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Biochemistry

Jun-D: A third member of the *Jun* gene family

(transcription factor AP-1/oncogene/growth response/DNA-binding protein)

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ABSTRACT **The protooncogene c-*jun* encodes a component of the transcription factor AP-1. Both murine c-*jun* and a related gene (*jun-B*) are rapidly activated in BALB/c 3T3 cells by serum growth factors. We report here the cloning and analysis of a cDNA encoding a third member of the murine *jun* family, *jun-D*. The amino acid sequence encoded by *jun-D* has two extensive regions of homology with the other Jun proteins. One homology region includes the DNA-binding domain and sequences required for dimer formation and interaction with the Fos oncoprotein; the other includes the acidic sequence thought to be involved in gene activation. All three jun mRNAs are present in a variety of murine tissues and cell lines. In resting 3T3 cells, *jun-D* is expressed at a higher level compared to c-*jun* and *jun-B*, and its transcription is stimulated only slightly by serum growth factors. Thus, *jun-D* appears to be regulated differently than c-*jun* and *jun-B*.**

The cellular homolog of the avian sarcoma virus oncogene v-*jun* (1–3) encodes a component of the complex transcription factor AP-1 (2–4). Both the murine protooncogene c-*jun* (also called *jun-A*) and a related gene (*jun-B*) have been identified as immediate early genes activated by serum growth factors in BALB/c mouse 3T3 cells (5–11). Based on restriction analysis of genomic DNA, it appeared that other *jun*-related genes are present in the mammalian genome (7). To search for these additional *jun*-related genes, we screened mouse cDNA and genomic libraries for clones that would hybridize to murine c-*jun* and/or *jun-B* cDNA probes. By this means we detected a third member of the *jun* family called *jun-D* (10). Here we report that jun-D cDNA encodes a protein closely related to c-Jun and Jun-B. Compared to c-*jun* and *jun-B*, *jun-D* is expressed at a higher level in nongrowing 3T3 cells and is activated only slightly by serum growth factors. Recently, we learned that jun-D cDNA has also been isolated by S.-I. Hirai, R. C. Ryseck, F. Mechta, R. Bravo, and M. Yaniv (personal communication).

MATERIALS AND METHODS

Cell Culture. BALB/c 3T3 cells and conditions of growth have been described (6).

Cloning of cDNA and Genomic DNA. A previously described λ bacteriophage cDNA library prepared from poly(A)$^+$ RNA of BALB/c 3T3 cells stimulated with serum for 3 hr in the presence of cycloheximide (6) was probed with ^{32}P-labeled murine c-jun or jun-B cDNA. Filters were washed at reduced stringency (0.5 M NaCl at 65°C) or at high stringency (0.1 M NaCl at 65°C) to detect phage that hybridized only at reduced stringency. Seventeen λ phage libraries (12) were prepared from size-selected *Eco*RI fragments of mouse genomic DNA, the sizes corresponding to fragments previously shown by Southern blotting to cross-hybridize with jun-B cDNA (7). The libraries were screened with ^{32}P-labeled jun-B or murine c-jun cDNA (or cloned fragments thereof) at 65°C in 1 M NaCl and washed with 0.3 M NaCl/0.03 M sodium citrate at 57°C.

Other Methods. DNA sequencing (13), mRNA mapping (7), nuclear run-on assays (14, 15), and blot-hybridization analysis of cellular or tissue RNA (Northern analysis; refs. 16–18) were performed as described (7, 8).

RESULTS

Isolation and Analysis of jun-D cDNA. To detect additional *jun*-related mouse genes, we screened cDNA libraries and genomic libraries with jun-B or c-jun cDNA probes, or both, under conditions of moderate to low stringency. A number of different genomic clones hybridized to one or more cDNA probes. One class of these corresponded to murine c-*jun* (8), another to *jun-B* (7), and another to a third member of the *jun* family (*jun-D*) whose cDNA was detected in a BALB/c 3T3 cell library (6). Of the remaining genomic clones, the most promising hybridizing isolates were found to have only short regions of sequence similarity to *jun-B* or c-*jun*.

