

## ***Drosophila* homolog of the mammalian *jun* oncogene is expressed during embryonic development and activates transcription in mammalian cells**

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**ABSTRACT** By means of low-stringency cross-species hybridization to Southern DNA blots, human *c-jun* sequences were used to identify a unique *Drosophila melanogaster* locus (*Djun*). The predicted DJun protein is highly homologous to members of the mammalian Jun family in both the DNA binding and leucine zipper regions. *Djun* was mapped by *in situ* hybridization to position 46E of the second chromosome. It encodes a 1.7-kilobase transcript constitutively expressed at all developmental stages. Functionally, *Djun* in cooperation with mouse *c-fos* can trans-activate activator protein 1 DNA binding site when introduced into mammalian cells. Taken together, these data suggest that *Djun*, much like its mammalian homolog, may activate transcription of genes involved in regulation of cell growth, differentiation, and development. Furthermore, the identification of *Djun* allows one to exploit the genetics of *Drosophila* to identify genes in signal transduction pathways involving *Djun* and thus *c-jun*.

The *c-jun* protooncogene is the normal cellular homolog of the transforming gene *v-jun* of the avian sarcoma virus 17 (1). A major step in understanding its function was the identification of c-Jun as a major component of the mammalian activator protein 1 (AP-1) complex (2, 3). This complex was originally found in mammalian cells as a factor that stimulates transcription of human metallothionein IIA and simian virus 40 early genes through its binding to the specific sequence TGACTCA (4). c-Jun and another protooncogene product c-Fos form a heterodimer, which in turn binds with high affinity to the same DNA sequence and activates AP-1 site-dependent transcription (5–7).

Investigations in a number of mammalian cell systems have strongly suggested that *c-jun* as well as *c-fos* play important roles in programs of cell growth and differentiation, in which cells integrate external physiological signals to bring about appropriate transcriptional changes (4, 8–10). Aspects of cell growth, differentiation, and development that are common to vertebrates and invertebrates are amenable to genetic analysis in *Drosophila* in a manner not feasible in higher eukaryotic organisms. As an initial step toward identifying genes interacting with *jun* in signal transduction pathways, and studying its role in development, we cloned and characterized the *Drosophila* homolog of the mammalian gene *jun* (*Djun*).

### **MATERIALS AND METHODS**

**Nomenclature.** In this paper, we refer to the *Drosophila* gene that encodes the mammalian *jun* homolog as *Djun* and the predicted protein as DJun. Nomenclature of the mam-

malian *jun* and *fos* genes and their proteins is according to Halazonetis *et al.* (5).

**Genomic Library Screening.** Using a DNA fragment containing the entire coding region of the human *c-jun* protooncogene (3), low stringency hybridization conditions were established for identifying cross-hybridizing bands on a genomic Southern blot of wild-type *Drosophila* DNA digested with *Hind*III. Using these hybridizing and wash conditions, a *Drosophila* genomic library constructed in the lambda dash vector (Stratagene) was screened using the entire *c-jun* coding region as a probe. Ten clones were isolated. The three strongest hybridizing clones corresponded to *Djun*. The remaining clones were not further characterized and possibly represent other *jun*-related genes.

**Northern and Southern Analyses.** Total RNA from staged *Drosophila* embryos raised at 25°C was obtained by the guanidinium/cesium chloride method (11). Poly(A)<sup>+</sup> RNAs were affinity purified on oligo(dT)-cellulose (type III; Collaborative Research). Northern blot analysis was by the method of Alwine *et al.* (12). Southern analysis was performed by standard procedures (11).

