Fos Family Members Induce Cell Cycle Entry by Activating Cyclin D1

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Expression of the *fos* family of transcription factors is stimulated by growth factors that induce quiescent cells to reenter the cell cycle, but the cellular targets of the Fos family that regulate cell cycle reentry have not been identified. To address this issue, mice that lack two members of the *fos* family, *c-fos* and *fosB*, were derived. The *fosB*^{-/-} *c-fos*^{-/-} mice are similar in phenotype to *c-fos*^{-/-} mice but are 30% smaller. This decrease in size is consistent with an abnormality in cell proliferation. Fibroblasts derived from *fosB*^{-/-} *c-fos*^{-/-} mice were found to have a defect in proliferation that results at least in part from a failure to induce cyclin D1 following serum-stimulated cell cycle reentry. Although definitive evidence that c-Fos and FosB directly induce cyclin D1 transcription will require further analysis, these findings raise the possibility that c-Fos and FosB are either direct or indirect transcriptional regulators of the cyclin D1 gene and may function as a critical link between serum stimulation and cell cycle progression.

The intricate mechanism by which a cell exactly replicates its DNA and divides into two cells has long been a subject of fascination. Recent studies have revealed a complex layering of control mechanisms that ensure that the DNA synthesis and mitosis phases of the cycle occur only at appropriate times. This exquisite regulation is mediated by the sequential activation of members of a family of serine-threonine kinases called cyclin-dependent kinases (cdk's) (26, 39, 40). As the cell cycle progresses, particular cdk's become activated by associating with an appropriate cyclin. One crucial step for G₁ progression appears to be the induction of cyclin D1 expression during growth factor-stimulated cell cycle reentry (19, 27, 32, 33, 44). Cyclin D1 mRNA expression is significantly enhanced 4 to 6 h after growth factor addition, and a minimum level of cyclin D1 protein appears to be required for progression through G₁ (19, 24, 27, 44). During growth factor-stimulated cell cycle reentry, the induction of cyclin D1 mRNA expression requires activation of the Ras-dependent mitogen-activated protein kinase pathway (1, 2, 42) and is temporally preceded by the activation of a class of genes known as immediate-early genes (IEGs) (16). Several IEGs encode transcription factors that may regulate transcription of genes such as the cyclin D1 gene.

Among the best-characterized IEGs are members of the *c-fos* proto-oncogene family. Expression of *fos* family genes, which include *c-fos*, *fosB*, *fra-1*, and *fra-2*, is induced within minutes of growth factor addition to quiescent cells (16). Fos family proteins form heterodimers with members of the Jun or ATF family, and these complexes bind to the sequence element TGA(G/C)TCA (AP-1 site) or TGACGTCA (ATF site), respectively (5, 13, 14). By binding to specific sites within the regulatory region of target genes, these Fos complexes may regulate the transcription of late-response genes whose expression might be critical for cell cycle reentry.

Specific Fos family targets that could control cell cycle progression and the mechanism by which these targets might cou-

ple to the cell cycle machinery are unknown, although indirect evidence suggests that the cyclin D1 gene could be a target of the Fos family (15, 25, 44). Overexpression of c-Fos in fibroblasts was found to enhance the level of cyclin D1 mRNA (25), and conditions such as cellular senescence which lead to reduced levels of *c-fos* transcription also lead to reduced levels of cyclin D1 expression (44). Cyclin D1 promoter analysis has also suggested that c-Jun may activate cyclin D1 expression through a cyclic AMP response element (CRE) site at -52, though no role for c-Fos was reported (15).

Despite this suggestive data, the importance of *fos* family gene induction for cell cycle reentry and progression into S phase has been difficult to establish. Early experiments with antibody microinjection and antisense RNA suggested that blocking *c-fos* function inhibited fibroblast proliferation (17, 28, 34). However, no growth abnormalities have since been found in *c-fos*^{-/-} embryonic stem cells (10) or in primary or 3T3 *c-fos*^{-/-} fibroblasts (4, 18). In addition, the growth of *fosB*^{-/-} fibroblasts was found to be unimpaired (12). Evidence that multiple *fos* family genes cooperate to induce S-phase progression was provided by antibody microinjection studies which showed that the inhibition of *c-fos* or *fosB* or *fra-1* function alone only partially blocked cell cycle reentry, while inhibiting all three genes together effectively abolished cell cycle progression (22). These results raised the possibility that several *fos* family members together play a critical role in growth factor-stimulated cell cycle reentry. Experiments were therefore initiated to determine whether disruption of two *fos* family members, *c-fos* and *fosB*, would uncover a role for the *fos* family in cell proliferation and facilitate the identification of the cell cycle targets of Fos proteins.

MATERIALS AND METHODS

Generation of *fosB*^{-/-} *c-fos*^{-/-} mice. *c-fos*^{+/-} (C57BL/6 × 129Sv) mice were bred to *fosB*^{+/-} (BALB/c × 129Sv) mice to generate double heterozygotes that were interbred. Weights were determined for an entire litter simultaneously between the ages of 19 and 23 days.

Preparation of primary embryonic fibroblast cultures. On day 14.5 after plug, pregnant females were sacrificed by cervical dislocation and each embryo was trypsinized by standard techniques (35) and plated onto one gelatinized 10-cm dish in Dulbecco modified Eagle medium (DMEM) with 15% fetal bovine serum

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(FBS), glutamine, antibiotics, and nonessential amino acids. One day after plating, each dish was harvested, counted, and split into 4.6×10^6 cells per 10-cm dish. Four days later, dishes derived from one embryo were harvested, pooled, counted, and frozen as passage 2 with 4×10^6 cells per vial.

Prior to an experiment, one vial per 6-cm dish or two vials per 10-cm dish were thawed and plated on gelatin. Three days later, the cells were plated for the experiment. Each genotype (except *fosB*^{+/+} *c-fos*^{-/-}, for which only one line was obtained) was represented by at least two independent fibroblast lines, and usually three.