The sequence of the longest jun-D cDNA detected in the 3T3 cell library and the sequence derived from a 5′ overlapping mouse genomic fragment of *jun-D* are shown in Fig. 1.* The complete cDNA is 1675 nucleotides long, corresponding to an electrophoretically estimated length of 1.8 kilobases for jun-D mRNA. The 5′ end of the mRNA (nucleotide 1 in Fig. 1) was mapped by primer extension on 3T3 cell RNA as well as by S1 nuclease analysis with a probe derived from genomic DNA as described (7). Sequencing of the overlapping genomic fragment revealed a typical TATA motif 25–30 nucleotides 5′ to the inferred transcription start site.

The first ATG at position 121 is preceded by a leader sequence with a G+C content of 85%. This ATG is followed by 1020 nucleotides of additional coding sequence, a termination codon, 532 nucleotides of 3′ noncoding sequence, and a poly(A) tail. Although there is no characteristic poly(A) addition signal, there is an AGTAAA sequence near the 3′ end that may serve as the signal. In addition, there is a single ATTTA element in the 3′ noncoding region (nucleotides 1434–1438), multiple copies of which are associated with mRNA instability (19).

Comparison of *jun* Sequences. When the predicted amino acid sequence of Jun-D is compared with those of murine c-Jun and Jun-B, two long homology regions (HR-1 and HR-2) are seen (Fig. 2). Between HR-1 and HR-2 and at the amino end are segments with only short stretches of sequence similarity. In each homology region, Jun-D is more similar to murine c-Jun than it is to Jun-B. The C-terminal 40–50% of each Jun protein (including HR-2) retains specific DNA-binding activity and is able to form dimers and interact with Fos (20). This segment has the heptad repeats of leucine,

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Abbreviation: HR-1 and HR-2, homology regions 1 and 2.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04509).