**Recombinant Plasmids.** Classical recombinant DNA technology was used to generate all the plasmids described below (11). Whenever incompatible restriction endonuclease ends had to be ligated, they were first filled in with *Escherichia coli* Klenow polymerase. Plasmids pSV2Djun, pSV2humjun, and pSV2murfos direct expression in eukaryotic cells of *Drosophila* Jun, human c-Jun, and mouse c-Fos proteins, respectively. They were derived from plasmid pSV2dhfr (13), which was linearized with *Hind*III and *Bgl* II to replace the dhfr insert, with inserts containing the *jun* or *fos* coding sequences. The *Djun* insert was derived from a 1.4-kilobase (kb) *Hind*III/*Eco*RI fragment. The human *c-jun* insert was derived by digestion of a 5.4-kb *Eco*RI genomic DNA fragment (5) with *Sal* I and *Bgl* II, while the mouse *c-fos* insert was derived from plasmid pGEMfos3 (5) by partial digestion with *Eco*RI and complete digestion with *Bam*HI. Plasmids pCONT/TKseap and pAP-1/TKseap direct the expression of a secreted form of placental alkaline phosphatase (SEAP). They were derived from plasmid pBC12/PLseap (14) and their structure will be described elsewhere (T.D.H. and P. Leder, unpublished data). Both plasmids have a herpes simplex virus thymidine kinase promoter and a synthetic oligonucleotide upstream that serves as an enhancer. Plasmid pAP-1/TKseap has a 54-mer containing an AP-1 DNA element, while pCONT/TKseap contains an identical 54-mer except that the AP-1 sequence is replaced by an unrelated sequence. These oligonucleotides have been described (5). Plasmid pRSVgh directs expression of the human growth hormone by the Rous sarcoma virus enhancer and promoter (T.D.H. and P. Leder, unpublished data).

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Abbreviations: AP-1, activator protein 1; SEAP, secreted form of placental alkaline phosphatase; REF, rat embryo fibroblast; PDGF, platelet-derived growth factor.

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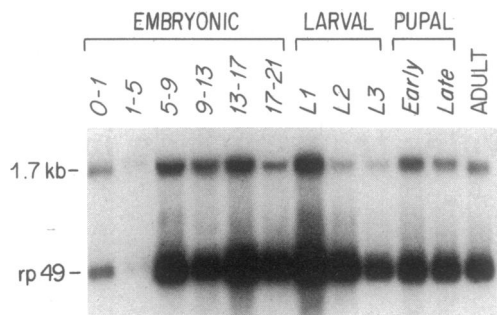


FIG. 1. Developmental Northern analysis of *Djun* expression. Lanes are marked according to the specific developmental stage: numbers during embryonic stages refer to hours of development after fertilization; larval stages L2 and L3 refer to second and third instar larvae, respectively; early pupae are 0–24 hr after pupation; late pupae are 96–120 hr after pupation; adult RNA is from a mixed population of both males and females. One transcript, 1.7 kb, is detected by the *Djun* probe. To control for quantity of RNA loaded in each lane, the same blot was hybridized with an *rp49* ribosomal protein gene sequence (22).

**Transactivation Assays.** Secondary rat embryo fibroblasts (REFs) were prepared as described (15) and grown in Dulbecco's modified Eagle's medium supplemented with 10%

fetal calf serum in 7% CO<sub>2</sub>/93% air. The cells were transfected as described by Chen and Okayama (16). For each transfection, 0.1 μg of pRSVgh plasmid was used, while for all other plasmids 10 μg of DNA was transfected. Whenever necessary, 10 μg of pSV7neo plasmid (17) was added, so the total amount of transfected DNA would be 30 μg. Seventy-six hours after transfection, medium supernatant was collected and assayed for alkaline phosphatase activity as described by Berger *et al.* (14). The concentration of growth hormone was determined by using the Allegro HGH kit (Nichols Institute, San Juan Capistrano, CA).

**In Situ Hybridizations.** *In situ* hybridization to sectioned wild-type Oregon R embryos was performed as described by Hafen and Levine (18). *In situ* hybridizations to polytene chromosomes were done as described (19).

