MOLECULAR AND CELLULAR BIOLOGY, Oct. 1992, p. 4654–4665
0270-7306/92/104654-12$02.00/0

Vol. 12, No. 10

Interactions among LRF-1, JunB, c-Jun, and c-Fos Define a Regulatory Program in the G_1 Phase of Liver Regeneration

JUI-CHOU HSU,[1] RODRIGO BRAVO,[2] AND REBECCA TAUB[1,3]*

Department of Human Genetics[1] and Howard Hughes Medical Institute,[3] University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6145, and Bristol-Myers Squibb Pharmaceutical Research Institute Molecular Biology Department, Princeton, New Jersey 08543-4000[2]

Received 10 April 1992/Returned for modification 22 June 1992/Accepted 14 July 1992

In regenerating liver, a physiologically normal model of cell growth, LRF-1, JunB, c-Jun, and c-Fos among Jun/Fos/LRF-1 family members are induced posthepatectomy. In liver cells, high levels of c-Fos/c-Jun, c-Fos/JunB, LRF-1/c-Jun, and LRF-1/JunB complexes are present for several hours after the G_0/G_1 transition, and the relative level of LRF-1/JunB complexes increases during G_1. We provide evidence for dramatic differences in promoter-specific activation by LRF-1- and c-Fos-containing complexes. LRF-1 in combination with either Jun protein strongly activates a cyclic AMP response element-containing promoter which c-Fos/Jun does not activate. LRF-1/c-Jun, c-Fos/c-Jun, and c-Fos/JunB activate specific AP-1 and ATF site-containing promoters, and in contrast, LRF-1/JunB potently represses c-Fos- and c-Jun-mediated activation of these promoters. Repression is dependent on a region in LRF-1 that includes amino acids 40 to 84 (domain R) and the basic/leucine zipper domain. As the relative level of LRF-1/JunB complexes increases posthepatectomy, c-Fos/Jun-mediated ATF and AP-1 site activation is likely to decrease with simultaneous transcriptional activation of the many liver-specific genes whose promoters contain cyclic AMP response element sites. Thus, through complex interactions among LRF-1, JunB, c-Jun, and c-Fos, control of delayed gene expression may be established for extended times during the G_1 phase of hepatic growth.

Liver regeneration provides one of the few systems for analysis of mitogenesis in the fully developed, intact animal. Following a two-thirds partial hepatectomy in which the remaining hepatic lobes undergo no injury, the majority of the hepatic cells, which are normally quiescent, rapidly reenter the cell cycle and initiate the first round of DNA synthesis in 12 to 16 h (16, 36). We have been interested in defining the liver-specific aspects of the growth response in regenerating liver and insulin-treated H35 cells, a hepatoma cell line that grows in response to insulin and has many properties of regenerating hepatocytes. To this end, as in other systems (2, 6, 10, 22, 30–32, 34, 57), we have identified immediate-early growth response genes induced in the absence of protein synthesis during the G_0-to-G_1 transition in regenerating liver and insulin-treated H35 rat hepatoma cells (37, 38). Immediate-early genes are felt to have important roles in regulating the growth response and driving cells through the G_1 phase of the cell cycle.

Some of the most highly induced immediate-early genes encode the Jun/Fos family of leucine zipper transcription factors, which include c-Jun, JunB, JunD, c-Fos, Fra-1, Fra-2, and FosB (11, 18, 24, 28, 35, 39, 46, 47, 49, 56). The multiple heterodimeric Fos/Jun complexes are felt to activate the transcription of delayed-early genes involved in later phases of the cell cycle and are postulated to have important roles in controlling subsequent G_1 events (26, 43). However, although Fos/Jun proteins have been strongly implicated in regulating delayed gene expression during the growth response, there has been little direct evidence to confirm their ability to activate target genes in cell systems reflective of a mitogen-stimulated cell (8, 9, 29, 50–52). Frequently, because of low endogenous AP-1 activity, these studies have been performed with F9 teratocarcinoma cells that may not have the same responses to Jun/Fos proteins as cells which demonstrate G_1 phase progression following mitogen stimulation (15, 40, 41, 52). Instead, these studies provide evidence for cell-type-specific transactivation by Jun/Fos family proteins.

We examined the expression of members of the Jun/Fos family in regenerating liver and insulin-treated H35 cells relative to that in serum-treated fibroblasts. We found that as in fibroblasts, *junB* and c-*jun* are induced in the liver systems during the G_0/G_1 transition, and *junD* is induced in H35 cells and to a much lesser extent in regenerating liver. Genes encoding related proteins, ATF-2/CRE-BP1 and ATF-4, are expressed at a low constitutive level in the first several hours posthepatectomy (54, 54a). Of Fos family members, c-*fos* is induced, while *fra-1* and *fosB* are not expressed in either liver cell system (38) and *fra-2* is induced at a very low level (24a). We wondered whether another protein induced in the liver systems might take the place of Fos family proteins.

In fact, using differential screening of regenerating liver cells, we identified a rapidly and highly induced gene encoding a novel 21-kDa leucine zipper-containing protein, designated LRF-1 (liver regeneration factor) (25). In previous studies we showed that LRF-1 has no strong homology with other leucine zipper proteins outside the basic domain. LRF-1 alone can bind DNA (25; see also reference 20 [ATF-3 appears to be the human homology]) but preferentially forms heteromeric complexes with c-Jun and JunB and does not interact with c-Fos (25). In solution, it binds with highest affinity to cyclic AMP (cAMP) response elements (CREs) but also has affinity for related AP-1 and ATF sites. In cotransfection studies, LRF-1 in combination with c-Jun strongly activates a CRE site-containing promoter. While *LRF-1* mRNA is rapidly induced in the absence of protein synthesis, its peak of induction is later than that of c-*fos* mRNA, suggesting that LRF-1 may regulate responsive genes at a later point in the cell cycle (25).

* Corresponding author.

In the studies presented here, we document that in vivo complexes between c-Fos/Jun proteins and LRF-1/Jun proteins are present for a large fraction of the G_1 phase of the cell cycle in hepatic cells and that the relative abundance of LRF-1/JunB complexes increases with time. Using a number of different target promoters that could serve as prototypes for delayed gene promoters, we have performed extensive analyses of the relative transactivating potentials of LRF-1, c-Fos, and Jun proteins. We find that in combination with Jun proteins, LRF-1 shows promoter-specific transactivation distinctly different from that of c-Fos. While strongly activating a CRE site-containing promoter, LRF-1/JunB is capable of potently repressing c-Fos and Jun transactivation of AP-1 and ATF site-containing promoters. These studies provide evidence for a regulatory program controlling delayed gene expression during the G_1 phase of regenerating liver and mitogen-stimulated cells.

MATERIALS AND METHODS

Rat tissue preparation and cell lines. For regenerating liver, Fischer rats (160 to 200 g; Bantin-Kingman) were ether anesthetized and subjected to midventral laparotomy with approximately 70% liver resection (left lateral and median lobes) (23). H35 cells were grown and induced with insulin as described elsewhere (55), and NIH 3T3 cells were grown as described previously (25).

Generation of antibodies. A polymerase chain reaction-generated DNA fragment containing the N-terminal 81 amino acids of LRF-1 was cloned into a modified hexahistidine PET expression vector (45). Cells transformed with this construct were induced to express the LRF-1 peptide by isopropyl-β-D-thiogalactopyranoside (IPTG) addition. The peptides were purified through an Ni^+ column (Qiagen) and used to generate LRF-1-specific rabbit polyclonal antibody by Cocalico biologicals. Anti-c-Fos, -c-Jun, and -JunB antibodies were as previously described (27).

Cell labeling and immunoprecipitation. For pulse-labeling experiments with ^{35}S-methionine (Amersham), quiescent H35 cells grown in 35-mm-diameter plates were stimulated with insulin. Thirty minutes before the indicated times, the cells were rinsed twice with Dulbecco modified Eagle medium without methionine or phosphate and labeled for 30 min with 500 μCi of ^{35}S-methionine per ml. For continuous labeling, quiescent H35 cells grown in 35-mm plates were rinsed twice and incubated with Dulbecco modified Eagle medium minus methionine or phosphate for 2 h. Cells were labeled with 500 μCi of ^{35}S-methionine per ml or $^{32}P_i$ (1 mCi/ml) in Dulbecco modified Eagle medium without methionine or phosphate supplemented with 2×10^{-8} M insulin for different times. At the indicated time, the cells were washed twice with cold saline and lysed by addition of 1 ml of radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.25 mM phenylmethylsulfonyl fluoride, 3 μg of aprotinin per ml). For immunoprecipitation, cell lysates were incubated with 3 μl of antiserum against c-Fos or LRF-1 for 1 h on ice and then incubated with 40 μl of protein A-Sepharose beads (Pharmacia) with shaking for 3 h at 4°C. The beads were spun and washed twice with 1 ml of buffer A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), once with buffer B (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), and once with buffer C (10 mM Tris-HCl, pH 7.5). To identify the specific component in the immunocomplexes, the washed protein A-Sepharose beads with immunocomplexes were boiled in denaturing buffer (50 mM Tris-HCl, pH 7.5, 0.5% SDS, 70 mM β-mercaptoethanol) for 10 min and diluted by addition of 4 volumes of radioimmunoprecipitation assay buffer without SDS. Antisera to specific components were added sequentially (see Fig. 1) and brought down by protein A-Sepharose beads as before. The samples were boiled and run on an SDS–12.5% polyacrylamide gel. Gels were fixed, and fluorography was performed by incubating the gel in En^3Hance (Du Pont) according to the instructions of the manufacturer.