Growth curves. For low-density growth, fibroblasts at passage 3 were plated at 10,600 cells/cm² on approximately 12 duplicate plates (day 0). On days 1, 3, 5, 7, 9, and 11, two plates of each genotype were trypsinized and counted with a hemacytometer. All remaining plates were refed every 3 days. For sixfold-higher density, cells were plated at 62,000 cells/cm² and counted only until day 9. The low-density experiment was performed seven times, and the high-density experiment was performed four times.

[³H]thymidine incorporation. Cells were plated at a density of 300,000 per well of a six-well plate. Twenty-four hours later, the cells were starved in DMEM with 0.5% FBS. After 24 to 30 h of starvation, the fibroblasts were stimulated with 20% FBS, pulsed with 1 μ Ci of [³H]thymidine per ml for the last hour, and harvested. Each well was washed, scraped in 0.5 ml of phosphate-buffered saline (PBS), transferred to 5 ml of 0.1 mg of bovine serum albumin, and incubated on ice. Five milliliters of 20% trichloroacetic acid was added, and the tube was vortexed for 20 s prior to incubation on ice for 30 min. The solution was vacuum filtered onto a glass filter, washed with 10% trichloroacetic acid followed by 100% ethyl alcohol, and dried in air for 20 to 30 min prior to counting. This experiment was performed four times.

Incorporation of 5-bromo-2'-deoxyuridine (BrdU). A total of 5.5×10^4 cells were plated on a 12-mm glass coverslip in one well of a 24-well plate. Twenty-four hours later, the cells were starved and stimulated as described above. The cells were pulsed with 10 μ M BrdU for the last 2 h prior to fixation in 75% methanol–25% acetic acid. The coverslips were removed and stored in PBS–Triton–glycine. For staining, the coverslips were postfixed in 70% ethyl alcohol, washed, permeabilized in 0.5% Triton X-100 for 30 min, washed, treated with 2 N HCl for 30 min, neutralized in 0.1 M sodium borate (pH 8.5) for 10 min, and washed. Blocking was for 1 to 2 h in 3% bovine serum albumin–0.3% Triton X-100 in PBS. Each coverslip was incubated overnight at 4°C in anti-BrdU antibody (Becton Dickinson 347580) diluted 1:10 in blocking buffer plus 1% normal goat serum. The coverslips were washed and incubated in fluorescein-conjugated anti-mouse secondary antibody for 1 to 2 h at room temperature. The coverslips were washed, stained with freshly diluted Hoechst stain at 10 ng/ml in PBS for 8 min, and washed. Each coverslip was mounted with glycerol gelatin plus para-phenylenediamine at 100 μ g/ml.

For transfected cells, the same procedure was employed, but the cells were fixed in 4% paraformaldehyde–8% sucrose and an additional primary antibody, rabbit anti- β -galactosidase, was added at 1:250. The additional secondary antibody was Texas Red-conjugated anti-rabbit antibody, at 1:100.

Cyclin D1 immune complex kinase assays. Cyclin D1 immunoprecipitation kinase assays were performed as previously described (42, 43). Cells were harvested in ice-cold PBS and extracted in lysis buffer (150 mM NaCl, 50 mM HEPES [pH 7.2], 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 μ g of leupeptin per ml, and 0.1 mM sodium orthovanadate [Sigma Chemicals, St. Louis, Mo.]) at 4°C. Lysates were centrifuged at 10,000 $\times$ g for 5 min. Protein content was normalized by the Bio-Rad protein assay, and 100 μ g was used for each sample. The supernatants were precipitated for 12 h at 4°C with protein A-agarose beads precoated with saturating amounts of the cyclin D1 antibody, DCS-11 (NeoMarkers, Fremont, Calif.). Immunoprecipitated proteins on beads were washed twice with 750 μ l of lysis buffer and twice with kinase buffer (50 mM HEPES [pH 7.0], 10 mM MgCl₂, 5 mM MnCl₂, 1 mM dithiothreitol). The beads were then resuspended in 40 μ l of kinase buffer containing the protein substrate (2 μ g of soluble glutathione S-transferase–RB fusion protein), 10 μ M ATP, and 5 μ Ci of [γ -³²P]ATP (6,000 Ci/mmol; 1 Ci = 37 GBq [Amersham Corp., Arlington Heights, Ill.]). The samples were incubated for 25 min at 30°C with occasional mixing. The samples were boiled in polyacrylamide gel sample buffer containing sodium dodecyl sulfate and separated by electrophoresis. Phosphorylated proteins were quantified after exposure to autoradiographic film (LabsScientific Inc., Livingston, N.J.) by densitometry with ImageQuant version 1.2 (Molecular Dynamics Computing Densitometer [Sunnyvale, Calif.]).

Cell cycle antibodies. Antibodies against cyclin D1 were a gift from Michael Rivkin and Li-Huei Tsai. Polyclonal antibodies against cyclins D1, D2, and D3 were obtained from Chuck Sherr. Antibodies against cyclins E and A were obtained from Santa Cruz Biotechnology.

Transfection of mouse embryo fibroblasts for rescue of growth defect. Fibroblasts were plated at 3×10^5 cells on 12-mm glass coverslips in 3.5-cm dishes. Plasmids employed in transfections were prepared by double banding on CsCl gradients and included pON260 (cytomegalovirus [CMV]-*lacZ*) (6), pRcCMV, CMV/*fos* Δ Xho (7), and pBBB and pF4 (38). pF4 contains the *c-fos* gene under the control of its native promoter; pBBB contains the β -globin gene under the control of the *c-fos* promoter. For rescue, 0.5 μ g of pON260 was mixed with 1.5 μ g of pRcCMV^{+/+} expression gene in 100 μ l of Opti-Mem buffer. Six microliters of Lipofectamine (Gibco) was mixed with 100 μ l of Opti-Mem buffer, transferred

to the DNA mix, and incubated for 45 min at room temperature prior to addition of 0.8 ml of serum-free DMEM. The fibroblasts were washed in serum-free DMEM, and the transfection mix was added. The fibroblasts were incubated for 5 h at 37°C and washed in complete medium. Medium was changed again approximately 4 h following transfection. For continuous cycling conditions, the fibroblasts were refed complete medium 4 h after transfection. Twenty-four hours later, BrdU was added to a 10 μ M concentration and incubation at 37°C was continued for an additional 16 h. The coverslips were fixed in 4% paraformaldehyde–8% sucrose in PBS prewarmed to 37°C and were stored at 4°C in PBS–Triton–glycine.

