MOLECULAR AND CELLULAR BIOLOGY, Dec. 1992, p. 5288–5300
0270-7306/92/125288-13$02.00/0

Vol. 12, No. 12

A Novel 7-Nucleotide Motif Located in 3′ Untranslated Sequences of the Immediate-Early Gene Set Mediates Platelet-Derived Growth Factor Induction of the JE Gene†

ROLF R. FRETER, JEAN-CLAUDE IRMINGER, JULIE A. PORTER, SUSAN D. JONES, AND CHARLES D. STILES*

Department of Microbiology and Molecular Genetics, Division of Clinical Oncology, and Division of Cellular and Molecular Biology, Harvard Medical School and the Dana-Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115

Received 23 July 1992/Accepted 3 September 1992

A cohort of the serum and growth factor regulated immediate-early gene set is induced with slower kinetics than c-*fos*. Two of the first immediate-early genes characterized as such, c-*myc* and JE, are contained within this subset. *cis*-acting genomic elements mediating induction of the slower responding subset of immediate-early genes have never been characterized. Herein we characterize two widely separated genomic elements which are together essential for induction of the murine JE gene by platelet-derived growth factor, serum, interleukin-1, and double-stranded RNA. One of these elements is novel in several regards. It is a 7-mer, TTTTGTA, found in the proximal 3′ sequences downstream of the JE stop codon. The 3′ element is position dependent and orientation independent. It does not function in polyadenylation, splicing, or destabilization of the JE transcript. Copies of the 7-mer or its inverse are found at comparable 3′ sites in 25 immediate-early genes that encode transcription factors or cytokines. Given its general occurrence, the 7-mer may be a required *cis*-acting control element mediating induction of the immediate-early gene set.

Platelet-derived growth factor (PDGF) stimulates transcription of the immediate-early gene set within its target cells. Included in the immediate-early gene class are numerous transcription factors such as c-*myc* and c-*fos* (11, 21, 28, 29, 34). Other immediate-early genes, including the M-CSF (43), KC/gro (2, 10, 37), and JE/MCP-1 (10, 43) (hereafter referred to as JE) genes, encode cytokines. *cis*-acting regulatory elements, required for transcriptional activation to occur, are present within these genes and define a useful endpoint(s) for analysis of PDGF signal transduction pathways. Identifying and characterizing nuclear, *trans*-acting proteins binding to these regulatory elements should allow reconstruction of the chain(s) of events initiated by activation of the PDGF receptor and ending with transcriptional activation of a gene.

Much progress has been made by using the c-*fos* gene as an experimental paradigm. Functionally distinct elements within the proximal 5′ flanking sequences of c-*fos* respond to serum, cyclic AMP, and PDGF B:B homodimers (3, 15, 49, 54, 58). A well-characterized 22-nucleotide region of dyad symmetry, termed the serum response element (SRE), confers serum inducibility onto the c-*fos* gene (16, 19, 20, 54, 55, 57). Nuclear *trans*-acting proteins binding to the SRE have been isolated and characterized (14, 33, 35, 40, 41, 56). In BALB/c 3T3 cells, induction of c-*fos* by PDGF is to a large extent dependent on activation of protein kinase C (24). The SRE and its binding proteins may therefore be an intranuclear apparatus responding to activation of protein kinase C in the cell cytoplasm.

Comparative sequence analysis and promoter function studies show that several other immediate-early genes, notably those encoding zinc finger transcription factors, contain *fos*-like SRE elements in their 5′ flanking sequences and require these SRE elements for induction in response to a stimulus (7, 42). The c-*fos* gene, however, does not stand as a prototype for all members of the immediate-early gene set. The JE gene was one of the first immediate-early genes to be cloned and characterized as such (10). Despite its early recognition, *cis*-acting genomic elements mediating the transcriptional response of JE to PDGF have never been defined. No *fos*-like regulatory elements are found within several kilobases of JE 5′ or 3′ flanking sequences or within the coding sequences of the JE gene. Furthermore, in BALB/c 3T3 cells, PDGF induces JE through a protein kinase C-independent signal transduction pathway (24). These data suggest that PDGF induction of JE may involve novel *cis*-acting genomic elements.

We set out to identify the *cis*-acting genomic element(s) mediating PDGF induction of JE. These elements are of interest for three reasons. First, they could provide access to a minimally characterized, protein kinase C-independent, PDGF signal transduction pathway. Second, since transcription of JE is also stimulated by double-stranded RNA and interleukin-1 (IL-1) (22, 23, 64), delineation of a PDGF-responsive control element(s) could provide insight into the control elements required for JE induction via these other two biological response modifiers. Finally, control elements comparable to those within the JE gene might be involved in induction of additional PDGF-inducible immediate-early genes. PDGF-responsive elements for c-*myc*, to cite one example, have never been identified (for a review, see reference 53). The c-*myc* gene, like JE, does not contain any *fos*-like SRE elements.

In this report we describe a discontiguous pair of *cis*-acting elements both of which are essential for induction of the murine JE gene. In the presence of both elements, induction of JE occurs in response to serum, PDGF, double-stranded RNA, and IL-1. One of the elements is a 7-base

* Corresponding author.

† This paper is dedicated to the memory of Gloria Goicochea Freter (1927–1991).

motif located in the proximal 3' JE untranslated sequences. Identical 7-mers are found in the proximal 3' untranslated sequences of c-*myc*, multiple zinc finger transcription factor genes, and multiple cytokines belonging to the immediate-early gene set. Given its widespread occurrence and essential function in mediating serum and PDGF induction of JE, this novel 7-nucleotide element may be a unifying feature in the regulation of expression of transcription factors and cytokines belonging to the immediate-early gene class.

MATERIALS AND METHODS

Growth factors and reagents. Recombinant A:A, A:B, and B:B isoforms of PDGF and recombinant IL-1 alpha were obtained from Upstate Biotechnology, Inc. Double-stranded RNA [poly(rI-rC)] and RNase A were obtained from Pharmacia. Proteinase K and RNase T1 were obtained from Boehringer Mannheim Biochemicals. Bovine calf serum was obtained from Hyclone. Human defibrogenated platelet-poor plasma was prepared as previously described (38).

Cell culture, DNA transfections, stimulation assays, and RNA preparation and analysis. BALB/c 3T3 cells (clone A31) were maintained in Dulbecco's modified Eagle medium (GIBCO Laboratories) supplemented with 10% bovine calf serum. Twenty-four hours prior to transfection, cells were plated at a density of 4.5×10^6 per 15-cm tissue culture dish. Calcium phosphate-mediated DNA transfections were performed as previously described (16) with the following modifications. Cells were exposed to DNA-containing precipitates for 6 h and were then given a 2-min 20% glycerin shock. Cells were then washed with Dulbecco's modified Eagle medium and placed in 5% platelet-poor plasma. Transfection mixtures included 50 μg of tagged JE reporter constructs together with 10 μg of alpha-globin reference construct (pSVα-1, a gift from M. Greenberg, Harvard Medical School) per 15-cm tissue culture plate. After 40 to 44 h in 5% platelet-poor plasma, stimulated groups of cells were exposed to 20% bovine calf serum, the three isoforms of PDGF (30 ng/ml), IL-1 (10 ng/ml), or poly(rI-rC) (50 μg/ml) for 2 h unless otherwise indicated in the figure legends. Total RNA was prepared by the guanidinium isothiocyanate method and purified on CsCl gradients (6).

Total RNA samples (30 μg per assay) were analyzed by RNase protection assay as previously described (63). RNase treatment was done with 60 μg of RNase A per ml and 2 μg of RNase T1 per ml for 105 min at 35°C. Nuclease resistant fragments were separated on 8-mm 6% polyacrylamide gels. Synthesis of labelled complementary RNA probes is as described elsewhere (32) and utilizes pGEM vectors (Promega). Probes were gel purified before use. The probe for tagged JE expression is a 272-bp *Hin*cII JE cDNA fragment spanning portions of the second and third exons. The fragment is elongated by addition of a 33-bp tag into a blunted *Sau*I site (see below) and inserted into the *Sma*I site of pGEM7. The length of the unprotected probe is 377 bases. This probe does not include the JE start of transcription. An 811-bp probe including the JE start of transcription was also used. The 272-bp *Hin*cII JE cDNA fragment, without the tag, inserted into the *Sma*I site of pGEM7 is the probe for the endogenous JE gene. Undigested, labelled RNA probes were used as size markers. Exposure and quantitation of gels were performed for all experiments on a phosphorimager (Molecular Dynamics).

