Promoter-specific trans-Activation and Inhibition Mediated by JunB

Jui-Chou Hsu,1 Drew E. Cressman, and Rebecca Taub2

Department of Genetics [J-C. H., D. E. C., R. T.] and Howard Hughes Medical Institute [J-C. H., R. T.], University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6145

ABSTRACT

Nuclear levels of c-Jun, JunB, c-Fos, and LRF-1 (liver regeneration factor) are high for a large fraction of the G1 phase in regenerating liver and mitogen-stimulated hepatic cells. Previously, JunB was regarded as a less potent transcriptional activator than c-Jun that could also function as a repressor. However, we found that, like c-Jun, JunB alone or LRF-1/JunB strongly transactivates a cAMP-responsive promoter. Unlike c-Jun, JunB represses several AP-1 or activator of transcription factor sites-containing promoters, and this inhibition is greatly enhanced in the presence of LRF-1. Here, we identify separate regions of JunB required for trans-activation and repression of these promoters. Deletion analysis shows that the region involved in trans-activation function is highly conserved among all Jun family members and corresponds to activator domain (A1) of c-Jun. In contrast, repression is maximal in the presence of both the DNA-binding domain and a region proximal to the basic region that is highly divergent among Jun proteins. Functional distinctions between Jun proteins during induction of the growth response and tumorigenesis may be accounted for by promoter-specific activation and repression mediated by regional differences in Jun family proteins.

INTRODUCTION

The Jun proteins, including c-Jun, JunB, and JunD, are members of the leucine zipper class of transcription factors and share extensive amino acid sequence homology in the DNA-binding and leucine zipper domain and the HR-1 segment, a transcriptional activation domain of c-Jun (1). Overall, JunD is more closely related by sequence to c-Jun than to JunB. Jun proteins can form homodimers in binding to AP-1 and CRE3 sites, but they differ in their binding affinity (2–6) and preferentially form heterodimers with LRF-1 (7, 8) and Fos family proteins, including c-Fos, Fra-1, Fra-2, and FosB (9–11).

Jun proteins have different biochemical and physiological properties. Although all three are required for cell cycle progression in fibroblasts (12), Jun proteins are differentially expressed during development (13, 14), in different cell lines (3, 15), and in different tissues (3, 5). As immediate-early genes, junB and c-jun are rapidly induced by extracellular stimuli (6, 16–18). In contrast, JunD transcription is only weakly activated when stimulated and is high in quiescent cells (1, 5). c-Jun and JunB have transforming potential, and thus far, JunD does not (19, 20). However, junB is a much weaker transforming gene than c-jun and may suppress c-Jun-mediated transformation (19, 20).

When examining the early growth response in regenerating liver (for reviews, see Refs. 21 and 22) and insulin-treated hepatic cells, we identified LRF-1, a 21-kD leucine zipper containing protein and member of the Jun-Fos family (7). LRF-1, along with c-Fos, c-Jun, and JunB mRNAs and proteins are rapidly and highly induced during liver regeneration (7, 23), while other Fos family members and related proteins are not induced. LRF-1 can form homodimers but preferentially forms heterodimeric complexes equally well with c-Jun and JunB in binding to CRE, AP-1, and ATF sites and does not interact with c-Fos (7). We have shown that c-Fos/c-Jun, c-Fos/JunB, and LRF-1/c-Jun, LRF-1/JunB complexes are present in vivo for a large fraction of the G1 phase of the cell cycle in hepatic cells, and the relative level of LRF-1/JunB complexes increases significantly with time posthepatectomy and following mitogen stimulation (8).