Immunoblotting. Liver nuclear extracts were prepared as previously described (14) with some modifications. Nuclear extracts from normal and regenerating liver were electrophoresed on an SDS–12.5% polyacrylamide gel, transferred to nitrocellulose, and detected by ECL (Amersham) according to the instructions of the manufacturer. Briefly, antisera diluted to 1:1,000 or 1:3,000 were incubated with the membrane for 1 h at room temperature. Horseradish peroxidase-linked secondary antibody was then added at a dilution of 1:9,000 for 1 h.

Plasmids. As described for Fos and c-Jun (52), we used a cytomegalovirus expression vector system to express LRF-1, c-Fos, c-Jun, JunB, and LRF-1 mutants which were constructed as described below. The *Eco*RI fragments of *LRF-1*, *c-fos*, and *junB* (approximately 1.8, 1.5, and 1.5 kb, respectively) and the *Hin*dIII-*Bam*HI fragment of *c-jun* (2.5 kb), all containing the initial methionine, were inserted into a cytomegalovirus type 5 expression vector (3). All clones are of rat origin except *junB* (47). All the *LRF-1* deletion mutants were generated by the polymerase chain reaction technique. Briefly, a 5′ overhang with a *Kpn*I site, 6 bp of *c-fos* ribosome binding site, and ATG was linked to 21 bp of sequence of the N-terminal region of each deletion mutant to create the forward primers. The backward primers were made by linking a 3′ overhang with a *Bam*HI site and stop codon to 21 bp of sequence complementary to the C-terminal region of the mutants. Referring to Fig. 7, *LRF-1* is the wild-type *LRF-1* polymerase chain reaction product. N39 and N84 contain 39- and 84-bp deletions from the N-terminal region, respectively, but both with an intact basic domain and leucine zipper region. The C34 mutant lacks the entire C-terminal region outside the leucine zipper region. The BL mutant contains only the leucine zipper and basic domain. The C66 mutant has a deletion of 66 amino acids (aa) from the C-terminal region of LRF-1 which includes the leucine zipper domain. The C90 mutant, without the leucine zipper and basic domain, contains the 90-aa deletion from the C terminus. All the deletion mutants have been sequenced. When gel shift assays were used, methods and sites were as described previously (25, 53).

The reporter genes used have been described elsewhere: ENK(CRE)cat (pENKAT 12) (12, 13) and 5xCRE2cat (42), courtesy of M. Comb; $(AP\text{-}1)_3$cat (33) and 21-4AP-1cat (36a), courtesy of R. Tjian and P. Mitchell; and PE3/4AP-1cat and PE3/4ATFcat (7), courtesy of R. Weinmann.

Cotransfections. NIH 3T3 cells were transfected with the indicated amounts of pCMV expression plasmids and 3 μg of pSV2A-PAP as a transfection control. In all cases, the amount of DNA transfected per dish was made constant with the addition of pCMV without an insert. At 16 to 18 h after $CaPO_4$ transfection, cells were serum deprived (0.5% fetal calf serum), and 24 h later cells were harvested and chloramphenicol acetyltransferase (CAT) assays were performed (17). Results were quantitated by densitometry after normalization for the level of placental alkaline phosphatase (21). None of the transfected pCMV expression plasmids had a

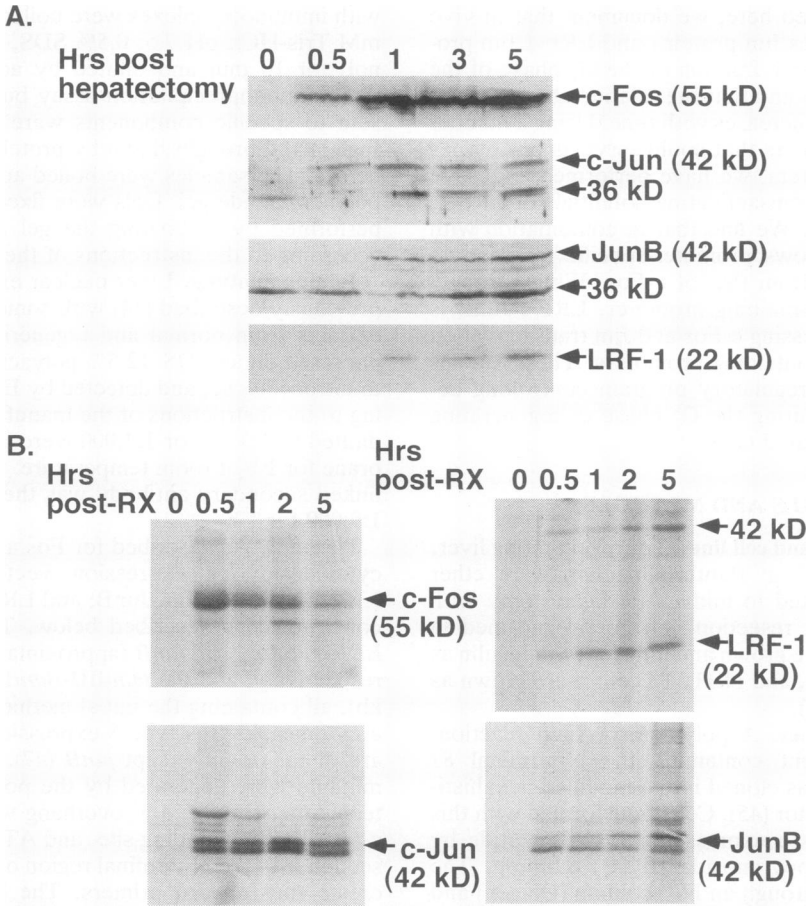


FIG. 1. (A) c-Fos, c-Jun, JunB, and LRF-1 remain elevated for extended times posthepatectomy in regenerating liver. Western blot of expression of c-Fos, c-Jun, JunB, and LRF-1 during liver regeneration was performed at the indicated times posthepatectomy. The 36-kDa band in the two Jun samples was not observed in immunoprecipitations and is probably nonspecific. (B) Protein synthesis of c-Fos, c-Jun, JunB, and LRF-1 parallels mRNA expression in insulin-treated H35 cells. Serum-deprived H35 cells were pulsed with ^{35}S-labeled methionine for 30 min prior to protein extraction at the indicated times post-insulin treatment, and following denaturation, proteins were identified by immunoprecipitation. In the LRF-1 panel, the 42-kDa band probably is the result of coimmunoprecipitation of Jun proteins.

strong transcriptional effect on the transfected pSV2A-PAP plasmid, as the alkaline phosphatase activity varied from the mean by only up to twofold and correlated with the number of cells per dish. For each condition, the same results were obtained at least two times, and data are presented as described in the figure legends. Using immunoblots, we demonstrated that various combinations of transfected *LRF-1*, c-*fos*, and *jun* pCMV expression plasmids did not have a large transcriptional effect on the pCMV promoter, because there was only a small deviation (maximum, twofold) from the predicted levels of transfected LRF-1 and Jun proteins. Because of the relatively low sensitivity and specificity of two anti-c-Fos antibodies, we were unable to demonstrate c-Fos protein in transfected cells. Additionally, in cells transfected with vector alone and then serum deprived, endogenous LRF-1, JunB, and c-Jun were not detectable. Using immunoblots with anti-LRF-1 antibodies, we also documented the presence of LRF-1 proteins LRF-1, LRF-1(dC34), and LRF-1(dC66) in transfected cells, the only deletion mutants which reacted with anti-LRF-1 antibody, which appears to be specific in immunoblots for aa 1 to 39.

