Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 3738–3742, June 1988
Biochemistry

Cloning and expression of *JE*, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties

(secretory proteins/growth factors)

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Communicated by Gordon H. Sato, February 1, 1988

ABSTRACT **The platelet-derived growth factor-inducible gene *JE* has been widely used as a molecular marker for the cellular response to growth factors, antimitogenic agents, and other biological response modifiers; however, the structure of the *JE* gene and the nature of its encoded protein have not been previously described. We present here structural and regulatory features of the *JE* gene and its product that link it to a family of cytokines, including macrophage colony-stimulating factor, interferon α, interleukin 6 (also known as interferon β_2, B-cell-stimulatory factor 2, 26-kDa protein, and hybridoma/plasmacytoma growth factor), and interleukin 2. Just as T lymphocytes secrete interleukins as a component of their response to mitogens, it appears that fibroblasts secrete cytokines as a component of their response to platelet-derived growth factor.**

In confluent growth-arrested BALB/c 3T3 cells, platelet-derived growth factor (PDGF) stimulates expression of a family of single-copy genes. The protooncogenes c-*myc* and c-*fos* are members of this gene family (1–5). However the PDGF-inducible genes detected and characterized as such were the "competence" genes, *JE*, *KC*, and *JB*. Partial cDNA clones for these three genes were isolated by differential screening of a cDNA library from PDGF-treated fibroblasts (6). The c-*myc* (7–12) and c-*fos* (13, 14) gene products can be shown to function as intracellular mediators of the mitogenic response to PDGF and other growth factors, but there is no direct evidence that the original PDGF-inducible genes play a similar role.

The most abundant of the known PDGF-inducible mRNAs is that encoded by *JE*. Treatment of quiescent fibroblasts with PDGF leads to the rapid accumulation of several thousand copies of JE mRNA per cell within 2 hr (6), and this induction occurs primarily at the transcriptional level (15, 16). A partial cDNA clone for *JE* has been used by a number of investigators as a molecular marker for the mitogenic response to growth factors, antimitogenic agents, and other biological response modifiers. In general, *JE* is not expressed in quiescent growth-arrested cell cultures, and its expression is stimulated by exposure to mitogens and growth factors (17–19). In these respects, *JE* is regulated like the c-*myc* and c-*fos* protooncogenes, suggesting that it, too, may function in the mitogenic response to PDGF. To determine whether this is the case, we began by undertaking a careful structural analysis of the *JE* gene.

MATERIALS AND METHODS

Cell Culture and Growth Factors. BALB/c 3T3 cells (clone A31) were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated bovine calf serum and antibiotics. Confluent density-arrested monolayer cell cultures were prepared as previously described (20) and then transferred to fresh DMEM supplemented with 5% platelet-poor plasma, which is free of PDGF (21). Cells were kept in platelet-poor plasma for 16 hr prior to analysis.

Due to growing safety concerns about processing thousands of units of clinically outdated human platelets each year, our laboratory now uses medium conditioned by v-*sis*-transformed NRK (normal rat kidney) cells (*sis*-conditioned medium) as the routine source of PDGF (B chain homodimer). All experimental results generated with *sis*-conditioned medium have been confirmed with either homogeneous PDGF from human platelets or recombinant v-*sis* protein (Amgen Biologicals, Thousand Oaks, CA). Recombinant human interleukin 1α (IL-1α) was obtained from Genzyme (Norwalk, CT).

