Activation of junB by PKC and PKA signal transduction through a novel cis-acting element

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ABSTRACT

The product of the junB gene, a gene homologous to the proto-oncogene c-jun, is a component of transcription factor AP-1. JunB expression is modulated by a wide variety of extracellular stimuli, such as serum, growth factors, phorbol esters (TPA) and activators of protein kinase A (PKA). In order to study the molecular basis of this complex regulation, we have cloned the mouse junB gene from a genomic testis library, and characterized the junB promoter. Here we show that the junB promoter is activated by serum, TPA, and activated PKA. Sequences located between -91 and -44 are necessary for induction. These sequences contain a CAAT box, a G-C rich region and a previously undescribed inverted repeat (IR). The IR element can mediate induction by TPA and PKA when coupled to a heterologous promoter, and specifically binds a protein of 110 kD.

INTRODUCTION

The c-jun proto-oncogene is the cellular homolog of v-jun, the transforming gene of avian sarcoma virus 17 (1), and encodes a component of the TPA-inducible transcription factor AP-1, JunB expression is modulated by a wide variety of extracellular stimuli, such as serum, growth factors, phorbol esters (TPA) and activators of protein kinase A (PKA). In order to study the molecular basis of this complex regulation, we have cloned the mouse junB gene from a genomic testis library, and characterized the junB promoter. Here we show that the junB promoter is activated by serum, TPA, and activated PKA. Sequences located between -91 and -44 are necessary for induction. These sequences contain a CAAT box, a G-C rich region and a previously undescribed inverted repeat (IR). The IR element can mediate induction by TPA and PKA when coupled to a heterologous promoter, and specifically binds a protein of 110 kD.

MATERIALS AND METHODS

Isolation of the junB promoter
A mouse balb-c testis genomic library, containing Sau3A partially digested fragments cloned into lambda EMBL-3 was screened with a mouse junB cDNA probe. Several JunB positive phages were characterized by restriction enzyme digestion, and junB containing DNA fragments were cloned into pGEM 3 plasmids (Promega) and were sequenced using the T7 polymerase sequencing kit (Promega).

Cells and plasmids
The mouse hepatoma cell line BW1 was a kind gift of Dr. Szpirer & Szpirer (28). P19 EC cells were a kind gift of Dr. McBurney (29). P19 EC and F9 EC cells were cultured in DF-Bic containing 7.5% fetal calf serum (FCS) as described earlier (30). Hep-G2 and BW1 cells were maintained in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum.

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As probes for hybridization studies, a 1.0 kb PstI mouse c-jun genomic fragment (de Groot et al., submitted), a 1.5 kb EcoRI cDNA fragment of junB (8), a 1.7 kb EcoRI cDNA fragment of junD (9), a 1.4 kb fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 31) and a 0.75 kb EcoRI-HindIII fragment of chicken βactin (32) were used.

The expression vectors pC-α-EV and pC-EV-X encode respectively for the wild type and mutant catalytic subunit of protein kinase A (33). Expression vectors for CREB (34), c-jun and junB (35) are described elsewhere.

JunB promoter fragments were cloned into the promoter-less CAT vector pKT. pJB1 was generated by cloning a partial BamHl-SmaI fragment from the junB 5’ regulatory region (−848 − +245) into the SmaI site of pKT. pJB1 contains the same fragment cloned in the reverse orientation relative to the CAT gene. pJB2 was generated from pJB1 by digestion with XbaI and religation. pJB3 consists of a 712 bp SmaI fragment cloned into the SmaI site of pKT. pJB4, 5 and 6 consist of a 436 bp PstI-SmaI fragment, a 331 bp BglII-SmaI fragment and a 284 bp PvuII(partial)-SmaI fragment respectively cloned in the SmaI site of pKT.

RNA isolation and Northern blotting
Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride method of Chirgwin et al. (36). 15 μg of total RNA was denatured for 10 min at 68°C in 50% (v/v) formamide, 2.2 M formaldehyde, 20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA, separated through 0.8% agarose/2.2 M formaldehyde gels, and subsequently transferred to nitrocellulose filters (BA 85, Schleicher & Schuell) in 20× SSC. RNA was immobilized by baking at 80°C for 2 h under vacuum. Hybridization was performed in 50% formamide, 5× SSC, 50 mM sodium phosphate pH 6.8, 10 mM EDTA, 0.1% NaDodSO4, 0.1 mg of sonicated salmon sperm DNA per ml, 2× Denhardt solution (1× Denhardt solution contains 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% sodium phosphate, 0.1% sodium EDTA and 0.1% polyvinylpyrrolidone) at 42°C overnight. 32P-labeled probes were generated using a multiprime DNA labeling kit (Amersham). After hybridization and washing, filters were exposed to Kodak XAR-5 film at −70°C using intensifying screens.