Cyclin D1 promoter analysis. Cyclin D1 promoter constructs employed included –1745CD1LUC, –964CD1LUC, –964CD1LUCmtAP-1, –163CD1LUC, –66CD1LUC, –66CD1LUCmtATF, and pA₃LUC (2, 42). Fibroblasts of the appropriate genotype were plated at 2×10^5 to 2.5×10^5 cells per 3.5-cm dish. Twenty-four hours later, each well was transfected with 1.5 to 2 μ g of DNA with 10 μ l of Lipofectamine as described above. Luciferase construct (0.75 to 1 μ g) was transfected with 0.5 to 0.75 μ g of empty vector (pRcCMV or pBBB) or *c-fos* expression vector (CMV/*fos* Δ Xho or pF4). In early experiments, 0.5 μ g of elongation factor-chloramphenicol acetyltransferase was included as a control for variations in transfection efficiency. Four hours after transfection, the fibroblasts were placed in starvation medium (0.5% FBS) for 12 to 14 h. Each well was then left unstimulated or stimulated for 7 h with 20% FBS. The fibroblasts were harvested and luciferase readings were obtained as described in Promega Technical Bulletin no. 161.

Electrophoretic mobility gel shift assays. Electrophoretic mobility gel shift assays with nuclear extracts or in vitro-translated proteins were performed as described previously (2, 14, 31). Nuclear extracts were prepared according to the method of Albanese et al. (2), and c-Fos and c-Jun proteins were generated with a rabbit reticulocyte lysate system (Promega).

Statistical analysis. Data analysis was performed with the program StatView. All data were analyzed by repeated-measures analysis of variance or paired *t* tests.

RESULTS

To generate mice carrying mutations in the *c-fos* and *fosB* genes, mice heterozygous for each single mutation were interbred (3, 20). *fosB*^{-/-} *c-fos*^{-/-} mice were born at the normal Mendelian frequency and found to have the same defects previously detected in the *c-fos*^{-/-} mouse, namely, osteopetrosis, small size, and a failure of tooth eruption (data not shown). The survival of *c-fos*^{-/-} mice was not influenced by their *fosB* genotype, and no obvious anatomic or pathologic differences were observed when *c-fos*^{-/-} and *fosB*^{-/-} *c-fos*^{-/-} mice were compared (data not shown). However, the measurement of body weight of *c-fos*^{-/-} and *fosB*^{-/-} *c-fos*^{-/-} mice revealed that *fosB*^{-/-} *c-fos*^{-/-} mice are significantly smaller than *c-fos*^{-/-} mice. On average, the *fosB*^{-/-} *c-fos*^{-/-} mice are 30% smaller than *c-fos*^{-/-} mice at approximately 3 weeks of age (Fig. 1). One possibility is that the decreased size of the *fosB*^{-/-} *c-fos*^{-/-} mice is due to an impairment in cell proliferation. Although we have not established whether impaired proliferation during development is the explanation for the decreased size of the *fosB*^{-/-} *c-fos*^{-/-} mice, the analysis described below of fibroblasts from these mice revealed that they are defective in their ability to reenter the cell cycle after growth arrest.

As previously shown, wild-type, *fosB*^{-/-} *c-fos*^{+/+}, and *fosB*^{+/+} *c-fos*^{-/-} fibroblasts were found to proliferate exponentially in culture and to efficiently reenter the cell cycle from G₀ upon serum stimulation (Fig. 2a). In contrast, *fosB*^{-/-} *c-fos*^{-/-} fibroblasts proliferated very poorly in continuous culture (Fig. 2a) and, upon serum stimulation following G₀ arrest, traversed G₁ inefficiently and failed to enter S phase at a significant rate as shown by BrdU staining and [³H]thymidine incorporation (Fig. 2b and c). This defect in S-phase entry was also observed with BrdU staining in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts in continuous cycling conditions (see Fig. 3c and 5b). Despite their failure to proliferate, *fosB*^{-/-} *c-fos*^{-/-} fibroblasts remained attached to the tissue culture dish, showed no evidence of apoptotic cell death, and appeared healthy for up to 2 weeks (data not shown). Interestingly, *fosB*^{-/-} *c-fos*^{+/+} fi-

FIG. 1. Average weights of *fosB*^{-/-} *c-fos*^{-/-} mice (double) and *c-fos*^{-/-} mice (*c-fos*) compared to *fosB*^{-/-} mice (*fosB*) and wild-type mice (wt). The weight of each animal in five litters containing *fosB*^{-/-} *c-fos*^{-/-} mice was determined between 19 and 23 days of age and combined with previous data on wild-type and *fosB*^{-/-} mice at the same ages. The difference in weight between *fosB*^{-/-} *c-fos*^{-/-} mice and *c-fos*^{-/-} mice is statistically significant ($P < 0.03$). Between wild-type or *fosB*^{-/-} mice and *fosB*^{-/-} *c-fos*^{-/-} mice or *c-fos*^{-/-} mice, all P values are <0.0001 .

broblasts proliferated normally in culture, while *fosB*^{+/-} *c-fos*^{-/-} fibroblasts were as defective in their proliferative capacity as *fosB*^{-/-} *c-fos*^{-/-} fibroblasts (Fig. 2a and c). This difference in the efficacy with which a single copy of *c-fos* or *fosB* promotes fibroblast proliferation may be due to a difference between *c-fos* and *fosB* either in their specific functions or in their levels of expression.

In contrast to *fosB*^{-/-} *c-fos*^{-/-} fibroblasts plated at low to moderate density, *fosB*^{-/-} *c-fos*^{-/-} fibroblasts plated at high density proliferate normally in culture (Fig. 2d). This observation suggests that, at lower plating densities, a *c-fos*- and *fosB*-dependent pathway is critical for G₁ progression and entry into S phase but that at high plating densities, other pathways that can substitute for the *c-fos*- and *fosB*-dependent pathway may be activated.

