remains to be determined.

We have no direct evidence that the function of the N terminus is regulated, but three observations suggest that it may be. First, the proteins encoded by FBJ murine sarcoma virus strains and FBR both contain mutations in the N-terminal region that we have defined (valine 58 to glutamic acid in FBR v-Fos; isoleucine 61 to threonine in FBJ v-Fos), although there is no evidence that these mutations are responsible for the increased transforming activity of the viral genes (11, 50). Second, an overlapping mutation in the c-Fos protein (amino acids 58 to 116) defines a negative regulatory region, as assayed by in vitro transcription using recombinant proteins expressed in bacteria (1, 2). Third, the N-terminal region that we have defined contains seven serine or threonine residues that may be targets for phosphorylation. Whether any of these observations are related to results that we have described remains to be determined. A precedent for regulatory control of transcriptional activation by proteins of the AP1 complex is provided by the phosphorylation-induced changes in the activity of the c-Jun activation domain (8, 45).

The transforming activity of the mutant FosB2-VP16 D2-141 deserves comment. This protein consists of only 95 amino acids derived from FosB, corresponding to the BLZ motif, fused to the VP16 activation domain. Transformation by this protein implies that the only functions required for transformation by Fos proteins are dimer formation, high-affinity DNA binding, and a transcriptional activation function. Whether there are other specialized functions present on the Jun partner that are required remains to be determined.

In summary, we have shown that an N-terminal region as well as the BLZ motif and a C-terminal activation domain are required for neoplastic transformation by the FosB protein. Mutations in the N-terminal region do not affect DNA binding, and this region does not itself function as an activation domain but instead appears to regulate the transcriptional activation properties of the FosB-Jun complex. The presence of a constitutive activation domain is sufficient to abolish the requirement for N-terminal sequences. Establishing the biochemical mechanisms underlying these effects is a challenge for the future.

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