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Transcriptional regulatory elements downstream of the JunB gene

(gene activation/growth factors/serum response element)

EVELIO D. PEREZ-ALBUERNE, GINA SCHATTEMAN*, LAURA K. SANDERS, AND DANIEL NATHANS

Howard Hughes Medical Institute and Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205-2185

Contributed by Daniel Nathans, August 12, 1993

ABSTRACT **JunB is an immediate early transcription factor that is induced by a variety of extracellular signaling agents, including growth factors, phorbol esters, and agents that elevate cyclic AMP. The mechanism of activation of the gene encoding JunB by these agents is not well understood. By using the JunB gene together with flanking DNA in transfection experiments, we show that a serum response element (SRE) and/or a cAMP response element (CRE) downstream of the gene mediate the response of the gene in mouse NIH 3T3 cells to serum, platelet-derived growth factor, basic fibroblast growth factor, phorbol ester, and forskolin. In addition, a segment of DNA just upstream of the TATA box is required for optimal activation of the gene.**

The long-term cellular effects of extrinsic signaling agents appear to be mediated in part by induced genetic programs, starting with the activation of transcription factor genes. Among the transcription factor genes rapidly activated by growth factors and other signaling agents are those encoding members of the Jun and Fos families. Of these, the activation of the gene encoding c-Fos has been studied in most detail (ref. 1 and references therein). Upstream of the transcription start site of c-*fos* is a symmetrical sequence—the serum response element (SRE)—that is required for serum and platelet-derived growth factor (PDGF) responsiveness of the gene and is capable of conferring responsiveness on otherwise inactive genes. The SRE binds a transcription factor termed the serum response factor (SRF) that is thought to mediate activation of c-*fos*. In addition to SRF, other proteins have been identified that bind to either the flanking sequence of the SRE or to other growth factor response elements near the c-*fos* promoter or to SRF itself (2–4).

Functional SREs whose sequences are similar to the c-*fos* SRE are also found upstream of other immediate early genes, including *egr1* (also called *zif268, NGFI-A, krox24*), which has four SREs (5), and *egr2* (*krox20*), which has two SREs, one of which is in the first intron (6). However, neither c-*jun* nor *junB*, both of which are activated in fibroblasts by serum, platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) (7), have been reported to have functional SREs. The gene encoding JunB in particular is rapidly activated by a variety of extracellular signaling agents in a number of different cells (e.g., refs. 7–10) and is likely to have several different response elements. Therefore, we set out to define the cis element(s) required for activation of *junB* by serum growth factors and certain other inducing agents in mouse NIH 3T3 cells. Our experiments indicate that a typical SRE is involved in *junB* activation by serum, PDGF, basic FGF (bFGF), and phorbol 12-tetradecanoate 13-acetate (TPA). However, unlike SREs for c-*fos*, *egr1*, and *egr2*, the SRE regulating *junB* is downstream of the gene. The same region of DNA also contains a cAMP response element (CRE) that partially mediates the *junB* response to forskolin.

MATERIALS AND METHODS

***junB* Plasmids.** *junB* genomic fragments were isolated from a bacteriophage λ library prepared from BALB/c mouse genomic DNA digested with *Eco*RI by using junB cDNA as a probe. The two *Eco*RI fragments detected by Southern blotting were isolated: an 8-kb fragment extending from the *Eco*RI site at nucleotide 1546 (in the 3′ untranslated cDNA sequence) upstream to 6.3 kb 5′ of the transcription start site (11), and a second 1.7-kb fragment extending downstream from the *Eco*RI site at 1546 to 1.5 kb 3′ of the end of the cDNA sequence (11, 12). For use in transfection experiments, these fragments were joined in the pBlueScript KS vector (Stratagene) in such a way as to distinguish it from the recombinant *junB* gene—namely, by using a *Not* I linker to join the two fragments at their *Eco*RI sites at nucleotide 1546. This resulted in a plasmid (referred to as "−6300/+3500") containing the entire *junB* with 6.3 kb of 5′ flanking sequence, 1.5 kb of 3′ flanking sequence, and a 51-base-pair insertion at nucleotide 1546 of the cDNA sequence. A derivative test plasmid (−91/+2176) contained a *Sac* II fragment of the −6300/+3500 insert comprising 91 bp 5′ of the transcription start site of *junB* through 395 nucleotides beyond the probable polyadenylylation signal. For constructing some of the variants with deletions at the 5′ end of the *junB* insert, it was more convenient to start with a test plasmid in which the −91/+2176 insert was inverted in the vector, as indicated in the figure legends. Inversion had no effect on the transcriptional activity of the test plasmid in transfected cells.