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 -53                                                                                                  CGGTGAGCTCATCGCGGGGGCCGAGGCTATAAGAGTGC
 -18  GCGCGGGCGGCGCGGGGCAGAGCGGCCGGCATCGGAGCCGGTCTGTACGGGCAGCGGACTGGGGGGCATACGGGGCCCCCCGGGGAGGCCGCGGCCACTC
  83  CCCCCGCCCCGGGGCCGAGCGCGGCGGGGGAGGTGGGG  ATG GAA ACG CCC TTC TAT GGC GAG GAG GCG CTG AGC GGC CTG GCT
(1)                                           MET Glu Thr Pro Phe Tyr Gly Glu Glu Ala Leu Ser Gly Leu Ala
 166  GCG GGT GCG TCG AGC GTC GCT GGT GCT ACT GGG GCC CCC GGC GGT GGT GGC TTC GCG CCC CCG GGC CGC GCT TTC
(16)  Ala Gly Ala Ser Ser Val Ala Gly Ala Thr Gly Ala Pro Gly Gly Gly Gly Phe Ala Pro Pro Gly Arg Ala Phe
 241  CCC GGG GCG CCC CCG ACG AGC AGC ATG CTG AAG AAA GAC GCG CTG ACG CTC AGC CTG GCG GAG CAG GGA GCG GCG
(41)  Pro Gly Ala Pro Pro Thr Ser Ser MET Leu Lys Lys Asp Ala Leu Thr Leu Ser Leu Ala Glu Gln Gly Ala Ala
 316  GGA TTG AAA CCA GGG TCG GCC ACT GCA CCT TCT GCG CTG CGC CCC GAC GGC GCC CCC GAC GGG CTG CTG GCT TCG
(66)  Gly Leu Lys Pro Gly Ser Ala Thr Ala Pro Ser Ala Leu Arg Pro Asp Gly Ala Pro Asp Gly Leu Leu Ala Ser
 391  CCG GAT CTT GGG CTG CTC AAA CTC GCG TCG CCG GAG CTG GAG AGG CTG ATC ATC CAG TCC AAC GGG CTG GTG ACC
(91)  Pro Asp Leu Gly Leu Leu Lys Leu Ala Ser Pro Glu Leu Glu Arg Leu Ile Ile Gln Ser Asn Gly Leu Val Thr
 466  ACT ACC CCG ACC AGT ACG CAG TTC CTC TAC CCG AAG GTG GCA GCC AGC GAG GAG CAG GAG TTC GCC GAA GGC TTC
(116) Thr Thr Pro Thr Ser Thr Gln Phe Leu Tyr Pro Lys Val Ala Ala Ser Glu Glu Gln Glu Phe Ala Glu Gly Phe
 541  GTC AAG GCG CTG GAG GAC CTG CAC AAG CAA AGC CAG CTG GGT GCG GCC ACC GCG GCC ACC TCA GGG GCT CCC GCG
(141) Val Lys Ala Leu Glu Asp Leu His Lys Gln Ser Gln Leu Gly Ala Ala Thr Ala Ala Thr Ser Gly Ala Pro Ala
 616  CCT CCC GCG CCC GCC GAC CTG GCC GCC ACC CCC GGG GCC ACG GAG ACC CCG GTC TAC GCC AAC CTG AGC AGT TTC
(166) Pro Pro Ala Pro Ala Asp Leu Ala Ala Thr Pro Gly Ala Thr Glu Thr Pro Val Tyr Ala Asn Leu Ser Ser Phe
 691  GCG GGT GGC GCC GGG CCC CCT GGG GGC GCG GCC ACC GTG GCT TTC GCC GCG GAG CCA GTG CCC TTC CCG CCG CCC
(191) Ala Gly Gly Ala Gly Pro Pro Gly Gly Ala Ala Thr Val Ala Phe Ala Ala Glu Pro Val Pro Phe Pro Pro Pro
 766  CCG GGC GCG CTG GGG CCG CCG CCA CCT CCG CAT CCA CCG CGC CTG GCC GCG CTC AAG GAC GAG CCG CAG ACC GTG
(216) Pro Gly Ala Leu Gly Pro Pro Pro Pro Pro His Pro Pro Arg Leu Ala Ala Leu Lys Asp Glu Pro Gln Thr Val
 841  CCG GAC GTG CCG AGC TTC GGC GAC AGC CCT CCG CTG TCG CCC ATC GAC ATG GAC ACG CAA GAA CGC ATC AAG GCG
(241) Pro Asp Val Pro Ser Phe Gly Asp Ser Pro Pro Leu Ser Pro Ile Asp MET Asp Thr Gln Glu Arg Ile Lys Ala
 916  GAG CGC AAG AGG CTG CGC AAC CGC ATC GCC GCC TCC AAA TGC CGC AAG CGC AAG CTG GAG CGT ATC TCG CGC CTG
(266) Glu Arg Lys Arg Leu Arg Asn Arg Ile Ala Ala Ser Lys Cys Arg Lys Arg Lys Leu Glu Arg Ile Ser Arg Leu
 991  GAG GAG AAA GTC AAG ACC CTC AAA AGC CAG AAC ACC GAG CTG GCG TCC ACC GCC AGC CTG CTG CGC GAG CAG GTG
(291) Glu Glu Lys Val Lys Thr Leu Lys Ser Gln Asn Thr Glu Leu Ala Ser Thr Ala Ser Leu Leu Arg Glu Gln Val
1066  GCG CAG CTC AAA CAG AAA GTC CTC AGC CAC GTC AAC AGC GGC TGC CAG CTG CTG CCC CAG CAC CAG GTC CCG GCG
(316) Ala Gln Leu Lys Gln Lys Val Leu Ser His Val Asn Ser Gly Cys Gln Leu Leu Pro Gln His Gln Val Pro Ala
1141  TAC TGA GCCCGAGCNCGGGGCGCATGCGCGGACTAGCTGCGGTGGGGGGGCGCCCCGGACTCTTTCGAGACTCGGTGCCCCCGGACTCGACAAGCCGG
(341) Tyr  *
1239  ACCCCCCTTAACTCTGGATGGGGAACCCGAGCGCACGACCCCCGCCCTCGCGCCGCCTCTCTACNCCCAGTCCTGCGCGTGGCTGCCCCTTTGTACCTCC
1339  GAGTAGGGGCTCTAAGGGGCGCGCAGCCTCACNCTCTGCCTTTCCTTTTTTTTTTGCCTTTTGGAAGAGAGAACGGGAGTGTCCGATTCCGCCCTATTTA
1439  TGTTTCTACTCGGGAACAAACGTTGGTTGCGTGTTGAGTGTGTGTTTCCTTGTGTTGGTTTTTTAAAGAAACGGGAAGAAGAAAAAAAAAACCCTCCCCC
1539  CTCCCCCCCCCCGCTCTCTCTCCTCCCGACACCATCCCCTCTCTTTTTGTTTGGTTTTGTTTTGCTACGAGTCCACATTCCTGTTCCGTAATCCTTGGTTC
1639  GCCGGTTTTGTGTTTTCAGTAAAGTCTTCGTTACGCCA(AN)
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FIG. 1. Nucleotide sequence of jun-D cDNA and the predicted Jun-D protein sequence. Also shown is the sequence from a genomic fragment that overlapped the 5′ end of the cDNA. Numbers at the left refer to the first nucleotide on each line or (in parentheses) to the first amino acid. The adenine at nucleotide 1 (mRNA start site) is indicated in relief. A TATAA sequence in the genomic DNA is underlined. The predicted termination codon is noted, and the poly(A) tail is indicated (A_N). The identity of two nucleotides (positions 1293 and 1371) was uncertain.