RESULTS

The initial appearance of LRF-1, c-Jun, JunB, and c-Fos proteins in regenerating liver and insulin-treated H35 cells parallels mRNA expression, but protein expression remains elevated for several hours. Our previous studies showed that during liver regeneration, c-*fos* mRNA is rapidly induced with peak expression at 30 min, c-*jun* and *junB* mRNAs are induced within 30 min but remain elevated for several hours, and *LRF-1* mRNA is induced with a peak level at 1 to 3 h (25, 38). To predict which heterodimeric complexes are likely to be present at any time posthepatectomy, we determined the temporal course of expression of LRF-1, c-Fos, c-Jun, and JunB proteins using specific antibodies to JunB, c-Jun, and c-Fos (27) and specific antibodies raised against the amino-terminal unique portion of LRF-1 reactant only with in vitro-translated LRF-1 and not c-Fos, c-Jun, or JunB. Although c-*fos* mRNA rapidly decreases, in an immunoblot of nuclear extracts from normal and regenerating liver, c-Fos protein appears at 30 min and remains elevated for at least 5 h posthepatectomy (Fig. 1A). c-Jun protein also is elevated at 30 min posthepatectomy and remains elevated at 5 h posthepatectomy. However, JunB and LRF-1 do not appear

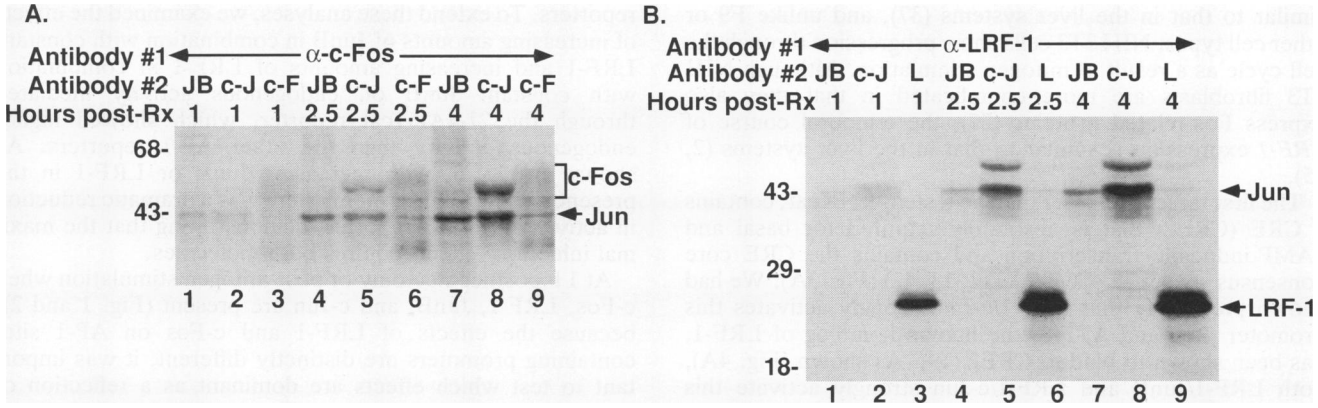


FIG. 2. Heterodimeric complexes of c-Fos/c-Jun, c-Fos/JunB, LRF-1/c-Jun, and LRF-1/JunB are detectable for several hours post-mitogen stimulation of hepatic cells. Cells were first labeled with ^{35}S-labeled methionine, and immunoprecipitations with antibody 1 α-c-Fos [A] or α-LRF-1 [B] were performed at the indicated times post-insulin treatment of serum-deprived H35 cells. The immunoprecipitates were then boiled to release complexes, and a second immunoprecipitation was performed with antibody 2 as indicated; JB, α-JunB; c-J, α-c-Jun; c-F, α-c-Fos; L, α-LRF-1. The products were electrophoresed on a 12.5 or 15% denaturing polyacrylamide gel and exposed to autoradiography.

until 1 h posthepatectomy, and their levels continue to increase even at 5 h posthepatectomy.

Previously, we had shown that the quantitative, qualitative, and temporal expression of *jun/fos/LRF-1* mRNAs observed in regenerating liver was roughly reflected in insulin-treated H35 cells (25, 38). To explore this similarity further, we examined the synthetic time course of the c-Fos, c-Jun, JunB, and LRF-1 proteins following insulin treatment. By pulse-labeling for the last 30 min with ^{35}S-methionine at the indicated times and immunoprecipitation with different antibodies, we found that protein synthesis roughly reflects mRNA expression and that all proteins continue to be synthesized at least 5 h post-insulin treatment (Fig. 1B). Of note, in H35 cells, c-*fos* mRNA peaks at 30 min but remains elevated for a more prolonged time than in other cells (55). The levels of newly synthesized LRF-1 and JunB are maximal 2 to 5 h post-insulin treatment, while the levels of newly synthesized c-Fos and c-Jun are highest at 0.5 to 1 h posttreatment. As in normal liver, quiescent H35 cells express very little c-Jun, JunB, c-Fos, or LRF-1 protein.

In vivo complexes between LRF-1 and c-Fos and both c-Jun and JunB are detected in H35 cells for several hours post-mitogen stimulation. Using gel shift assays, we have shown that LRF-1 can form heterodimers with Jun proteins (25). Using proteins translated in rabbit reticulocyte lysate, we next demonstrated that we could detect in vitro LRF-1/c-Jun and LRF-1/JunB complexes by using specific antibodies. We found that the relative formation of complexes between LRF-1 and either JunB or c-Jun is roughly equivalent. However, we found that anti-LRF-1 antibodies partially dissociate LRF-1/Jun heterodimers and that both anti-c-Jun and anti-JunB antibodies incompletely immunoprecipitate the denatured c-Jun and JunB molecules. In addition, the methionine content in c-Jun and JunB is very different. The results of these in vitro analyses indicated to us that we would be able to document specific complex formation in vivo and measure changes in the relative, not absolute, levels of individual complexes.

In gel shift assays using CRE, AP-1, and ATF site oligonucleotides and nuclear extracts from regenerating liver and insulin-treated H35 cells, we observed little complex formation in serum-deprived H35 cells and normal liver and a dramatic induction in protein complexes bound to these sites by 30 min posttreatment which persisted for several hours (not shown). To precisely identify the relevant complexes, we used immunoprecipitation assays to detect LRF-1/c-Jun, LRF-1/JunB, c-Fos/c-Jun, and c-Fos/JunB complexes as a function of time in insulin-treated H35 cells continuously labeled with ^{35}S-methionine. As shown (Fig. 2A), we first immunoprecipitated complexes with anti-c-Fos antibody, dissociated the complexes, and then performed a second immunoprecipitation with JunB, c-Jun-, or c-Fos-specific antibody. c-Fos/c-Jun and c-Fos/JunB complexes were detectable by 1 h and had increased 5- to 10-fold by 4 h posttreatment. We then examined LRF-1 complex formation (Fig. 2B) and found that LRF-1-specific complexes with c-Jun were present at all times posttreatment but that LRF-1/JunB complexes were barely apparent at 1 h (lane 1). LRF-1/c-Jun complexes increased 10-fold from 1 to 4 h (lanes 2, 5, and 8), and LRF-1/JunB complexes increased 40-fold during this period (lanes 1, 4, and 7).

LRF-1 and c-Fos show distinctly different transactivation potentials in combination with Jun proteins. On the basis of the findings in Fig. 1 and 2, we predict that in regenerating liver, predominantly c-Fos/c-Jun complexes are present within minutes posthepatectomy. With time posthepatectomy, all four complexes, c-Fos/c-Jun, c-Fos/JunB, LRF-1/c-Jun, and LRF-1/JunB, are present, and the relative increase in LRF-1/JunB is most dramatic. The relative increase in LRF-1 and JunB is even more significant in regenerating liver than in H35 cells, because the levels of nuclear LRF-1 and JunB increase significantly between 3 and 5 h while the levels of c-Jun and c-Fos are maximal by 1 h (Fig. 1A). We wondered whether the four heterodimers that predominate in the liver systems would show differential activity with respect to prototypic promoter elements that could be present in delayed-early genes. We reasoned that because of the relatively high abundance of LRF-1 in the liver systems, LRF-1-containing complexes might have a unique role in regulating delayed gene expression during hepatic growth. To begin to explore the relative function of the various heterodimers expressed in the liver systems, we used transient transfection assays in serum-deprived NIH 3T3 cells. The immediate-early response in NIH 3T3 cells is

similar to that in the liver systems (37), and unlike F9 or other cell types, NIH 3T3 cells show progression through the cell cycle as a result of mitogen stimulation. Although NIH 3T3 fibroblasts are more complicated in that they also express Fos-related proteins (27), the temporal course of *LRF-1* expression is similar to that in the liver systems (2, 25).

The first target promoter that we tested, ENKcat, contains a CRE (CRE2) that is absolutely required for basal and cAMP-inducible transcription and contains the CRE core consensus sequence, CGTCA (12, 13, 42) (Fig. 3A). We had shown previously that LRF-1/c-Jun strongly activates this promoter (25), and ATF-3, the human homolog of LRF-1, has been shown to bind to CRE2 (19). As shown (Fig. 4A), both LRF-1/JunB and LRF-1/c-Jun strongly activate this promoter more than LRF-1 or Jun alone. Of note, JunB alone was consistently an activator of this promoter (Fig. 3B), and activation was demonstrated by using CRE2 in five tandem copies (Fig. 3B). On the other hand, c-Fos in combination with either Jun protein did not activate the ENKcat reporter (Fig. 4B), and activation of 5xCRE2cat with c-Fos plus either Jun protein was not above the activation achieved by c-Jun or JunB alone (Fig. 3B). This is in agreement with earlier findings in which Fos/c-Jun activated this promoter in F9 cells but not in 3T3 cells (52), again emphasizing that F9 cells may not reflect the same activation seen during the growth response.