Plasmid construction. A murine JE genomic clone (pGMJE-1) with 464 bp of 5' flanking sequences and 1.9 kb of 3' flanking sequences and a murine JE cDNA clone (pcJE-1) were gifts from B. Rollins (Dana-Farber Cancer Institute). Both JE constructs were originally inserted into the *Eco*RI site of pGEM 1 (43). Tagged JE constructs were formed by ligation of a blunted 33-bp *Xho*I-*Cla*I fragment of the pGEM7 polylinker to blunted *Sau*I sites in the third exon of pGMJE-1 (1,049 nucleotides downstream of the transcription start site), pcJE-1, and the RNA probe vector described above. A 2.8-kb segment of 5' JE flanking sequences was isolated from a lambda gt11 murine fibroblast library and ligated to the *Eco*RI site of pGEM7. The longest tagged JE genomic construct used (TG; see Fig. 1 and 3) was formed by ligation of a 3.9-kb *Sau*I-*Eco*RI fragment of tagged pGMJE-1 to *Sau*I-*Eco*RI fragment of the pGEM7 vector containing the 2.8-kb JE 5' flanking sequences. The length of TG is approximately 9.4 kb. The longest tagged JE cDNA used (TC; see Fig. 1 and 3) was formed by ligation of a 3.2-kb *Nae*I fragment of TG (containing the 5' JE flanking sequences and part of the first exon) to *Nae*I-digested, tagged pcJE-1. A tagged JE genomic construct lacking 3' flanking sequences (TG3'−) was formed by ligation of a 200-bp *Eco*RI fragment of TC (containing third exon sequences and 49 bp of 3' flanking sequences including the first polyadenylation signal) to a 7.5-kb *Eco*RI fragment of TG. A tagged JE cDNA construct including 3' flanking sequences (TC3'+) was formed by ligation of a 2.1-kb *Eco*RI fragment of TG (containing third exon sequences and 1.9 kb of 3' flanking sequences) to a 6.4-kb *Eco*RI fragment of TC.

Constructs for the 5' deletion series (see Fig. 4) were obtained by ligation of a 2.1-kb *Sma*I-*Sau*I fragment of TG or a 1.85-kb *Hin*cII-*Sau*I fragment of TG (with 5' ends 2,538 and 2,298 bp upstream of the JE start of transcription, respectively) to a 6-kb *Sau*I-*Nae*I fragment of tagged pGMJE-1. Constructs for the 3' deletion series were obtained by progressive *Exo*III digestion of *Xho*I- and *Aat*II-opened TG. The 3' endpoints of the deletions were determined by DNA sequencing (48).

Multiple 5' and 3' JE flanking sequence fragments are used as shown in the schematics of Fig. 5, 6, 9, and 10. Fragments of 5' flanking sequences include a 468-bp *Xba*I-*Hin*cII fragment (with ends at −2766 and −2298) and a 240-bp *Sma*I-*Hin*cII fragment (with ends at −2538 and −2298 bp). Fragments of 3' flanking sequences include a 317-bp *Bam*HI-*Ple*I fragment (with ends at +104 and +421 relative to the stop codon), a 221-bp *Bam*HI-*Hin*dIII fragment (with ends at +104 and +325 relative to the stop codon), a 117-bp *Bam*HI-*Ssp*I fragment (with ends at +104 and +221 relative to the stop codon), a 104-bp *Ssp*I-*Hin*dIII fragment (with ends at +221 and +325 relative to the stop codon), and a 68-bp *Mse*I-*Dde*I fragment (with ends at +245 and +313 relative to the stop codon). Complementary 14-bp oligonucleotides, 5'-ATGAATTTTGTAAA-3' and 5'-TTTACAAAATTCAT-3', and 7-bp complementary oligonucleotides, 5'-TTTT GTA-3' and 5'-TACAAAA-3', were chemically synthesized. Genomic 5' and 3' fragments and annealed 14- and 7-bp double-stranded oligonucleotides were blunt ligated to a 5' *Sal*I polylinker site of tagged pGMJE-1 or a 3' *Xho*I polylinker site of TG3'− as shown in the schematics of the figures.

The basal truncated tagged JE cDNA shown in Fig. 7 and 8 was formed by blunt ligation of a 0.9-kb *Sau*I-*Bam*HI fragment of TC3'+ into the *Sma*I site of the pGEM7 polylinker. Additional constructs listed in Fig. 7 and 8 were formed by blunt ligation of the 240-bp 5' *Sma*I-*Hin*cII and/or 221-bp 3' *Bam*HI-*Hin*dIII fragments described above into a 5' *Xho*I site (position 1) or 3' *Cla*I site (position 2).

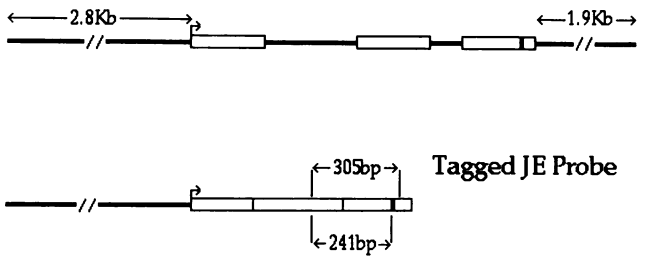


FIG. 1. Structures of the tagged murine JE genomic and cDNA genes. At the top is shown the structure of the longest tagged JE genomic construct used consisting of 2.8 kb of JE 5′ flanking sequences, three exons denoted by open boxes, two introns represented by the dark lines between exons, and 1.9 kb of JE 3′ flanking sequences. At the bottom is shown the structure of the tagged JE cDNA construct used which lacks introns and 3′ flanking sequences. Exon boundaries are highlighted by thin vertical lines. The tag is a 33-bp DNA spacer inserted into the third exon denoted by the dark vertical band in both constructs. The transcription start site for both constructs is marked by the bent arrow. The RNA probe used in all RNase protection assays protects the shown 305-nucleotide fragment of the tagged JE constructs (covering portions of the second and third exons and including the 33-bp tag). Since the difference between tagged and endogenous JE genes is only the presence of a 33-bp spacer, this probe also protects a 241-nucleotide fragment of the endogenous JE gene shown at the bottom. An additional 31-nucleotide protected fragment of the endogenous JE gene (distal to the tag) is run off the gel and therefore never seen in the reported experiments.

All constructs were checked for accuracy by DNA sequencing (48).

RESULTS

Tagged murine JE and endogenous JE behave identically upon serum or PDGF stimulation. We constructed JE reporter vectors by inserting a 33-bp tag into the third exon of a genomic clone or the equivalent position in a cDNA clone (Fig. 1). Expression of these tagged JE reporter constructs generates transcripts that are 33 bases longer than endogenous JE transcripts. Using a probe which spans the tag (Fig. 1), we can discriminate between transcripts of the endogenous JE gene and the JE reporter vectors by RNase protection assay. Inductions of transfected JE reporter constructs and endogenous JE genes can be compared directly on the same gel. The RNA probe used in all succeeding figures protects a 305-nucleotide fragment specific for the transfected tagged JE constructs (covering portions of the second and third exons and including the 33-bp tag [Fig. 1]). The same RNA probe also protects a 241-nucleotide fragment specific for the endogenous JE gene (Fig. 1). Since the 33-bp tag is added to a *Sau*I site 31 bp from the 3′ end of the JE cDNA fragment used to synthesize the riboprobes, the protected tagged JE fragment (305 nucleotides) is 64 nucleotides longer than the protected endogenous JE fragment (241 nucleotides) instead of simply 33 nucleotides longer (Fig. 1).

Transient transfection experiments are prone to titration artifacts because multiple copies of the transfected reporter gene are often introduced into the nucleus (47). For this reason, we first validated use of tagged JE genomic constructs in transient transfections by comparing their induction to that of the endogenous JE gene in serum and PDGF-stimulated BALB/c 3T3 cells. In Fig. 2 (top), we show the results of a dose-response experiment. Cells were

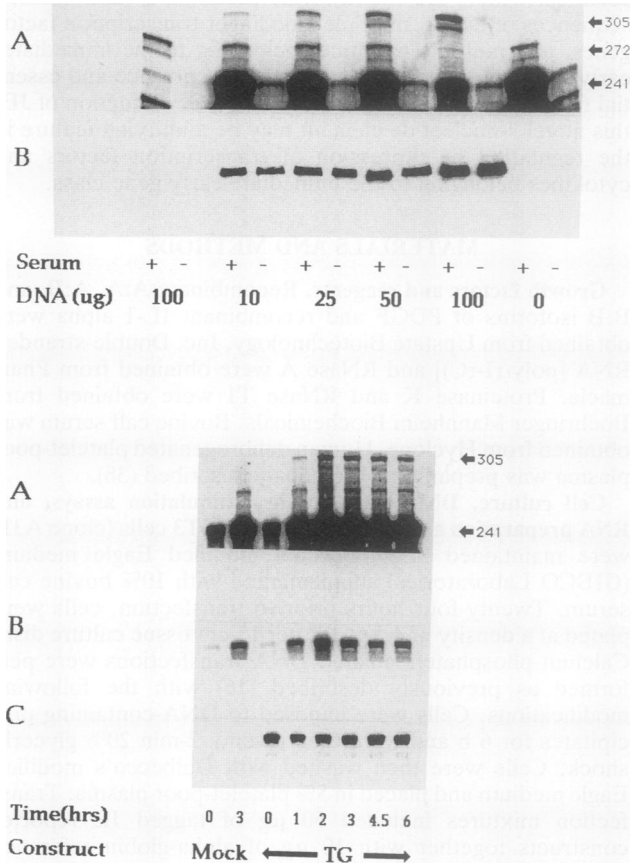


FIG. 2. Tagged JE and endogenous JE genes behave identically upon serum or PDGF stimulation. Top panel. (A) Dose response of tagged JE genomic vector induction in response to serum. Shown are RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with the indicated amounts of the tagged JE genomic construct described in Fig. 1, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum for 2 h. Lanes 0, mock-transfected cells. The 305- and 241-nucleotide bands are protected RNA fragments transcribed from the tagged JE gene and endogenous JE gene, respectively, as described in the legend to Fig. 1. In this and all subsequent experiments an inducible protected fragment appears between the 305- and 241-base protected fragments. This additional protected fragment is observed in all stimulated groups of cells, including all stimulated groups of mock-transfected cells, and represents a nonspecific digestion artifact of the RNase protection assay. Shown in the leftmost two lanes only are the results of RNase protection assay on 1 μg of total cellular RNA with a probe specific for a 272-base fragment of the endogenous JE gene. (B) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel A with an alpha-globin riboprobe. Bottom panel. (A) Time course of tagged JE genomic vector induction in response to the A:B isoform of PDGF. Shown are RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the tagged JE genomic construct described in the legend to Fig. 1 (TG) or mock transfected, allowed to become quiescent, and then exposed to PDGF A:B (30 ng/ml) for the indicated times. The 305- and 241-base bands are as in the top panel. (B) Shown are different phosphorimager settings of the 241-base protected fragments in panel A, representing the endogenous JE gene, to facilitate direct comparisons with the intensities and changes with time of the 305-base band in panel A. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel A with an alpha-globin riboprobe.