Early studies suggested that JunB plays a dominant negative role in the stimulation of transcription. Most of these studies were performed in P9 or HeLa cells which do not demonstrate G0/G1 transition (24, 25). However, in cotransfection studies in serum-deprived NIH 3T3 cells, we demonstrated that, like c-Jun, JunB alone or in combination with LRF-1 activates a cAMP-responsive promoter. Unlike c-Jun, JunB represses several different AP-1 or ATF site-containing promoters (8), and this promoter-specific repression is greatly enhanced in the presence of LRF-1. Given the relative increase in LRF-1/JunB complexes post-mitogen stimulation, and the distinct effects on different promoters, we postulated that LRF-1/JunB may have an important role in regulating delayed-early gene expression during the G1 phase of liver regeneration and mitogen-stimulated cells (8). We have mapped an inhibitory domain in LRF-1, which, together with the leucine zipper/basic domain of the molecule, is required for LRF-1/JunB-mediated repression (8). Here, we describe the contribution of regions of JunB in mediating promoter-specific trans-activation and inhibition.

MATERIALS AND METHODS

Construction of Deletion Mutants. The series of deletion mutants was constructed using the polymerase chain reaction, followed by cloning into a PCMV expression vector as we have described previously (8). The following PCR oligonucleotides were used:

1 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
2 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
3 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
4 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
5 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
6 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
7 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
8 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
9 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;
10 CCAGGTACCACACGACCATGTGCAAGAAATGGAACAG;

Each mutant has only a single additional methionine codon and the downstream region ends in the 3′-untranslated region of mouse JunB. The primer pairs used were as follows: JunB 1, 6; JunB 2, 6; JunB 3, 6; JunB 4, 6; JunB 5, 6. All of the constructs have been sequenced.

Electrophoretic Mobility Shift Assays. A similar amount of each JunB deletion mutant mRNA was cotransfected with LRF-1 mRNA and used in gel shift analysis. Premade high-performance liquid chromatography-purified double-strand AP-1 and CRE oligonucleotides were radiolabeled and mixed with 2 μg of in vitro translated proteins, in a binding buffer containing 10 μM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM Mg-mercaptoethanol, and 4% glycerol. The reaction was incubated for 30 min and was then electrophoresed on a 5% Tris-glycine polyacrylamide (7). The AP-1 oligonucleotide TATACGATCTGCTACTCTGGAGTTA is from the human choriogonadotropin α-chain gene (26).

Transfection Analyses. Exponentially growing NIH 3T3 cells were transfected with the pENKAT construct (5 μg) (27), 5×CRE2cat (28) 21–4AP-1cat construct (4 μg) (29), AP-1cat (30) or PE3/4AP-1cat (5 μg) (31), and expression plasmid of either different junB deletion mutants alone or each junB mutant in combination with LRF-1 or c-fos. The exact amounts of expression constructs used are: CRE, CAMP response element; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; ATF, activator of transcription factor.
RESULTS

Although several studies have identified activation domains in c-Jun (32–34), to date no such analyses of JunB have been performed. To analyze whether the distinct transcriptional activity of JunB and JunB/LRF-1 could be attributable to specific regions in JunB, we examined the ability of several amino-terminal deletion derivatives of JunB to transactivate or repress specific promoters. As shown (Fig. 1), N64 was created by deleting the NH2-terminal 64 amino acids of JunB, which share 36% homology with c-Jun. This region of c-Jun encompasses the cell type-specific inhibitory region, δ (33). Recently, it has been shown that phosphorylation of specific amino acids within the δ region of c-Jun may be important for trans-activation function (35). The corresponding region within JunB does not appear to be kinase sensitive and does not function as a transcriptional activator (36). Additionally, ε, which decreases activation mediated by activator region A1 of c-Jun has been recently identified (37) and is not conserved in JunB. N142 removed the highly conserved HR-1 segment (79% homology) containing the a1 (subdomain of A1 responsible for activation) and e regions of c-Jun. N185, in addition to the basic and leucine zipper, contains a region with only 25% sequence homology to the corresponding activator domain 2 (A2) of c-Jun. The BL deletion contains only the basic and leucine zipper regions which are highly conserved between Jun proteins.