Another promoter element that could potentially be found in delayed genes is the AP-1 site. In our studies, three different AP-1cat constructs, each of which contains core AP-1 sites (TGACTC) in a different context (Fig. 3A), were tested. The results are summarized in Fig. 3B and presented for $(AP\text{-}1)_3$cat in Fig. 4C and D. We found that LRF-1 in the presence of c-Jun was a very weak transactivator of AP-1 reporters, stimulating activity only slightly more than c-Jun alone. In the presence of JunB, LRF-1 appeared to repress endogenous promoter activity, and LRF-1 alone had little effect. On the contrary, c-Fos plus c-Jun or JunB resulted in strong activation of AP-1 promoters, well above the effect of c-Fos or either Jun protein alone. At low concentrations of c-Fos, JunB showed lower activation of $(AP\text{-}1)_3$cat relative to c-Jun, but at higher c-Fos concentrations, the transactivation was roughly equal. Of note, although other AP-1 constructions showed stimulation by c-Fos/Jun (Fig. 3), there was construct-specific variation and, in particular, PE3/4AP-1cat was strongly activated by c-Jun alone. However, in all cases LRF-1/JunB repressed these promoters, confirming that repression was mediated through AP-1 sites.

Furthermore, we tested an ATF reporter, PE3/4ATFcat, containing consensus ATF sites (TGACGA) and found very strong cooperativity in transactivation mediated by c-Jun and LRF-1 and by c-Fos with either Jun protein (Fig. 4E and F). No protein alone activated this reporter, but with protein combinations the activation was greater than 100-fold. This is the greatest level of cooperativity that has been observed between Jun/Fos/LRF-1 proteins in transfected NIH 3T3 cells. However, like AP-1cat, ATFcat was not activated by LRF-1/JunB. Interestingly, although the core sequences of the ATF and AP-1 elements in the reporters used here are substantially different, the activities of ATF and AP-1 promoters with respect to LRF-1, Jun, and Fos proteins were similar (Fig. 3, 4, and 7B).

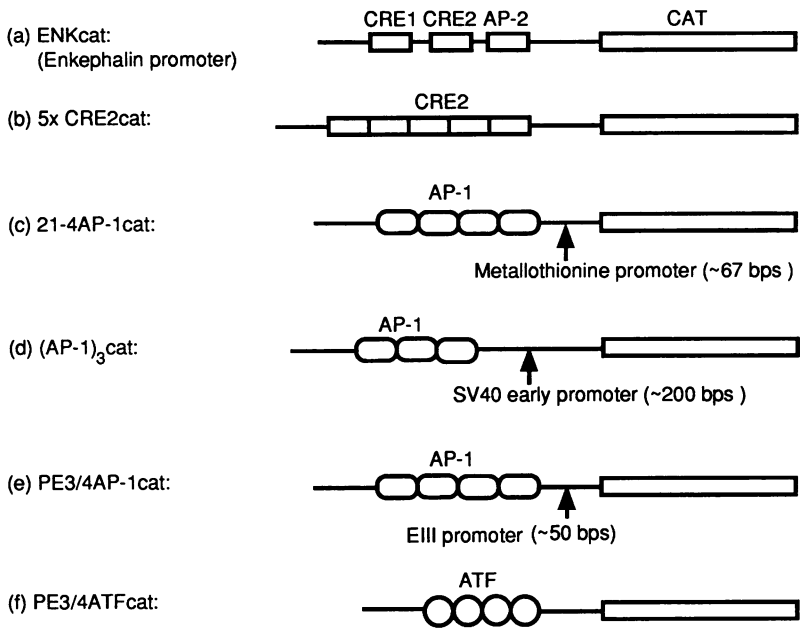
LRF-1/JunB represses c-Jun- and c-Fos-mediated transactivation of ATF and AP-1 site-containing promoters. In the studies mentioned above, it appeared that LRF-1 in combination with JunB reduced endogenous activity from AP-1 reporters. To extend these analyses, we examined the effects of increasing amounts of JunB in combination with constant LRF-1 and increasing amounts of LRF-1 in combination with constant JunB on endogenous activity mediated through the 21-4AP-1cat reporter, which showed higher endogenous activity than the other AP-1 reporters. As shown in Fig. 5, with increasing JunB or LRF-1 in the presence of the other protein, there was a dramatic reduction in activity from this reporter, demonstrating that the maximal inhibitory effect requires both molecules.

At 1 h posthepatectomy or post-mitogen stimulation when c-Fos, LRF-1, JunB, and c-Jun are present (Fig. 1 and 2), because the effects of LRF-1 and c-Fos on AP-1 site-containing promoters are distinctly different, it was important to test which effects are dominant as a reflection of relative protein concentration. We stimulated the $(AP\text{-}1)_3$cat promoter with JunB plus c-Fos and then examined the effects of increasing amounts of LRF-1 (Fig. 6A). We found that LRF-1 could repress c-Fos/JunB-mediated transactivation of $(AP\text{-}1)_3$cat. Furthermore, as shown (Fig. 6B), stimulation of PE3/4AP-1cat in the presence of LRF-1 and c-Jun could be dramatically reduced by small amounts of JunB. Because of different efficiencies of specific antibodies in Western blots (immunoblots), we could not accurately measure relative cellular protein levels in transfected cells. However, in general, the level of JunB appeared somewhat higher than the levels of LRF-1 and c-Jun for a corresponding amount of DNA. The amount of *LRF-1* and *junB* DNA needed to achieve complete repression was approximately equal to the amount of c-*fos* and c-*jun* DNA, respectively. In a similar assay, we also demonstrated that LRF-1 and JunB can block transactivation of PE3/4AP-1cat and PE3/4ATFcat by c-Fos/JunB and LRF-1/c-Jun, respectively (not shown). These results suggest that the repressive effects of JunB and LRF-1 can be dominant.

LRF-1-induced repression is mediated by a region including aa 40 to 84 and the leucine zipper/basic domain. To identify the region of LRF-1 responsible for ATF/AP-1 promoter repression, we performed deletion analyses of LRF-1 (Fig. 7). We documented that LRF-1 deletion constructs (LRF-1, dN39, dN84, and dC34) were similarly able to form heterodimers with Jun proteins and bind to ATF and AP-1 sites in vitro (not shown). We then tested the LRF-1 deletions for their ability to repress the c-Fos/JunB-induced activity of the $(AP\text{-}1)_3$cat reporter. The values given in Fig. 7 represent percent repression relative to that of the intact LRF-1 protein, which is arbitrarily assigned a value of 100. Only the molecules (LRF-1, dN39, and dC34) which contain the leucine zipper/basic domain and aa 40 to 84 (domain R) showed maximal inhibition of c-Fos/JunB-induced activity. The mutants without domain R (dN84 and BL), although bearing the leucine zipper/basic domain, had only a 30% inhibitory effect compared with that of LRF-1. The findings with other AP-1 reporters were similar.

Activation is dominant in the LRF-1/c-Jun heterodimer. c-Jun itself is an activator of some, although not all, reporter genes used in our assay condition, and previously the activation domains of c-Jun have been mapped (1, 4). A question that arises is which domain will be dominant in the LRF-1/c-Jun heterodimer which contains both an inhibitory region from LRF-1 and an activation domain from c-Jun. As shown in Fig. 3B, it is clear that the activation domain contributed by c-Jun is dominant over any inhibitory domain of LRF-1, because LRF-1/c-Jun transactivated all of the reporter genes tested. We then asked how significant a role different regions of LRF-1 played in LRF-1/c-Jun-mediated

A. Reporter Genes:



B. Transfection Analyses:

Reporter \ Expression vector	c-jun	junB	LRF-1	c-fos	c-jun/LRF-1	c-jun/c-fos	junB/LRF-1	junB/c-fos
(a) ENKcat	◀▶	▲	◀▶	◀▶	▲▲▲	◀▶	▲▲▲	◀▶
(b) 5xCRE2cat	▲▲	▲▲	◀▶	◀▶	▲▲▲	▲	▲▲	▲▲
(c) 21-4AP-1cat	◀▶	▼	▼	◀▶	◀▶	◀▶	▼▼▼	◀▶
(d) (AP-1)3cat	▲	▼	◀▶	▲	▲▲	▲▲▲	▼▼▼	▲▲▲
(e) PE3/4AP-1cat	▲▲▲	▼	◀▶	▲▲	▲▲▲	▲▲▲	▼▼▼	▲▲▲
(f) PE3/4ATFcat	◀▶	◀▶	◀▶	◀▶	▲▲▲	▲▲▲	▼▼▼	▲▲▲