Variants with deletions of *junB* flanking sequences were prepared by controlled exonuclease III digestion, and variants with base pair substitutions were prepared by oligonucleotide-directed mutagenesis procedures (13). Another *junB* plasmid was constructed to serve as an internal control in the RNase protection assays. It contained *junB* genomic sequence from −91 to +2176 but had a deletion of 59 bp between the *Xho* I site and *Eco*RI site in the 3′ untranslated region of the gene. Finally, a plasmid was constructed to serve as template for an antisense RNA probe to be used in RNase protection experiments that could distinguish transcripts from the test, control, and endogenous *junB* genes. For this purpose the 265-bp *Xho* I/*Dra* III fragment, derived from the −6300/+3500 plasmid and containing 3′ untranslated junB cDNA sequence (including the *Not* I linker insert described above) was cloned into the *Sal* I site of pBluescript KS so that the T7 RNA polymerase transcript was comple-

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Abbreviations: SRE, serum response element; CRE, cAMP response element; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; TPA, phorbol 12-tetradecanoate 13-acetate; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; CREB/ATF, CRE binding protein/activating transcription factor.

*Present address: St. Elizabeth's Hospital, Tufts University School of Medicine, Boston, MA 02135.

mentary to junB mRNA. The structure of all constructs was confirmed by nucleotide sequence analysis of relevant segments.

A plasmid containing a 3′ *junB* segment downstream of TK-CAT (chloramphenicol acetyltransferase gene under control of a thymidine kinase promoter) was prepared by inserting *junB* DNA corresponding to nucleotides 2059–2139 (see Fig. 3) with *Kpn* I linkers into pBLCAT-2 (14) at a unique *Kpn* I site downstream of the CAT gene. pBLCAT-2 with a c-*fos* SRE or *egr1* (*zif268*) SRE1 upstream of the promoter have been described (5). CAT assays were carried out and normalized as described (5).

Cell Culture, Transfection, and RNase Protection Assay. NIH 3T3 cells were maintained and transfected as described (5). Unless otherwise indicated, 50 μg of internal control plasmid and 50 μg of the test construct were used to transfect 1–2 $\times 10^6$ NIH 3T3 cells in a 10-cm Petri dish. After transfection, the cells were incubated for 3 days in medium containing 1% fetal bovine serum (FBS) and then stimulated with 20% FBS, 20 ng of PDGF BB (human recombinant; Collaborative Research) per ml, 50 ng of bFGF (human recombinant; Collaborative Research) per ml, 10 μM forskolin (Sigma), or 300 ng of TPA (Sigma) per ml. For preparation of RNA, cells were lysed with 4 M guanidinium isothiocyanate/25 mM sodium citrate, pH 7/0.5% sarcosyl/0.1 M 2-mercapatoethanol; total RNA was purified as described (15).

The RNase protection assay was performed as described (16) with a ^{32}P-labeled T7 RNA polymerase transcript as a probe. After electrophoresis, RNA species corresponding to the probe protected by various transcripts were cut out of the gel and assayed in a liquid scintillation counter. In later experiments the individual bands were quantitated by use of a phosphor imager. To calculate the normalized transgene activity, the net radioactivity of a test transgene band was divided by the net radioactivity of the internal control band in the same lane. The final results were then expressed as the ratio of the normalized activity of a given plasmid to the normalized activity of the −91/+2176 standard plasmid in the same experiment and under the same experimental conditions.