To demonstrate whether different deletion mutants of JunB have similar ability to form heterodimers with LRF-1 and bind to CRE or AP-1 sites, we transfected RNA encoding LRF-1 and JunB mutants and then cotranslated similar amounts of LRF-1 mRNA and each junB deletion mRNA in rabbit reticulocyte lysates. Rabbit reticulocyte lysate alone showed an endogenous binding activity to labeled AP-1 and CRE oligonucleotides (Fig. 2). Additionally, we showed that LRF-1 homodimers migrate at the position of the endogenous band. When both LRF-1 and JunB are present in the gel shift assay, only the LRF-1/JunB heterodimer, and neither the LRF-1 or JunB homodimer, is detected (7). Specific band shifts were detected upon incubation with LRF-1 plus various JunB deletion mutants that were competed by excess cold oligonucleotides (data not shown). In complexes with LRF-1, lysates with smaller sized JunB deletion mutants showed bands with correspondingly faster mobilities (asterisks, lanes 2–6 and 8–12). Since the intensity of specific band shifts was similar among deletion mutants, the basic/leucine zipper domain of JunB is sufficient for heterodimer formation, with LRF-1 and binding to either AP-1 or CRE sites.

The amino-terminal region of JunB is important for transcriptional activation. The proenkephalin promoter and reporter, ENKcat, contains a cAMP response element (CRE2) that is absolutely required for basal and cAMP-inducible transcription and contains the CRE core consensus sequence, CGTCA (27, 28). Previously, we showed that JunB alone or LRF-1/JunB complexes can transactivate proenkephalin reporters, ENKcat and 5XCRE2cat (8; data not shown). It has been shown that LRF-1 (human ATF-3) binds to the CRE2 element in the proenkephalin promoter (38). To analyze the functional domains of JunB in a cellular context, NIH 3T3 cells were cotransfected with the proenkephalin reporter, ENKcat, along with expression vectors encoding various JunB deletion mutants alone (Fig. 3A). The amount of JunB DNA was chosen to achieve a high level of trans-activation, and JunB alone activated ENKcat about 6-fold (Fig. 3A). N64 still contained 90% of the activation function as compared to wild-type JunB. N142, N185, and BL, all of which lack the A1 homology region, showed no ability to transactivate the ENKcat promoter. Previously, we showed that LRF-1 can potentiate the trans-activation of ENKcat mediated by JunB by 2- to 3-fold (8). However, in those studies, the JunB vector used contained the natural translation control region of JunB (“non-PCR JunB”), whereas all of the JunB deletion mutants, including intact JunB (“PCR JunB”), contained the control region of c-Fos introduced by PCR. Therefore, the relative amounts of LRF-1 and PCR JunB required to achieve maximal trans-activation were retarded (Fig. 3B, ○) relative to non-PCR JunB (●). Most likely because of small differences in transfected protein levels, PCR JunB showed less cooperativity with LRF-1 that extended over a narrow range. Whereas 8 μg LRF-1/4 μg non-PCR JunB always showed some cooperativity (Fig. 3B), 8 μg LRF-1/4 μg PCR JunB routinely resulted in a 2-fold decrease in the amount of trans-activation ob-
Fig. 3. Identification of a transactivating region in JunB. NIH 3T3 cells were transfected with pENKAT construct (5 μg; A, D, and E) or 5xCRE2cat (F) and expression plasmid of either different JunB deletion mutants (5 μg) alone (A), 0.5 μg LRF-1 in combination with 2 μg each JunB mutation (D), 4 μg of each JunB mutant in combination with LRF-1 (8 μg; E and F). Results were quantitated by densitometry after normalization for the level of placental alkaline phosphatase and standard deviations of identical experiments were determined. Fold activation is relative to the CAT activity of the reporters alone. B, transfection as above with indicated amounts of LRF-1 DNA with either non-PCR JunB (4 μg; □) or PCR JunB (2 μg; ○). C, Western blot showing levels of non-PCR JunB and LRF-1 obtained after cotransfecting the indicated amounts of each expression plasmid.