▲▲▲ = >10 fold activation
▲▲ = 5-10 " "
▲ = 2-5 " "
◀▶ = 0-2 fold activation
▼ = 0-<0 " "
▼▼▼ = <<0 " "

FIG. 3. Summary of transcriptional effects of LRF-1, c-Jun, JunB, and c-Fos on CRE, AP-1, and ATF-containing reporter genes. (A) Schematic diagram of reporter genes used in panel B. SV40, simian virus 40. (B) A series of independent cotransfection assays similar to those in Fig. 4 were performed with the indicated combinations of expression vectors and reporter constructs. Each transfection contained 4 to 5 μg of reporter constructs and increasing amounts of *LRF-1* or *c-fos* (from 1 to 16 μg) along with a constant amount (4 μg) of *c-jun* or *junB* expression plasmid. The relative CAT activities from each set of experiments were transformed into the indicated symbols on the basis of induction or repression of the endogenous reporter construct activity. However, PE3/4AP-1cat and PE3/4ATFcat had to be activated by c-Fos/JunB or LRF-1/c-Jun before repression by LRF-1/JunB could be observed. In most instances, results reflect simultaneous duplicate determinations.

transactivation. To address this question, we performed cotransfection analyses with intact c-Jun and deletion mutants of LRF-1 in the presence of PE3/4ATFcat. This reporter is cooperatively activated by LRF-1/c-Jun greater than 100-fold over endogenous promoter activity or activity with c-Jun alone or LRF-1 alone (Fig. 4E). Although full activation in the presence of c-Jun requires the entire LRF-1 molecule, all of the LRF-1 deletion mutants that contained at least a leucine zipper/basic domain resulted in strong activation ranging from 30- to 55-fold (Fig. 7B).

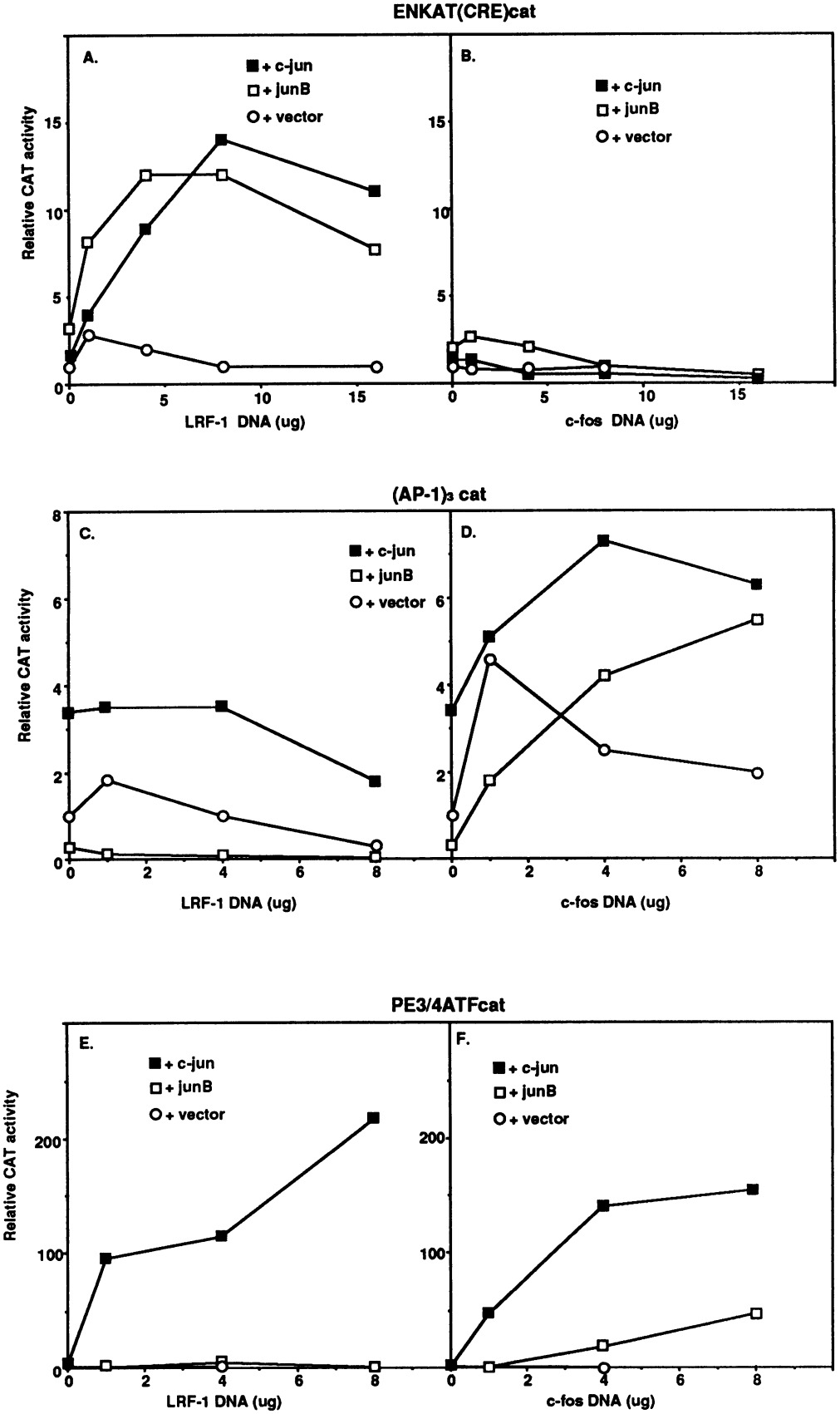

ENKAT(CRE)cat
A.
B.
+ c-jun
+ junB
+ vector
Relative CAT activity
LRF-1 DNA (ug)
c-fos DNA (ug)
(AP-1)3 cat
C.
D.
PE3/4ATFcat
E.
F.

FIG. 4. Target promoters containing CRE, AP-1, and ATF sites are differentially activated or repressed by LRF-1 and c-Fos in the presence of JunB and c-Jun. Serum-starved NIH 3T3 cells were cotransfected with constant amounts of ENKAT(CRE)cat (5 μg) (A and B), $(AP\text{-}1)_3$cat (4 μg) (C and D), or PE3/ATFcat (4 μg) (E and F) in the presence of c-*jun* or *junB* expression plasmid (4 μg) and various amounts of *LRF-1* (A, C, and E) or c-*fos* (B, D, and F) expression plasmid. The amount of expression vector in each sample was adjusted to the same level by addition of pCMV vector without an insert (see Materials and Methods); CAT activity was normalized to alkaline phosphatase activity and expressed relative to the activity from the corresponding reporter gene plus pCMV alone, which was given a value of 1.0. Experiments represent triplicate determinations (A and B), duplicate determinations performed simultaneously (C and D), or a representative experiment (E and F), but in all cases, the same results were obtained several times. In panel E, the value of >200 for activation by *LRF-1* (8 μg)/c-*jun* (4 μg) was higher than the average (150-fold) from three experiments.

DISCUSSION

c-Fos/Jun and LRF-1/Jun complexes are present in hepatic cells for extended times posthepatectomy or post-mitogen stimulation. Previous studies with 3T3 fibroblasts indicated that complexes between Fos and Jun proteins are present for extended times post-mitogen treatment (27), and our studies with hepatic cells confirm these findings and extend these analyses to include the LRF-1 protein. These results allow us to make predictions (Fig. 8) about heterodimer formation during the hepatic growth response. Within the first hour, c-Fos/c-Jun complexes are predominant (Fig. 1 and 2), and later, c-Fos/c-Jun, c-Fos/JunB, LRF-1/c-Jun, and LRF-1/JunB complexes are abundant, the relative level of LRF-1/JunB complexes continuing to increase. As DNA synthesis initially occurs 12 to 16 h posthepatectomy in regenerating liver and 9 to 10 h post-insulin treatment in H35 cells, it is likely that Jun/Fos/LRF-1 proteins activate delayed gene promoters for a significant fraction of the G_1 phase of the cell cycle.

The transactivation potential of LRF-1/Jun is distinctly different from that of c-Fos/Jun heterodimers. We have clearly demonstrated dramatic differences in the activation of six target promoters by different combinations of c-Fos, c-Jun, JunB, and LRF-1. LRF-1 in combination with c-Jun or JunB activates a CRE site-containing promoter, and LRF-1/c-Jun, c-Fos/c-Jun, and c-Fos/JunB activate specific ATF and AP-1 site-containing promoters. However, LRF-1/JunB strongly represses the ATF and AP-1 site-containing promoters used in these studies in a dose-dependent and dominant fashion. Although CRE and ATF core sequences in the reporters used here are similar, in agreement with results of this study, such ATF and CRE elements have previously been shown to have different activities (7). We can make predictions about activation of delayed gene promoters regulated by CRE, AP-1, and ATF elements with similar sequences and contexts. Given the relative abundance of these proteins at different times posthepatectomy, we predict that transcriptional activity of promoters regulated by AP-1 and ATF sites is high for the first hour posttreatment in regenerating liver and mitogen-stimulated cells. As levels of LRF-1 and JunB increase, these promoters will become less active and promoters regulated by CREs should become more active and remain active for many hours (Fig. 8).