**Sequencing Strategy.** The 1.4-kb *Hind*III/*Eco*RI *Djun* genomic fragment was sequenced by the combined methods of Henikoff (20) and Sanger *et al.* (21). The complete sequence was determined for both DNA strands.<sup>§</sup>

## RESULTS

**Cloning of *Djun*.** Using a human *c-jun* probe, we performed low-stringency genomic Southern analysis with DNA pre-

<sup>§</sup>The sequence reported in this paper has been deposited in the GenBank data base (accession no. M36181).

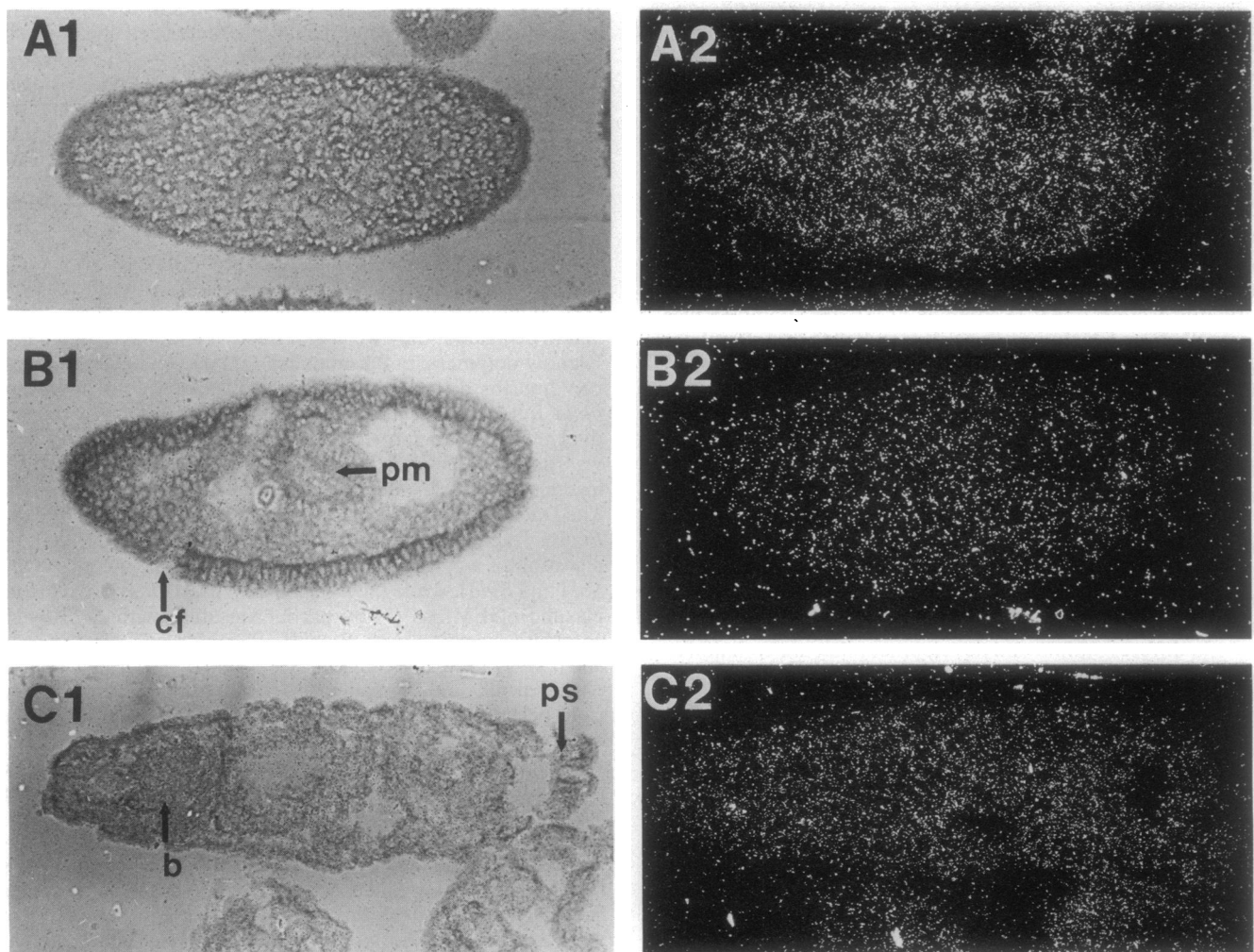


FIG. 2. Embryonic expression pattern of *Djun*. Sagittal and parasagittal sections with anterior at the left and ventral at the bottom. This figure illustrates bright-field (1) and dark-field (2) views of *Djun* expression in a preblastoderm stage embryo (A), expression in a germ band elongating embryo at 5 hr of embryonic development (B), and expression in a fully mature embryo (C). The hybridization pattern at all of these stages (and all remaining embryonic stages not shown) is uniform. *In situ* hybridization using a *fushi tarazu* probe (23) was used as a control (data not shown). cf, Cephalic furrow; pm, posterior midgut; b, brain; ps, posterior spiracles.

pared from various species. Hybridization signals were detected with genomic DNA from chicken, *Xenopus*, *Drosophila*, *Caenorhabditis elegans*, and yeast (data not shown). Under the same hybridization conditions, a *Drosophila* genomic phage library was screened to isolate genomic fragments that cross-hybridize with the human *c-jun* probe. We isolated and characterized three positive clones. Subsequent restriction mapping indicated that they were likely derived from the same genomic locus. Further mapping defined the *c-jun* homologous region to a 1.4-kb *HindIII/EcoRI* fragment. This fragment was used in all subsequent experiments. Using this genomic fragment, we cytologically mapped *Djun* at position 46E on the salivary gland polytene chromosomes (data not shown).