Fig. 4. Regions in JunB important for inhibition of AP-1 site-regulated reporters. NIH 3T3 cells were transfected with 21-4AP-1cat construct (4 μg) and expression plasmid of JunB deletion mutants alone (A). Fold repression indicates the activity relative to the activity of the 21-4AP-1cat reporter alone standardized to placental alkaline phosphatase activity or JunB deletion protein level (B) and standard deviations (bars) of separate experiments were determined. B, Western blot of extracts from cells transfected with indicated JunB deletion mutant plasmids and probed with an anti-JunB antibody. C, results of cotransfection experiment using the AP-1cat reporter (5 μg), c-fos expression plasmid (8 μg), and indicated JunB deletion mutants (4 μg). Relative CAT activity is indicated. bps, base pairs.
transcriptional repressor in the absence of these elements has low activity (29). Alone, BL had little inhibitory activity. These results suggest that the activator region is responsible for both JunB alone and JunB/LRF-1-mediated repression of AP-1 site-containing promoters (8). To gain insight into the contribution of JunB in mediating inhibition, we carried out cotransfection experiments with JunB alone in the presence of 21-AP-1cat [referred to as (AP1) 1 MCAT in Ref. 30], a reporter which has high endogenous activity. Although the transactivators responsible for basal activity have not been identified, activation is clearly mediated through the AP-1 elements because the reporter in the absence of these elements has low activity (29). Alone, JunB and N64 repressed the endogenous activity of this reporter by 3-fold (Fig. 4A). N142 and N185 which lack the putative activation domain were much more potent repressors than wild-type JunB. However, BL had little inhibitory activity. These results suggest that the region between amino acids 142 and 257 together with the basic/leucine zipper domain cause maximal inhibition. The NH2-terminal activator region seems to counteract the negative effect of this inhibition. Without the activator region, JunB can be a much stronger transcriptional repressor.

To establish the level of transfected proteins, we performed immunoblots using JunB-specific antibodies on the same samples in which CAT assays were performed. Two different antibodies that were tested only detected epitopes within the wild type and the two larger deletion proteins, N64 and N142. The relative levels of these proteins obtained after transfection varied by no more than 1.5-fold in three separate determinations (Fig. 4B). Thus, although the increase in repression between N64 and N142 was >10-fold, the level of the two JunB deletion mutant proteins was approximately equal in the transfected cells. We were not able to verify the level of N185 and BL proteins in transfected cells. However, both mutants translated well in vitro, N185 consistently demonstrated activity in multiple different experiments, and BL demonstrated trans-activation function in the presence of c-Fos (Fig. 4C). As shown in Fig. 4C, c-Fos-mediated trans-activation of AP-1 sites is dominantly present in the presence of JunB. Maximal c-Fos-mediated trans-activation requires only the BL domain of JunB which presumably potentiates heterodimer formation. Also of note, c-Fos, which is an inhibitor of the ENKcat promoter (8), blocks activation of the ENKcat promoter by wild-type JunB and the N64 deletion mutant (not shown).

When JunB deletion mutants were cotransfected with LRF-1, similar results were obtained using three different AP-1 site-containing reporters (Fig. 5). When 21-AP-1cat was used, cotransfection of LRF-1 with each of the JunB deletion mutants including BL resulted in enhanced repression relative to JunB mutants alone (Fig. 5A) or LRF-1 alone (Fig. 5A, lane 1). In the presence of 21-AP-1cat, the JunB activator region appeared to counteract the repressive effects of LRF-1/JunB, because BL showed a similar level of inhibition as JunB wild type and N64. N142 and N185, which do not have the activator region, were much more potent repressors. However, this counteraction by the activator region was not observed with other AP-1 (Fig. 5, B and C) or ATF-site reporters (not shown). These reporters are transactivated by LRF-1/c-Jun and are strongly inhibited (in most experiments >100-fold) by all JunB deletion mutants (wild type, N64, N142, N185) containing the proximal and BL regions. BL alone had a much smaller inhibitory effect, again implying that maximal inhibition requires a region proximal to the basic region and the dimerization/DNA-binding domain. Any inhibition mediated by JunB BL is likely due to the potentiation of JunB/LRF-1 heterodimer formation which effectively decreases the amount of LRF-1/c-Jun.