present in all three Jun proteins, postulated to form an amphipathic helix and a dimerizing "leucine zipper" (25). HR-1, which is not required for specific DNA binding (2, 3, 20), contains the stretch of acidic amino acids of c-Jun that is able to substitute for the activator domain of the yeast transcription activator protein GCN4 (21); all 5 of the acidic amino acids in a 16-amino acid segment of c-Jun HR-1 are also present in Jun-B and Jun-D.

Serum Stimulation of Murine *jun* Genes. Both *jun-B* and c-*jun* are immediate early genes in 3T3 cells, characterized by rapid and transient activation by serum growth factors independent of new protein synthesis (5–9). Compared with *jun-B* and c-*jun*, *jun-D* mRNA is more abundant in nongrowing 3T3 cells and is stimulated less by serum (Fig. 3*A*) or by platelet-derived growth factor or fibroblast growth factor (results not shown). Moreover, there is little increase in transcription of *jun-D* after serum stimulation of resting cells, in contrast to what is found for c-*jun* and *jun-B* and other immediate early genes (Fig. 3*B*). In addition, jun-D mRNA is more stable than the other jun mRNAs after serum stimulation (data not shown). These results suggest that regulation of *jun-D* expression by serum growth factors is different from regulation of c-*jun* and *jun-B*.

Presence of jun-D mRNA in Tissues and Cell Lines. We have shown (8) that jun-B and c-jun mRNAs are present in many adult mouse tissues and in mouse placenta at days 8–14 of gestation. Similar results have been obtained with jun-D mRNA (Fig. 4*A*). With the exception of spleen and thymus, the distribution in tissues is similar to that found for c-jun and jun-B mRNAs; jun mRNAs are also present in many cell lines, but the relative amount of each jun RNA is different in some cell lines compared with others (Fig. 4*B*). These observations suggest that the Jun proteins play an important regulatory role in many cell types and that they are often not regulated coordinately. Moreover, since different Jun proteins interact *in vitro* to form heterodimers (20), it is possible that in cells expressing more than one *jun* gene, heterodimers contribute distinct regulatory activities.

DISCUSSION

Three members of the mammalian *jun* family have now been identified: murine c-*jun*, *jun-B*, and *jun-D*. The three Jun proteins are closely related in amino acid sequence, particularly in the region (HR-2) containing the DNA-binding domain (2, 3, 20), the heptad leucine repeats (25), and domains for dimerization and interaction with Fos (20, 22–24), and in the segment (HR-1) containing a putative transcription activation domain (21). Jun-D is more closely related by sequence to c-Jun than to Jun-B. Functionally, the three Jun proteins show similar nucleotide sequence specificity for binding to a series of oligonucleotides, and DNA-binding by each protein is stimulated markedly by Fos (20). Although no activities unique to a particular Jun protein have been observed so far, the fact that regions of dissimilar sequence in the Jun proteins are evolutionarily conserved [in the case of c-Jun (8)] suggests that these regions are functionally important and therefore that each protein has distinctive biological properties.