DNA Binding Assay. To compare the activities of the c-*fos* and *junB* SREs for binding by nuclear proteins, 0.5 nM ^{32}P-labeled double-stranded c-*fos* SRE oligonucleotide (CGGATGTCCCATATTAGGACATCTA) or *junB* SRE oligonucleotide (CCTCTGCCCATATATGGGCCTATA) was incubated with nuclear extract from 3T3 cells stimulated with FBS for 30 min, and the degree of binding was assessed by gel-mobility retardation assay (17). In the competition experiments, unlabeled oligonucleotide was incubated with nuclear extract prior to addition of radioactive nucleotide. Binding of nuclear protein to a *junB* CRE oligonucleotide (GCTCAGTGACGCCAGCGCGG) or an oligonucleotide containing a canonical CRE (GGTATCGATAAGCTCTGACGTCAGCCGGG) was determined in a similar way, with nuclear extract from cells stimulated with 10 μM forskolin for 30 min.

RESULTS

Activation of an Intact *junB* Transgene Assayed by RNase Protection. In preliminary experiments undertaken to identify regulatory elements upstream of mouse *junB*, we tested NIH 3T3 cells transfected with *junB* promoter-CAT plasmids containing *junB* 5′ genomic segments of various lengths from 91 bp to 3 kb for response to serum stimulation in transfected NIH 3T3 cells. Little or no increase in CAT activity was observed after 4 hr, in contrast to the brisk stimulation of transfected c-*fos* promoter-CAT or *egr1* promoter-CAT plasmids (data not shown). Since these results indicated that 5′ elements were either not required or were not sufficient to account for the activation of *junB* by serum, we turned to assaying the intact, intronless *junB* transgene by the RNase protection procedure (15). For this purpose a BALB/c mouse genomic *junB* clone was isolated that contained 6.3 kb upstream of the transcription start site and 1.5 kb downstream of the end of the cDNA sequence (11, 12).

Transfection experiments with this clone and others with shorter 5′ and 3′ segments revealed that they were all activated by serum to an extent comparable to activation of endogenous *junB* (see below). Therefore, we concentrated on a fully serum-responsive test transgene (−91/+2176) containing 91 bp upstream of the transcription start site and 395 bp downstream of the probable polyadenylylation signal (through nucleotide 2176). To distinguish transcripts of the endogenous *junB* gene, the test transgene, and an internal control transgene, the transgenes contained a short insert or deletion in the 3′ nontranslated sequence as described in *Materials and Methods*. RNA levels were determined after 1 hr of cellular exposure to serum or other stimulating agent, since at this time junB mRNA derived from the transgene or the endogenous gene was at or near its maximal level. A typical experimental result is shown in Fig. 1, and the extent of stimulation of the control transgene and the endogenous gene by serum, PDGF-BB, bFGF, forskolin, or TPA is presented in Table 1. For each of these agents, the extent of stimulation of the transgene vs. the endogenous gene was very similar. Therefore, the major response elements appear to be present in the test and control transgenes—i.e., between nucleotides −91 and +2176.

Effect of Upstream Mutations on the Activation of *junB*. Although our *junB*-CAT experiments referred to above had failed to detect regulatory SREs upstream of *junB*, we nonetheless tested the response of a series of transgenes with alterations in sequences upstream of the transcription start