DISCUSSION

Although JunB has been considered to be a transcriptional inhibitor relative to c-Jun, we have found that JunB transactivates a cAMP-activated promoter at a level comparable to c-Jun (8). This trans-activation is mediated by the cAMP response element (CRE2). We were able to identify a activating region in JunB responsible for trans-activation of a cAMP element-regulated promoter that corresponds and is similar to the A1 activator region in c-Jun. We found that the same activating region is responsible for both JunB alone and JunB/LRF-1-mediated trans-activation of this promoter. In contrast, we were not able to identify any major activation domain in LRF-1 (8). Deletion of the amino or carboxyl terminus of LRF-1 with an.
intact leucine zipper/basic domain had little effect on JunB/LRF-1-mediated trans-activation of the same reporter. Thus, it is likely that the contribution to JunB/LRF-1 activation by LRF-1 is simply due to potentiation of heterodimer formation. When the levels of JunB are high such that JunB homodimers can form, LRF-1 is not necessary for trans-activation, and in fact, very high levels of LRF-1 may be inhibitory. This finding is consistent with what previously has been shown with c-Jun and c-Fos. The most active molecules are c-Jun homodimers, not c-Jun/Fos heterodimers (39), and highest levels of trans-activation are achieved at levels of c-Jun at which homodimers can form.

In extension of earlier studies (8), the results presented here demonstrate that JunB/LRF-1 can be a dominant negative inhibitor of c-Jun-mediated trans-activation of AP-1 site-regulated promoters. Maximal inhibition mediated by JunB or LRF-1 deletion mutants occurs when a region proximal to the basic region accompanies the basic/leucine zipper domain. In the presence of several AP-1- or ATF-regulated promoters, deletion of a single proximal region from either LRF-1 or JunB results in only a 20% inhibitory effect relative to intact LRF-1/JunB (Fig. 5, B and C; 8). All of the AP-1- and ATF-regulated promoters we tested behaved similarly. However, JunB-mediated inhibition of 21-AP-1-cat endogenous activity was less than JunB-mediated inhibition of LRF-1/c-Jun AP-1 activation (Figs. 4 and 5). Deletion of the activating region of JunB eliminated this difference. This finding suggests that the amino-terminal region of JunB can be a modulator of inhibition with respect to certain AP-1 promoters. However, because the etiology of endogenous activity of the 21-AP-1-cat reporter is unknown, the mechanism of this modulation is not clear.

Previously, we showed that this proximal region in LRF-1 has no inhibitory activity in the absence of the dimerization/DNA-binding region (8). From the similarity in the results obtained with JunB deletion mutants, it is likely that maximal JunB-mediated inhibition also requires both the proximal and basic/leucine zipper regions. There is no sequence homology between these regions in LRF-1 and JunB, but both are just proximal to the basic domain. It will be interesting to find out how these two regions cooperate with each other to cause inhibition of AP-1 site-mediated activation. It is possible that these regions are important in establishing distinctly different DNA-binding conformations on AP-1 sites and CRE sites which result in repression or activation, respectively.

JunB is responsible for mediating promoter-specific trans-activation and repression in serum-deprived NIH 3T3 cells which show G0/G1 transition and G1 phase progression following mitogen stimulation. Separate regions in JunB correlate with the ability to activate or repress the ENKcat/CREZ promoter and AP-1 site-containing promoters, respectively. The activation domain resides in a region which is highly conserved among c-Jun, JunB, and JunD. In fact, this region has been demonstrated to be critical for c-Jun- and JunD-mediated trans-activation (5, 33, 40) and is required for transformation of rat embryo cells (41). In contrast, the region of JunB required for maximal inhibition is very divergent from the corresponding region of c-Jun and JunD. Deletion of this region in c-Jun results in the complete loss of its ability to transactivate AP-1 site-containing promoters in F9 cells (33). In contrast, almost no effects in transforming activity and transactivating activity were found in rat embryo cells (41). Differences between corresponding regions of Jun proteins in the contribution to promoter-specific trans-activation may be responsible for distinct phenotypic effects mediated by different Jun proteins.

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