**Developmental Expression of *Djun*.** Developmental Northern analysis with the 1.4-kb genomic fragment described above reveals a major 1.7-kb transcript, which is expressed throughout development at relatively constant levels (Fig. 1). *Djun* is expressed maternally since the *Djun* transcript is observed in RNA isolated from 0- to 1-hr embryos, prior to the initiation of zygotic transcription. The spatial distribution of *Djun* transcripts during embryonic development was examined with a <sup>35</sup>S-labeled 0.6-kb *Apa I/EcoRI* fragment that contains mostly *Djun* coding sequence and part of the 3'

untranslated region. *Djun* expression is observed in all tissues from fertilization to hatching at relatively constant levels (Fig. 2).

**The Predicted DJun Protein.** The 1.4-kb genomic fragment that hybridized to the human *c-jun* probe was entirely sequenced and shown to contain only one sizable open reading frame, of 867 base pairs, capable of encoding a protein of 289 amino acids. The nucleotide sequence and predicted amino acid sequence of the open reading frame are shown in Fig. 3. Like the mammalian *c-jun* (5), *Djun* does not contain any introns. Fig. 4 shows the alignment of DJun sequence with sequences of other mammalian Jun family proteins. At the protein level, with the evolutionary conservative changes, the overall homology between DJun and human c-Jun is 58%. However, both the DNA binding and leucine zipper regions show the highest homology with 66% identity at the amino acid level and 84% similarity when evolutionarily conservative changes are included. Similar conservation is found with JunD and JunB, two other members of the Jun family (Fig. 4).

