Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3511–3515, May 1991
Biochemistry

Identification of LRF-1, a leucine-zipper protein that is rapidly and highly induced in regenerating liver

(transcription factor/immediate early gene/mitogenesis)

JUI-CHOU HSU*, THOMAS LAZ*†, KENNETH L. MOHN*, AND REBECCA TAUB*†‡

*Department of Human Genetics and †Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6145

Communicated by Robert E. Forster, February 8, 1991 (received for review September 11, 1990)

ABSTRACT **Liver regeneration provides one of the few systems for analysis of mitogenesis in the fully developed, intact animal. Several proteins have been identified as part of the primary growth response in regenerating liver and in mitogen-stimulated cells. Some of these proteins, such as the Jun and Fos families of transcription factors, are thought to have a role in activating transcription of genes expressed subsequently in the growth response. Through differential screening of a regenerating-liver cDNA library, we have identified a rapidly and highly induced gene encoding a 21-kDa leucine-zipper-containing protein that we have designated liver regeneration factor 1 (LRF-1). LRF-1 has no homology with other leucine-zipper proteins outside the basic and leucine-zipper domains. LRF-1 alone can bind DNA, but it preferentially forms heteromeric complexes with c-Jun and Jun-B and does not interact with c-Fos. In solution, it binds with highest affinity to cAMP response elements but also has affinity for related sites. In cotransfection studies, LRF-1 in combination with c-Jun strongly activates a c-Jun-responsive promoter. The induction of the LRF-1 gene in regenerating liver greatly increases the potential variety of heterodimeric combinations of leucine-zipper transcription factors. While LRF-1 mRNA is rapidly induced in the absence of protein synthesis, its peak induction is later than c-*fos* mRNA, suggesting that LRF-1 may regulate responsive genes at a later point in the cell cycle. As such, LRF-1 may have a unique and critical role in growth regulation of regenerating liver and mitogen-stimulated cells.**

The liver is a multicellular organ consisting mainly of epithelial cells. It is normally quiescent but has the capacity to regenerate following partial hepatectomy, liver transplant, or toxic injury (1, 2). In the rat, following a 70% hepatectomy in which the smaller liver lobes are left completely intact, the majority of the remaining liver cells rapidly reenter the growth cycle and initiate the first round of DNA synthesis at 12–16 hr so that the liver regains its original mass in about 10 days. Multiple factors including circulating hormones, growth factors, and nervous input participate in the regulation of this mitogenic response (1, 2), but the actual mechanism remains incompletely understood. As in other mitogen-stimulated cells, primary or immediate early growth-response genes induced in the absence of prior protein synthesis are likely to play an important regulatory role in the regenerative process (3, 4).

Immediate early growth-response genes fall into three known categories, encoding (*i*) transcription factors such as Jun (5–7), Fos (8–10), and zinc-finger proteins (11–13), (*ii*) secreted proteins, or (*iii*) structural proteins such as actin (14, 15). Many of these genes are induced in a variety of mitogen-treated cells, including regenerating liver (4, 14–16) and insulin-treated H35 rat hepatoma cells (4). Among the most highly studied in this group are the Jun and Fos families of leucine-zipper-containing transcription factors, which include c-Jun, Jun-B, Jun-D, c-Fos, Fra-1, Fra-2, and Fos-B (5–10, 17). The multiple heterodimeric Fos/Jun complexes (18, 19) are thought 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 that drive cells through the cell cycle.

Here we describe the isolation of liver regeneration factor 1 (LRF-1) cDNA and characterization of the LRF-1 cDNA and protein.§ LRF-1 is a leucine-zipper protein that is rapidly and highly induced in regenerating liver and is likely to have an integral role in regulating the growth response in regenerating liver and some mitogen-stimulated cells.

MATERIALS AND METHODS

Rat Tissue Preparation and Cell Lines. For regenerating liver, female Fischer rats [160–200 g, Bantin & Kingman (Fremont, CA)] were anesthetized with ether and subjected to midventral laparotomy with ≈70% liver resection (left lateral and median lobes) (20). For cycloheximide-treated samples, rats were pretreated 15 min prior to laparotomy with cycloheximide (50 mg/kg of body weight; 5% solution in phosphate-buffered saline, i.p.). H35 cells were grown and induced with insulin and cycloheximide as described (21, 22), and BALB/c 3T3 cells were treated with 20% serum and cycloheximide as described (11). Sham surgery was performed by subjecting rats to midventral laparotomy and closure, followed by removal of the liver for RNA extraction at specific times after surgery.