The failure of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts grown at lower densities to efficiently enter S phase was not due to a general impairment in the response of these cells to serum. Northern analyses indicated that the patterns of gene expression that characterize the proliferative response to serum are intact in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts grown at low density. The timing and extent of induction of the IEGs *c-myc*, *nur77*, *zif268*, and *junB* are similar in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts and in wild-type fibroblasts, although IEG expression did remain elevated for a longer period of time in the mutant fibroblasts (Fig. 3a and data not shown). This prolonged elevation in IEG levels in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is consistent with the results of transient transfection studies showing that c-Fos and FosB regulate the shutoff of IEG transcription (11, 29). The sustained induction of IEGs is unlikely to be related to our finding that S-phase entry is impaired because elevated levels of IEGs usually correlate with improved entry into S phase (25, 37, 45).

In addition to IEG induction, several later events in G₁ occur normally in the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. The delayed-response genes encoding cyclin D2 and the protease transin are induced normally when quiescent *fosB*^{-/-} *c-fos*^{-/-} fibroblasts are exposed to serum (Fig. 3b). Growth factor stimulation of transin has been shown previously to be mediated by an AP-1 site that is present within the regulatory region of the transin gene (8, 21). The induction of transin in response to platelet-derived growth factor and epidermal growth factor,

but not 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), was found to be impaired in a *c-fos*^{-/-} established 3T3 cell line (18). Our finding that serum induces transin normally in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is most likely consistent with these observations, since addition of serum to *fosB*^{-/-} *c-fos*^{-/-} fibroblasts probably mimics the effect of factors such as TPA that are capable of inducing normal transin expression in the established *c-fos*^{-/-} cell line used by Hu et al. (18). The efficacy with which transin and other delayed-response genes are induced in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts may reflect the ability of *fra-1*, *fra-2*, or other genes to compensate for the loss of *c-fos* and *fosB*. Taken together, these experiments suggest that the program of gene expression induced during serum stimulation of wild-type fibroblasts is primarily intact in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts and that the growth abnormality in the mutant fibroblasts may be due to a specific rather than a global defect.

If, as expected, the growth defect in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is a direct result of the loss of *c-fos* and *fosB*, and not secondary to a developmental defect or a random mutation, the expression of *c-fos* or *fosB* in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts should restore the ability of these double mutant fibroblasts to enter S phase. To explore this possibility, *c-fos* was reintroduced into the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. *c-fos* was chosen because the presence of a single allele of *c-fos*, but not *fosB*, was sufficient for normal cell proliferation in the cell cycle studies described above. Fibroblasts were transfected with either a CMV-*c-fos* expression vector or an empty CMV expression vector, together with CMV-*lacZ* to mark the transfected cells. DNA synthesis was assessed in two experimental paradigms, one in which the fibroblasts were continually cycling for approximately 42 h after transfection (Fig. 3c) and another in which the fibroblasts were serum starved for 12 h and then stimulated by the addition of 20% FBS (data not shown). The results were the same in both paradigms. Transfection of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts with CMV-*c-fos* led to a significant increase in the percentage of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts incorporating BrdU while having little effect on BrdU incorporation in wild-type fibroblasts. The *fosB*^{-/-} *c-fos*^{-/-} fibroblasts transfected with CMV-*c-fos* entered S phase at a rate similar to that of the wild-type fibroblasts (Fig. 3c). These findings suggest that *c-fos* expressed by transfection can act in G₁ to rescue the growth defect of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. Thus, the defect in cell cycle progression in the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is strictly due to the absence of *c-fos* and *fosB* and is not secondary to some other perturbation of these cells.

To identify the critical targets of c-Fos and FosB, we investigated whether the regulation of components of the cell cycle machinery was altered in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts during the G₁ phase of the cell cycle. We found that, although the levels of cyclin D1 mRNA and protein are induced in wild-type fibroblasts within a few hours of serum stimulation (Fig. 4a), the exposure of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts to serum failed to induce cyclin D1 mRNA or protein (Fig. 4a and c), even though cyclin D2 was induced in the mutant cells (Fig. 3b). We therefore assessed the level of cyclin D1-associated kinase activity with a truncated retinoblastoma protein as substrate. In wild-type fibroblasts, kinase activity was induced approximately fourfold by 8 to 12 h of serum stimulation, while no induction was seen in the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts (Fig. 4d). Although cyclin D1-associated kinase activity in wild-type fibroblasts falls at 18 h before rising again at 24 to 28 h (Fig. 4d and data not shown), cyclin D1 mRNA levels remain elevated throughout this period. This prolonged elevation in cyclin D1 mRNA may reflect an effect on mRNA stability or a loss of synchrony of the serum-stimulated cell population.

FIG. 2. (a) Fibroblast growth at low density. On the indicated day after plating, two plates were harvested and counted. *fosB*^{-/-} *c-fos*^{-/-} versus *fosB*^{+/-} *c-fos*^{-/-}, no significant difference by repeated-measures analysis of variance (see Materials and Methods). Between the four genotypes that grow well and the two genotypes that grow poorly, all *P* values are <0.0001 . (b) BrdU incorporation following 20 h of stimulation (upper panels) and Hoechst staining of the same fields (lower panels). Magnification, ca. $\times 33$. (c) Incorporation of [^3H]thymidine into DNA following serum starvation and stimulation for the indicated number of hours. *fosB*^{+/+} *c-fos*^{+/+} versus *fosB*^{+/-} *c-fos*^{-/-}, *P* = 0.001; *fosB*^{+/+} *c-fos*^{+/+} versus *fosB*^{-/-} *c-fos*^{-/-}, *P* = 0.0003; *fosB*^{+/-} *c-fos*^{-/-} versus *fosB*^{-/-} *c-fos*^{-/-}, no significant difference. (d) Fibroblast growth at high density. No significant differences in cell number were found.

FIG. 3. (a and b) Northern blots. The upper panels include the gene of interest, and the lower panels include glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each lane contains 10 μ g of total RNA, and all times are hours of stimulation. Shown are two representative IEGs, *zif268* and *junB* (a), and two late genes, cyclin D2 and transin (b). (c) Rescue of S-phase entry by expression of *c-fos* in continuous cycling conditions. White bars, vector-transfected fibroblasts; black bars, *c-fos*-transfected fibroblasts. Vector-transfected versus *c-fos*-transfected *fosB*^{-/-} *c-fos*^{-/-} fibroblasts, $P = 0.006$.