transfected with increasing amounts of tagged genomic JE vector ranging from 10 to 100 μg of reporter DNA per culture. Transfected cultures were later stimulated with serum for 2 h. Analysis in all cases was performed on a phosphorimager (Molecular Dynamics) which allows accurate quantitation of the inductions obtained in each experiment. Transfection of increasing amounts of the tagged JE genomic construct results in increasingly intense 305-nucleotide protected fragments, i.e., no plateau in the absolute amounts of transcribed tagged JE after serum stimulation is observed (Fig. 2, top). Reporter JE and endogenous JE transcripts are both induced 75- to 150-fold by serum in all groups. The experiment was performed three times with similar results. Thus within the range of 10 to 100 μg of DNA per culture, we are not titrating out a regulatory factor(s) necessary for inducible transcription of the JE gene. Furthermore, a single start site for serum-inducible transcription of the tagged JE gene was noted by using a probe spanning the endogenous JE start of transcription (data not shown). Controls for minor variations in transfection efficiency were included in these and all subsequent experiments (see below and also Fig. 2 legend).

The kinetics of PDGF induction of transfected tagged JE and endogenous JE genes were also compared directly and shown to be identical. Using PDGF A:B heterodimers as the inducing agent, we observed a maximum 30- to 40-fold induction at 2 h after PDGF addition (Fig. 2, bottom). This is followed by a slow decrease in the amounts of RNA present over a subsequent 4-h period. This can best be seen by comparing changes in the JE reporter bands in panel A with changes in intensities of the endogenous JE bands presented in panel B of Fig. 2 (bottom). In the latter instance the phosphorimager settings were adjusted to achieve comparable intensities of the endogenous and reporter-protected fragments.

The possibility that an apparent induction of the transfected tagged JE gene might be the result of differences in transfection efficiencies between tissue culture plates was addressed with the use of a cotransfected, constitutively active, alpha-globin construct. As shown in Fig. 2B (top) and Fig. 2C (bottom), equal amounts of protected alpha-globin fragments were noted in unstimulated and stimulated cells. This control was run in all experiments presented in this paper and demonstrates that differences in transfection efficiencies among tissue culture plates cannot account for the observed inductions of tagged JE.

Serum induction of JE requires the presence of 3′ flanking sequences. Preliminary experiments using chimeric murine-human JE constructs suggested a role for the 3′ JE flanking sequences in serum induction of JE (data not shown). To evaluate the role of 3′ flanking sequences in transcriptional induction of JE, tagged reporter vectors, including or lacking JE 3′ flanking sequences, were constructed. The structures of these constructs and their responses to serum stimulation are shown in Fig. 3. Genomic and cDNA reporters which include JE 3′ flanking sequence are induced 250- and 150-fold, respectively, by serum. In contrast, equivalent genomic or cDNA constructs lacking JE 3′ flanking sequences are not induced by serum (Fig. 3). The experiment was performed four times with similar results. Tagged JE constructs which lack 3′ flanking sequences do include the first JE polyadenylation signal. Hence lack of a functional polyadenylation signal cannot explain the loss of serum induction upon removal of 3′ JE flanking sequences.

Serum induction of JE requires both a 5′ and a 3′ genomic element. The results of deletion analysis on serum inducibility of tagged JE genomic constructs are presented in Fig. 4. Deletion of progressively greater portions of distal 5′ JE flanking sequences results in complete loss of serum inducibility after deletion of a 240-bp genomic piece 2,298 bp upstream of the transcription start site (Fig. 4, top). Deletion of progressively greater portions of distal 3′ JE flanking sequences results in complete loss of serum inducibility after deletion of a 299-bp genomic piece found 119 bp distal to the JE stop codon (Fig. 4, bottom). The first noninducible construct (D3 in Fig. 4, bottom) retains the first JE polyadenylation signal (data not shown). In both deletion series loss of only one genomic element is sufficient to abolish serum inducibility of the tagged JE construct.

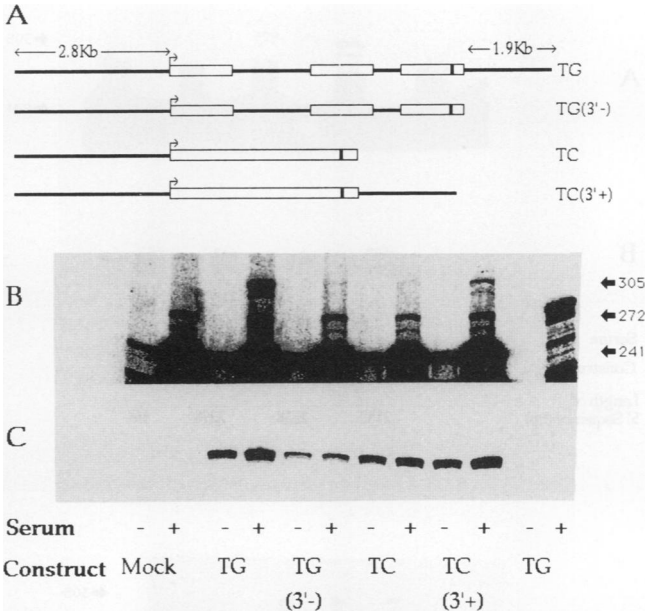


FIG. 3. Serum induction of JE requires the presence of 3′ flanking sequences. (A) Shown in schematic form are the structures of the tagged JE genomic construct described in the legend to Fig. 1, abbreviated TG; the same tagged JE genomic construct from which the 3′ flanking sequences have been removed (with the exception of 49 bp beyond the JE stop codon which includes the first JE polyadenylation signal), abbreviated TG(3′−); the tagged JE cDNA construct described in the legend to Fig. 1, abbreviated TC; and the same tagged JE cDNA construct (TC) to which 1.9 kb of JE 3′ flanking sequences have been added, abbreviated TC(3′+). (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum for 2 h. The rightmost two lanes only are RNase protection assays on 1 μg of total cellular RNA with a probe specific for a 272-bp fragment of the endogenous JE gene. The 305- and 241-base bands are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe.

Further evidence supporting an essential role for 5′ and 3′ genomic elements in serum induction of JE is derived from "add back" experiments. Data in the top panel of Fig. 5 depict the result of 5′ add back studies. Readdition of a 240-bp 5′ element to a noninducible tagged JE gene (including the 3′ flanking sequences) in both orientations restores serum inducibility to the tagged JE gene (Fig. 5, top panel, compare the stimulated lanes of constructs 2 and 3 with construct 1). Readdition of a longer 468-bp 5′ genomic segment (including an additional 228 bp of contiguous 5′

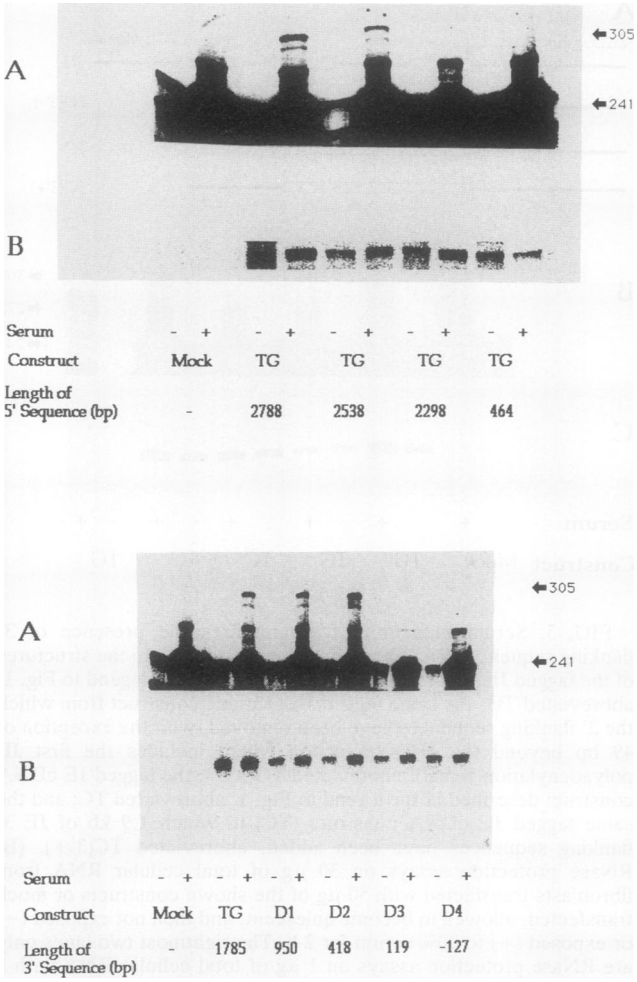


FIG. 4. Serum induction of JE requires both a 5′ genomic element and a 3′ genomic element. Top panel. (A) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of tagged JE genomic constructs (TG) containing progressively greater 5′ flanking sequence deletions or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum for 2 h. The length of 5′ sequence is relative to the JE transcription start site. The construct containing 2,788 bp of 5′ flanking sequences is the one described in the legend to Fig. 1. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. All constructs include the full 1.9 kb of JE 3′ flanking sequences. (B) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel A with an alpha-globin riboprobe. Bottom panel. (A) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of tagged JE genomic constructs containing progressively greater 3′ deletions (TG and D1 through D4) or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum for 2 h. The length of 3′ sequence is relative to the JE stop codon. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. All constructs include the full 2.8 kb of JE 5′ flanking sequences. (B) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel A with an alpha-globin riboprobe.

sequence) to a noninducible JE gene in both orientations also restores serum inducibility (Fig. 5, top panel, compare the stimulated lanes of constructs 4 and 5 with construct 1). Note that the restoration of serum inducibility is to a large degree position independent, i.e., addition of the 240-bp element to construct 1 in Fig. 5 (top panel) effectively moves the 5′ element 1.8 kb closer to the JE start of transcription.