RNA and Blot Preparation. Total RNA was prepared by the guanidinium thiocyanate/CsCl method (23). For Northern blots, 10 μg of total RNA per lane was electrophoresed in a 1% agarose/2.2 M formaldehyde gel and transferred to Optibind (Schleicher & Schuell) supported nitrocellulose (22).

Probes and Hybridization. Recombinant plasmids or isolated cDNA inserts were labeled through the incorporation of [α-^{32}P]dCTP (New England Nuclear) by nick-translation (BRL nick-translation reagent kit). Hybridization buffer was 10% (wt/vol) dextran sulfate/40% (vol/vol) formamide/0.6 M NaCl/0.06 M sodium citrate/7 mM Tris, pH 7.6/0.8× Denhardt's solution/0.002% heat-denatured, sonicated salmon sperm DNA. Blots were hybridized at 42°C overnight and washed at 60°C in 15 mM NaCl/1.5 mM sodium citrate/0.1% SDS prior to exposure to film (22).

cDNA Library Construction, Screening, and Sequencing of LRF-1 cDNA. Two libraries were prepared from poly(A)$^+$ RNA [selected by passage over an oligo(dT)-cellulose (Col-

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Abbreviations: ATF, activating transcription factor; CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; CREB, CRE-binding protein; LRF-1, liver regeneration factor; TRE, phorbol 12-tetradecanoate 13-acetate (TPA) response element.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base [accession no. M63282 (*Rattus rattus* LRF-1)].

laborative Research) column] isolated as described above from regenerating livers 3 hr after 70% partial hepatectomy in the presence of cycloheximide (50 mg/kg). One library was made using the InVitrogen (San Diego) Lambda Librarian kit and the other was subtraction-enriched for differentially expressed inserts by using the InVitrogen Subtractor kit and a quiescent rat liver cDNA library purchased from InVitrogen. Differential screening was performed essentially as described (14). The DNA sequence of several near-full-length LRF-1 cDNA clones was the result of bidirectional sequencing by the dideoxy chain-termination method (24). The sequence of the first 11 base pairs was determined by direct mRNA sequencing (25).

Gel Mobility-Shift Analyses. Preannealed HPLC-purified double-stranded oligonucleotides were radiolabeled and mixed with 2–3 μl of *in vitro* translated proteins in binding buffer [10 mM Tris, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM 2-mercaptoethanol/4% (vol/vol) glycerol]. The mixtures were incubated for 30 min and then electrophoresed in a 5% polyacrylamide gel with Tris/glycine buffer (26).

Cotransfection Studies. Both LRF-1 and c-*jun* cDNAs were cloned into the pCMV-5 vector (27). NIH 3T3 cells were transfected with the indicated amounts of pCMV-LRF-1 and pCMV-c-jun or pCMV without insert, 4 μg of pENKAT-12 reporter, and 3 μg of pSV2A-PAP (28) as a transfection control. In all cases, the amount of DNA transfected per dish was made constant to 30 μg with the addition of pCMV without insert. Sixteen to eighteen hours after calcium phosphate-mediated transfection, cells were serum-deprived (0.5% fetal bovine serum); 24 hr later the cells were harvested and chloramphenicol acetyltransferase (CAT) assays were performed (29). Results were quantitated by densitometry after normalization for the level of placental alkaline phosphatase (28).

RESULTS AND DISCUSSION

Through differential screening and subtraction cloning of regenerating rat liver cDNA libraries prepared from remnant liver 3 hr after 70% hepatectomy in the presence of cycloheximide, we isolated several clones encoding LRF-1. The number of independent isolates indicated that LRF-1 is one of the most highly expressed immediate early genes in regenerating liver. Fig. 1*A* shows a time course of induction of its 2-kilobase mRNA. Like other immediate early genes it is induced in the absence of prior protein synthesis and superinduced by cycloheximide. LRF-1 mRNA remains undetectable at all time points following sham surgery (data not shown). LRF-1 mRNA is also expressed at a high level in insulin-treated H35 cells, a minimal-deviation hepatoma cell that appears to have many properties of regenerating liver (21, 22), and to a lesser extent in mitogen-treated BALB/c 3T3 cells. Fig. 1*B* shows the posthepatectomy level of LRF-1 mRNA relative to that of another immediate early gene, c-*fos*. LRF-1 mRNA expression peaks at 2–3 hr and is still high 8 hr after hepatectomy. LRF-1 mRNA is expressed at high levels in tissues containing skeletal muscle or smooth muscle, such as intestine and stomach (Fig. 1*C*), and at low levels in some tumor cells (data not shown).