The failure of serum to induce cyclin D1 mRNA, protein, and associated cdk activity in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts may explain the defect in cell cycle progression observed in these mutant cells. In support of this possibility, the failure of serum to induce cell cycle reentry correlated with the failure of serum to induce cyclin D1 mRNA expression. Serum stimulation of single mutant *fosB*^{-/-} or *c-fos*^{-/-} fibroblasts effectively induced cyclin D1 mRNA expression and cell cycle reentry (data not shown). By contrast, *fosB*^{-/-} *c-fos*^{-/-} fibroblasts failed to induce either cyclin D1 mRNA expression or cell cycle reentry in response to serum (data not shown).

To determine whether the decreased expression of cyclin D1 in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is specific, the levels of expression of several additional cyclins were determined in wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. Both cyclin D2 and cyclin E proteins were expressed at similar levels in wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts (data not shown). However, the induction of cyclin A protein was delayed for several hours in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts, and its peak level was substantially reduced relative to the level detected in wild-type cells (Fig. 4c). Since the induction of cyclin A protein is reduced in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts, the levels of cyclin A mRNA in

FIG. 4. (a) Northern blot of cyclin D1 mRNA following serum starvation and stimulation for the indicated number of hours, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. The graphed values are means $\pm$ standard errors of cyclin D1 values normalized to GAPDH values for four Northern blots quantitated on a phosphorimager. *fosB*^{+/+} *c-fos*^{+/+} versus *fosB*^{-/-} *c-fos*^{-/-}, $P < 0.04$. (b) Cyclin A mRNA induction following serum starvation and stimulation, with GAPDH control. (c) Western blots of cyclins D1 (top) and A (bottom) following serum starvation and stimulation for the indicated number of hours. (d) Cyclin D1-associated cdk activity, with glutathione *S*-transferase-RB as substrate. Immune-complex kinase assays were performed on lysates from wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts following serum starvation and stimulation for the indicated number of hours. The graphed values represent quantitation of the experimental data shown in the figure, which is representative of three performed; in this case, the wild type is actually *fosB*^{+/+} *c-fos*^{+/+}. Horizontal axis, number of hours of stimulation.

wild-type fibroblasts and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts were compared. No difference was seen in levels of cyclin A mRNA (Fig. 4b) between the mutant and wild-type cells, suggesting that the disruption of *c-fos* and *fosB* does not affect cyclin A transcription. Thus, the effect of the *c-fos* and *fosB* mutations on cyclin A protein expression may be a secondary conse-

quence of the alteration in cyclin D1 mRNA and protein expression that occurs earlier in G₁.

The analysis of cyclin expression in wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts suggests that a critical function of *c-fos* and *fosB* in wild-type fibroblasts is to promote cell cycle progression by either directly or indirectly stimulating the expression

FIG. 5. (a) Growth curve of cyclin D1^{-/-} fibroblasts cultured at densities similar to those in Fig. 2a. Cell numbers were determined on the indicated days. Each point for cyclin D1^{-/-} fibroblasts represents the mean value $\pm$ standard error of four experiments conducted at least in duplicate. Each point for wild-type fibroblasts represents the mean value $\pm$ standard error of at least two experiments conducted in duplicate. Cyclin D1^{+/+} versus cyclin D1^{-/-}, $P < 0.03$. (b) Rescue of S-phase entry by expression of cyclin D1 in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts in continuous cycling conditions. The ordinate shows the percentage of transfected cells that had incorporated BrdU. CMV indicates vector-transfected fibroblasts, and CMV cyclin D1 indicates cyclin D1-transfected fibroblasts. P is 0.008 for the difference between vector-transfected and cyclin D1-transfected *fosB*^{-/-} *c-fos*^{-/-} fibroblasts, and P is 0.0002 for the same difference in wild-type fibroblasts.

of cyclin D1 mRNA and protein. If the failure to induce cyclin D1 mRNA during cell cycle reentry is the primary explanation for the defect in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts, expression of cyclin D1 should be able to rescue the fibroblasts' defect in cell cycle progression. To address this question, fibroblasts were transiently transfected with a CMV-cyclin D1 or empty CMV expression vector and studied both while they were continuously cycling and following serum starvation and stimulation. Transfection of the cyclin D1 expression vector, but not the empty vector, effectively rescued S-phase entry in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts following serum stimulation (data not shown) or while they were continuously growing (Fig. 5b). Expression of cyclin D1 was not sufficient to initiate S-phase entry in serum-starved fibroblasts of either genotype (data not shown), suggesting that cyclin D1 acts together with other serum-inducible factors to regulate S-phase entry. Ectopic expression of cyclin D1 also significantly increased the percentage of wild-type fibroblasts entering S phase (Fig. 5b). Since increasing the level of cyclin D1 in wild-type fibroblasts also stimulates cell cycle reentry, the effect of ectopic cyclin D1 expression on the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts may reflect a function of cyclin D1 other than cyclin D1's ability to rescue the defect in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. Nevertheless, the ability of cyclin D1 to enhance S-phase entry in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is consistent with our hypothesis that loss

of cyclin D1 induction in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is a significant contributor to their proliferation defect.

If the low level of cyclin D1 expression in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is a determining factor that prevents these fibroblasts from entering S phase, then fibroblasts from a cyclin D1-deficient mouse (9, 41) should display a similar defect in proliferation when cultured in vitro. Although a previous study failed to identify such a defect (9), our analysis revealed that when cyclin D1^{-/-} fibroblasts are plated at lower densities, they proliferate poorly and reenter the cell cycle inefficiently, as was seen with *fosB*^{-/-} *c-fos*^{-/-} fibroblasts (Fig. 5a). Our preliminary results suggest that plating cyclin D1^{-/-} fibroblasts at higher densities improves their ability to grow (data not shown), similar to our findings with *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. These observations indicate that, at least under certain conditions of growth in culture, cyclin D1 expression is critical for efficient cell cycle reentry. Taken together, these results suggest that the proliferative defect detected in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is due at least in part to the failure to induce cyclin D1 expression as the mutant fibroblasts traverse G₁.