The lower panel of Fig. 5 shows the results of 3′ add back experiments. Readdition of a 317-bp 3′ genomic segment to a noninducible tagged JE gene (including the 5′ flanking sequences) restores serum inducibility to the tagged JE gene (Fig. 5, bottom panel, compare the stimulated lanes of construct 2 with construct 1). Readdition of the proximal 221 bp of the 317-bp 3′ genomic element to a noninducible JE gene yields similar results (Fig. 5, bottom panel, compare the stimulated lanes of constructs 4 and 5 with construct 1). Readdition of an unrelated 328-bp genomic JE fragment (consisting of 5′ flanking sequences not containing the active 240-bp 5′ element) to the same noninducible tagged JE gene shown in Fig. 5 (construct 1, bottom panel) results in no induction of the modified construct upon serum stimulation (data not shown).

Induction of JE in response to PDGF, IL-1, and double-stranded RNA requires the same 5′ and 3′ genomic elements as serum induction of JE. We next established that the two genomic elements required for serum induction of JE are also required for JE induction in response to PDGF, double-stranded RNA, and IL-1. Add back experiments identical in design to those conducted with serum were performed. In Fig. 6, the left hand panels (upper and lower) demonstrate the role of the 5′ and 3′ genomic elements in the response to the A:A, A:B, and B:B isoforms of PDGF. The right-hand panels of Fig. 6 (upper and lower) demonstrate the roles of the 5′ and 3′ genomic elements in the response to double-stranded RNA and IL-1.

The three isoforms of PDGF are not equally effective at inducing JE. Although equal concentrations of the three isoforms of PDGF are added to all groups of transfected cells (30 ng/ml), the B:B isoform of PDGF is a more effective inducer of JE than the A:A isoform (Fig. 6, top left panel, compare the BB-stimulated lanes of constructs TG, 2, and 3 with the AA-stimulated lanes of the same constructs). This differential may reflect the reported fourfold-greater number of PDGF B:B receptors than PDGF A:A receptors on 3T3 cells (36). Since the B:B isoform of PDGF activates the three known PDGF receptors (9, 25), only the B:B isoform is tested to demonstrate that construct 1 is not PDGF inducible (Fig. 6, top left panel).

Roles of position and orientation in the function of the 5′ and 3′ genomic elements. Delineation of the active configuration(s) of 5′ and 3′ genomic elements yields insights into their mechanism(s) of action. To establish constraints on the active positions and orientations of the 5′ and 3′ elements, we started with a truncated tagged JE cDNA construct as the basal reporter (Fig. 7A). The basal, tagged cDNA is not induced in response to serum stimulation (Fig. 7B, third and fourth lanes from the left). We then inserted the 5′ and/or 3′ genomic fragments upstream (position 1) or downstream (position 2) of the reporter cDNA. The 5′ and 3′ fragments were tested singly or in pairs in each of 15 informative configurations. Figure 7B shows a representative example of the induction data. Serum inducibility of the complete set of 15 truncated JE cDNA constructs is summarized in Fig. 8. Similar results were obtained with PDGF (B:B isoform) and IL-1 stimulation of the listed series of constructs (data not shown).

The data summarized in Fig. 8 make several points. Two 5′ elements alone or two 3′ elements alone do not restore serum inducibility to the cDNA (Fig. 8, lines 10 and 11). Hence the 5′ and 3′ JE genomic elements are not equivalent

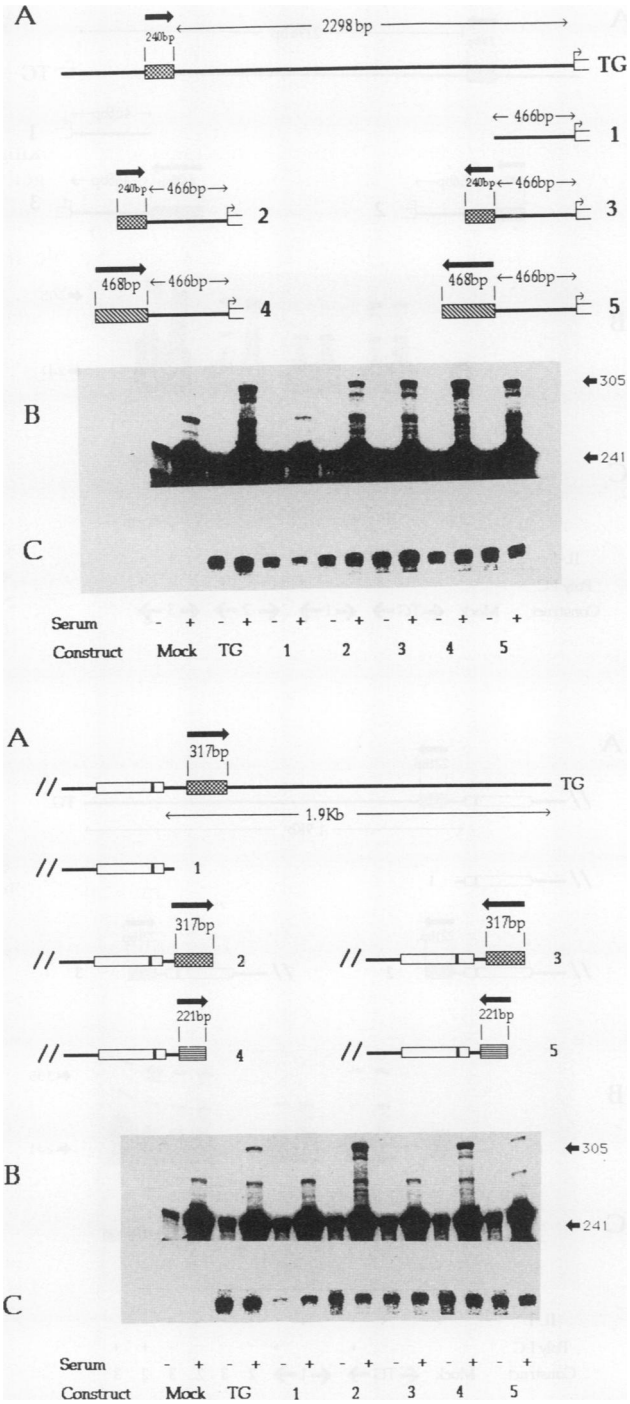


FIG. 5. Readdition of 5′ and 3′ flanking sequences restores serum induction of tagged murine JE. Top panel. (A) Shown in schematic form is the structure of the 5′ end of the tagged JE genomic construct described in the legend to Fig. 1 (TG). A 240-bp segment, suggested to be required for serum induction of JE from the 5′ deletion analysis shown in Fig. 4, is marked by the hatched box. The proximal (3′) end of the 240-bp segment is 2,298 bp upstream of the JE start of transcription. The rightward-pointing bold arrow denotes the orientation of the 240-bp segment in the endogenous JE gene. A tagged genomic construct containing only 466 bp (relative to the JE start of transcription) of 5′ flanking sequences is denoted 1. Constructs 2 and 3 are derived from construct 1 by addition of the highlighted 240-bp segment distal to the 466-bp upstream region in the in vivo (construct 2) and reverse (construct 3) orientations. Constructs 4 and 5 are derived from construct 1 by addition of a 468-bp segment (containing the complete 240-bp segment and a further 228 bp of contiguous 5′ flanking sequences) distal to the 466-bp upstream region in the in vivo (construct 4) and reverse (construct 5) orientations. All constructs include the full 1.9 kb of JE 3′ flanking sequences. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe. Bottom panel. (A) Shown in schematic form is the structure of the 3′ end of the tagged JE genomic construct described in the legend to Fig. 1 (TG). A 317-bp segment, suggested to be required for serum induction of JE from the 3′ deletion analysis shown in Fig. 4, is marked by the hatched box. The proximal (5′) end of the 317-bp segment is 104 bp downstream of the JE stop codon. The rightward-pointing bold arrow denotes the orientation of the 317-bp segment in the endogenous JE gene. A tagged genomic construct containing only 49 bp of 3′ untranslated sequences, relative to the JE stop codon and including the first JE polyadenylation signal, is denoted 1. Constructs 2 and 3 are derived from construct 1 by addition of the highlighted 317-bp segment to the 49-bp 3′ segment in the in vivo (construct 2) and reverse (construct 3) orientations. Constructs 4 and 5 are derived from construct 1 by addition of a 221-bp segment (consisting of the proximal 221 bp of the 317-bp region) to the 49-bp 3′ segment in the in vivo (construct 4) and reverse (construct 5) orientations. All constructs include the full 2.8 kb of JE 5′ flanking sequences. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe.