On sequence analyses of multiple overlapping and near-full-length cDNA clones, LRF-1 was found to be a 20.7-kDa protein containing basic and leucine-repeat regions, characteristic of the leucine-zipper family of transcription factors (Fig. 2*A*). With the exception of the mRNA cap site, the sequence of the first 11 bases of LRF-1 mRNA was determined by primer extension in the presence of dideoxynucleotides, and it was ascertained that there was no methionine codon upstream of nucleotide 163. Further confirmation of the size of the open reading frame was obtained by translating the LRF-1 mRNA *in vitro* and assessing the product's size by polyacrylamide gel electrophoresis, where it migrated as a 21-kDa band (Fig. 1*D*). In genomic blot analyses the LRF-1 cDNA hybridized to a single band, even at low stringencies (data not shown).

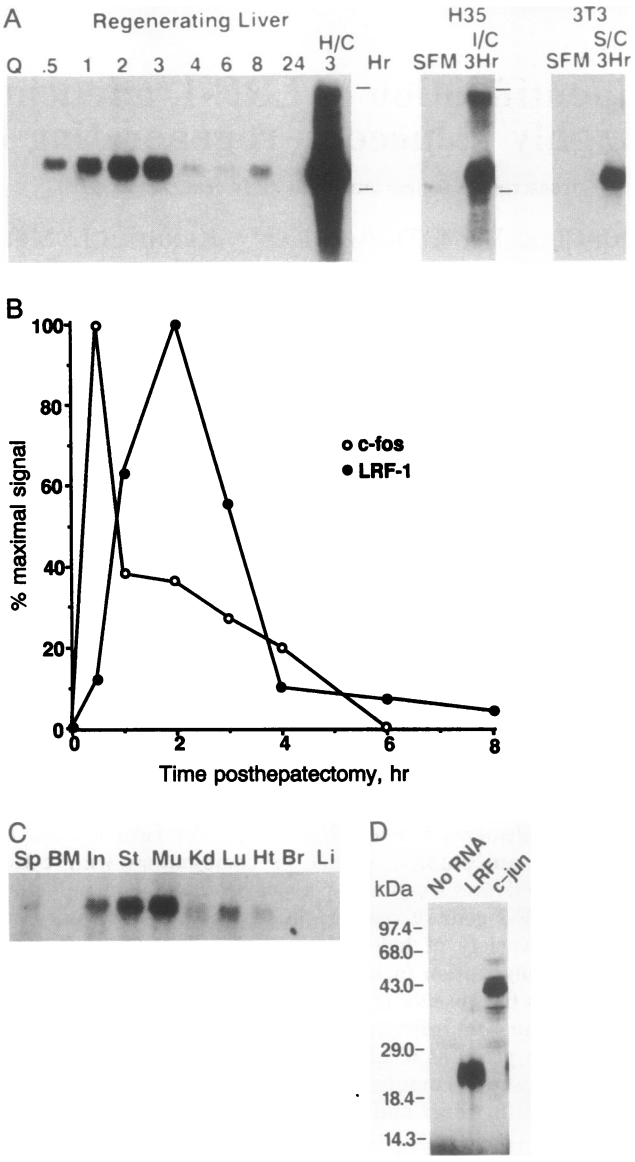

FIG. 1. (*A*) LRF-1 mRNA is rapidly induced in regenerating liver and is expressed in other mitogen-treated cells. Northern blots contained 10 μg of total RNA per lane immobilized on nitrocellulose and hybridized with a ^{32}P-labeled LRF-1 cDNA probe and were exposed overnight. (*Left*) RNA from quiescent, untreated liver (lane Q) or from regenerating liver 0.5–24 hr after 70% hepatectomy (lanes .5 to 24) or 3 hr after hepatectomy with cycloheximide (50 mg/kg) pretreatment (lane H/C 3). (*Center*) RNA from H35 cells grown for 3 days in serum-free medium (lane SFM) or treated for 3 hr with insulin (10 nM) and cycloheximide (10 μg/ml) (lane IC 3HR). (*Right*) RNA from BALB/c 3T3 cells grown for 2 days at 0.5% fetal bovine serum (lane SFM) or 3 hr with 20% fetal bovine serum and cycloheximide (10 μg/ml) (lane S/C 3HR). Markers indicate positions of 28S and 18S rRNAs. (*B*) LRF-1 and c-*fos* mRNA expression during liver regeneration. After densitometric scanning of autoradiograms, values were normalized for β_2-microglobulin expression. (*C*) LRF-1 expression is tissue-specific. Northern blot of 