Gene and cDNA Cloning. For the isolation of *JE* genomic DNA, 10^6 plaques from the BALB/c germ line DNA library of Davis *et al.* (22) were transferred to nitrocellulose and probed as described (23). The probe was the 750-base-pair (bp) *Pst* I fragment of pBC-JE (6) nick-translated to 10^8 cpm/μg. Four hybridizing clones (λJE-1, λJE-2, λJE-3, and λJE-4) were identified and subcloned in pGEM-1 (Promega Biotec, Madison, WI) for sequence analysis. For the isolation of JE cDNA, poly(A)-selected RNA was prepared from BALB/c 3T3 cells that had been treated with PDGF (v-*sis* conditioned medium) for 2 hr. cDNA was synthesized by the RNase H method (24) and cloned in λgt10. About 10^6 plaques were screened as above. Seventy-eight hybridizing plaques were identified, three of which (cJE-1, cJE-2, and cJE-3) were subcloned in pGEM-1. Sequence determination was performed by using the chain-termination method (25). Oligonucleotides complementary to the SP6 and T7 promoters flanking the cloning sites of pGEM-1 were used as primers. The cap site for JE mRNA was determined by primer extension. The 5′-phosphate-labeled, single-stranded *Hpa* I–*Nae* I fragment (bases +166 to +127 in Fig. 1) was annealed with 1 μg of poly(A)$^+$ RNA and extended by using reverse transcriptase from avian myeloblastosis virus. Extension products were analyzed on an 8% polyacrylamide/8 M urea gel.

RNA Analysis. Cells were scraped directly into 4 M guanidine isothiocyanate/25 mM sodium citrate, pH 7.0/100 mM 2-mercaptoethanol, and the RNA was purified by centrifugation through a cushion of 5.7 M CsCl and precipitation with ethanol (26). Twenty micrograms of purified RNA was electrophoresed through a 1.5% agarose/2.2 M formaldehyde gel and transferred to nitrocellulose filters in 20× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7). Baked filters were prehybridized and hybridized at 42°C in solutions

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Abbreviations: PDGF, platelet-derived growth factor; M-CSF, macrophage colony-stimulating factor; IL, interleukin; IFN, interferon.

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described in ref. 3. Final washes for all blots were 0.1× SSC at 65°C for 1 hr. Probes were nick-translated to a specific activity of greater than 10^8 cpm/μg and used at 3×10^6 cpm/ml. Probes were as follows: c-*myc*, 600-bp *Pst* I fragment of p54 (27), containing the third exon of mouse c-*myc* cDNA, a gift of K. Marcu (State University of New York at Stony Brook, NY); *JE*, a 650-bp *Eco*RI fragment of pcJE-1; and macrophage colony-stimulating factor 1800-bp *Hin*dIII–*Eco*RI fragment of psp64T-M-CSF, representing the coding region of 3ACSF-69 (28), a gift of G. G. Wong and S. C. Clark (Genetics Institute, Cambridge, MA).

COS Cell Expression. cJE-1 was subcloned in pmt2 in both orientations. This vector is a derivative of pXM (29) and was a gift of G. G. Wong and S. C. Clark. Ten micrograms each of pSV_2-neo, cJE-1 in the sense orientation, and cJE-1 in the antisense orientation were transfected into COS-1 cells at 60% confluence by the DEAE-dextran method, followed by chloroquine treatment (30, 31). After 48 hr, medium was replaced by methionine-free medium for 30 min. Cells in 100-mm dishes were then incubated for 4 hr in 0.5 ml of methionine-free medium to which 500 μCi of [^{35}S]methionine (800 Ci/mmol; 1 Ci = 37 GBq) had been added. Medium was collected and made 1 mM in phenylmethylsulfonyl fluoride, and cells were spun out on a microcentrifuge.

Carbohydrate Linkage Analysis. For N-linked carbohydrates, the COS cell supernatant was made 0.2 M Na_2HPO_4 (pH 8.6). Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (EC 3.5.1.5.2; previously called peptide:N-glycosidase F; N-Glycanase; Genzyme) was added to 10 units/ml (as defined by the supplier) and incubated for 12 hr at 37°C. Denaturation in 0.17% $NaDodSO_4$ followed by 1.25% Nonidet P-40 did not alter digestion patterns. For O-linked carbohydrates, the COS cell supernatant was made 20 mM Tris acetate (pH 6.5), 10 mM D-galactono-γ-lactone, and 1 mM calcium acetate. Sialic acid residues were first removed by adding neuraminidase (EC 3.2.1.18; Genzyme) at 1 unit/ml (as defined by the supplier) and incubating at 37°C for 1 hr. This was followed by addition of endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97; O-Glycanase; Genzyme) at 80 units/ml (as defined by the supplier) and incubating for 6 hr at 37°C.