Since serum induction of c-Fos and FosB proteins occurs within 1 to 2 h and persists for at least several hours (data not shown), the temporal course of Fos family protein and cyclin D1 induction is such that Fos family members are active for a significant period prior to cyclin D1 induction. Experiments were therefore undertaken to determine if a reporter gene driven by the cyclin D1 promoter was differentially responsive to serum when transfected into wild-type versus *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. A reporter plasmid containing 1,745 bp of the cyclin D1 upstream regulatory region linked to the firefly luciferase gene (-1745CD1LUC) was transfected into wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts together with the appropriate Fos expression constructs or empty vector controls. In wild-type fibroblasts, the cyclin D1 promoter was reproducibly induced by serum within 6 h (Fig. 6a). Although the induction of luciferase activity is only approximately twofold, this effect is similar in magnitude to the induction of cyclin D1 seen with Northern blotting (Fig. 4a) and is highly reproducible. In the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts, basal expression from the cyclin D1 promoter-driven luciferase gene was reduced by 30% compared to that for wild-type fibroblasts, and serum failed to induce the promoter (Fig. 6a). However, expression from -1745CD1LUC could be rescued in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts by cotransfection with *c-fos*, indicating that cyclin D1 promoter induction can be restored by *c-fos* function (Fig. 6a). The absolute levels of luciferase activity in *c-fos*-transfected and serum-stimulated *fosB*^{-/-} *c-fos*^{-/-} fibroblasts were generally comparable to the absolute levels of luciferase activity in stimulated wild-type fibroblasts. These results are consistent with the hypothesis that c-Fos and FosB induce the cyclin D1 promoter, either directly or indirectly via activation of other transcriptional regulators. However, these findings do not rule out the existence of other mechanisms of cyclin D1 induction or the possibility that additional Fos family targets contribute to cell cycle progression.

The cyclin D1 promoter contains two sites that could potentially mediate a direct induction by Fos family proteins in response to serum; a classic AP-1 site at approximately -950 and a CRE/ATF site at approximately -60. To identify the serum-responsive element in these primary fibroblasts, a series of cyclin D1 promoter deletion constructs were transfected into wild-type and mutant fibroblasts (2, 42). Although the absolute level of expression decreased incrementally as the promoter was shortened, the serum inducibility of the cyclin D1 promoter constructs in wild-type fibroblasts was reproduc-

FIG. 6. (a) Induction of the cyclin D1 promoter. Wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts were cotransfected with a cyclin D1-luciferase reporter construct (-1745CD1LUC) and an empty vector control (CMV or pBBB) or a *c-fos*-containing expression vector (CMV *c-fos* or pF4). Fold induction is the mean $\pm$ standard error of luciferase activity in serum-stimulated samples normalized to the mean $\pm$ standard error of luciferase activity in serum-starved samples, for two experiments repeated in triplicate. (b) (Left) Induction of -66CD1LUC. Wild-type (wt) and *fosB*^{-/-} *c-fos*^{-/-} (mt) fibroblasts were cotransfected with a cyclin D1-luciferase reporter construct (-66CD1LUC) and an empty vector control (CMV or pBBB) or a *c-fos*-containing expression vector (pF4). "stim" represents luciferase activity of serum-stimulated samples divided by luciferase activity of serum-starved samples, all cotransfected with CMV or pBBB; *P* is 0.003 for the difference between wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. pF4 represents luciferase activity of serum-stimulated samples cotransfected with pF4 divided by luciferase activity of serum-starved samples; no significant difference between the wild type and the mutant was found. (Right) Effect of mutation of the CRE/ATF site of -66CD1LUC on luciferase activity in serum-starved (unstim) and serum-stimulated (stim) wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. wt66 is -66CD1LUC with a wild-type CRE/ATF site, and mut66 is -66CD1LUC with three point mutations in the CRE/ATF site. The ordinate shows the absolute value of luciferase activity. *P* is <0.0001 for the difference between the wt66 and mut66 constructs; *P* is 0.001 for the difference between wild-type and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. (c) Gel mobility shift analyses with the CRE/ATF element of the cyclin D1 promoter and lysates from fibroblasts serum starved and stimulated for the indicated times. Lysates were incubated with either nonimmune serum or antibodies to c-Jun, c-Fos, or CREB/CREM. F, c-Fos-containing complexes; C, CREB/CREM-containing complexes; SS, supershifted complexes. 1, wild type; 2, *fosB*^{-/-} *c-fos*^{-/-}. (d) Gel mobility shift analysis with in vitro-transcribed and -translated c-Fos and c-Jun. F and SS are as defined above.

ibly maintained for each of the constructs tested, including a construct (-66CD1LUC) that contained only 66 nucleotides 5' of the start site of initiation of cyclin D1 mRNA synthesis (Fig. 6b). In contrast to the results with wild-type fibroblasts, the -66CD1LUC construct was only minimally induced when transfected *fosB*^{-/-} *c-fos*^{-/-} fibroblasts were stimulated with serum (Fig. 6b). The failure of serum to induce the -66CD1LUC construct in the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts was due to the disruption of *c-fos*, since cotransfection of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts with the *c-fos* expression plasmid pF4 restored the inducibility of the -66CD1LUC construct (Fig. 6b).