Two observations indicate that regulation of the different murine *jun* genes is not coordinate. First, the relative amounts of c-jun, jun-B, and jun-D mRNAs in different cells or organs vary, in some cases markedly. Second, the level of jun-D mRNA in nongrowing 3T3 cells is higher than that of c-jun and jun-B mRNAs, and jun-D mRNA is more stable after serum stimulation. Moreover, transcriptional activation

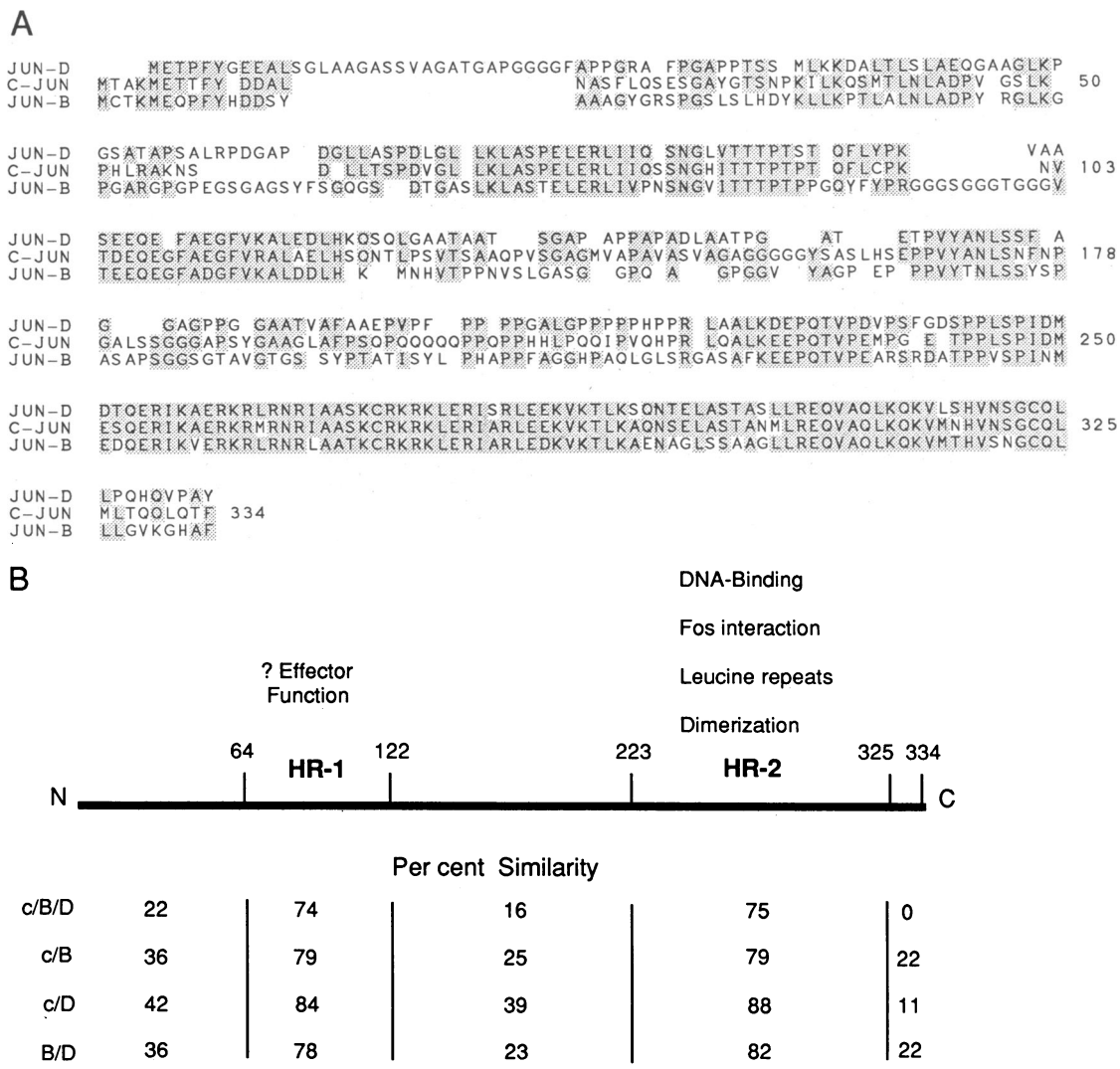

FIG. 2. (*A*) Comparison of murine Jun-D, c-Jun, and Jun-B sequences in the single-letter amino acid code. Sequence identities and similarities (D = E, I = V, K = R, and T = S) are shaded. Numbers to the right refer to the last residue of c-Jun on each line. (*B*) Linear representation of murine c-Jun (heavy line, numbers above the line indicating amino acid residue number) with the percent similarity between Jun proteins for each region taken from *A*. HR-1 and HR-2 are the homology regions 1 and 2 referred to in the text. Above the line are functions assigned to HR-1 and HR-2 (refs. 2, 3, 20–24).

of *jun-D* in 3T3 cells by serum growth factors is much less than activation of c-*jun* and *jun-B*. Therefore, each of the genes is likely to have unique controlling elements that govern responses to specific intracellular or extracellular signals.