Potential mechanisms of LRF-1/Jun-mediated activation and repression. In the context of the above discussion, LRF-1 may regulate specific gene expression by several mechanisms. First, LRF-1 may compete with c-Fos for c-Jun or JunB binding, in which both the concentration and the relative affinity of c-Fos and LRF-1 for c-Jun or JunB molecules are the major factors. As we have shown, the relative levels of the individual proteins and therefore of the various heterodimers will change during G_1. Second, promoter specificity may be determined by heterodimer competition for CRE, AP-1, and related elements in which the affinity for specific DNA sequences is a contributing factor. For example, we have previously demonstrated that both LRF-1/c-Jun and LRF-1/JunB heterodimers have higher affinity for a specific CRE site than for an AP-1 site (25), and similar analyses using Jun/Fos proteins have demonstrated site-specific differences in affinity (48). Third, inhibitory and activation domains may be brought into relative proximity by heterodimer formation and binding to a specific DNA sequence. This interaction may determine the final effect of a specific heterodimer as either an activator or a repressor. Composite interactions between these elements and DNA-

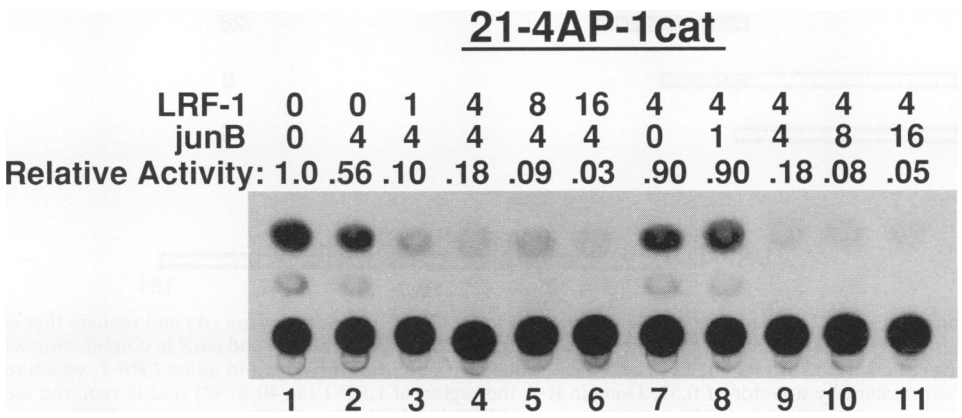


FIG. 5. LRF-1 in combination with JunB can suppress the basal activity of 21-4AP-1CAT. 21-4AP-1CAT (5 μg) was cotransfected with the indicated amounts of *LRF-1* or *junB* (in micrograms of DNA), and the results of the CAT assay are shown. The CAT activity observed with 21-4AP-1CAT alone was arbitrarily set at 1.0.

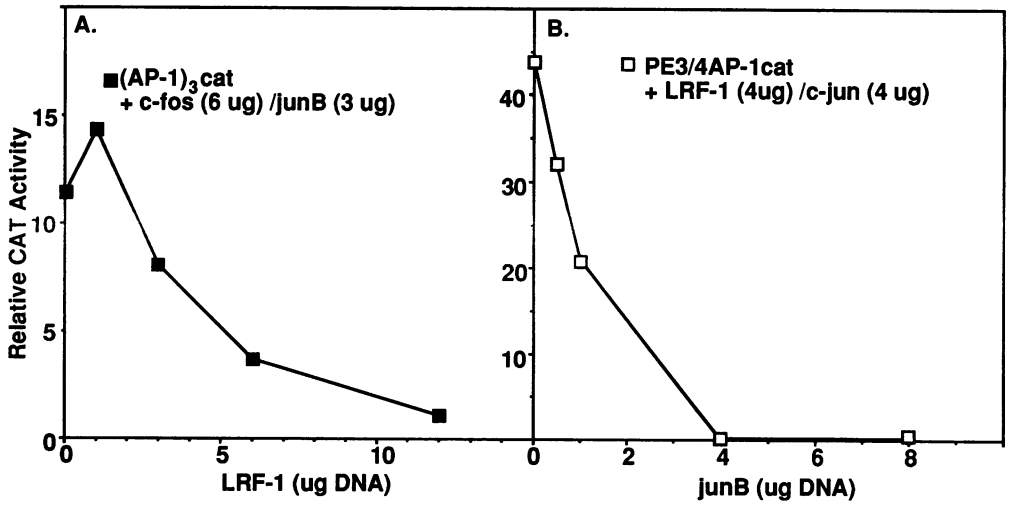


FIG. 6. (A) LRF-1 can repress Fos/JunB-mediated activation on an AP-1-containing reporter. $(AP\text{-}1)_3$cat (5 μg) was cotransfected with a fixed amount of c-*fos* and *junB* and increasing amounts of *LRF-1* expression plasmid as indicated. (B) JunB can suppress activation by LRF-1/c-Jun of an AP-1-containing reporter gene. PE3/4AP-1cat (5 μg) was cotransfected with a fixed amount of *LRF-1* and c-*jun* and increasing amounts of *junB* as indicated.

binding proteins will determine the strength of a particular target promoter at different times following mitogen stimulation, thus fine-tuning the growth response.

In previous studies, JunB and ΔFosB (FosB/SF) were predicted to be repressors of c-Jun and Fos transactivation, respectively, in F9 cells (8, 41), although not necessarily in 3T3 or other cells (15, 40). However, both JunB and ΔFosB are themselves weak activators (8, 15, 40) and could de-