halves of a single larger genomic element. Although a small 5- to 10-fold induction is always observed with the 5′ element alone (Fig. 7B, fifth and sixth lanes from the left, and Fig. 8, line 2), both elements must be present for the full 30- to 50-fold serum induction to occur (Fig. 7B, fifth and sixth lanes from the right, and Fig. 8, compare lines 2 through 5 with line 6). The occurrence of a small serum induction in the presence of the 5′ element alone (Fig. 8, line 2) probably results from using a JE cDNA in these experiments, which lacks the two large JE introns and is therefore a significantly altered reporter gene, rather than the tagged JE genomic constructs used for the experiments shown in Fig. 2 through 6. These data demonstrate that neither element is functioning in splicing of the JE transcript. The 3′ element displays little if any activity when inserted into position 1 (Fig. 8, line 8). Maximal serum induction occurs with the 5′ and 3′ elements added in their relative in vivo positions and orientations (Fig. 8, line 6). Inversion of the relative positions of the 5′ and 3′ elements results in nearly total loss of serum inducibility (Fig. 7B, first and second lanes from the right, and Fig. 8, compare lines 6 and 8). These latter results argue that at least one element is not acting as a classical transcriptional enhancer in response to a given stimulus. If both elements acted as classical transcriptional enhancers, it would be expected that the relative positions of the two elements could be inverted without significant loss of activity.

Two heterologous reporter genes were tested for serum and PDGF inducibility in the presence of the 5′ and 3′ JE

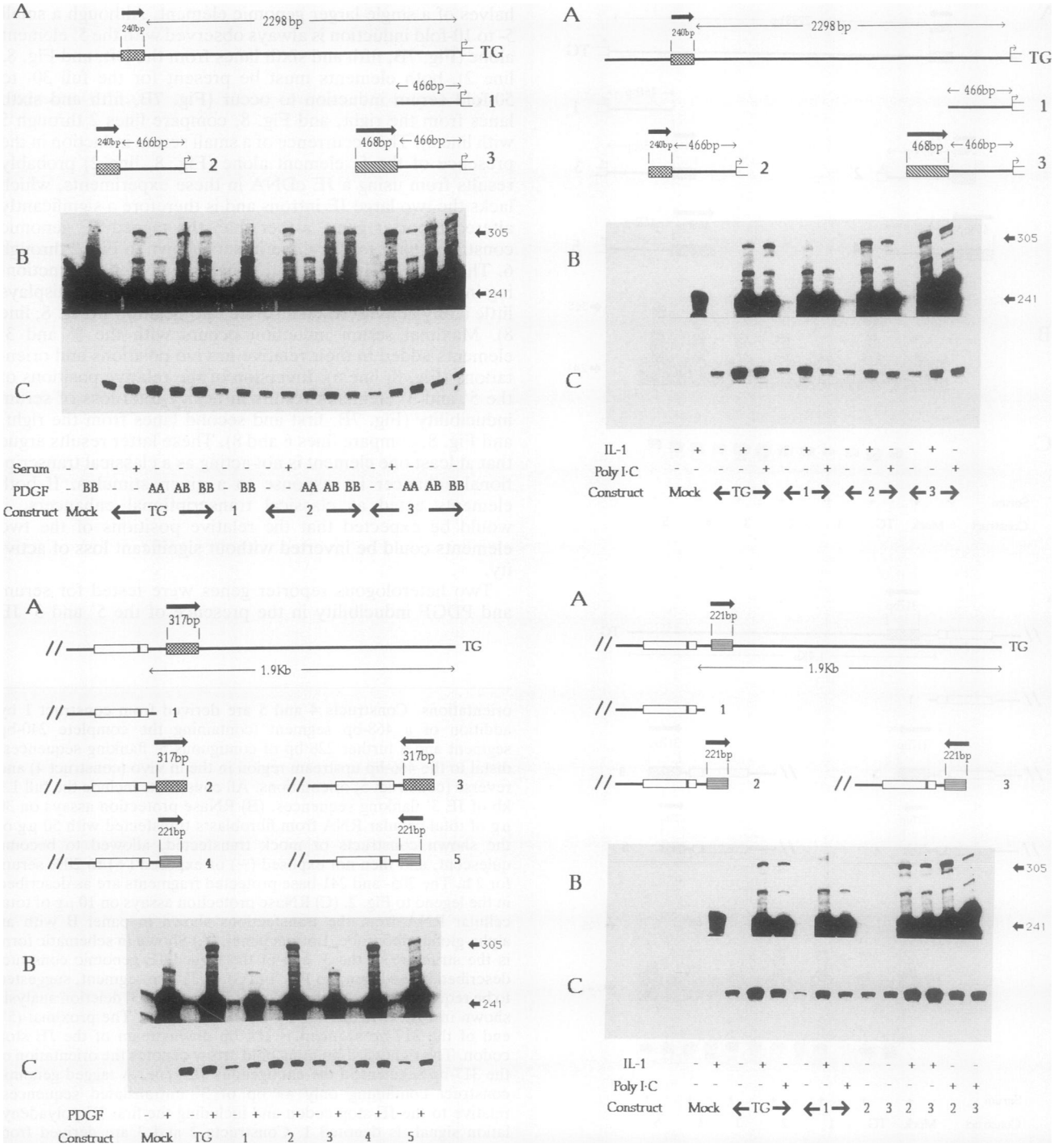


FIG. 6. The 5′ and 3′ serum-responsive genomic elements also mediate JE induction by PDGF, IL-1, and double-stranded RNA. Top left panel. (A) Shown in schematic form is the structure of the 5′ end of the tagged JE genomic construct described in the legend to Fig. 1 (TG). Constructs 1, 2, and 3 correspond to constructs 1, 2, and 4, respectively, in the top panel of Fig. 5. All constructs include the full 1.9 kb of JE 3′ flanking sequences. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum, or the A:A, A:B, or B:B isoforms of PDGF (30 ng/ml) for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe. Top right panel. (A) Shown in schematic form is the structure of the 5′ end of the tagged JE genomic construct described in the legend to Fig. 1 (TG). Constructs 1, 2, and 3 correspond to constructs 1, 2, and 4, respectively, in the top panel of Fig. 5. All constructs include the full 1.9 kb of JE 3′ flanking sequences. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to IL-1 (10 ng/ml) or double-stranded RNA (50 μg/ml) for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe. Bottom left panel. (A) Shown in schematic

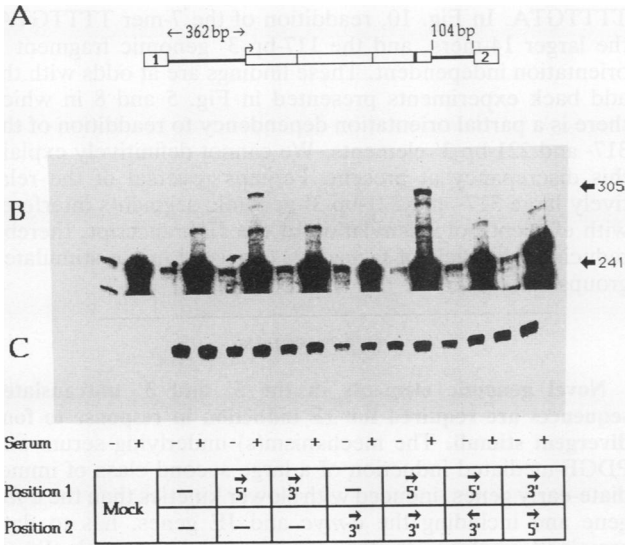


FIG. 7. Roles of position and orientation in the function of the 5′ and 3′ genomic elements. (A) Shown in schematic form is the structure of a truncated tagged JE cDNA construct (TC) consisting of 362 bp of 5′ JE flanking sequences (relative to the start of transcription) and 104 bp of 3′ JE untranslated sequences (relative to the stop codon and including the first JE polyadenylation signal). The cDNA and 33-bp tag are as described in the legend to Fig. 1. DNA elements are added to either position 1, directly upstream of the 362-bp 5′ flanking sequences, or position 2, directly downstream of the 104-bp 3′ untranslated sequences, as shown in the schematic. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. Positions 1 and 2 are as shown in panel A. The designations 5′ and 3′ imply addition of the 240-bp 5′ and 221-bp 3′ JE genomic elements described in the legend to Fig. 5, respectively, to the shown positions. A rightward-facing bold arrow denotes an element added in the in vivo orientation. A leftward-facing bold arrow denotes an element added in the reverse orientation. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe.

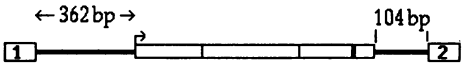
genomic elements. A chloramphenicol acetyltransferase (CAT) gene under control of the thymidine kinase promoter and a *fos*-beta-globin fusion gene under control of a truncated *fos* promoter were employed (52). In neither case was addition of the 5′ and 3′ JE elements to their respective positions sufficient to confer serum or PDGF inducibility onto the heterologous gene (data not shown). These data suggest that there is an additional JE element(s), present within the very proximal 5′ flanking sequences, or within the JE coding sequences, which is also required for serum and PDGF induction of JE to occur.