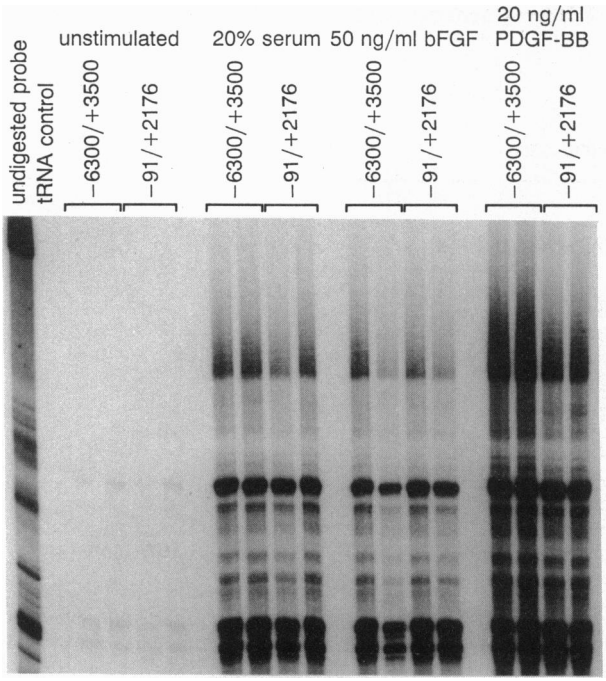

FIG. 1. Stimulation of the *junB* test transgene (T), internal control transgene (C) and the endogenous gene (E), measured by the RNase protection assay. Shown is an autoradiogram of the ^{32}P-labeled probe protected by transcripts from each of the genes after electrophoresis under denaturing conditions. The "tRNA control" lane contains digested probe after incubation with tRNA under annealing conditions. The transgenes are described in the text. Protected probes are 2181 nucleotides (T), 218 (C), and ≈164 (E).

Table 1. Stimulation of the *junB* internal control transgene and endogenous gene

Stimulating agent	Fold stimulation of *junB* transcription	
	Transgene	Endogenous gene
Serum	17.9 ± 11 (143)	13.9 ± 7.7 (143)
PDGF-BB	22.3 ± 12 (38)	23.0 ± 11 (38)
bFGF	12.0 ± 6.5 (23)	15.1 ± 7.9 (23)
Forskolin	4.6 ± 2.8 (46)	4.5 ± 2.1 (46)
TPA	7.9 ± 4.1 (30)	12.0 ± 6.3 (30)

Results are presented as the level of transcript in cells stimulated for 1 hr divided by the level of transcript in nonstimulated cells and are the averages of all samples analyzed ± SD. Numbers in parentheses are the numbers of samples analyzed. The experiments included in the tabulation were done on different batches of NIH 3T3 cells over a period of 2 yr.

site by the RNase protection procedure. The constructs used are diagrammed in Fig. 2, where the results with each variant are expressed as the ratio of transcripts of the test transgene to that of the reference transgene (−91/+2176) as described in *Materials and Methods*. Removal of 5′ sequence from base pair −6300 to −91 and 3′ sequence from base pair 3500 to 2176 resulted in a drop in the basal level of transcript but little or no change in stimulation by any of the agents tested. Further 5′ deletion to nucleotide −37 reduced the basal level of transcript and also the stimulation by serum, PDGF, and TPA but not by forskolin. Since the segment between nucleotides −91 and −37 contains an inverted repeat sequence (AGTGCACT) implicated in the response of *junB* to TPA and to activated protein kinase A (18), we determined whether a point mutation in the inverted repeat sequence reported to abolish responsiveness (18) would affect the response of the transgene. Such a mutation had little or no effect on stimulation by serum, forskolin, or TPA (Fig. 2). We conclude that the DNA segment between nucleotides −37 and −91 contains