DNA transfection and transient expression assays
P19 EC and 3T3 cells were transfected as described previously (30, 37). HepG2 and BW-1 cells were plated in DMEM-Bic/7.5% FCS at 6.105 cells per 50 mm tissue culture dish 24 hrs prior to transfection. Two hours before transfection, the dishes received fresh medium. Cells were incubated for 16−20 hrs with calcium phosphate precipitated DNA’s (10−20 μg plasmid per 50 mm dish), followed by addition of fresh medium. 16−24 hours later, the cells were harvested followed by measuring CAT activity. CAT activity was determined as described by Gorman et al. (38), and was quantitated by liquid scintillation counting of TLC-plate 14C spots.

Gel shift assay and UV crosslinking
Double stranded oligonucleotides were labeled with α-32PdATP and α-32PdCTP (5000 Ci/mol) using Klenow fragment of DNA polymerase I. The sequence of the oligonucleotides used are : mouse c-jun TRE, 5′-GATCGGGTGCACATCAGG-3′ (de Groot et al., submitted); mouse junB IR, 5′-GATCTCGGAGTGGCAGTTCCG-3′. Isolation of nuclear extracts and gel shift assays were performed as described previously (35).

For UV crosslinking experiments, protein-DNA complexes were allowed to form for 20 min at 20°C. Samples were then treated with UV light (254 nm) for another 20 min at a distance of 5 cm from the light source. After this, samples were boiled in Laemmli sample buffer, and analyzed on 8−12% denaturing polyacrylamide gels.

DNase I Footprinting
DNase I footprinting reactions were performed as described by Jones et al. (39) with some modifications using nuclear extracts of P19 EC, NIH-3T3 or BW-1 cells. In short, DNA fragments were end-labeled by filling in with Klenow fragment of DNA polymerase I. Fragments were incubated in a total volume of 50 μl containing 5% v/v glycerol, 20 mM Hapes-KOH pH 7.5, 10 mM MgCl2, 75 mM NaCl, 1 mM DTT, 0.18% v/v NP40 and 1 μg poly(d-I-C)) with 10−20 μg of nuclear extract. After incubation for 30−45 min. at room temperature, 4 μl of freshly prepared DNase I diluted in 50 mM MgCl2 was added. Digestion was allowed for 1.5 min. at room temperature, after which 3.5 μl stop buffer (0.2 M EDTA, 2% w/v SDS) was added. Reactions were analyzed on 6% polyacrylamide/7.5 M urea gels after extraction with phenol:chloroform and precipitation with isopropanol.

RESULTS
Isolation of the mouse junB gene
Expression of the junB gene is rapidly induced in response to serum, growth factors, phorbol esters and agents that raise the cytoplasmic concentration of cAMP in a wide variety of cells (8, 24, 35, 37). To study the molecular mechanisms underlying this versatile regulation, we set out to clone the junB gene from a mouse balb-c testis genomic library using a mouse junB cDNA fragment (8) as a probe. A number of positive clones were isolated, of which one (XJB31) contained the complete junB gene including about 8 kb of 5′ sequences (not shown). Restriction enzyme analysis and partial sequence analysis indicated that, like the human c-jun and junB genes (26, 40), the mouse junB gene is devoid of intervening sequences (not shown). To further study junB regulation, a 1100 bp BamHl-SmaI (−848 to + 245) fragment was subcloned and sequenced. The sequence of this fragment (−200 to + 100) is shown in figure 1. This fragment contains a TATA box, a CAAT box and a GC-rich region that probably is a binding site for transcription factor zif268 (42). No TRE or SPI binding site, both of which are present

Figure 1. Nucleotide sequence of the mouse junB promoter. The 1100 bp BamHI (partial)−SmaI (partial) fragment (−848 to + 245, see figure 2A) of the junB promoter was subcloned and sequenced. Only part of the sequence is shown (−200 to + 100). The major transcription start site as determined by Ryder et al. (37) was assigned position +1. The TATA box is underlined, and the GC-rich region and the CAAT-box are double underlined.
in the human (40) and mouse c-jun promoters (de Groot et al., submitted), can be found in this fragment, nor in sequences up to \(-848\) (not shown).