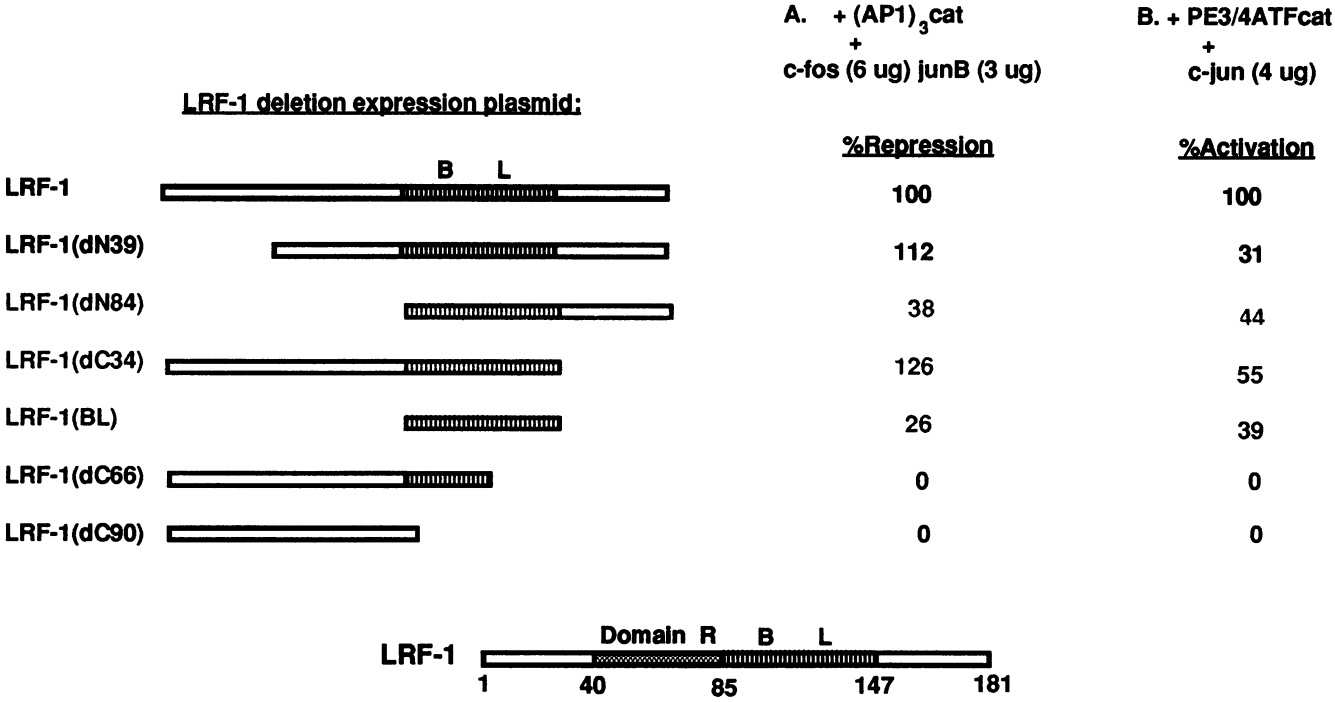


FIG. 7. Deletion analysis of LRF-1 identifies a region important for ATF/AP-1 site repression (A) and regions that cooperate with c-Jun in transactivation (B). (A) The reporter plasmid $(AP\text{-}1)_3$cat (5 μg) was cotransfected with c-*fos* and *junB* in combination with different deletion mutants of *LRF-1* (6 μg) into serum-starved NIH 3T3 cells. The value of 100 is arbitrarily given to intact *LRF-1*, which repressed the activity of $(AP\text{-}1)_3$cat in this experiment by a factor of 0.57. Domain R is the region of LRF-1 (aa 40 to 84) that is required along with the leucine zipper/basic domain for maximal inhibition. (B) The reporter plasmid PE3/4ATFCAT (4 μg) was cotransfected with c-*jun* alone, *LRF-1* (8 μg) alone, or c-*jun* (4 μg) in combination with different deletion mutants of *LRF-1* (8 μg) into serum-starved NIH 3T3 cells. The transactivation by wild-type LRF-1 in the presence of c-Jun was 100-fold over that by either vector alone (c-Jun alone or LRF-1 alone) and was arbitrarily assigned a value of 100.

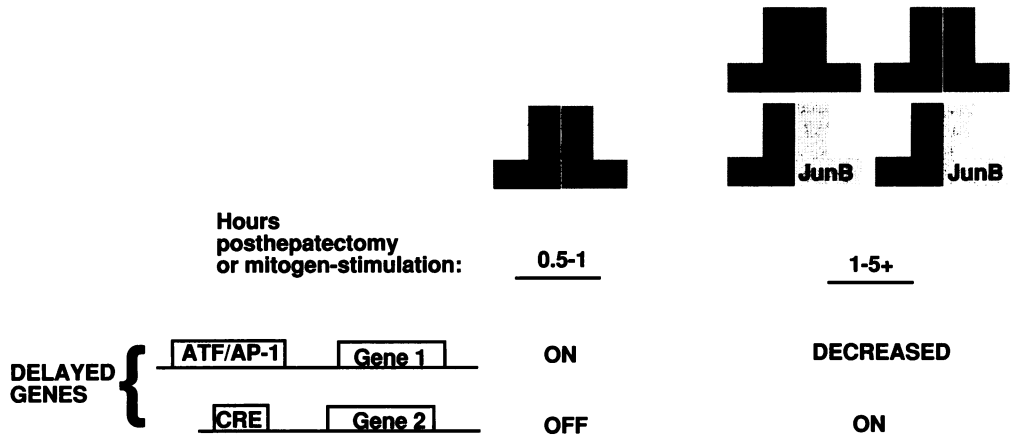


FIG. 8. Regulation of the expression of AP-1 and CRE site-containing promoters during the G_1 phase of regenerating liver and mitogen-stimulated cells. Various heterodimers were found at the indicated times posthepatectomy and post-mitogen stimulation. Relative activation (on, off, or decreased) of delayed gene promoters is indicated.

crease c-Jun- or Fos-mediated transactivation simply by competing for promoter sites which they more weakly activate. In the studies presented here, LRF-1/JunB does not even weakly activate the AP-1/ATF reporters tested and in fact represses any endogenous activity of these reporters. Our data are most consistent with the conclusion that LRF-1/JunB-mediated repression is due to site-specific DNA binding by LRF-1/JunB complexes. First, LRF-1/JunB can repress transactivation in a sequence-specific manner when the AP-1 site is situated around 200 bp upstream of transcriptional initiation sites, as in $(AP\text{-}1)_3$cat, or around 50 bp upstream, as in PE3/4AP-1cat. Second, the repression is independent of promoter context, as $(AP\text{-}1)_3$cat contains simian virus 40 early promoter and 21-4AP-1cat contains a metallothionine promoter. Third, only the deletion mutants which contain domain R cause maximal inhibition of c-Fos/JunB-mediated activation, implying that simple competition between LRF-1 and c-Fos binding to JunB is not sufficient.

LRF-1 contributions to LRF-1/c-Jun-mediated activation most likely occur through the potentiation of c-Jun heterodimer formation and DNA binding and participation of LRF-1 activating regions, notably aa 1 to 39 (Fig. 7B), which are required for full activation. Interestingly, in the presence of c-Jun, the region that includes aa 40 to 147 of LRF-1 is not an inhibitory domain. Instead, together with other portions of LRF-1, this region plays a supporting role in LRF-1/c-Jun-mediated activation.

Implications of the role of LRF-1 and Jun in stimulating hepatic growth. Particularly in mitogen-stimulated hepatic cells and regenerating liver, in which both LRF-1 and Jun proteins are abundant, transactivation of promoters regulated by CREs may be an important component of the growth response. During liver regeneration, even though the overwhelming majority of the hepatic cells reenter the cell cycle, normal liver function, synthetic function, and metabolic homeostasis are maintained. Therefore, following a 70% partial hepatectomy, the relative expression of liver-specific genes must increase. Many liver-specific genes, such as PEPCK and TAT, contain CRE sites in their promoters and are cAMP inducible (5, 44). Recently, it has been shown that extinction of the liver-specific phenotype in hybrid cells is caused by the presence of a regulatory subunit of protein kinase A which represses basal protein kinase A activity and reduces cAMP-dependent CRE site activity (5). In addition to specific effects on cell growth, LRF-1 in concert with Jun proteins may be responsible for high levels of cAMP-independent CRE site transactivation and maintenance of normal liver function during regeneration.

ACKNOWLEDGMENTS

We thank Frank Yue for expert surgical and computer assistance and Manorama Tewari for help with preparation and analysis of nuclear extracts. We thank Nancy Grow for help with manuscript preparation. We are grateful to Tom Kadesch, Robert Ricciardi, Michael Atchison, and Frank Rauscher III for helpful discussions and Frank Rauscher III, Roberto Weinmann, Michael Comb, and Karla Kovary for use of many reagents.

REFERENCES

1. **Abate, C., D. Luk, and T. Curran.** 1991. Transcriptional regulation by Fos and Jun in vitro: interaction among multiple activator and regulatory domains. Mol. Cell. Biol. **11**:3624–3632.
2. **Almendral, J. M., D. Sommer, H. Macdonald-Bravo, J. Burckhardt, J. Perera, and R. Bravo.** 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8**:2140–2148.
3. **Andersson, S., D. L. Davis, H. Dahlback, H. Jornvall, and D. W. Russell.** 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. **264**:8222–8229.
4. **Baichwal, V. R., and R. Tjian.** 1990. Control of c-jun activity by interaction of a cell-specific inhibitor with regulatory domain delta: differences between v- and c-jun. Cell **63**:815–825.
5. **Boshart, M., F. Weih, M. Nichols, and G. Shutz.** 1991. The tissue-specific extinguisher locus TSE1 encodes a regulatory subunit of cAMP-dependent protein kinase. Cell **66**:849–859.
6. **Bravo, R.** 1990. Growth factor inducible genes, p. 324–343. *In* A. Habenicht (ed.), Growth factors, differentiation factors, and cytokines. Springer-Verlag KG, Berlin.
7. **Buckbinder, L., V. J. Miralles, and D. Reinberg.** 1989. TPA can overcome the requirement for E1a and together act synergistically in stimulating expression of the adenovirus EIII promoter. EMBO J. **8**:4239–4250.
8. **Chiu, R., P. Angel, and M. Karin.** 1989. Jun-B differs in its biological properties from, and is a negative regulator of, c-jun. Cell **59**:979–986.
9. **Chiu, R., W. J. Boyle, J. Meek, T. Smeal, T. Hunter, and M. Karin.** 1988. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell **54**:541–552.