The 3′ genomic element is duplicated and does not function in polyadenylation of the JE transcript. Given its apparently unique properties we focused on the nature of the 3′ element. The JE gene possesses two polyadenylation signals (43). The second polyadenylation signal occurs 229 bp downstream of the first polyadenylation signal (43). In all preceding figures the noninducible JE construct(s) includes a short segment of 3′ JE untranslated sequences containing the first JE polyadenylation signal. The active 221-bp 3′ genomic element includes the second JE polyadenylation signal. It is possible, therefore, that the function of the 221-bp 3′ element in the preceding experiments is simply to provide a preferred polyadenylation signal for serum and PDGF induction of JE. To test this possibility, the 221-bp 3′ genomic element was bisected into 117- and 104-bp nonoverlapping pieces. The 104-bp distal piece contains the second JE polyadenylation signal. The individual pieces were then added to the 3′ end of a noninducible tagged JE gene, and serum inducibility of the resulting constructs was determined.

Addition of either the 117-bp proximal portion or 104-bp distal portion of the 221-bp 3′ genomic element restores serum inducibility to a noninducible tagged JE gene (Fig. 9, compare the stimulated lanes of constructs TG, 2, 3, and 5 with the stimulated lane of construct 1). Hence the 3′ genomic element is functionally duplicated. Furthermore, the 117-bp segment (which does not include the second polyadenylation signal) is as effective at restoring serum inducibility as the 104-bp segment (Fig. 9, compare the stimulated lanes of constructs 3 and 5). Identical results are obtained with IL-1 and the B:B isoform of PDGF (data not shown). In summary, the 221-bp 3′ genomic element necessary for serum and PDGF induction of JE is functionally duplicated. Furthermore, the experiments suggest that the 3′ JE element does not function in polyadenylation of the JE transcript.

The 7-mer, TTTTGTA, is a JE 3′ element. The results presented in Fig. 9 demonstrate that the 3′ JE genomic element is functionally duplicated. Fully functional elements are present in the 104- and 117-bp 3′ fragments. Given the small sizes of the functionally equivalent 3′ elements, computer comparisons of the fragments for sequence identity were undertaken. An identical 7-mer, TTTTGTA, was found in both 3′ elements. Similar 14-mers with 11 matches of 14, ATGAATTTTGTAAA, are also found in both 3′ elements. Addition of a 14-mer or the 7-mer, in either orientation, to a noninducible tagged JE gene restores the full magnitude of PDGF inducibility (Fig. 10, compare the stimulated lanes of constructs TG, 2, 3, 5, 6, 7, and 8 with the stimulated lane of construct 1). Addition of a 68-bp 3′ JE genomic fragment,

form is the structure of the 3′ end of the tagged JE genomic construct described in the legend to Fig. 1 (TG). Constructs 1 through 5 correspond to constructs 1 through 5, respectively, in the bottom panel of Fig. 5. All constructs include the full 2.8 kb of JE 5′ flanking sequences. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to the B:B isoform of PDGF (30 ng/ml) for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe. Bottom right panel. (A) Shown in schematic form is the structure of the 3′ end of the tagged JE genomic construct described in the legend to Fig. 1 (TG). Constructs 1, 2, and 3 correspond to constructs 1, 4, and 5, respectively, in the bottom panel of Fig. 5. All constructs include the full 2.8 kb of JE 5′ flanking sequences. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to IL-1 (10 ng/ml) or double-stranded RNA (50 μg/ml) for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe.



Position		Fold Induction	
1	2	1	2
0	0	0	0
5′→	0	9.9	6.3
0	5′→	—	0
3′→	0	0	0
0	3′→	0	0
5′→	3′→	49.0	29.1
5′→	3′←	7.3	—
3′→	5′→	8.8	0
3′→	5′←	0	—
5′→	5′→	—	5.5
3′→	3′→	—	0
3′→ 5′→	0	10.0	—
3′← 5′→	0	6.8	—
0	3′→ 5′→	—	43.0
0	3′→ 5′←	—	10.6

FIG. 8. Roles of position and orientation in the function of the 5′ and 3′ genomic elements. Shown in schematic form at the top is the structure of a truncated tagged JE cDNA. The details of this reporter construct are given in the Fig. 7 legend. DNA elements are added singly or together to position 1, directly upstream of the 362-bp 5′ flanking sequences, or to position 2 directly downstream of the 104-bp 3′ untranslated sequences. The designation 5′ refers to the 240-bp 5′ element added to a position. The designation 3′ refers to the 221-bp 3′ element added to a position. 0, no addition at the position. A rightward-pointing bold arrow denotes a fragment inserted in the in vivo orientation; a leftward-pointing bold arrow denotes a fragment inserted in the reverse orientation. Transfected 3T3 cells were exposed to 20% serum for 2 h. For both experiments, control mock-transfected 3T3 cells were exposed to serum for an equivalent time. Inductions of the indicated constructs were displayed and quantitated on a phosphorimager apparatus as outlined in Materials and Methods. The numerical data indicate fold induction (stimulated/quiescent) after subtraction of background counts from the mock-transfected groups. 0, no induction above background; —, a construct not tested in a given experiment.

centered over the active 7-mer, also restores PDGF inducibility to the tagged JE gene (Fig. 10, compare the stimulated lanes of constructs 4 and 1). Identical results are obtained with serum stimulation of the shown constructs (data not shown). Since the full extent of serum and PDGF inducibility of the JE gene is restored by the addition of the 7-mer alone, these data demonstrate that a 3′ JE element is the 7-mer TTTTGTA. In Fig. 10, readdition of the 7-mer TTTTGTA, the larger 14-mers, and the 117-bp 3′ genomic fragment is orientation independent. These findings are at odds with the add back experiments presented in Fig. 5 and 8 in which there is a partial orientation dependency to readdition of the 317- and 221-bp 3′ elements. We cannot definitively explain this discrepancy at present. Perhaps reversal of the relatively large 317- and 221-bp 3′ genomic segments interferes with efficient polyadenylation of the JE transcript, thereby reducing the levels of tagged JE observed in the stimulated groups of cells.

DISCUSSION

Novel genomic elements in the 5′ and 3′ untranslated sequences are required for JE induction in response to four divergent stimuli. The mechanism(s) underlying serum and PDGF-mediated induction of a large second class of immediate-early genes, induced with slower kinetics than the c-*fos* gene and including the c-*myc* and JE genes, has to date remained unclear. In the experiments presented above, using a tagged murine JE genomic construct, we have duplicated the magnitude of and kinetics of serum and PDGF induction of the endogenous JE gene. We have further demonstrated that induction of the murine JE gene in response to serum, PDGF, IL-1, and double-stranded RNA requires two widely separated genomic elements. The elements are present in the 5′ and 3′ untranslated JE sequences and are separated by 4 kb of intervening flanking and coding sequences. We have shown that the 7-nucleotide sequence, TTTTGTA, present at the 3′ end of the JE gene is a 3′ element. Addition of this 7-mer alone to the 3′ end of a noninducible JE gene is sufficient to restore the full extent of serum and PDGF induction of JE.

The most interesting control feature of the JE gene appears to be the active 3′ 7-mer. Computer searches of the sequence data bases reveal a significant overrepresentation of the JE 3′ 7-mer in 3′ untranslated sequences of immediate-early genes. Summarized in Table 1 are 25 immediate-early genes containing the JE 3′ element, TTTTGTA, in their proximal 3′ sequences. In all examples cited, the 7-mer occurs 138 to 1,161 bp downstream of the stop codons of each gene (Table 1). The striking finding is that multiple transcription factors and cytokines belonging to the immediate-early gene class possess the active 7-mer in similar positions within their proximal 3′ untranslated sequences (Table 1). The list includes human, murine, rat, and chicken homologs of various immediate-early genes. In contrast, other serum- and growth factor-inducible genes functioning as cytoplasmic signal transduction proteins or structural proteins do not contain the 7-mer within their proximal 3′ untranslated sequences. As an additional control for the selectivity of occurrence of the 7-mer, multiple "housekeeping" genes were surveyed and found not to contain the 7-mer in their proximal 3′ untranslated sequences (Table 1). The genes listed in the 7-mer absent column include long-enough segments of 3′ untranslated sequences beyond their respective stop codons to allow valid comparisons with the immediate-early genes listed in the present column. We recognize that, if one searches the 3′ untranslated sequences of enough non-immediate-early genes, one would expect eventually to find copies of the active JE 3′ 7-mer within the sequences of some of these genes. This does not mean that the 7-mer is active within these non-immediate-early genes or that it is a nonspecific sequence in immediate-early genes. Rather, experiments presented here demonstrate that inclusion of 3′