The proximal 66 nucleotides of the cyclin D1 promoter include the CRE/ATF-like sequence 5' TAACGTC 3', which is capable of binding CREB/CREM proteins in electrophoretic mobility shift assays (42). Fos-Jun complexes are known to

bind to a similar sequence, 5' TGA(C/G)TCA 3' (5). The CRE/ATF element of the cyclin D1 promoter differs by only one nucleotide from a sequence that binds heterodimers of Fos family members and the CREB-related transcription factor ATF4 (14). To examine the importance of the CRE/ATF element for serum induction, clustered point mutations were introduced into the region of the -66CD1LUC construct that binds CREB proteins (42). The basal activity of this -66CD1LUC-ATF mutant reporter construct was reduced by 90%, and its inducibility was decreased, although some induction remained (Fig. 6b). These results indicate that the CRE/ATF element at -60 contributes to basal expression and may contribute to serum induction of cyclin D1. Although these experiments suggest that the Fos family may directly regulate cyclin D1 via the proximal 66 nucleotides of the cyclin D1

promoter, the findings should be viewed with caution. Given the complexity of the cyclin D1 promoter, the incremental decrease in absolute promoter induction that is observed as the cyclin D1 promoter is truncated, and the small magnitude of the effect of c-Fos expression on -66CD1LUC, it is likely that transcription factors in addition to Fos regulate cyclin D1 transcription. In addition, although the cyclin D1 promoter truncation experiments did not provide evidence for the involvement of the AP-1 site at -950 in serum induction of cyclin D1 transcription (data not shown), it remains possible that the AP-1 site and/or other promoter sites may play a role in the induction of endogenous cyclin D1 expression.

To determine if Fos family members might be capable of regulating cyclin D1 transcription by interacting directly with the CRE/ATF element, we characterized the nature of the complexes present in fibroblast nuclear extracts that bind to this cyclin D1 promoter element and assessed whether these complexes contain c-Fos. Electrophoretic mobility shift assays were performed with a 23-bp double-stranded oligonucleotide that encompasses the CRE/ATF site. Two protein complexes, which were present at very low levels in extracts from serum-starved wild-type fibroblasts but were expressed at higher levels following serum stimulation and bound specifically to the CRE/ATF site, were identified (Fig. 6c, complexes C and F). By contrast, extracts from serum-stimulated *fosB*^{-/-} *c-fos*^{-/-} fibroblasts expressed very low levels of these protein complexes (Fig. 6c). Addition of anti-c-Fos specific antibodies to the extracts of wild-type fibroblasts significantly reduced the formation of both complex C and complex F (Fig. 6c), suggesting that both complexes contain c-Fos. One of the complexes also contains Jun family proteins, since anti-c-Jun antibodies were able to cause a supershift. Complex C contains CREB-related proteins since anti-CREB/CREB antibodies both reduced the abundance of and caused a partial supershift of complex C. Since multiple protein complexes clearly interact with the 5' TAACGTC 3' element, verification of the significance of Fos family-containing complexes detected in vitro will require further analysis. However, our observation that among the protein complexes that bind to the CRE/ATF element there are some that are serum inducible and are recognized by anti-c-Fos antibodies suggests that members of the Fos family, c-Fos and FosB, can bind directly to the cyclin D1 promoter. The capacity of Fos-containing complexes to bind this promoter site in vitro was demonstrated by mobility shift analysis with in vitro-transcribed and -translated c-Fos and c-Jun. While neither c-Fos nor c-Jun alone nor a mixture of c-Fos and ATF4 bound to the CRE/ATF element, c-Fos and c-Jun together bound specifically to this sequence (Fig. 6d). Taken together, these DNA mobility shift analyses suggest that Fos family members are capable of binding to the cyclin D1 CRE/ATF element in vitro, albeit more weakly than to an AP-1 site. Given the complexity of the cyclin D1 promoter, the probability that multiple factors are binding to the cyclin D1 promoter simultaneously, and the relatively weak binding of Fos family proteins to the CRE/ATF site, further experimentation will be needed to determine definitively whether the Fos family plays a role directly in the regulation of the cyclin D1 gene.

DISCUSSION

In this study, we have described the generation and initial characterization of *fosB*^{-/-} *c-fos*^{-/-} mice, which are similar in phenotype to *c-fos*^{-/-} mice but significantly smaller in size. Their smaller size does not appear to reflect worsened osteopetrosis or other organ dysfunction. Further studies will be required to determine whether the size of *fosB*^{-/-} *c-fos*^{-/-}

mice reflects a cell-autonomous effect on cell growth or proliferation or a nonspecific effect. However, the decreased size of the *fosB*^{-/-} *c-fos*^{-/-} mice may be a manifestation in vivo of a mild impairment in cell proliferation. Consistent with this hypothesis is our observation that dense cultures of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts grow normally in culture, while the growth of lower-density *fosB*^{-/-} *c-fos*^{-/-} and *fosB*^{+/-} *c-fos*^{-/-} fibroblasts is severely impaired. This impairment in the growth of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is due to a defect in S-phase entry which correlates with a specific loss of cyclin D1 induction following serum stimulation.

The loss of normal cyclin D1 expression and D1-associated kinase activity in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts may explain the growth impairment seen in these cells. Our observations that cyclin D1^{-/-} fibroblasts have a proliferation defect and that cyclin D1 expression in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts restores proliferation are consistent with a significant role for cyclin D1 in the growth deficiency of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. Assays of cyclin D1 promoter activity suggest that serum induction of the cyclin D1 promoter in primary fibroblasts is dependent on intact *c-fos* and *fosB* genes. These findings establish a functional role for c-Fos and FosB in the cell cycle and suggest that one mechanism by which these two IEGs promote S-phase entry is via cyclin D1.

It remains unclear whether the failure of cyclin D1 induction in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is sufficient to explain the severe growth defect detected in these cells. *fos* family genes may have as-yet-unidentified targets which are also critical for S-phase entry. In addition, other pathways may converge on the activation of cyclin D1. Nonetheless, a number of lines of evidence support our hypothesis that failure of cyclin D1 induction is a major contributor to growth failure in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts. First, the only other cell cycle-related molecular abnormality that we have identified in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is the reduced level of cyclin A protein. Since the reduction in cyclin D1 precedes the reduction in cyclin A, the cyclin A changes may be secondary to the changes in cyclin D1. Second, we have found that expression of cyclin D1 in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is sufficient to restore their progression through the cell cycle, consistent with the hypothesis that c-Fos and FosB activate cell cycle progression via cyclin D1. Third, we have shown that fibroblasts that lack cyclin D1 show a similar density-dependent defect in cell cycle progression. Given that a threshold abundance of cyclin D1 is known to be critical for G₁ progression (30, 32, 33), the reduction in basal and serum-induced cyclin D1 expression in the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts could be responsible for their failure to progress into S phase. That the reduction in cyclin D1 levels is due to the loss of c-Fos and FosB is consistent with previous studies, which have shown that Fos overexpression induces cyclin D1 (25). In addition, promoter analyses have suggested a role for Fos and/or Jun family proteins in the activation of cyclin D1 (30, 44).