**DJun in Cooperation with Mouse c-Fos Activates Transcription in REFs.** To examine the transcriptional activity of DJun, secondary REFs were transfected with a reporter plasmid and plasmids directing expression of *Djun* and mouse *c-fos*. The reporter plasmid contains a SEAP as the indicator gene

1	GGAATTCGATATCAAGCTTCTTGTGGTCAATGCTGCCATTGTTTAAACATCGCATT	60
61	TAAAGACCAGTATCGATAGCAATATCGAATAAATAATCGACACTTGACCACCGCTAGCA	120
121	CTCATTCTGAAAACAACCTTTGCGCGTAAATAAATATTTTCGTAATAATTATTTAGCGAAA	180
181	CCAAAATAAATAAATCAAACGATTCGTTGGCCGATAAAACCGGTGATTAAGCAGTGGCAGTG	240
241	CAGAAAGCAGAAAACACAAAAGTACAGATTGTGCTAATCAAATTTTGAAGCAAAGTTCC	300
301	CACCCACTGGTGAGTAAGAAGAAGAGCGAGTTATTTCGCATGACTCATCGCGAAACGAGTT	360
361	TTTATCAGATTGTTTTATTCCGTTTTCGTCTCTTTCACTTCATCCGAATCAGATTGACG	420
421	TCATTGCTTGAGCAAACATGAAAACCCCGTTTTCCGCTGCTGCGAACTTAAGTATTGAGA	480
	M K T P V S A A A N L S I Q N	
481	ATGCTGGCAGTTCGGGAGCAACTGCCATTAGATCATACTAAAACCGAGCCGTTGGAG	540
	A G S S G A T A I Q I I P K T E P V G E	
541	AAGAAGCCCATGTGCTGGACTTTCAGTCGCGAACCTGAACACATCCACCCGAATC	600
	E G P M S L D F Q S P N L N T S T P N P	
601	CTAACAAAGCGTCCCGGCTCGCTGGATCTGAACAGCAAGAGTGCCAAGAACAAGCGCATCT	660
	N K R P G S L D L N S K S A K N K R I F	
661	TCGCACCACTGGTTCATCAACTACCGGATCTGTTCATCCAAGACGGTAAACACACCCGATT	720
	A P L V I N S P D L S S K T V N T P D L	
721	TGGAGAAGATCTGCTATCCAACAATCTGATGCAAAACCCGACGCGGAAAGGTGTTCC	780
	E K I L L S N N L M Q T P Q P G K V F P	
781	CCACCAAGGCGGGGCCCGTACCGTGGAGCAGTTGGACTTCGGCAGGGGATTTCGAGGAGG	840
	T K A G P V T V E Q L D F G R G F E E A	
841	CCTACACAATCTTCACTAACTCCAGGCATTTCCGTCGCGCAATTCGCGCGTAATT	900
	L H N L H T N S Q A F P S A N S A A N S	
901	CCGCCCAATAACCAACTGCGGCAGCCATGACAGCGGTGAACAATGGCATCAGCGGAG	960
	A A N N T T A A A M T A V N N G I S G G	
961	GCACCTTACCTACCAACATGACCGAGGGCTTCTCGGTGATTAAGGACGAGCCCGTCA	1020
	T F T Y T N M T E G F S V I K D E P V N	
1021	ATCAAGCCAGCTCGCCACCGTTAATCCCATGACATGGAAGCGCAGGAGAAGATCAAGC	1080
	Q A S S P T V N P I D M E A Q E K I K L	
1081	TGGAGCGCAAGAGGCGAGCGTAACCGTGTGGCTGCATCCAAGTGCCGCAAGCGCAAGCTGG	1140
	E R K R Q R N R V A A S K C R K R K L E	
1141	AGCGCATCTCAAAGCTGGAGGATCGCGTGAAGGTACTTAAGGGCGAGAACGTCGACCTGG	1200
	R I S K L E D R V K V L K G E N V D L A	
1201	CTAGCATCGTGAAGAACCTCAAGGACCATGTGGCGCACGTGAAGCAGCAGGTGATGGAGC	1260
	S I V K N L K D H V A H V K Q Q V M E H	
1261	ACATTGCGGGCTGCACGGTCCGCGAACTCGACAGACCAATAACATTTGGAGTTGT	1320
	I A A G C T V P P N S T D Q *	
1321	CAGCCGGGAGAATGATGAGGAGGACGTGGCACTGGAGACTGAAACCCCTCGAATCCTG	1380
1381	AAGATCCCGAGCAACCCATGCCTCTGGAATTCTTTTCAAGTGCTAGCACCGGTGCCTTGG	1440

FIG. 3. Nucleotide and predicted amino acid sequences of *Djun*. The open reading frame is 867 nucleotides long and stops at the TAA stop codon at nucleotide 1305. The predicted protein is 289 amino acids long. The DNA binding domain and leucine zipper region are underlined. The putative AP-1 binding site in the 5' untranslated region is underlined.