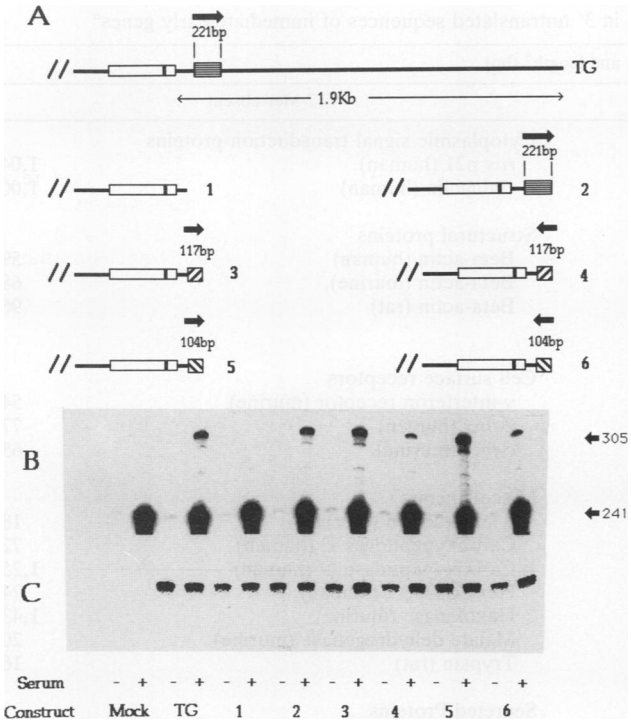


FIG. 9. The 3′ JE genomic element is duplicated and does not function in polyadenylation of the JE transcript. (A) Shown in schematic form is the structure of the 3′ end of the tagged JE genomic construct described in the legend to Fig. 1 (TG). The 221-bp element marked by the striped box corresponds to the 221-bp element described in the bottom panel of Fig. 5 (construct 4). The proximal (5′) end of the 221-bp fragment is 104 bp downstream of the JE stop codon. The righward-pointing bold arrow denotes the orientation of the 221-bp fragment in the endogenous JE gene. Constructs 1 and 2 correspond to constructs 1 and 4, respectively, in the bottom panel of Fig. 5. Constructs 3 and 4 are derived from construct 1 by addition of a 117-bp segment (the proximal portion of the highlighted 221-bp fragment not including the second JE polyadenylation signal) to the 49-bp 3′ segment of construct 1 in the in vivo (construct 3) and reverse (construct 4) orientations. Constructs 5 and 6 are derived from construct 1 by addition of a 104-bp segment (the distal portion of the highlighted 221-bp fragment including the second JE polyadenylation signal) to the 49-bp 3′ segment of construct 1 in the in vivo (construct 5) and reverse (construct 6) orientations. All constructs include the full 2.8 kb of JE 5′ flanking sequences. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to 20% serum for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe.

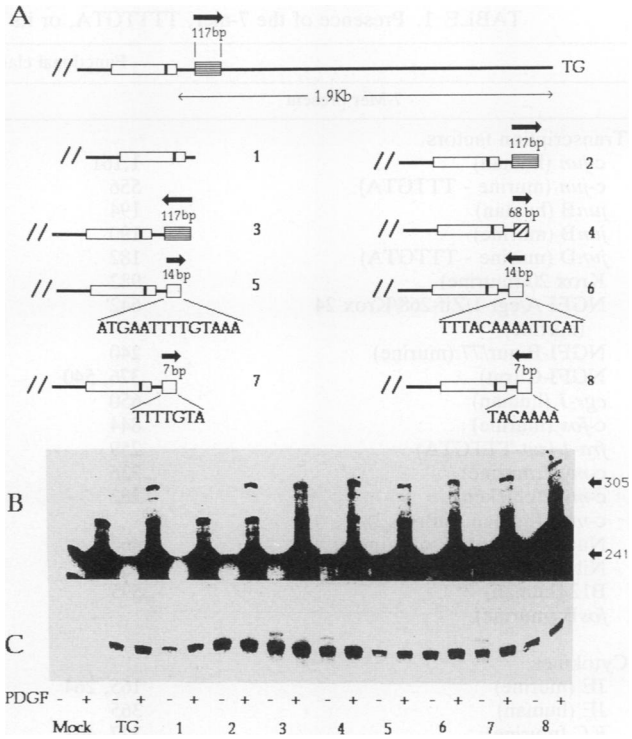


FIG. 10. Addition of the 7-mer TTTTGTA to the 3′ end of a noninducible tagged JE gene restores PDGF inducibility. (A) Shown in schematic form is the structure of the 3′ end of the tagged JE genomic construct described in the legend to Fig. 1 (TG). The 117-bp element highlighted by the striped box corresponds to the 117-bp element described in the legend to Fig. 9 (construct 3). The proximal (5′) end of the 117-bp fragment is 104 bp downstream of the JE stop codon. The rightward-pointing bold arrow denotes the orientation of the 117-bp fragment in the endogenous JE gene. Constructs 1, 2, and 3 correspond to constructs 1, 3, and 4, respectively, in Fig. 9. Construct 4 is derived from construct 1 by addition of a 68-bp JE 3′ flanking segment (centered over the 7-mer TTTTGTA in the proximal JE 3′ flanking sequences) to the 49-bp 3′ segment of construct 1 in the in vivo orientation. Constructs 5 and 6 are derived from construct 1 by addition of the 14-mer, ATGAATTTTGTAAA, to the 49-bp 3′ segment of construct 1 in the in vivo (construct 5) and reverse (construct 6) orientations. Constructs 7 and 8 are derived from construct 1 by addition of the 7-mer, TTTTGTA, to the 49-bp 3′ segment of construct 1 in the in vivo (construct 7) and reverse (construct 8) orientations. The sequences of the 14- and 7-mers shown are those of the coding strand. All constructs include the full 2.8 kb of JE 5′ flanking sequences. (B) RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 50 μg of the shown constructs or mock transfected, allowed to become quiescent, and then not exposed (−) or exposed (+) to the B:B isoform of PDGF (30 ng/ml) for 2 h. The 305- and 241-base protected fragments are as described in the legend to Fig. 2. (C) RNase protection assays on 10 μg of total cellular RNA from the transfections shown in panel B with an alpha-globin riboprobe.

untranslated sequences of the murine JE gene alone (including up to three copies of the active 7-mer) confer no serum or PDGF inducibility onto the JE gene in the absence of the active JE 5′ element (Fig. 4, 5, 6, and 7). Hence, the specificity of serum and PDGF induction of JE, and by extension other immediate-early genes in which the 3′ 7-mer is active, results from the presence of a specific 5′ element(s) in addition to the 3′ 7-mer.

The minimal JE 3′ element conferring inducibility functions equally well in both orientations in mediating serum and PDGF induction of JE (Fig. 10). This orientation independence suggests that the 3′ 7-mer acts at the DNA level. To postulate a primary function for the 3′ element at the RNA level in PDGF and serum induction of JE would be to require that the element, TTTTGTA, and its equally active inverse, TACAAAA, mediate the same process. This is an unlikely possibility given the total dissimilarity of the two sequences at the RNA level. Secondary functions of the 3′ 7-mer at the RNA level are ruled out by experiments presented herein. The 3′ element is required for induction of a tagged JE cDNA reporter vector in response to serum,

TABLE 1. Presence of the 7-mer, TTTTGTA, or its inverse in 3′ untranslated sequences of immediate-early genes[a]

Functional class, gene, and length[b] (bp)			
7-Mer present		7-Mer absent	
Transcription factors		Cytoplasmic signal transduction proteins	
c-*jun* (human)	1,161	*ras* p21 (human)	1,045
c-*jun* (murine - TTTGTA)	556	Calcyclin (human)	1,003
*jun*B (human)	194		
*jun*B (murine)	190	Structural proteins	
*jun*D (murine - TTTGTA)	182	Beta-actin (human)	591
Krox 20 (murine)	982	Beta-actin (murine)	684
NGFI-A/egr-1/Zif-268/Krox 24 (murine)	642	Beta-actin (rat)	966
NGFI-B/nur/77 (murine)	240		
NGFI-C (rat)	376, 540	Cell surface receptors	
egr-1 (human)	650	γ-interferon receptor (murine)	546
c-*fos* (murine)	844	c-*fms* (human)	773
fra-1 (rat-TTTGTA)	259	c-*fms* (murine)	659
c-*myc* (murine)	336		
c-*myc* (chicken)	282	Miscellaneous	
c-*myc* (human - intron 2)	—	Alpha-tubulin (murine)	189
Nuclear factor IL-6 (human)	468	Carboxypeptidase E (human)	725
Nil-2-a (human)	138	Carboxypeptidase N (human)	1,251
B12 (human)	595	Hexokinase 1 (human)	745
*fos*B (murine)	—	Hexokinase (murine)	1,437
		Malate dehydrogenase (murine)	202
Cytokines		Trypsin (rat)	169
JE (murine)	165, 264		
JE (human)	365	Secreted Proteins	
KC (murine)	327, 461	Enkephalin (murine)	302
gro (human)	464	Enkephalin (human)	356
IL-2 (human)	150		
M-CSF 1 (human)	658		

[a] Immediate-early genes found to have the 7-mer, TTTTGTA, present within their proximal 3′ untranslated sequences are listed by functional classes. Since the minimal constraints on the function of the 7-mer are not yet known, listed also are immediate-early genes including the 6-mer, TTTGTA, within their 3′ untranslated sequences; these exceptions are noted. The presence of the active 7-mer within a distal intron is noted and listed as such. Listed in the absent column are a variety of growth factor-inducible (but not necessarily immediate-early) genes, and housekeeping genes, not possessing the 7-mer within their 3′ untranslated sequences. Most sequence information was obtained by accession of GenBank sequences. Sequences for zif/268 (8), *fra-1* (12), nur/77 (26), Krox-24 (30), *jun*B (45), *fos*B (62), human JE (44), KC (37), Nil-2-a (60), NGFI-C (13), B12 (61), and M-CSF (27) were obtained from the original reports.