RESULTS

Isolation of Genomic and cDNA Clones for JE. We isolated genomic and nearly full-length cDNA clones for JE by using the original partial cDNA clone, pBC-JE, as probe. These clones were completely sequenced. The structure of the *JE* gene and the DNA sequence of the longest cDNA clone are shown in Fig. 1. The authenticity of the cDNA sequence data was confirmed in three ways: first, its sequence is matched, base for base, in the genomic clone, and, where introns appear, there are consensus splice donor and acceptor sequences (36) at the intron/exon boundaries; second, on a genomic Southern blot, labeled cDNA hybridized only to bands predicted by the sequence of the genomic clone (thus also confirming the sequence determination of the genomic clone); and, third, *in vitro* translation of SP6-generated RNA, using the cDNA as template, yielded a protein that has an apparent molecular weight identical to that of the *in vitro* translation product of hybrid-selected JE mRNA described in our original studies (6).

The *JE* gene has three exons spaced over 2000 bp. When spliced, the mRNA is either 594 or 797 bases [exclusive of poly(A)], depending on which of the alternative polyadenylylation signals is used. The sequence of 466 bases 5′ to the mRNA cap shows little homology to the promoter regions of the c-*fos* and c-*myc* genes. In particular, the serum response element of c-*fos* described by Treisman and others (37–40) is not represented within the DNA that we have sequenced so far. From position −179 to −163, there is a perfectly conserved copy of the so-called interferon response sequence (IRS) (41) identified in interferon-inducible genes such as class I major histocompatibility complex antigens and metallothionein. While JE is not interferon-inducible *per se* (42), preliminary data suggest that JE mRNA can be "primed" to higher levels [similar to interferon-β (IFN-β) mRNA] by prior treatment with a mixture of IFN-α and IFN-β.

The cDNA clone, cJE-1, contains a single long open reading frame that presumably encodes a protein of 148 amino acids. Assignment of the N-terminal methionine was based on the presence of the eukaryotic consensus sequence for translation initiation (43). There is a single consensus sequence for N-linked glycosylation at amino acid 126, but the predicted protein is rich in serine and threonine, allowing ample potential for O-linked glycosylation. The 3′ untranslated region is A+T-rich and contains one copy of the ATTTA sequence described for cytokines and rapidly turning over mRNAs (34, 44). Also shown in Fig. 1 is the hydropathy plot for the predicted JE protein. It shows a hydrophobic N-terminal sequence of 29 residues that could serve as a leader sequence.

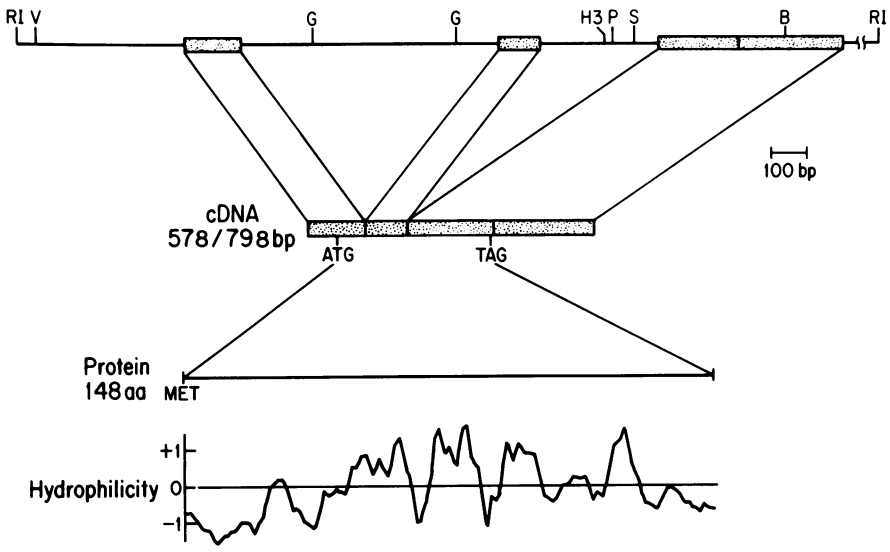
Expression of JE Protein in COS Cells. The cDNA sequence of JE predicts a secretory glycoprotein with a processed polypeptide core of about 12 kDa. These predictions are borne out by COS cell expression studies shown in Fig. 2. The cDNA clone cJE-1 was subcloned in the expression vector pmt2 in both sense and antisense orientations. As shown in Fig. 2*A*, COS cells that received a sense-oriented JE plasmid secreted a 25-kDa microheterogeneous protein. The cells also secreted a 12-kDa protein that migrated as a sharp band. This is the predicted size of the unmodified core protein. Glycosylation causes the increased size and heterogeneity of the 25-kDa protein. Fig. 2*B* shows that digesting the COS cell supernatant with endo-*N*-acetylgalactosaminidase removes most of the 25-kDa material and causes an accumulation of 12-kDa material. This demonstrates the presence of a sizeable amount of O-linked polysaccharide. Digestion of this material with an enzyme specific for N-linked carbohydrate has little effect on the apparent molecular weight of the JE protein.