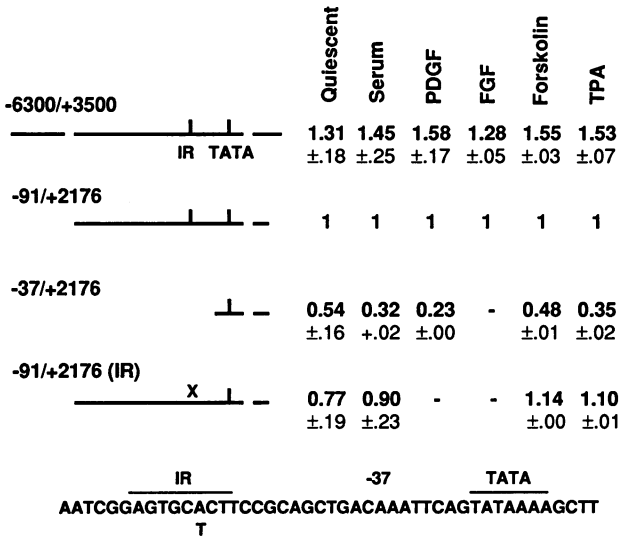

FIG. 2. The effect of mutations upstream of the *junB* test transgene on stimulation of the transgene. The transgenes shown diagrammatically at the left are described in the text. The positions of the TATA box at nucleotide −28 and the inverted repeat sequence (IR) at −57 are indicated, as is the sequence of this region in the mouse genome. (The mouse *junB* sequence from nucleotide −849 to the transcription start has been deposited in GenBank, accession no. X57154). X indicates a T-for-A substitution in the IR, as shown below the sequence. The orientation of transgenes −6300/+3500 and −37/+2176 in the plasmid vector was reversed relative to the others. Results are expressed as the ratios of normalized transcript values ± SD for from two to six samples. -, Not done.

one or more elements needed for optimal basal transcription of *junB* and probably for optimal stimulation of the gene by serum, PDGF, and TPA. However, such a signal by itself cannot account for the major stimulation observed with any of these agents.

Response Elements Downstream of *junB*. Based on the foregoing results we looked for regulatory elements elsewhere in *junB* and in the 3′ flanking sequence. Testing of four large deletion constructs implicated sequences in the 3′ noncoding region of *junB* or in the 3′ flanking sequence in serum responsiveness (data not shown). Nucleotide sequence of DNA flanking the 3′ end of *junB* revealed a typical SRE starting at nucleotide 2084 and a probable CRE starting at nucleotide 2109 (Fig. 3). In addition, there is a CRE-related sequence (overlined in Fig. 3) starting at nucleotide 2100; however, this sequence has an inverted central CG relative to an authentic CRE and is asymmetric.

Effect of Downstream Mutations on the Activation of *junB*. To determine whether the 3′ flanking elements detected by sequencing were functional or whether other response elements were present, we tested the effect of a series of 3′ deletions and base substitutions on the responsiveness of the *junB* transgene to serum, PDGF, FGF, forskolin, or TPA (Fig. 4 and data not shown). Deletion from the 3′ end including part of the putative CRE (−91/+2113) resulted in a slight fall in basal activity of the transgene and a greater decrease in serum and forskolin stimulation. Somewhat more extensive 3′ deletion including the CRE-related sequence (−91/+2099) was without further effect. When the 3′ deletions included part or all of the putative SRE (−91/+1854), the response to serum fell further, but the forskolin response did not.

More specific mutagenesis was carried out by constructing double base substitutions in the CRE, CRE-related sequence, and SRE that would be expected to abolish binding by CREB/ATF (CRE binding protein/activating transcription factor) or SRF proteins (Fig. 4). When these transgenes were tested, it was found that mutation in the CRE led to a decrease only in forskolin stimulation and mutation in the SRE resulted in a decrease in stimulation by serum, PDGF, FGF, and TPA but increased stimulation by forskolin. Mutations within both the SRE and CRE led to a slight decrease in basal transcription and a further decrease in stimulation by all agents.

To determine whether the transgene mutated in both SRE and CRE lost all response to serum, we repeated the comparison of the activities of −91/+2176 transgenes with and without point mutations in both of these elements and measured stimulation over the basal level of transcript. Relative to the basal level of transcript from the reference transgene, the serum-stimulated counterpart was 15.6 ± 2.5; the basal

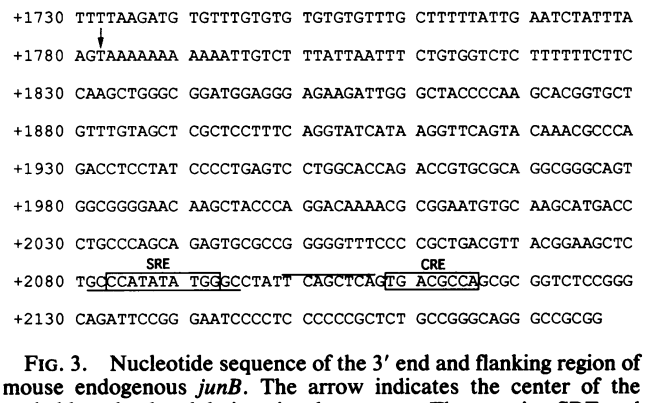

FIG. 3. Nucleotide sequence of the 3′ end and flanking region of mouse endogenous *junB*. The arrow indicates the center of the probable polyadenylylation signal sequence. The putative SRE and CRE are indicated. The overlined sequence resembles a CRE. The human junB cDNA ends at nucleotide 1806 (12).