10 μg of total RNA per lane was hybridized with ^{32}P-labeled LRF-1 cDNA. Sp, spleen; BM, bone marrow; In, intestine; St, stomach; Mu, skeletal muscle; Kd, kidney; Lu, lung; Ht, heart; Br, brain; Li, liver. This is a 1-day exposure. (*D*) *In vitro* translated LRF-1 migrates at 21 kDa. Full-length linearized LRF-1 and c-*jun* cDNAs were transcribed from T3 and T7 promoters and translated in the presence of [^{35}S]methionine *in vitro* using kits and methods from Stratagene. The products were electrophoresed in an SDS/12.5% polyacrylamide gel.

A

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TGTCTCACTCAGCGAGACGCGCGGTGCACGGTGCTTCCCCGGCGGACGACCGAC   54
CAACCCGCGCTCCGGCAGAGTCCTTGGCGCTCGCCTGCCGGCGGGACAGACAGC  108
CCGCCTCTAGCCGCTCTCTGGACCCTGGCCGCCCCGAGCGAAGACTGGAGCAAA  162

ATGATGCTTCAACATCCAGGCCAGGTCTCTGCCTCAGAAGTCAGCGCGACCGCC  216
METMetLeuGlnHisProGlyGlnValSerAlaSerGluValSerAlaThrAla   18

ATCGTCCCCTGCCTCTCACCTCCTGGGTCACTGGTGTTTGAGGATTTTGCTAAC  270
IleValProCysLeuSerProProGlySerLeuValPheGluAspPheAlaAsn   36

CTGACACCTTTTGTCAAGGAAGAGCTGAGATTCGCCATCCAGAACAAGCACCTT  324
LeuThrProPheValLysGluGluLeuArgPheAlaIleGlnAsnLysHisLeu   54

TGCCATCGGATGTCCTCTGCGCTGGAGTCAGTCACCATCAACAACAGACCTCTG  378
CysHisArgMetSerSerAlaLeuGluSerValThrIleAsnAsnArgProLeu   72

GAGATGTCAGTCACCAAGTCTGAGGTGGCCCCTGAAGAAGATGAGAGAAAAAGG  432
GluMetSerValThrLysSerGluValAlaProGluGluAspGluArgLysArg   90

AGGCGGCGGGAAAGAAACAAAATTGCTGCTGCCAAGTGTCGAAACAAGAAAAAA  486
ArgArgArgGluArgAsnLysIleAlaAlaAlaLysCysArgAsnLysLysLys  108

GAGAAGACAGAGTGCCTGCAGAAGGAGTCAGAGAAACTGGAGAGTGTGAATGCC  540
GluLysThrGluCysLeuGlnLysGluSerGluLysLeuGluSerValAsnAla  126

GAACTGAAGGCCCAGATCGAGGAGCTGAAGAATGAGAAGCAGCATCTGATTTAC  594
GluLeuLysAlaGlnIleGluGluLeuLysAsnGluLysGlnHisLeuIleTyr  144

ATGCTCAACCTGCACCGGCCCACGTGTATCGTCCGGGCTCAGAACGGGCGGACG  648
MetLeuAsnLeuHisArgProThrCysIleValArgAlaGlnAsnGlyArgThr  162

CCGGAAGACGAGAGGAACCTTTTATCCAACAGATAAAAGAAGGAACATTGCAG  702
ProGluAspGluArgAsnLeuPheIleGlnGlnIleLysGluGlyThrLeuGln  180