**Activation of the junB promoter by serum, TPA and PKA**

To determine whether the observed effects of serum and TPA on junB expression are caused by transcriptional activation of the junB promoter, we coupled the BamHI-Smal 5' junB fragment (\(-848\) to +245) to the bacterial chloro-ampenicol acetyl transferase (CAT) gene (Fig. 2A, pBJ1) and studied its activity in transient transfection assays in a number of different cell lines. As shown in figure 2B, treatment of mouse 3T3 fibroblasts with 20% FCS causes a strong increase (8-fold) in the activity of the junB promoter. Similar effects were observed in human HeLa cells and mouse BW-1 hepatoma cells (not shown). In addition, TPA treatment also induces a pronounced increase in the activity of the junB promoter in BW-1 (Fig. 2C) and HepG2 cells (not shown). A construct containing the junB promoter fragment in the reverse orientation (pBJ1) was neither activated by serum (Fig. 2B) nor by TPA (Fig. 2C). Furthermore, co-transfections with the PKA expression vector p-\(\alpha\)-EV (33) in BW-1 cells caused a strong increase in the activity of the junB promoter, an effect enhanced by cotransfection of a CREB expression vector (not shown). These results show that the cloned fragment contains the functional junB promoter, and that this promoter is activated by serum, TPA and activated PKA.

To further pinpoint the sequences responsible for the observed effects of serum, TPA and PKA on junB promoter activity, a number of CAT plasmids containing progressive 5'-deletions of the junB promoter were constructed (Fig. 2A, pJB2-6). These plasmids were transiently transfected in 3T3 and BW-1 cells, and the effects of serum, TPA and activated PKA was determined. As shown in table I, a fragment spanning junB sequences from \(-91\) to +245 (pJB5) is still fully responsive to all three stimuli. Further 5' deletion of 47 bp (pJB6, \(-44\) to +245) however completely abolishes this inducibility, indicating that sequences between \(-91\) and \(-44\) are necessary for the observed effects of serum, TPA and activated PKA.

**Identification of three protein binding sites in the junB promoter**

In an effort to define the cis-acting elements present in the junB promoter, we performed in vitro DNasel footprinting experiments using nuclear extracts from both BW1 and HepG2 cells. As shown in figure 3, three protected sites in this fragment were detected. No differences between extracts from junB-expressing or -non-expressing cells was observed. In the region from \(-100\) to \(-77\), a G-C rich region is protected, which is a potential

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<th>Table I. Activity of the junB deletion constructs</th>
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<td>JBI (-848)</td>
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<td>JB2 (-574)</td>
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<td>JB3 (-472)</td>
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Serum experiments were performed in 3T3 cells, and TPA and PKA experiments were performed in BW1 cells. Data are indicated as fold induction relative to unstimulated samples (serum and TPA) or samples co-transfected with mutant PKA expression vector (PKA), and are the mean of three independent experiments.

Figure 2. The junB promoter is activated by serum and TPA. A. Structure of a number of junB promoter-CAT fusion constructs. These constructs were made as described in the materials and methods section. Abbreviations of restriction sites are as in 1B, and K - KpnI, H - HindIII, B - BglII, P - PvuII. B - Activation by serum. Mouse 3T3 fibroblasts were transiently transfected with pBJ1 (see A) or pBJ1, a construct containing the junB promoter fused in reverse orientation to the CAT gene (10 \(\mu\)g per 5cm dish). 16 hours after transfection, the cells were cultured in medium containing 0.5% FCS for 8 hours, after which the dishes received fresh medium (7.5% FCS), followed after 8 hours by addition of TPA (+TPA, 100 ng/ml) or carrier alone (+TPA) for another 14 hours. CAT activity was determined as in B.

Figure 3. Identification of protein binding sites in the junB promoter. The \(-196\) to +36 PstI-BamHI junB promoter fragment was end-labeled with Klenow polymerase or T4 polynucleotide kinase. The probes were incubated with BSA or nuclear extract from BW1 or HepG2 cells, and subjected to DNase I footprinting as described in the materials and methods section. Protected sites and the TATA-box are underlined. The G-C rich region is located between \(-96\) and \(-79\), the CAAT box between \(-67\) and \(-61\), and an inverted repeat (IR) between \(-57\) and \(-50\). Differences with the human junB promoter are indicated by asterisks.
The IR element specifically binds a 110 kD protein