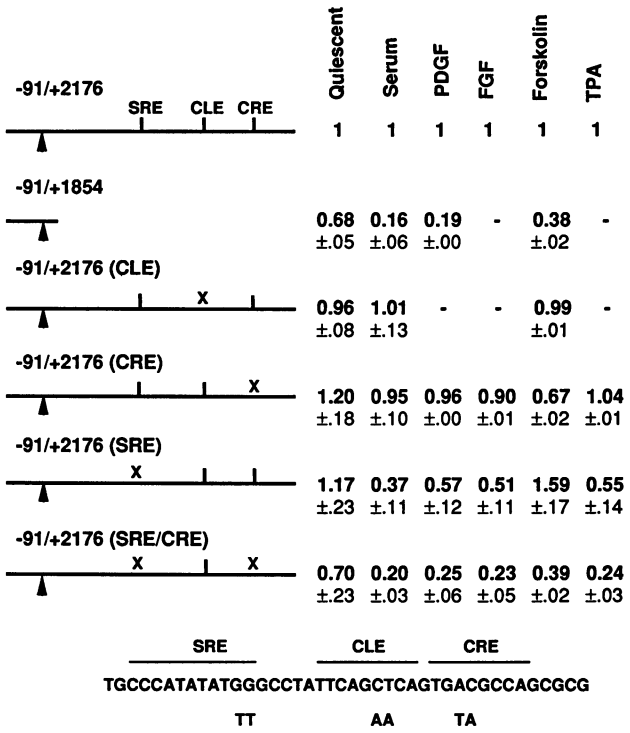

FIG. 4. The effect of mutations downstream of the *junB* test transgene on the stimulation of the transgene. Each transgene begins at −91; only the 3′ end is diagrammed. Arrowheads indicate the probable polyadenylylation signal. The DNA sequence shown is between nucleotides 2080 and 2120 (Fig. 3). The positions of the SRE, CRE, and CRE-like sequence (CLE) are indicated. X represents a double base pair substitution in each of the elements, as indicated at the bottom of the figure. Results are expressed as the ratios of normalized transcript values ± SD for from two to six samples. -, Not done.

level of transcript from the 3′ SRE/CRE mutant transgene was 0.62 ± 0.23, and its serum-stimulated counterpart was 2.97 ± 0.58. In a separate experiment we determined whether combining the SRE and CRE point mutations with a 5′ deletion to −37 abolished serum stimulation of the transgene. Relative to the basal level of transcript from the reference transgene, the serum-stimulated counterpart was 16.2 ± 0.65; the basal level of transcript from the combined 3′ SRE/CRE/5′Δ37 transgene was 0.31 ± 0.03, and its serum-stimulated counterpart was 0.48 ± 0.11. The point mutation in the inverted repeat sequence was also tested in a construct that had both the SRE and CRE mutations; it had no effect.

Finally, to determine whether the segment of DNA containing the 3′ *junB* SRE and CRE can confer serum responsiveness on a nonresponsive transgene, a DNA fragment from +2059 to +2139 was inserted downstream of a TK-CAT transgene, and serum or PDGF responsiveness was determined by CAT assay at various times after addition of serum or PDGF BB. In addition, TK-CAT transgenes with either the c-*fos* SRE or *egr1* (*zif268*) SRE1 placed upstream of the TATA box (5) were also tested. Only the transgenes with 3′ or 5′ inserts were stimulated by serum or PDGF. The relative CAT activities 6 hr after PDGF stimulation were 0, 0.2, 0.9, and 1.0 for TK-CAT with no SRE insert, TK-CAT with 3′ *junB* SRE/CRE insert, TK-CAT with 5′ *egr1* SRE1 insert, and TK-CAT with 5′ c-*fos* SRE insert, respectively; and the respective relative CAT activities 6 hr after serum stimulation were 0, 0.1, 0.8, and 1.0.