AGCTAAGCAGAGGTGGCATGGGGGCAATTGGGGAGTCCTTACTGAATCCTCCTT  756
Ser                                                      181

TTCCACCCCAAACCCTGAAGCCATTGGAAAACTGGCTTCCTGTGCACTTCTAGA  810
ATCTCAGCAGCCACGAGCTGTTGGGTCAGGAGGGCCTGCGGTGACTACTGCGTT  864
GTCCCACTCTGTCCCCGAGTGAACCGTGGAGCAGGCAGGAGCATCCTTTGTCTC  918
ACCGGCTCCAGGATTTAGGCCTTACCATCCCGGCCATTCTCAGATGACCTAGCT  972
GGCCCCAGGCTGGGGTCCCATGCAAAGCAGGATCGCACTAATGGGATGCAGGCA 1026
GAAGTGTCTACCTTGACAGGTGGGGTGGACCACGTCCTCCACTGCGGCTGACAA 1080
CATCCCTCCTAGGGAAGATGGAGTGAGAACATTCATCATTGAAGTTGTCCAATG 1134
GCCAGGGTATGCTTTCTAGAAACTATGCTGTTCTGTCCTAGACTGACTGTGCAT 1188
AGGGCATTCATTTCTGAGCCTGGTGTTGTGCTATTTAGATGTTTGTCTTGCACA 1242
ACATTGGCGTGATTTTTTTCCGGGAGTTTCATCAGACCTGATTTCCGAGAGTTT 1296
GGGGGTCTGCCACTGTGGACAATATCCCCCAAAAGTGTTTGGGTGGCCATGTAA 1350
ACTGGCTGATGACCAGCTGTGCTACTCTGTGCTGACCGAGGACTGATGCCTCCT 1404
TCCCCTGTACCCACTGCTGAGGAAGAACCCGGGCACAGCAGCTGTCCTTGGCTA 1458
CAAACTGTTACAATGTCACAGAACGAAGGCACAAAGTCCCGCTTTCAAAGGGCG 1512
TAGGACTCCACACTCAGTGACAGGGCAGGAAGAGCCAAGGATTCTCCGTTTTCC 1566
CTTCCTTCCCACCAAAAACCACAGCCCGTGGAGACTGGTATTTGAAGCCAGGAG 1620
TGGGGCAAGGAAGGTGTCTGCACTGTGGGATGTAACTGCGCTTTGTCTTGAA 1674
GCTATTTTGAGATGCGGTCCAGAGTATTTCAGCTGGGAGGTCCCTCCCACTGGC 1728
CACCAGGGCTCTGGCTACTGTTAAAATTCTGATGTTTCTGTGAAATCCTCAGTG 1782
TTCAATCCAGACTCAGTAGTATATTACAGTTTTCTGTAAGAGAGAACGTTACTT 1836
ATTTATCCCAGTATTCCTAGCCTGTCAACGTAATAAAATATCAGAATGAGACCT 1890
GGT                                                      1893
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B

Basic Region

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LRF-1  E R K R R R R E R N K I A A A K C R N K K K E K T E C
C-FOS  E K R R i R R E R N K h A A A K C R N R R R E l T D t
CREB   r K R e v R l m K N R e A A r e C R r K K K E y v k C
C-JUN  i K a e R K R m R N R I A A s K C R k R R l E R i a r
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Leucine Repeat

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LRF-1  L Q K E S E K L E S V N A E L K A Q I E E L K N E K Q H L
C-FOS  L Q a E T D q L E d e k s a L q t e I a n L l k E K e k L
CREB   L e n r v a v L E n q N k t L i e e l k a L K d l y c E k
C-JUN  L e e k v k t L k a q N s E L a s t a n m L R e q v a q L
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FIG. 2. LRF-1 contains basic and leucine-zipper regions. (*A*) DNA sequence and predicted translation of LRF-1. (*B*) LRF-1 aligns with the basic and leucine-zipper regions of c-Fos, cAMP response element (CRE)-binding protein (CREB), and c-Jun (30). Amino acids that differ from the LRF-1 sequence are shown by lowercase letters.

We compared the sequence of the LRF-1 gene with sequences that Bravo and coworkers (14) had determined from the 3′ ends of cDNA clones induced in mitogen-stimulated BALB/c 3T3 cells and found that it aligned with U56, a low-abundance clone in mitogen-treated fibroblasts (R. Bravo, personal communication). LRF-1 also aligns precisely with portions of a minimally characterized clone, ATF-3, which was isolated by ATF (activating transcription factor)-site expression screening of a HeLa cell cDNA library (31). However, ATF-3 is unlike LRF-1 at its amino terminus and could only be the human homolog of LRF-1 if ATF-3 resulted from alternative splicing or if sequence differences in ATF-3 were caused by cDNA cloning artifacts.