JE Is Homologous to Several Cytokines. The coding sequence of *JE* shows extensive nucleic acid sequence similarity to a number of cytokine genes. It is most closely related to the IFN-α gene, sharing 61% of its sequence, and to the M-CSF gene, sharing 59% of its sequence over the first 460 bases of the M-CSF coding sequence. JE shows more limited similarity to IL-6 [also known as IFN-β_2, 26-kDa protein, or hybridoma/plasmacytoma growth factor (45–48)] over the first 300 bases of *JE*, and to IL-2 over the last 300 bases of *JE*. These sequence relationships were analyzed by using an algorithm for which rigorous statistical measures have been developed (49). Table 1 shows that the similarity scores generated by comparing the coding sequences of JE to those

Table 1. Similarity between the coding sequence of JE and the coding sequences of IFN-α, M-CSF, IL-6, and IL-2

	Similarity score (SD)			
	IFN-α	M-CSF	IL-6	IL-2
JE	30 (9)	31 (8)	29 (8)	28 (8)

The nucleic acid sequence of the coding region of cJE-1 was compared to the sequences of the indicated genes by using the local sequence comparison algorithm (49). The similarity score generated by the algorithm [number of matches − (0.9 × number of mismatches + (1.01 + 0.9 × length of gaps] is shown. The mean similarity score for two random sequences of given length was derived as described in ref. 48, with a standard deviation of 1.78. The value in parentheses is the number of standard deviations the similarity score lies from this mean.

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  -460              -440              -420              -400              -380
gaattcatgcctgataaccaaggactcagccgacttactgggggtcctttcccagctgcaaggttccttgagccaggggcaagctaggctgtgtttccaa

  -360              -340              -320              -300              -280
gcatcttcttcctaaggactttaggttccttggccacttccttttatttcagtgaaagcagagccactccattcacacttgtggtcacagtagtacaatt

  -160              -240              -220              -200              -180
actgccaattcttccctctttccccccccccccctactcctgcgcagttgatttgtccaggagtggtagaaaaatacaaattccaaccacagtttctctc

  -160              -140              -120              -100               -80
ttccacttcctggaaacaccccagggctctgcacttactcagcggattcaacttccactttccatcacttatccagggtgatgctactccttggcaccaa

   -60               -40               -20               +1               20 *
gcaccctgcctgactccacccccctggcttacataaaaggctgcctcagagcagccagaagtgcAGAGAGCCAGACGGAGGAAGGCCAGCCCAGCACCA
                                                              ‡
   40                60                80               100               120
GCACCAGCCAACTCTCACTGAAGCCAGCTCTCTCTTCCTCCACCACCATGCAGGTCCCTGTCATGCTTCTGGGCCTGCTGTTCACAGTTGCCGGCTGGAG
                                                MetGlnValProValMetLeuLeuGlyLeuLeuPheThrValAlaGlyTrpSe