We conclude from this series of experiments that (*i*) sequences approximately 2.1 kb downstream of the transcription start site of *junB* contain a functional SRE and a functional CRE; (*ii*) one or both of these elements are major determinants of the responsiveness of *junB* to serum, PDGF-BB, bFGF, TPA, and forskolin; and (*iii*) 5′ sequences between nucleotides −91 and −37 play a role in both basal transcription of *junB* and in the response of *junB* to serum, PDGF-BB, and TPA.

Binding of 3T3 Nuclear Proteins to the *junB* 3′ SRE and CRE. To determine whether the *junB* SRE and CRE are binding sites for SRF-related or CREB/ATF-related 3T3 nuclear proteins, respectively, we tested nuclear extracts of serum- or forskolin-treated 3T3 cells for proteins that bind to oligonucleotides containing these elements or canonical SRE and CRE sequences (Fig. 5). Nuclear extracts from serum-stimulated cells, but not forskolin-stimulated cells, showed increased binding activity for both SRE- and CRE-containing oligonucleotides relative to extracts from nonstimulated cells. The *junB* 3′ SRE-binding protein appears to be identical to the c-*fos* SRE-binding protein (presumably SRF), and each of the SRE oligonucleotides binds with similar affinity (Fig. 5*A*). The *junB* 3′ CRE oligonucleotide binds more weakly than a canonical CRE oligonucleotide to the major protein species that bind the canonical CRE but binds more strongly than the canonical CRE to a lower mobility protein in the 3T3 extract (Fig. 5*B*). We conclude from these experiments that the 3′ *junB* SRE and the c-fos SRE bind SRF or an SRF-related protein and that the *junB* CRE binds weakly to multiple protein species in 3T3 nuclear extract, some of which also bind a canonical CRE. Whether any of these proteins plays a role in activating *junB* is not known.

DISCUSSION

JunB is a member of the Jun family of transcription factors that resembles c-Jun and JunD in its interaction with Fos proteins and in the DNA-binding properties of Fos/Jun dimers (19, 20). Since expression of the gene encoding JunB is stimulated by a variety of extracellular signaling agents in different cell types, JunB is thought to be involved in the regulation of the cellular response to a large number of signaling agents. Therefore, mechanisms of activation of *junB* by extracellular ligands are of considerable interest.

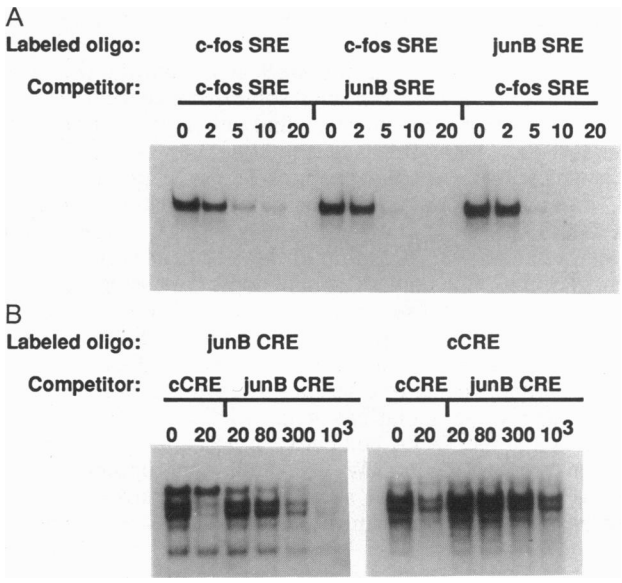

FIG. 5. Binding of nuclear proteins to *junB* SRE and CRE, assessed by electrophoresis of protein–oligonucleotide complexes. The numbers indicate the molar ratios of unlabeled competitor oligonucleotide to labeled oligonucleotide. In *B*, the electrophoresis gel with labeled *junB* CRE was exposed to x-ray film 10 times as long as the gel with labeled canonical CRE (cCRE).