LRF-1 is much smaller than other leucine-zipper proteins, with weak consensus sites for phosphorylation by protein kinase A and C and no evident casein kinase II sites (32). The alignment of LRF-1 with c-Fos, CREB, and c-Jun is shown in Fig. 2*B*. Of well-characterized gene products, LRF-1 is most similar to c-Fos, but unlike Fos-family proteins and Fos-related antigens (17), LRF-1 has no homology with Fos outside of the basic domain, and LRF-1 is able to bind DNA as a homomer (see below).

In liver regeneration, c-*fos* and *jun-B* (4), and to a lesser extent *jun-D* (4) and c-*jun* (4, 33, 34), are induced following hepatectomy. The *fos-B* and *fra-1* genes are induced in mitogen-stimulated fibroblasts but not in regenerating liver (4). As we have shown (Fig. 1*B*), while LRF-1 mRNA expression peaks at 2–3 hr, c-*fos* expression peaks at 30 min and disappears more rapidly posthepatectomy. Like LRF-1, c-*jun*, *jun-B*, and *jun-D* expression are elevated for extended times posthepatectomy (4). It was important to determine the potential of LRF-1 to complex with itself and the c-Fos, Jun-B, and c-Jun proteins that are present simultaneously during regeneration.

Leucine-zipper proteins have been shown to bind to various elements including the CRE, the phorbol ester [phorbol 12-tetradecanoate 13-acetate (TPA)] response element (TRE), and the ATF element found in viral promoters (30–32, 35, 36). We used mobility-shift analyses to assess the ability of *in vitro* translated LRF-1 and c-Jun to bind to oligonucleotides containing these elements (Fig. 3*A*). Alone, LRF-1 bound to all of these sites and migrated at a position close to endogenous extract proteins that bind to the TRE and CRE sites (19, 30). Interestingly, the ATF oligonucleotide did not bind endogenous extract proteins. With all three oligonucleotides, when c-Jun was present, there was preferential binding of a LRF-1/c-Jun complex relative to the LRF-1 complex. Alone, the c-Jun complex was present at its highest level with the CRE, was barely detectable with the TRE, and was not detectable with the ATF site. Preliminary crosslinking experiments confirmed homo- and heterodimer formation of LRF-1 and LRF-1/c-Jun. Similar experiments demonstrated LRF-1/Jun-B complex formation (CRE, Fig. 3*B*; ATF and TRE, data not shown), while c-Fos formed no complex with LRF-1 on the CRE (Fig. 3*B*) or on the ATF or TRE sites (data not shown). LRF-1/Jun-B migrated slightly more slowly than the c-Fos/Jun-B complex (Fig. 3*C*). With all three proteins present, and either a CRE (Fig. 3*C*) or TRE (data not shown) oligonucleotide, both LRF-1/Jun-B and c-Fos/Jun-B complexes appeared to be present, but the relative affinities of LRF-1 and c-Fos for Jun-B have not been assessed.

Recently, c-Fos/Jun and CRE-BP2/Jun complexes have been shown to have higher relative affinities for TRE and CRE sites, respectively (35–37). These relative *in vitro* affinities could be critical in determining which genes are regulated by these proteins *in vivo*. Hence, we examined which sites had greatest affinity for LRF-1 alone or for LRF-1/Jun complexes. In competition studies with various amounts of identical and heterologous unlabeled oligonucleotides, the CRE site, relative to the TRE and ATF sites, consistently had a 6- to 8-fold, 2- to 3-fold and 2- to 3-fold higher affinity for LRF-1 alone, LRF-1/c-Jun and LRF-1/Jun-B, respectively (Fig. 3*D*). These studies suggest that homo- and heteromeric LRF-1 complexes have some preference for CRE sites, which are present in the promoter regions of many genes.

As an initial assessment of the transactivating potential of LRF-1, we transfected NIH 3T3 cells with pCMV-LRF-1 and/or pCMV-c-jun and an enkephalin promoter–CAT gene reporter (pENKAT-12) that had previously been shown to be transactivated by c-Jun (38, 39). This reporter construction was chosen because unlike others tested, it had low endogenous activity in NIH 3T3 cells. Alone, pCMV-LRF-1 and