  140                           160               180               200               220
CATCCACGTGTTGGCTCAGCCAGgtgag...ctccagATGCAGTTAACGCCCCACTCACCTGCTGCTACTCATTCACCAGCAAGATGATCCCAATGAGTA
rIleHisValLeuAlaGlnProA              spAlaValAsnAlaProLeuThrCysCysTyrSerPheThrSerLysMetIleProMetSerA

            240               260               280               300
GGCTGGAGAGCTACAAGAGGATCACCAGCAGCAGGTGTCCCAAAGAAGCTGTAGTgtgag...ccacagTTTTGTCACCAAGCTCAAGAGAGAGGTCTGT
rgLeuGluSerTyrLysArgIleThrSerSerArgCysProLysGluAlaValVa              lPheValThrLysLeuLysArgGluValCys

         320               340               360               380               400
GCTGACCCCAAGAAGGAATGGGTCCAGACATACATTAAAAACCTGGATCGGAACCAAATGAGATCAGAACCTACAACTTTATTTAAAACTGCATCTGCCC
AlaAspProLysLysGluTrpValGlnThrTyrIleLysAsnLeuAspArgAsnGlnMetArgSerGluProThrThrLeuPheLysThrAlaSerAlaL

         420               440               460               480               500
TAAGGTCTTCAGCACCTTTGAATGTGAAGTTGACCCGTAAATCTGAAGCTAATGCATCCACTACCTTTTCCACAACCACCTCAAGCACTTCTGTAGGAGT
euArgSerSerAlaProLeuAsnValLysLeuThrArgLysSerGluAlaAsnAlaSerThrThrPheSerThrThrThrSerSerThrSerValGlyVa
                                                                o
         520               540               560               580               600
GACCAGTGTGACAGTGAACTAGTGTGACTCGGACTGTGATGCCTTAATTAATATTAAACTTATTTAACTTATTGATGTTATGGTATTCCCTTTGATGAAT
lThrSerValThrValAsn ·

         620               640               660               680               700
ACTAAAATTTCTTAAATGCAAGGTGTGGATCCATTTTTCCCTCTCTGTGAATCCAGATTCAACACTTTCAATGTATGAGAGATGAATTTTGTAAAGATGA

         720               740               760               780                8
ATGGGTAAACTTTGTGTTTGAGATTCCAAGGTATTGTTTAAAATATTATTATGGATATTCCTTATTATTAAAAGAAATATATTATTTTTGT
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FIG. 1. (*Upper*) Structure of the *JE* gene. Restriction sites and exons (stippled boxes) derived from the 4.0-kbp *Eco*RI fragment of λJE-1. Vertical bar in the third exon represents the more 5′ of the two polyadenylylation sites. RI, *Eco*RI; V, *Pvu* II; G, *Bgl* II; H3, *Hin*dIII; P, *Pst* I; S, *Sac* I; B, *Bam*HI. Hydrophilicity plot was derived from the Hopp–Woods algorithm (32), averaging over five amino acids. (*Lower*) Nucleotide sequence of *JE* 5′ flanking region and exons with predicted amino acid sequence. The 4.0-kbp *Eco*RI fragment of JE-1 was sequenced from the 5′ *Eco*RI site to 100 bp 3′ to the end of the pBC-JE sequence; cJE-1 was completely sequenced. Lowercase letters indicate boundary sequences of the two introns. Candidate TATA sequence is boxed; RNA cap site, determined by primer extension, is number +1; polyadenylylation signals (33) are overlined; ATTTA sequence (34) is underlined; the Ala-Pro dipeptide (35) and possible N-glycosylation site are underlined. *, 5′ end of cJE-1; ‡, 5′ end of pBC-JE (6); o, polyadenylylation site of cJE-1; §, 3′ end of pBC-JE [there is no poly(A) sequence in pBC-JE].

of IFN-α, M-CSF, IL-6, and IL-2 are 8 to 9 standard deviations above the score generated by two random sequences of the same lengths. These homologies exist at the nucleic acid level and are not apparent at the amino acid level, a situation reminiscent of the relationship between human and murine IL-3 (29).