Since the IR element is a previously undescribed cis-acting element, we set out to characterize the protein(s) that interacts with this sequence. For this purpose we performed gel shift experiments using the IR element as a probe. As shown in figure 5A, 2 protein-DNA complexes (I and II) were observed with nuclear extracts from BW-1 cells. Competition experiments with a 100-fold molar excess of unlabeled oligonucleotide shows that formation of the slower migrating complex (I) is completely inhibited under these conditions, while complex II was reduced about 70%. Treatment of BW-1 cells with forskolin (fig. 5A) or TPA (not shown), which rapidly induce junB expression in these cells, did not result in changes in the retarded complexes. The same complexes were observed using extracts from HepG2 cells (fig. 5B), although complex I is more abundant in these cells. Both complex I and II were not inhibited by pre-incubation with a non-specific oligonucleotide (c-jun TRE, fig. 5B; and somatostatin CRE, not shown). However, competition with increasing amounts of unlabeled IR element shows that formation of complex I is efficiently inhibited at low concentrations of competitor, while complex II is much more resistant to competition, suggesting that complex I is more specific than complex II. Furthermore, no effects of preincubation with antibodies to CREB or Fos on binding and migration of complex I and II were observed (not shown), indicating that the IR binds protein(s) distinct from the classical PKA (CREB) and TPA (Jun/AP-1) inducible transcription factors.

To determine the molecular weight of the protein factor(s) binding to the IR, we performed UV crosslinking experiments. Nuclear extracts from BW-1 and HepG2 cells were incubated with labeled IR oligonucleotide, followed by UV treatment. Samples were then boiled in sample buffer and analyzed on a denaturing gel. With both cell types, a complex of 110 kD (complex I) and a complex of 75 kD (complex II) were observed (fig. 5C). Complex I was efficiently competed with unlabeled IR oligonucleotide (lanes S), but not with non-specific competitor DNA (lanes N). By contrast, complex II was inhibited by specific as well as non-specific competitor, indicating that complex II is probably an-a-specific DNA binding protein. Similar results were observed with nuclear extracts from HeLa, P19 EC and F9 EC cells (data not shown). Taken together, these data show that a
JunB promoter is induced by serum, TPA, and activated PKA. JunD, and JunB are components of transcription factor AP-1, that mediates serum and TPA responsiveness to a heterologous promoter.

**Figure 5.** Specific binding of proteins to the IR element. A. Gel shift analysis with nuclear extracts from untreated BW1 cells (BW1) or BW1 cells treated for 1 hr with forskolin (10 μM, FOR) using an 32P-labeled oligonucleotide encompassing the IR element from the junB promoter as a probe. Two protein-DNA complexes were detected (I & II), of which complex I was completely inhibited by pre-incubation of the nuclear extracts with a 100-fold molar excess of unlabeled IR element (+ COMP.). No differences were observed between untreated and forskolin-treated cells. B. Competition with homologous, but not with heterologous oligonucleotide inhibits formation of complex I. Nuclear extracts from human HepG2 cells were incubated with the labeled IR oligo alone (lanes 1 and 6), or with labeled IR oligonucleotide in combination with increasing amounts (2-50 fold molar excess) of unlabeled homologous (JB-IR) or heterologous (cJ-TRE, see figure 6A) oligonucleotide. The formation of protein-DNA complexes was monitored on a low ionic strength acrylamide gel. Only the retarded complexes are shown. Complex I is rapidly competed by unlabeled JB-IR, while complex II is only slightly competed with high amounts (50 fold molar excess) of this oligonucleotide. No competition of either complex was observed with unlabeled cJ-TRE. C. A protein of 110 kD binds specifically to the IR element. UV cross-linking experiment with nuclear extracts from BW-1 and HepG2 cells using the IR element as a probe. Protein-DNA complexes were allowed to form for 20 min. at 20°C in the absence (lanes marked -) or presence of a 25-fold molar excess of non-specific (cJ-TRE, lanes marked N) or specific (JB-IR, lanes marked S) competitor oligonucleotide, after which the reactions were treated with UV light (254 nm at a distance of 5cm) for another 20 min. Samples were then boiled in Laemmli sample buffer, and analyzed on a 8% denaturing acrylamide gel. Arrows indicate the positions of the protein markers. Large arrows indicate the position of two protein-DNA complexes (I and II), and are distinct from I and II from panels A and B.

A protein of 110 kD, which is expressed in a number of different cell lines, interacts specifically with the IR from the junB promoter. This protein will be referred to as IR binding protein (IRBP) hereafter.