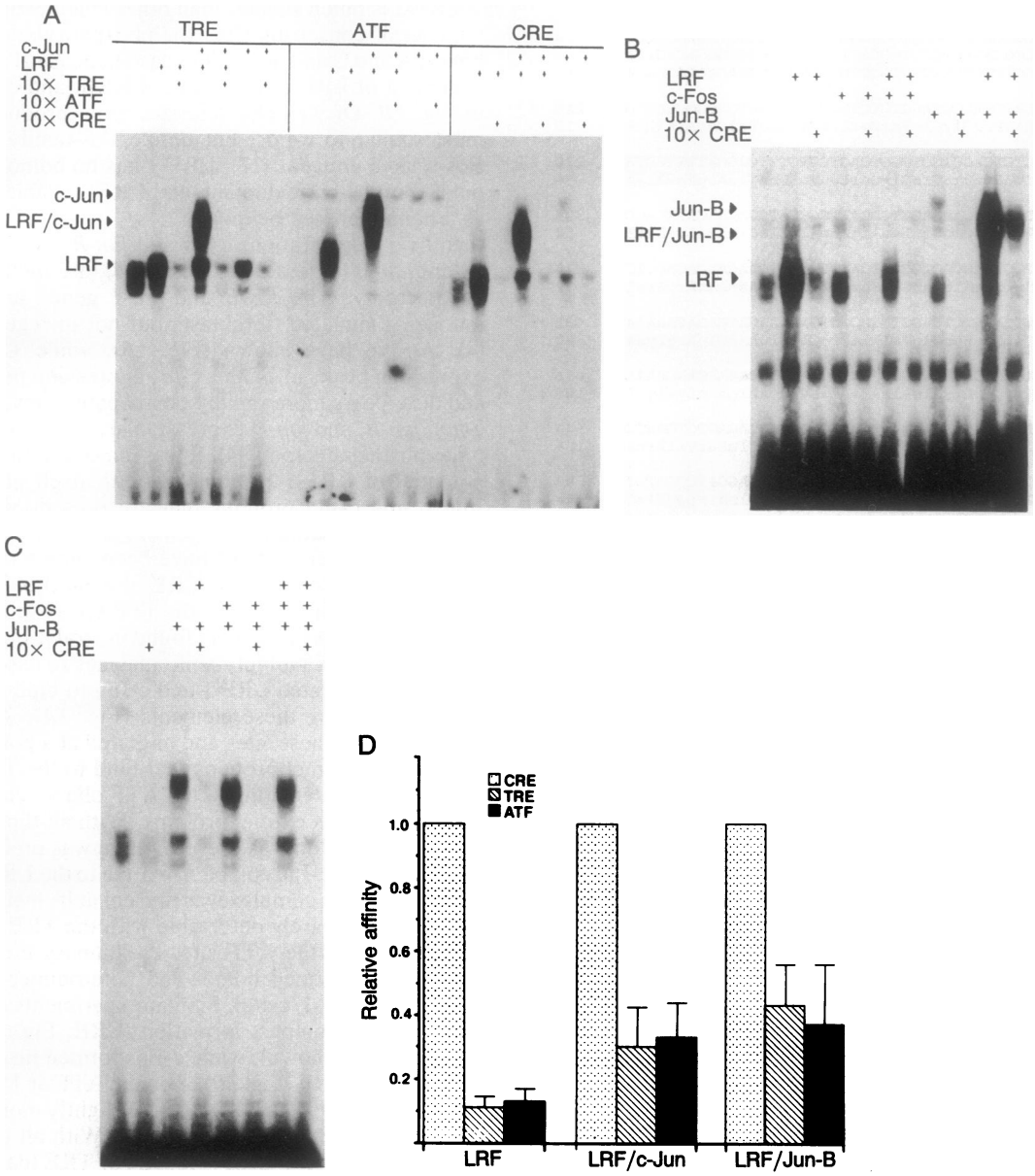

FIG. 3. (*A*) LRF-1 binds to TRE, ATF, and CRE sites alone or as a complex with c-Jun. LRF-1 and rat c-Jun (complete cDNA from regenerating liver library) were translated *in vitro* with rabbit reticulocyte lysate and bound to radiolabeled TRE (consensus site for AP1 protein, TATCGATAAGC-TATGACTCATCCGGGGGA), ATF (adenovirus type 5E4 gene, nucleotides −65 to −35, TGGACTTTAACCGTTACGTCATTTTTTAGT), or CRE (human choriogonadotropin α-chain gene, TCATGGTAAAAATGACGTCATGGTAATTA) oligonucleotides (30). Plus signs indicate the presence of c-Jun, LRF-1, or 10-fold excess of nonradioactive competitor. At far left, the control lane contained rabbit reticulocyte lysate and, as has been found previously (18, 30), shows some specific binding to TRE and CRE oligonucleotides that varied between different lots of lysate. All lanes contained rabbit reticulocyte lysate. (*B*) Jun-B complexes with LRF-1 whereas c-Fos does not. *In vitro* synthesized RNAs from *jun-B* (ATCC no. 63025) and rat c-*fos* (complete coding region from H35 cell cDNA library) were translated *in vitro*. All lanes contained rabbit reticulocyte lysate and radiolabeled CRE oligonucleotide. (*C*) c-Fos/Jun-B and LRF-1/Jun-B form similar-size complexes. Synthetic RNAs from *jun-B*, LRF-1 cDNA, and rat c-*fos* were translated *in vitro* as above. c-Fos/Jun-B or LRF-1/Jun-B were translated separately and then mixed together for 30 min in the experiment with all three proteins. All lanes contained a radiolabeled CRE oligonucleotide and rabbit reticulocyte lysate. (*D*) LRF-1-containing complexes have higher relative affinity for the CRE oligonucleotide. Relative affinity is expressed as the ratio of identical to heterologous competitor needed to achieve equivalent reduction of binding of indicated proteins (LRF-1 alone, LRF-1/c-Jun, and LRF-1/Jun-B) within the linear range of the assay after densitometric scanning of autoradiograms. The value of the affinity of the CRE was arbitrarily set to 1.0; relative affinity and standard deviation are indicated for the TRE and ATF oligonucleotides.

pCMV-c-jun stimulated this promoter slightly above baseline in serum-deprived cells, while increasing amounts of pCMV-LRF-1 in the presence of constant amounts of pCMV-c-jun greatly increased relative CAT activity (Fig. 4). pCMV-LRF-1 in the presence of pCMV-c-jun also strongly activated a minimal AP1–CAT reporter construct (data not shown). Although it remains to be seen whether LRF-1 acts as a transcriptional activator under different conditions, in these studies, LRF-1 had an activating effect comparable to what has been observed with c-Fos (39).