(14). A herpes simplex virus thymidine kinase promoter directs expression of SEAP, while an oligonucleotide just upstream of the promoter serves as an enhancer. In one form of the plasmid, pAP-1/TKseap, the oligonucleotide contains an AP-1 DNA sequence, while in another form, pCONT/TKseap, a control oligonucleotide is used. To control for differences in transfection efficiencies, a second reporter plasmid was used that directs synthesis of human growth hormone under control of the Rous sarcoma virus promoter and enhancer. DJun, when expressed in the presence of mouse c-Fos, activates transcription from the reporter plasmid containing the AP-1 DNA element. The level of activation is 25-fold higher than the one achieved by cotransfection of the reporter plasmid with pSV7neo (Fig. 5), a plasmid directing expression of a neomycin-resistance gene (17). Moreover, DJun, in cooperation with c-Fos, is equally as active as human c-Jun, with activation of transcription being dependent on the presence of an AP-1 DNA element upstream of the promoter of the reporter plasmid (Fig. 5). These results demonstrate that, like human c-Jun, DJun can interact with c-Fos to form a transcription complex with sequence-specific DNA binding activity.

**DISCUSSION**

**The *Drosophila* Homolog of the *jun* Oncogene.** We report the isolation of *Drosophila jun* (*Djun*) by its cross-hybridization to sequences from the human *c-jun* protooncogene. We have identified a single genetic locus that cytologically maps to position 46E on the second chromosome. Unfortunately, this region has not yet been studied genetically and there are no available mutations or deficiencies.

The uniform expression of *Djun* during *Drosophila* embryonic development may indicate that it is required in all cells as they undergo growth and differentiation. Alternatively, the transcriptional activity of DJun could be regulated by differ-

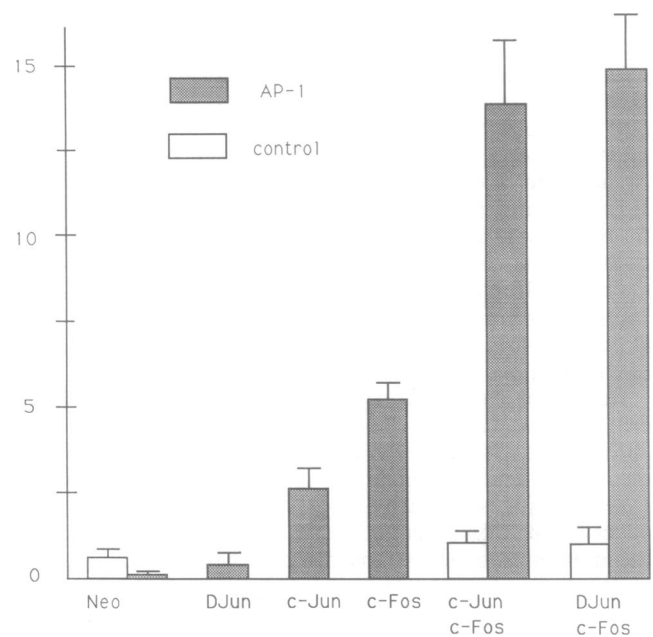


FIG. 5. Trans-activation of the AP-1 site by *Djun* in mammalian cells. Results are expressed as arbitrary units of alkaline phosphatase activity corrected for transfection efficiency. Means  $\pm$  1 SE are indicated. Each data point represents three independently assayed transfections.

ential expression of c-Fos, which dimerizes with DJun and increases DNA binding and transcriptional activity dramatically.