The constitutive expression of *jun-D* in resting 3T3 cells raises the possibility that Jun-D is involved in the activation of immediate early genes induced by growth factors and by tumor promoters. Several tumor promoter- and growth factor-responsive genes are downstream of AP-1 binding sites (26–28). Ligand-induced modification of preexisting Jun-D could play a role in the activation of these genes.

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1. Maki, Y., Bos, T. J., Davis, C., Starbuck, M. & Vogt, P. K. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2848–2852.
2. Bohman, D., Bos, T. J., Admon, A., Nishiimurg, T., Vogt, P. K. & Tjian, R. (1987) *Science* **238,** 1386–1392.
3. Angel, P., Allegretto, E. A., Okoni, S. T., Hattori, K., Boyle, W. J., Hunter, T. & Karin, M. (1988) *Nature (London)* **332,** 166–171.
4. Bos, T. J., Bohman, D., Tsuchie, H., Tjian, R. & Vogt, P. K. (1988) *Cell* **52,** 705–712.
5. Lamph, W. W., Wansley, P., Sassone-Corsi, P. & Verma, I. M. (1988) *Nature (London)* **334,** 629–631.
6. Lau, L. F. & Nathans, D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1182–1186.
7. Ryder, K., Lau, L. F. & Nathans, D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1487–1491.
8. Ryder, K. & Nathans, D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8464–8467.
9. Ryseck, P.-P., Hirai, S. I., Yaniv, M. & Bravo, R. (1988) *Nature (London)* **334,** 535–537.
10. Nathans, D., Lau, L. V., Christy, B., Hartzell, S., Nakabeppu, Y. & Ryder, K. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53,** 893–900.
11. Lau, L. F. & Nathans, D. (1985) *EMBO J.* **4,** 3145–3151.
12. Frishauf, A. M., Lehrach, H., Poustka, A. & Murray, N. (1983) *J. Mol. Biol.* **170,** 827–842.

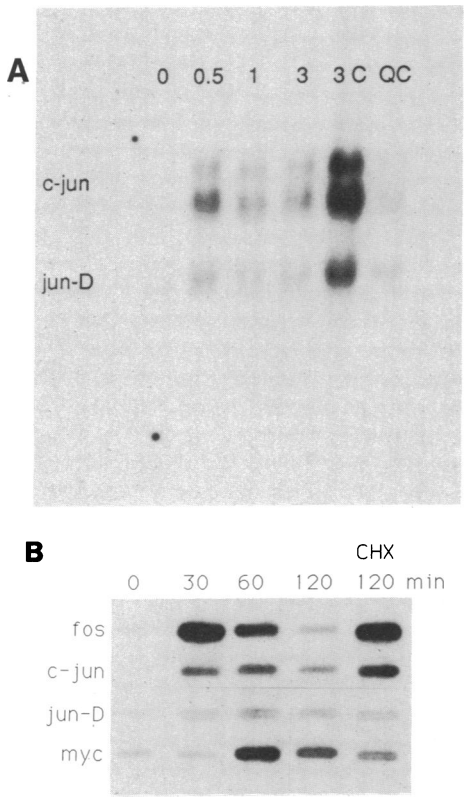

FIG. 3. (*A*) Changes in jun-D and c-jun mRNA levels after stimulation of 3T3 cells with serum. Total RNA (10 μg) was fractionated by electrophoresis, blotted, and hybridized with ^{32}P-labeled c-jun or jun-D cDNA. Times above each lane refer to hours after addition of serum to resting cells; 3C refers to treatment with serum plus cycloheximide for 3 hr, and QC refers to quiescent cells treated with cycloheximide alone for 3 hr. (*B*) Changes in transcription of *jun-D* and *c-jun* genes after serum stimulation of 3T3 cells. ^{32}P-labeled nuclear run-on transcripts were hybridized to jun-D, c-jun, fos, and myc cDNA immobilized on nitrocellulose filters. Nuclei were prepared at the times after addition of serum indicated above each lane. In the last lane, the cells were treated with serum plus cycloheximide (CHX) for 120 min.