In our initial experiments to localize regulatory elements responsive to serum in NIH 3T3 cells, we used the common practice of constructing a *junB* promoter-CAT plasmid to transfect cells, assessing promoter activation by measuring CAT activity. With *junB* upstream sequences of various lengths (up to 3 kb), we observed little or no stimulation by serum in contrast to the marked stimulation seen with c-*fos* promoter-CAT or *egr1* promoter-CAT plasmids. These results, which are different from those reported by Kruijer *et al.* (18) but are similar to those reported briefly by Apel *et al.* (21), led us to search for regulatory elements within *junB* or downstream of it by using the entire gene together with 5′ and 3′ flanking DNA and determining the response to signaling agents by measuring gene transcripts. The *junB* transgene containing 91 bp upstream of the transcription start site and 395 bp beyond the probable polyadenylylation signal was sufficient to respond to serum, PDGF-BB, bFGF, TPA and forskolin about as well as endogenous *junB*.

The effect of large deletions localized response elements to the 3′ end of the gene or adjacent sequence. The sequence of this region revealed a typical SRE sequence and a probable CRE sequence downstream of the gene. More detailed mutagenesis demonstrated that the 3′ SRE and CRE play an important role in the response of *junB* to serum, PDGF-BB, bFGF, and TPA and that the CRE is responsive to forskolin. Moreover, the segment of DNA that contains the SRE and CRE was able to confer responsiveness to serum or PDGF when placed downstream of a recombinant TK promoter-CAT gene. Functional interaction of the SRE and CRE is suggested by the reduced response of the *junB* transgene to serum, growth factors, or TPA when CRE mutation was coupled with SRE mutation (in contrast to the lack of effect of CRE mutation alone) and by the consistent increase in forskolin response when the SRE alone was mutated. We conclude that major elements ≈2.1 kb downstream of the transcription start site mediate the response of *junB* to a number of signaling agents. The finding that the *junB* SRE competes well with the c-*fos* SRE for binding to a nuclear protein (presumably SRF) suggests that the *junB* element is the functional equivalent of SREs derived from the c-*fos* promoter and other immediate early genes. On the other hand, the *junB* CRE appears to be a weak forskolin response element; it is not clear what protein(s) might act at the *junB* CRE. The remoteness of the *junB* SRE from the promoter indicates that the SRE can act at some distance from the start of transcription and suggests that other immediate early genes lacking an SRE near the promoter may also have distant SREs that mediate responsiveness to growth factors.

In addition to the 3′ signals, optimal stimulation of *junB* by serum, PDGF-BB, and TPA requires DNA between base pairs −91 and −37. Deletion of this segment reduces basal transcription, but reduces stimulation by these agents even more. In the case of serum stimulation, which was examined in more detail, double point mutations in the SRE and CRE expected to abolish binding of SRF and CREB-related proteins markedly decreased serum responsiveness of *junB*, and combining these mutations with deletion of the segment upstream of the TATA box between −91 and −37 abolished serum responsiveness. We conclude that optimal serum responsiveness requires sequences just upstream of the TATA box. Whether the upstream and downstream sites act coordinately via protein–protein interactions remains to be determined.

Recently Kitabayashi *et al.* (22) identified two SREs approximately 1.5 and 3 kb upstream of rat *junB* that were active in cells stably transformed by *junB*-CAT plasmids but not in cells transiently transfected by the plasmids. Since we have found that the JunB transgene from base pair −91 to +2197 is about as responsive to serum and growth factors in transiently transfected cells as is endogenous *junB*, we suggest that *junB* may have redundant SREs distant from the transcriptional start site.

Finally we point out that the use of promoter-containing DNA segments linked to reporter genes may often be inadequate for identifying relevant transcriptional regulatory elements of a given gene. Measurement of transcripts from a transgene containing the intact gene together with long 5′ and 3′ sequences allows a quantitative comparison of the activities of the transgene and endogenous gene and makes it possible to identify regulatory elements within and downstream of the gene that would otherwise escape detection.

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