We have shown that DJun, when introduced into mammalian cells, can cooperate with c-Fos and trans-activate AP-1

Djun	MKTPVSAANLSIQNAGSS	GATAIQ	IIPKTEP	VGEEGPMSLDFQSPNLNT	50
Jun-D	METFFYGEALSGLAAGASSVAGATGAPGGGGFAPPGR	FPGAPPTSSMLKKDALTL			
c-Jun	MTAKMETTFY	DDAL	NASFLQSESGAYGTSNPKILKQSMTL		
Jun-B	MCTKMEQPFYHDSY		AAAGYGRSPGSLSLHDYKLLKPTLAL		
	* * *			*	
Djun	STPNPNKRPGSLDLNLSKSAKNRIF	AP LVINSPDLSS	KTVNTPDLEKILL	SNNLMQ	106
Jun-D	SLAEQ	GAAGLKPGSATAPALRPDGPDLGL	LKLAPELERLIQ	SNGLVT	
c-Jun	NLADP	VGSLK PHLRAKNS	DLTSPDVGL	LKLAPELERLIQSSNGHIT	
Jun-B	NLADP	YRGLKPGARGPPEGSAGSYFSGQSDTGASLKLASTELERLIVPNSNGVIT			
		*	***	*****	**
Djun	TPQPG	KVFPK	AGPVTVEQLDFGRGFEEALHNLHTNSQAFPSANSAA	NSA	156
Jun-D	TPPTST	QFLYPK	VAASEEQE	FAEGFVKALEDLHKQSQ	LGAATAA
c-Jun	TPPTPT	QFLCPK	NVTDEQEGFAEGFVRALAEHLHSQNT	LPSVTSAAAGPVSQ	
Jun-B	TPPTPPGQYFYPRGGSGGGTGGGVTEEQEGFADGFYKALDDLHKMNH	VTPPNVSLGASG			
	** *	*	** * *	* * * *	* * *
Djun	AN NTTAAAMTAVNNGISGG	T FTYTNM			182
Jun-D	AP APPADLAATPG	ATET PVYANLSSF	AGGAGPPGGAATVAFAA		
c-Jun	AGMVAPAVASVA	GAGGGGYSASLHSEP	PVYANLSNPNFGALSSGGGAPSYGAAGLAFPS		
Jun-B	GP	QAGPGGVYAGP	EPPPVVTLSSYSPASAPSGGSGTAVGTGS	SYPT	
	*	* * *	* * *		
Djun		TEGFSVIKDEPVNQ	ASSPTVNPIDMEAQEKIKL		215
Jun-D	EPYFP	PP PPGALGPPPPHPPR	LAALKDEPQTVPDVPSFGDSPPLSPIDMDTQERIK		
c-Jun	QPQQQQPPPPHLLPQQIPVQHPR	LQALKEEPQTVPEMPG	ETPPLSPIDMESQERIK		
Jun-B	ATISYL	PHAPPFAGGHPAQLGLSRGASAFKEEPQTVPEARSRDATPPVSPINMEDQERIKV			
		****	**	*****	*****
Djun	ERKRQRNRVAASKCRKRKLERISKLEDRVKVLKGENVDLASIVKNLKDVAHVQVMEHIA				277
Jun-D	ERKRLRNRIAAASKCRKRKLERISRLEEKVTKLSQNTELASTASLLREQVAQLKQKVLSHVN				
c-Jun	ERKRMRNRIAAASKCRKRKLERIARLEEKVTKLKAQNSSELASTANMLREQVAQLKQKVMNHVN				
Jun-B	ERKRLRNRLAATKCRKRKLERIARLEDKVTKLKAENAGLSSAAGLLREQVAQLKQKVMTHVS				
	****	***	*****	**	*****
Djun	AGCTVPPNSTDQ				289
Jun-D	SGCQLLPQHQPAY				
c-Jun	SGCQLMLTQQLQTF				
Jun-B	NGCQLLLGVKGHAF				
	**				