Further sequence evidence consistent with the identification of JE as a member of a cytokine family is that the hydrophobic leader sequence is followed by an Ala-Pro dipeptide. This is the initial dipeptide found in a number of cytokines (granulocyte–macrophage colony-stimulating factor, erythropoietin, IL-1, IL-2, and IL-3) (35), and we predict that this will be the initial dipeptide found in secreted JE.

IL-1 Induces *JE* Expression. Previous work from our laboratory has shown that *JE* expression is regulated in opposition to that of c-*myc* (16). This finding plus the sequence and expression analysis described above argue that JE is not an intracellular mediator of the growth response to PDGF. We would predict, then, that *JE* expression should be modulated by other agents that have little effect on cell growth or on c-*myc* expression. Fig. 3 shows that recombinant IL-1α fulfills this prediction. It is a potent inducer of *JE* expression, but unlike PDGF, IL-1α is a poor competence factor for 3T3 cells and is unable to induce c-*myc* expression (Fig. 3). Thus *JE* expression is not strictly dependent upon a strong mitogenic stimulus. In addition, double-stranded RNA [poly(rI)·poly(rC)], an agent that induces interferon expression and is not a growth factor for 3T3 cells, also induces *JE* expression (50).

These data suggest that there is a close relationship between the actions of PDGF, those of other cytokines, and inducible cytokine expression (46). There are at least two more examples of such a relationship. First, PDGF stimulates expression of the same multiple mRNAs of M-CSF as IL-1 does in 3T3 cells (data not shown). The induction of M-CSF mRNA has functional consequences as documented by the fact that conditioned medium from PDGF-treated 3T3 cells can support mouse bone marrow monocyte/macrophage colony formation *in vitro* (J. Griffin, Dana–Farber Cancer Institute, Boston; personal communication). Second, in mu-

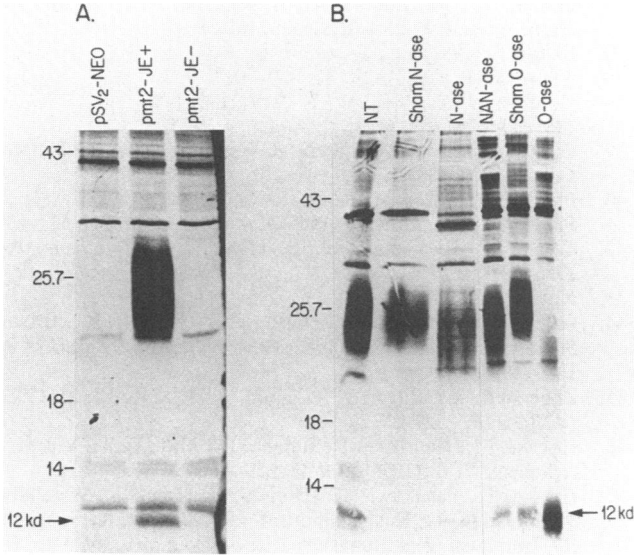

FIG. 2. (*A*) COS cell expression of JE cDNA. cJE-1 was subcloned in pmt2 in both orientations. Ten micrograms of pSV2-neo, cJE-1 in the sense orientation (pmt2-JE+), or cJE-1 in the antisense orientation (pmt2-JE−) was transfected into COS-1 cells at 90% confluence by the DEAE-dextran method, followed by chloroquine treatment. After 48 hr, medium was replaced by methionine-free medium for 1 hr. Cells in 100-mm dishes were then incubated for 4 hr in 0.5 ml of methionine-free medium to which 500 μCi of [^{35}S]methionine (800 Ci/mmol) had been added. Medium was collected and cells were spun out. Media were electrophoresed on a 17% polyacrylamide gel in $NaDodSO_4$ under reducing conditions. The gel was dried and exposed without enhancement. Positions of molecular mass markers are given on the left, and the position of the predicted 12-kDa core peptide is marked. (*B*) Glycosidase treatment of transfected COS cell supernatant. [^{35}S]Methionine-labeled COS cell supernatant was prepared from pmt2-JE+-transfected cells as in *A*. Aliquots were subjected to glycosidase treatment and electrophoresed on a 17% polyacrylamide gel as in *A*. NT, no treatment; Sham N-ase, sham digestion with N-Glycanase; N-ase, N-Glycanase digestion; NAN-ase, digestion with neuraminidase: Sham O-ase, sham digestion with neuraminidase and O-Glycanase; O-ase, neuraminidase and O-Glycanase digestion. Position of the predicted 12-kDa core peptide is shown.