**DISCUSSION**

The products of the c-jun proto-oncogene and two related genes, junB and junD, are components of transcription factor AP-1, that regulate the expression of a number of TPA-inducible genes by binding to the TRE (for a review, see refs. 6 and 7). Both c-jun and junB are rapidly induced by a wide variety of extracellular stimuli such as serum, growth factors and phorbol esters (8, 9, 16–18). In this paper we describe the isolation of the intronless mouse junB gene and its 5′ regulatory region. We show that the junB promoter is induced by serum, TPA, and activated PKA. Sequences between -91 and -44 are involved in the regulation of junB expression. In this region, three different protein binding sites were defined, a G-C rich region, a CAAT box and an inverted repeat (IR) element. Furthermore, we show that the IR, which specifically binds a protein of 110 kD (IRBP), can confer TPA and PKA responsiveness to a heterologous promoter.

The finding that the murine junB gene does not contain intervening sequences is not completely surprising, since the human c-jun gene is also intronless (40). However, since the DNA binding domain of all three Jun proteins is highly homologous to the DNA binding domain of the yeast transcription factor GCN4 (43), one might expect it to be located on a separate and evolutionary conserved exon. It is therefore likely that the ancestral jun gene, from which all three jun genes have probably evolved, might have lost its introns during evolution.

Using transient transfection assays, we have shown that sequences located between -91 and -44 are necessary for the induction of the junB promoter by serum. Although the junB gene is induced with similar kinetics as c-fos and βactin, this fragment does not contain a serum response element (SRE), which is responsible for serum induction of these early response genes (44, 45). The induction of junB by serum is therefore unlikely to be mediated by p67-SRF, one of the factors that bind to the SRE and is involved in serum responses (45). Footprinting analysis showed that three protein binding sites are present in the junB promoter, a G-C rich region (-100 to -77), a CAAT box (-66 to -61) and an inverted repeat (IR) (-57 to -50). Since only part of the G-C rich region is present in the fragment important for serum induction of junB, it seems not essential for this process. The CAAT box is usually recognized by transcription factor CTF (39), while the IR is a novel cis-acting element. Since CTF was not previously indicated to be involved...
in serum effects on transcription, and the IR is insufficient to confer full serum inducibility to a heterologous promoter, it will be interesting to further determine the mechanism of junB induction by serum by molecular analysis of its regulatory factors.

Our results suggests that the IR can mediate induction of the junB promoter by TPA and cAMP. Signal transduction via the adenylate cyclase/cAMP system involves activation of cAMP-dependent protein kinase A (PKA). Activation of PKA relays signals generated by stimulation of the cAMP/adenylate cyclase system to the cell nucleus (41, 46) as does protein kinase C (PKC) in reaction to TPA or receptor-linked phospholipid turnover (47). Activation of these kinases results finally in the modulation of the activity of transcription factors such as NF-kB or members of the AP-1 gene family after stimulation of PKC (7, 48, 49) or the CREB/ATF family after activation of PKA (50–53; for review see refs. 41 and 46). The best characterized protein of the CREB/ATF transcription factor family is CREB. CREB was identified as a 43 kD nuclear transcription factor, which binds as a dimer to the CRE of the somatostatin gene and activates transcription (52, 54). Unlike the recently described tyrosine amino transferase-CRE (TAT-CRE) binding factor (55), but like the classical CREB protein (54), the binding activity of IRBP is not enhanced upon induction of the adenylate cyclase system. Transcriptional activation of IRBP, in analogy with CREB, might therefore be mediated by a PKA mediated phosphorylation event (54, 56). The binding of IRBP is furthermore only competed by a PKA mediated phosphorylation event (54, 56). The binding of IRBP is not likely to be a member of the Jun/AP-1 family, since: 1—the apparent molecular weight of IRBP is 110 kD; 2—pre-incubation with a Fos antibody did not result in an altered mobility of IRBP; and 3—no competition of IRBP in a gel shift was observed with TRE oligonucleotides. We therefore hypothesize that the IRBP might represent a novel transcription factor involved in nuclear signalling by both PKC and PKA. Further characterization of IRBP is currently in progress.

In contrast to the data presented here, a recent paper by Lamph et al. shows that the repression of the c-jun TRE by CRE is relieved when PKA is co-transfected (57). However, since these studies were performed in NIH-3T3 cells, the discrepancy with the data from F9 cells presented here might be based on cell type specific differences in proteins binding to the c-jun TRE.

The observed versatile regulation of junB is apparently necessary to correctly control the timing and magnitude of junB expression in response to a wide variety of extracellular stimuli. Since JunB is a trans-repressing component of AP-1 (25, 26), this complex regulation is probably of major importance for the correct regulation of TRE containing target genes by Jun/AP-1. The availability of the junB promoter therefore offers new possibilities for investigation of the complex genomic response to phorbol esters and other extracellular stimuli.

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