FIG. 4. Comparative alignment of the amino acid sequence of DJun to the mammalian Jun family (Jun-D, c-Jun, and Jun-B). Sequence identities and similarities (D = E, K = R, T = S) are indicated by asterisks. An asterisk is shown whenever an amino acid in DJun is found at the same position in at least two of three other Jun protein sequences.

site-dependent transcription. These results establish that DJun, like the mammalian c-Jun, can function as a transcription factor. Recently, a Jun-related protein was copurified with a Fos-related protein by AP-1 sequence-specific DNA affinity chromatography from *Drosophila* embryonic extracts (24). Most likely, *Djun* encodes the protein identified in these biochemical experiments.

**Sequence Comparison of DJun with the Mammalian Jun Family.** *Djun* encodes a predicted protein of 289 amino acids, which displays very high sequence similarity to members of the mammalian Jun family in both the DNA binding and the leucine zipper regions. These similarities suggest that they bind to similar DNA sequences and that they dimerize with c-Fos. Interestingly, the last leucine in the leucine zipper domain of DJun is changed to a valine. Our results are consistent with the results of mutagenesis studies of the leucine zipper region of the mouse c-Jun by Ransone *et al.* (25), indicating that this single leucine change has no effect on c-Jun function.

Upstream of the DNA binding and leucine zipper regions, the predicted DJun sequence diverges from mammalian Jun sequences. This may reflect low evolutionary pressure on the activation domain and suggests that the primary sequence may not be very important for the trans-activating function. Nevertheless, one region that extends from position 82 to 130 in DJun (Fig. 4) shows high similarity among all the Jun sequences, including Jun-B and Jun-D. This region has been referred to as HR-1 (homology region 1) by Ryder *et al.* (26). Finally, sequence divergence may reflect differential regulation of the transcriptional activation domains of *Djun* versus the mammalian Jun family members.

**Using *Drosophila* Genetics to Dissect *jun* Function and Signal Transduction Pathways.** In mammalian cells, *c-jun* is activated in response to a mitogenic program activated by growth factors—e.g., platelet-derived growth factor (PDGF; ref. 9). Activated PDGF receptor forms a specific complex with Raf-1, a serine/threonine kinase protooncogene product, resulting in phosphorylation of tyrosine in Raf-1 and an increase in its kinase activity. A mutant PDGF receptor defective in transmitting its mitogenic signal, fails to complex with Raf-1 and increase its kinase activity (27). In addition, *v-raf*, which is constitutively activated, trans-activates AP-1 site-linked reporter gene expression (28). The above data suggest that *c-jun* expression responds to growth factor stimulation, which may be mediated through *raf-1*. Interestingly, a similar pathway may exist in *Drosophila* embryonic development. A group of genes, known as the terminal class genes, are required for development of the most anterior and posterior structures of the embryo. All null mutations in this class of genes produce deletions of the most anterior and posterior structures, suggesting that they probably act through the same biochemical pathway. To date, two components of this pathway have been well characterized; *torso* encodes a putative receptor tyrosine kinase that shares similarities with the mammalian PDGF receptor (29) and the *l(1)pole hole* gene product is the homolog of the mammalian *raf* oncogene (30). Genetic analysis indicates the *torso* functions by interacting with *l(1)pole hole* (30). By analogy to the mammalian system, it is likely that *Djun* may be one of the downstream target genes involved in terminal determination. A combination of genetic and molecular studies will most likely provide useful information concerning how *jun* functions in cell growth regulation and development.

**Note Added in Proof.** An independent analysis of the *Drosophila*

homolog of mammalian *jun* has been recently reported by Perkins *et al.* (31).

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