rine and human fibroblasts, PDGF has been shown to induce the IL-6 gene (46).

DISCUSSION

We have cloned and expressed the full-length cDNA for the PDGF-inducible gene *JE*. We showed the following: (*i*) *JE* encodes a small secretory glycoprotein: (*ii*) the coding sequences of *JE* are homologous to those of several cytokines; and (*iii*) the *JE* gene is induced by IL-1 and synthetic

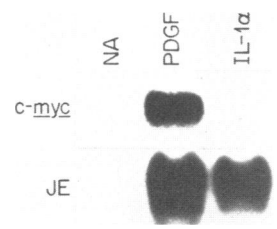

FIG. 3. Induction of *JE* expression by IL-1α. Confluent density-arrested monolayers of BALB/c 3T3 cells were prepared and made quiescent by placing them in 5% platelet-poor plasma and then treated for 2 hr with PDGF at 300 units/ml or recombinant IL-1α at 2.5 units/ml. RNA was collected and analyzed by electrophoresis and blot hybridization. NA, no addition to medium during the 2-hr treatment.

double-stranded RNA, both potent inducers of cytokine expression in fibroblasts. Because of these structural and regulatory linkages to known cytokines and because PDGF induces two authentic cytokines, M-CSF and IL-6, we conclude that the *JE* gene product does not function like c-*myc* and c-*fos* as an intracellular mediator of growth factor action. Rather, JE is probably a cytokine.

If JE is a cytokine, its target cell and biological activity remain to be discovered. In the sense that *JE* encodes a ligand in search of a receptor, it is a mirror image of the c-*trk* (51) and *HER-2*/c-*neu* protooncogenes (51–54). These protooncogenes encode putative receptors for growth factors that have yet to be discovered. We can state with certainty that *JE* does not encode any currently known murine cytokine. With the exception of IL-6, sequence data for the murine counterparts of most human cytokines are already entered into the GenBank data base, which we have searched (on February 11, 1987). JE is homologous to several of these cytokines but identical to none of them. No DNA sequence data on the murine counterpart of IL-6 have yet been reported; however, the human mRNA and protein are much larger than JE. Also, an N-terminal sequence for purified murine hybridoma growth factor has been published (55), and it bears no relation to the predicted JE amino acid sequence.

Stern and Smith (56) have drawn attention to striking similarities between T-cell activation and the mitogenic response of fibroblasts to serum growth factors. A two-stage mechanism that we have termed "competence" and "progression" (20) is involved for both cell types and, in each case, competence is associated with induction of c-*myc*, c-*fos*, and other single-copy genes (1–6, 56–59). The data shown here, together with work from Vilcek, Sehgal, and their co-workers (46) documenting induction of IL-6 by PDGF, extend the analogy between T-cell and fibroblast activation. In both cell types, the secretion of cytokines is an integral component of the mitogenic program.

Note. Burd *et al.* (60) have recently reported the sequence of a T-cell gene, called *TCA-3*, whose expression is selectively induced during activation via the antigen-receptor pathway. The deduced amino acid sequence indicates a small secretory glycoprotein. From amino acids 50 to 93 of both proteins, there is 44% sequence identity and 91% sequence similarity if conserved amino acid changes are considered. All cysteine residues in JE are in conserved positions in TCA-3. Thus the JE protein is similar to a T-cell activation protein.

B.J.R. is supported by a Medical Foundation Fellowship and the Smith Professorship Fund. C.D.S. is supported by grants from the American Cancer Society and the National Institutes of Health.

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