In summary, we have identified LRF-1, a leucine-zipper protein encoded by a highly induced immediate early gene in regenerating liver and mitogen-stimulated cells. The identification of LRF-1 as a member of the family of immediate early leucine-zipper proteins that, like Fos, can interact with Jun and, unlike fos, can bind DNA directly adds another level of complexity to the immediate early growth response. Because LRF-1 mRNA is expressed at peak levels in regenerating liver later than c-*fos* mRNA, while *jun* expression is still elevated, it is likely that maximal LRF-1 interaction with Jun

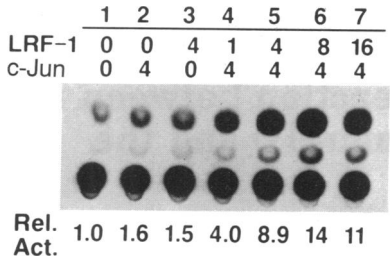

FIG. 4. In cotransfection studies, LRF-1 and c-Jun are strong transactivators. Shown for each lane are the results of CAT assays for the indicated plasmids (amount in micrograms) that were co-transfected with the pENKAT-12 reporter (38, 39): LRF-1, pCMV-LRF-1; c-Jun, pCMV-c-jun. Lane 1 was obtained by using pCMV-5 without insert instead of pCMV-c-jun or pCMV-LRF-1. The relative CAT activity (Rel. Act.) shown below each lane was calculated on the basis of three separate experiments.

occurs later than Jun/Fos interactions. The temporal course of delayed-gene induction by leucine-zipper proteins in the G_1 phase of the cell cycle is not known. The possibility that LRF-1 acts as a transcriptional activator could be particularly important in this context, because progression through the cell cycle may require continued stimulation of delayed-gene expression beyond the time that *fos* expression has ceased. Thus far, LRF-1 is the smallest member of this leucine-zipper family that has been described, and its small size may help delineate the minimal functional transactivation region of leucine-zipper proteins. Since it is both highly induced and distinct from Jun and Fos, LRF-1 is likely to have a unique and important role in regulating the growth response in regenerating liver and mitogen-stimulated cells.

We thank Frank Yue and A. E. Melby for technical help, Susan Kelchner for secretarial help, and Frank Rauscher III and Michael Comb for reporter plasmids. We are grateful to Rodrigo Bravo for sharing data prior to publication and to Sean Stevens, Holger Beckmann, and Tom Kadesch for helpful discussions. K.L.M. is supported by Juvenile Diabetes Foundation Postdoctoral Fellowship 389303.

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