10. **Cochran, B. H., A. C. Reffel, and C. D. Siles.** 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell **33**:939–947.
11. **Cohen, D. R., and T. Curran.** 1988. *fra*-1, a serum-inducible, cellular immediate-early gene that encodes a Fos-related antigen. Mol. Cell. Biol. **8**:2063–2069.
12. **Comb, M., N. C. Birnberg, A. Seasholtz, E. Herbert, and H. M. Goodman.** 1986. A cyclic AMP- and phorbol ester-inducible DNA element. Nature (London) **323**:353–356.
13. **Comb, M., N. Mermond, S. E. Hyman, J. Pearlberg, M. E. Ross, and H. M. Goodman.** 1988. Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. EMBO J. **7**:3793–3805.
14. **Costa, R. H., D. R. Grayson, K. G. Xanthopoulos, and J. E. Darnell, Jr.** 1988. A liver-specific DNA binding protein recognizes multiple nucleotide sites in the regulatory regions of transthyretin, alpha-1 antitrypsin, albumin and simian virus 40 genes. Proc. Natl. Acad. Sci. USA **85**:3840–3844.
15. **Dobrzanski, P., T. Noguchi, K. Kovary, C. A. Rizzo, P. S. Lazo, and R. Bravo.** 1991. Both products of the *fos*B gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. Mol. Cell. Biol. **11**:5470–5478.
16. **Fausto, N., and J. E. Mead.** 1989. Regulation of liver growth: proto-oncogenes and transforming growth factors. Lab. Invest. **60**:4–13.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
18. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311**:433–438.
19. **Hai, T., and T. Curran.** 1991. Fos/Jun and ATF/CREB cross-family dimerization alters DNA binding specificity. Proc. Natl. Acad. Sci. USA **88**:3720–3724.
20. **Hai, T., F. Liu, W. J. Coukos, and M. R. Green.** 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. **12b**:2083–2090. (Erratum, **4**:682, 1990.)
21. **Henthorn, P., P. Zervos, M. Raducha, H. Harris, and T. Kadesch.** 1989. Expression of a human placental alkaline phosphatase gene in transfected cells: use as a reporter for studies of gene expression. Proc. Natl. Acad. Sci. USA **85**:6342–6346.
22. **Herschman, H. R.** 1991. Primary response genes induced by growth factors and tumor promoters. Annu. Rev. Biochem. **60**:281–319.
23. **Higgins, G. M., and R. M. Anderson.** 1931. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch. Pathol. **12**:186–202.
24. **Hirai, S. I., R.-P. Ryseck, F. Mechte, R. Bravo, and M. Yaniv.** 1989. Characterization of junD: a new member of the jun oncogene family. EMBO J. **8**:1433–1439.
24a. **Hsu, J.-C., and R. Taub.** Unpublished data.
25. **Hsu, J.-C., T. Laz, K. L. Mohn, and R. Taub.** 1991. Identification of LRF-1, a leucine-zipper protein that is rapidly and highly induced in regenerating liver. Proc. Natl. Acad. Sci. USA **88**:3511–3515.
26. **Kerppola, T. K., and T. Curran.** 1991. Transcription factor interactions: basics on zippers. Curr. Opin. Struct. **1**:71–79.
27. **Kovary, K., and R. Bravo.** 1991. Expression of different Jun and Fos proteins during the G_0-to-G_1 transition in mouse fibroblasts: in vitro and in vivo associations. Mol. Cell. Biol. **11**:2451–2459.
28. **Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein. Nature (London) **312**:711–716.
29. **Lamph, W. W., P. Wamsley, P. Sassone-Corsi, and I. M. Verma.** 1988. Induction of proto-oncogene jun/AP-1 by serum and TPA. Nature (London) **334**:629–631.
30. **Lau, L. F., and D. Nathans.** 1985. Identification of a set of genes expressed during the G_0/G_1 transition of cultured mouse cells. EMBO J. **4**:3145–3151.
31. **Lau, L. F., and D. Nathans.** 1987. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. Proc. Natl. Acad. Sci. USA **84**:1182–1186.
32. **Lau, L. F., and D. Nathans.** 1991. Genes induced by serum growth factors, p. 257–293. *In* P. Cohen and J. G. Foulkes (ed.), The hormonal regulation of gene transcription. Elsevier, New York.
33. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49**:741–752.
34. **Lim, R. W., B. C. Varnum, and H. R. Herschman.** 1987. Cloning of tetradecanoyl phorbol ester-induced primary response sequences and their expression in density-arrested Swiss 3T3 cells and a TPA nonproliferative variant. Oncogene **1**:263–270.
35. **Matsui, M., M. Tokuhara, Y. Konuma, N. Nomura, and R. Ishizaki.** 1990. Isolation of human fos-related genes and their expression during monocyte-macrophage differentiation. Oncogene **5**:249–255.
36. **Michalopoulos, G. K.** 1990. Liver regeneration: molecular mechanism of growth control. FASEB J. **4**:176–187.
36a. **Mitchell, P.** Unpublished data.
37. **Mohn, K. L., T. M. Laz, J.-C. Hsu, A. E. Melby, R. Bravo, and R. Taub.** 1991. The immediate-early growth response in regenerating liver and insulin-stimulated H-35 cells: comparison with serum-stimulated 3T3 cells and identification of 41 novel immediate-early genes. Mol. Cell. Biol. **11**:381–390.
38. **Mohn, K. L., T. M. Laz, A. E. Melby, and R. Taub.** 1990. Immediate-early gene expression differs between regenerating liver, insulin-stimulated H-35 cells, and mitogen-stimulated Balb/c 3T3 cells. J. Biol. Chem. **265**:21914–21921.
39. **Muller, R., R. Bravo, J. Burckhardt, and T. Curran.** 1984. Induction of c-fos gene and protein by growth factors precedes activation of c-myc. Nature (London) **312**:716–720.
40. **Mumberg, D., F. C. Lucibello, M. Schuermann, and R. Muller.** 1991. Alternative splicing of fosB transcripts results in differentially expressed mRNAs encoding functionally antagonistic proteins. Genes Dev. **5**:1212–1223.
41. **Nakabeppu, Y., and D. Nathans.** 1991. A naturally occurring truncated form of fosB that inhibits fos/jun transcriptional activity. Cell **64**:751–759.
42. **Nguyen, T. V., L. Kobierski, M. Comb, and S. E. Hyman.** 1990. The effect of depolarization on expression of the human proenkephalin gene is synergistic with cAMP and dependent upon a cAMP-inducible enhancer. J. Neurosci. **10**:2825–2833.
43. **Ransone, L. J., and I. M. Verma.** 1989. Association of nuclear oncoproteins fos and jun. Curr. Opin. Cell Biol. **1**:536–540.
44. **Roesler, W. J., G. R. Vandenbark, and R. W. Hanson.** 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J. Biol. Chem. **263**:9063–9066.
45. **Rosenberg, A. H., B. N. Lade, D.-S. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene **56**:125–135.
46. **Ryder, K., A. Lanahan, E. Perez-Albuerne, and D. Nathans.** 1989. Jun-D: a third member of the jun gene family. Proc. Natl. Acad. Sci. USA **86**:1500–1503.
47. **Ryder, K., L. F. Lau, and D. Nathans.** 1988. A gene activated by growth factors is related to the oncogene v-jun. Proc. Natl. Acad. Sci. USA **85**:1487–1491.
48. **Ryseck, R. P., and R. Bravo.** 1991. c-Jun, JunB, and JunD differ in their binding affinities to AP-1 and CRE consensus sequences: effect of Fos proteins. Oncogene **6**:533–542.
49. **Ryseck, R. P., S. I. Hirai, M. Yaniv, and R. Bravo.** 1988. Transcriptional activation of c-jun during the G_0/G_1 transition in mouse fibroblasts. Nature (London) **334**:535–539.
50. **Schonthal, A., P. Herrlich, H. J. Rahmsdorf, and H. Ponta.** 1988. Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell **54**:325–334.
51. **Schutte, J., J. Viallet, M. Nau, S. Segal, J. Fedorko, and J. Minna.** 1989. Jun-B inhibits and c-Fos stimulates the transforming and transactivating activities of c-jun. Cell **59**:987–997.
52. **Sonnenberg, J. L., F. J. Rauscher III, J. I. Morgan, and T. Curran.** 1989. Regulation of proenkephalin by Fos and Jun.

Science **246:**1622–1625.

53. **Staudt, L. M., H. Singh, R. Sen, T. Wirth, P. A. Sharp, and D. Baltimore.** 1986. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. Nature (London) **323:** 640–643.
54. **Takeda, J., T. Maekawa, T. Sudo, Y. Seino, H. Imura, N. Saito, C. Tanaka, and S. Ishii.** 1991. Expression of the CRE-BP1 transcriptional regulator binding to the cyclic AMP response element in central nervous system, regenerating liver, and human tumors. Oncogene **6:**1009–1014.

54a. **Taub, R.** Unpublished data.

55. **Taub, R., A. Roy, R. Dieter, and J. Koontz.** 1987. Insulin as a growth factor in rat hepatoma cells: stimulation of proto-oncogene expression. J. Biol. Chem. **262:**10893–10897.
56. **Zerial, M., L. Toschi, R. P. Ryseck, M. Schuermann, R. Muller, and R. Bravo.** 1989. The product of a novel growth factor activated gene, fos B, interacts with Jun proteins enhancing their DNA binding activity. Nature (London) **8:**805–813.
57. **Zipfel, P. F., S. G. Irving, K. Kelly, and U. Siebenlist.** 1989. Complexity of the primary genetic response to mitogenic activation of human T cells. Mol. Cell. Biol. **9:**1041–1048.