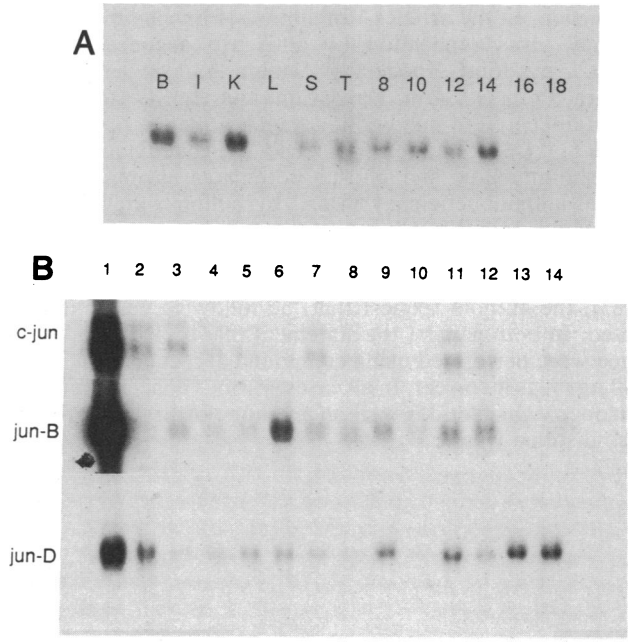

FIG. 4. (*A*) Distribution of jun-D mRNA in mouse tissues. Total RNA (10 μg) was analyzed by Northern blotting. Lanes: B, brain; I, intestine; K, kidney; L, liver; S, spleen; T, thymus; 8–18, placental RNA at the indicated day of gestation. (*B*) Distribution of c-jun, jun-B, and jun-D mRNAs in cultured cell lines. Total RNA (10 μg) was analyzed by Northern blotting with one jun probe at a time, and the three autoradiograms were photographed together. Lanes: 1, 3T3 cells treated with serum plus cycloheximide for 3 hr; 2, quiescent 3T3 cells; 3, NIH 3T3 cells; 4, C127I cells transformed by bovine papilloma virus; 5, $C_3H/10T1/2$ mouse fibroblasts; 6, mouse L 929 cells; 7, simian virus 40-transformed mouse testicular cells; 8, BNL CL.2 fetal hepatocytes; 9, chemically transformed BNL CL.2; 10, HEPA-1 hepatoma cells; 11, RAW 264.7 mouse macrophages; 12, Friend erythroleukemia cells; 13, undifferentiated F9 cells; 14, differentiated F9 cells.

13. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
14. Groudine, M., Peretz, M. & Weintraub, H. (1981) *Mol. Cell. Biol.* **1,** 281–288.
15. Greenberg, M. & Ziff, E. (1984) *Nature (London)* **311,** 433–438.
16. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
17. Goldberg, D. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5794–5798.
18. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5201–5205.
19. Shaw, G. & Kamen, R. (1986) *Cell* **46,** 659–667.
20. Nakabeppu, Y., Ryder, K. & Nathans, D. (1988) *Cell* **55,** 907–915.
21. Struhl, K. (1988) *Nature (London)* **332,** 649–651.
22. Kouzarides, T. & Ziff, E. (1988) *Nature (London)* **336,** 646–651.
23. Halazonetis, T. D., Geogopoulos, K., Greenberg, M. E. & Leder, P. (1988) *Cell* **55,** 917–924.
24. Rauscher, F. S., III, von Papas, P., Franza, B. R., Jr., & Curran, T. (1988) *Cells Dev.* **2,** 1687–1699.
25. Landschulz, W. H., Johnson, P. F. & McKnight, S. L. (1988) *Science* **240,** 1759–1763.
26. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. & Karin, M. (1987) *Cell* **49,** 729–739.
27. Lee, W., Mitchell, P. & Tjian, R. (1987) *Cell* **49,** 741–752.
28. Sassone-Corsi, P., Sisson, J. C. & Verma, I. M. (1988) *Nature (London)* **334,** 314–319.