The only other molecular defect observed to date in the *fosB*^{-/-} *c-fos*^{-/-} fibroblasts is a prolonged elevation in IEG levels following serum stimulation. The down-regulation of IEGs has been proposed to be important for effective cell cycle control, since increased IEG levels due to overexpression have been associated with enhanced S-phase entry. In *fosB*^{-/-} *c-fos*^{-/-} fibroblasts where the induction of cyclin D1 expression is compromised, this prolonged elevation of IEGs is not sufficient to lead to enhanced cell proliferation. However, the fact that IEG transcription remains elevated in *fosB*^{-/-} *c-fos*^{-/-} fibroblasts provides evidence that c-Fos and FosB play a critical role in the shutoff of transcription of multiple IEGs, as has been suggested by transfection assays in which both

c-Fos and FosB were shown to repress the activity of the *c-fos* and *fosB* promoters (11, 23).

Our observation that *fosB*^{+/-} *c-fos*^{-/-} fibroblasts are as impaired as *fosB*^{-/-} *c-fos*^{-/-} fibroblasts suggests that a single allele of *fosB* is not sufficient to lead to activation of cyclin D1 or other target genes to levels high enough to restore cell cycle progression. The observation that the gene dosage of *fosB* is critically important is interesting but not unprecedented. In the case of *MyoD* and *myf-5*, although *MyoD*^{-/-} *myf-5*^{+/+} mice are normal, *MyoD*^{-/-} *myf-5*^{+/-} mice show a partial impairment in muscle formation that is severe enough to result in their perinatal death (36). The apparent difference in the potency of single alleles of *c-fos* and *fosB* implies that the two loci must have some functional difference(s). One possibility is that the alternatively spliced short *fosB* mRNA encodes a protein that acts in a dominant negative manner and therefore reduces the net activity of full-length FosB. Alternatively, it may be that a certain minimum total amount of c-Fos and FosB is required and that c-Fos is expressed at a higher level than FosB.

Our observation that the growth defect of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts can be overcome by plating at very high densities raises interesting questions. First, it is important to note that the lowest plating density used in our experiments is comparable to or higher than the plating densities used in many previously described studies of cell cycle progression (4, 18), and our observation of impaired growth is quite obvious and dramatic under standard experimental conditions. Why this proliferation defect would be overcome at high cell density is unclear: the cells may secrete more growth factors to condition the medium, or they may be stimulated by cell-cell contact. A preliminary experiment suggested that conditioned medium from cells growing at high density was not sufficient to rescue the growth of cells at low density (27a). Furthermore, in BrdU incorporation experiments we have noticed a sometimes striking local effect of cell density, where individual cells within clumps have a higher rate of S-phase entry than do well-spread cells. These observations suggest that cell-cell interactions may be critical to the ability of *fosB*^{-/-} *c-fos*^{-/-} fibroblasts to effectively progress into S phase. Whatever the mechanism, S-phase entry occurs in a *fos*-independent manner in dense cultures. Interestingly, we have observed that the growth of cyclin D1^{-/-} fibroblasts also appears to be improved by plating them at high density (data not shown). This result suggests that the mechanism of S-phase entry at high density is not only *fos* independent, but also cyclin D1 independent, and may instead involve the activation of other cyclins.

Since the growth deficiencies of cyclin D1^{-/-} and *fosB*^{-/-} *c-fos*^{-/-} fibroblasts may share some properties, it is of interest that both cyclin D1^{-/-} and *fosB*^{-/-} *c-fos*^{-/-} mice are substantially growth impaired. At approximately 3 weeks of age, both cyclin D1^{-/-} and *fosB*^{-/-} *c-fos*^{-/-} mice are approximately 50 to 60% smaller than wild-type mice (9, 41). Although the interpretation of this result is complicated by osteopetrosis in the *fosB*^{-/-} *c-fos*^{-/-} mice, the finding is striking and suggests the possibility that Fos family-mediated activation of cyclin D1 may have significance in vivo. Nevertheless, there are clearly alternative pathways for activating cyclin D1, as evidenced by retinal and mammary defects in cyclin D1^{-/-} mice that have not as yet been detected in the *fosB*^{-/-} *c-fos*^{-/-} mice (27b). The lack of coincidence of the retinal defects in the two different mutant mice may be explained by the continued expression of other Fos family members in the retina of the *fosB*^{-/-} *c-fos*^{-/-} mice or by an alternative Fos family-independent mechanism for the upregulation of cyclin D1 in retinal cells. It would not be surprising if the mechanisms of activation of cell cycle genes were cell type specific or if the cell-type-specific

abundance of particular Fos family proteins dictated the phenotype observed in animals lacking one or two family members. In support of this hypothesis, distinct protein complexes bind to the serum-responsive regions of the cyclin D1 promoter in different cell types (31a).

Given the striking effect on fibroblast proliferation, the phenotype of the *fosB*^{-/-} *c-fos*^{-/-} mouse is relatively subtle. Although the size difference between *c-fos*^{-/-} and *fosB*^{-/-} *c-fos*^{-/-} mice is significant and suggestive of an effect on cell proliferation in vivo, any associated loss of viability seems minimal. It may be that compensation by *fos*-independent pathways exists in vivo just as it does at high cell density in vitro. If so, additional experiments might identify other manifestations of this proliferative defect in vivo. For example, wound healing may be an in vivo equivalent of low growth density and might therefore be abnormal in the *fosB*^{-/-} *c-fos*^{-/-} mice. In addition, *fosB*^{-/-} *c-fos*^{-/-} mice may be found to be unusually resistant to tumorigenesis or to show impaired proliferation in other cell types such as lymphocytes. Further experiments will be required to identify the full range of manifestations of the defect in cell proliferation in *fosB*^{-/-} *c-fos*^{-/-} mice.

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