[b] The numbers in the present column refer to the position of the 7-mer relative to the stop codons of a given gene. The numbers in the absent column refer to the length of 3′ untranslated sequences available for analysis, relative to the stop codons of a given gene.

[c] A number of minimally altered 7-mers are found in the proximal 3′ untranslated sequences of these genes, including TTTTGTG, TTTTGGA, TTTTGTT, TTTTGTC, TTTTATA, TTTTGAA, AACAAAA, and TCCAAAA.

PDGF, and IL-1 (Fig. 7 and 8). These data demonstrate that the 3′ element is not required for correct splicing of the JE transcript. The 7-mer has been disassociated from the two polyadenylation signals present within the JE gene (Fig. 9), suggesting that the 3′ element does not promote polyadenylation of the JE transcripts. Moreover, since the inverse of the 3′ 7-mer, TACAAAA, confers serum and PDGF responsiveness to the JE gene (Fig. 10) the additional possibility that the 3′ element is functioning as a downstream G/T cluster sequence necessary for correct polyadenylation of nascent transcripts to occur is unlikely (4, 5). Finally, both 7-mer sequences are clearly different from the reported RNA destabilizing element ATTTA (51) and from the c-*fos* ARE element (52).

The distal JE 5′ element is position and orientation independent and may therefore function as a regulated transcriptional enhancer. At present the distal JE 5′ element is contained within a 240-bp genomic segment. The 240-bp segment does not contain NF-kB, SRE, or SIF sequences (50, 55, 58). The segment does not contain sequences which could bind RSRF proteins (39). It also does not contain the double-stranded RNA response element described for the interferon gene (17, 18, 31), the IL-1 response element described for the IL-6 gene (1), or a PEA 3 element (59). It appears therefore that the 240-bp 5′ JE element includes a new sequence(s) responsive to at least four divergent stimuli. These data do not address whether one discrete 5′ subsequence is the target element for the four stimuli or whether there are (up to) four separate response elements within the 240-bp genomic segment. Preliminary dissections of the 240-bp 5′ segment demonstrate that the PDGF-responsive element lies within a 71-bp subfragment (data not shown).

Role of the 7-mer, TTTTGTA, in serum and PDGF induction of transcription factors and cytokines of the immediate-early gene class. We propose that the novel 3′ 7-nucleotide element, TTTTGTA, is a unifying feature in the control of serum and PDGF induction of transcription factors and cytokines belonging to the immediate-early gene class. Consistent with this proposal is the selective occurrence of the 7-mer (or the 6-mer TTTGTA) in 3′ untranslated sequences of 25 transcription factors and cytokines of the immediate-early class and the dramatic function of the 7-mer in mediating serum and PDGF induction of murine JE (Fig. 10). For convenience we will hereafter refer to the 3′ 7-mer as the immediate response box (IRB).

Catalogued among the immediate-early genes possessing an IRB are the proto-oncogenes c-*myc* and c-*fos* (Table 1).

Although one PDGF-responsive 5′ genomic element has been shown to mediate a small induction of c-*myc*-CAT fusion constructs in response to PDGF (46), *cis*-acting elements mediating the full induction of c-*myc* in response to PDGF have never been defined (for a review, see reference 53). Serum and PDGF inducibility of c-*myc* may require, like JE, two widely separated elements including the 7-mer, TACAAAA (the inverse of the JE IRB), located 336 bp downstream of the murine c-*myc* stop codon (Table 1).

The presence of an IRB in the 3′ untranslated sequences of murine c-*fos* (844 bp downstream of the stop codon [Table 1]) raises a different issue. A number of independent studies have shown that the 5′ c-*fos* SRE alone is sufficient to confer serum inducibility onto a heterologous reporter gene such as CAT (16, 19, 54). It is possible that the 3′ IRB in c-*fos* is a vestigial control element with no essential function. Alternatively, the c-*fos* 3′ IRB may enhance the action of the 5′ SRE without being strictly essential for function. Experiments are now in progress in our laboratory to determine whether the IRB, TTTTGTA, or its inverse plays a role in serum and PDGF induction of the c-*fos* and c-*myc* genes.

ACKNOWLEDGMENTS

We thank John Alberta for invaluable late-night assistance with computer sequence analysis; Julia Alberta, Alan D'Andrea, Briggs Morrison, and Barrett Rollins for helpful discussions; and Alan D'Andrea, Rolf G. Freter, Michael Greenberg, Barrett Rollins, and Thomas Roberts for critical reading of this manuscript. We also thank M. C. Cardoso for assistance with sequencing of the 3′ flanking sequences of the murine JE gene, and László Kondor for assistance with preparation of the figures.

R.R.F. is a recipient of a fellowship from the National Institutes of Health (5 F32 GM14091-02). This research was supported by a grant from the National Cancer Institute (CA22042) to C.D.S.

REFERENCES

1. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9**:1897–1906.
2. **Anisowicz, A., L. Bardwell, and R. Sager.** 1987. Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. Proc. Natl. Acad. Sci. USA **84**:7188–7192.
3. **Berkowitz, L. A., K. T. Riabowol, and M. Z. Gilman.** 1989. Multiple sequence elements of a single functional class are required for cyclic AMP responsiveness of the mouse c-*fos* promoter. Mol. Cell. Biol. **9**:4272–4281.
4. **Birnstiel, M. L., M. Busslinger, and K. Strub.** 1985. Transcription termination and 3′ processing: the end is in site! Cell **41**:349–359.
5. **Brown, P. H., L. S. Tiley, and B. R. Cullen.** 1991. Effect of RNA secondary structure on polyadenylation site selection. Genes Dev. **5**:1277–1284.
6. **Chirgwin, J. M., A. E. Przybyla, R. J. Macdonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
7. **Christy, B., and D. Nathans.** 1989. Functional serum response elements upstream of the growth factor-inducible gene *zif268*. Mol. Cell. Biol. **9**:4889–4895.
8. **Christy, B. A., L. F. Lau, and D. Nathans.** 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. USA **85**:7857–7861.
9. **Claesson-Welsh, L., A. Eriksson, B. Westermark, and C. H. Heldin.** 1989. cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. Proc. Natl. Acad. Sci. USA **86**:4917–4921.
10. **Cochran, B. H., A. C. Reffel, and C. D. Stiles.** 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell **33**:939–947.
11. **Cochran, B. H., J. Zullo, I. M. Verma, and C. D. Stiles.** 1984. Expression of the c-fos gene and of a fos-related gene is stimulated by platelet-derived growth factor. Science **226**:1080–1082.
12. **Cohen, D. R., and T. Curran.** 1988. *fra-1*: a serum-inducible, cellular immediate-early gene that encodes a *fos*-related antigen. Mol. Cell. Biol. **8**:2063–2069.
13. **Crosby, S. D., J. J. Puetz, K. S. Simburger, T. J. Fahrner, and J. Milbrandt.** 1991. The early response gene NGFI-C encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding family. Mol. Cell. Biol. **11**:3835–3841.
14. **DeBelle, I., P. R. Walker, I. C. P. Smith, and M. Sikorska.** 1991. Identification of a multiprotein complex interacting with the c-*fos* serum response element. Mol. Cell. Biol. **11**:2752–2759.
15. **Fisch, T. M., R. Prywes, M. C. Simon, and R. G. Roeder.** 1989. Multiple sequence elements in the c-fos promoter mediate induction by cAMP. Genes Dev. **3**:198–211.
16. **Gilman, M. Z.** 1988. The c-fos serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. Genes Dev. **2**:394–402.
17. **Goodbourn, S., H. Burstein, and T. Maniatis.** 1986. The human β-interferon gene enhancer is under negative control. Cell **45**:601–610.
18. **Goodbourn, S., K. Zinn, and T. Maniatis.** 1985. Human β-interferon gene expression is regulated by an inducible enhancer element. Cell **41**:509–520.
19. **Graham, R., and M. Gilman.** 1991. Distinct protein targets for signals acting at the c-fos serum response element. Science **251**:189–192.
20. **Greenberg, M. E., Z. Siegfried, and E. B. Ziff.** 1987. Mutation of the c-*fos* gene dyad symmetry element inhibits serum inducibility of transcription in vivo and the nuclear regulatory factor binding in vitro. Mol. Cell. Biol. **7**:1217–1225.
21. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311**:433–437.
22. **Hall, D. J., C. Brownlee, and C. D. Stiles.** 1989. Interleukin-1 is a potent regulator of JE and KC gene expression in quiescent Balb/c fibroblasts. J. Cell. Physiol. **141**:154–159.
23. **Hall, D. J., S. D. Jones, D. R. Kaplan, M. Whitman, B. J. Rollins, and C. D. Stiles.** 1989. Evidence for a novel signal transduction pathway activated by platelet-derived growth factor and by double-stranded RNA. Mol. Cell. Biol. **9**:1705–1713.
24. **Hall, D. J., and C. D. Stiles.** 1987. Platelet-derived growth factor-inducible genes respond differentially to at least two distinct intracellular second messengers. J. Biol. Chem. **262**:15302–15308.
25. **Hart, C. E., J. W. Forstrom, J. D. Kelly, R. A. Seifert, R. A. Smith, R. Ross, J. J. Murray, and D. F. Bowen-Pope.** 1988. Two classes of PDGF receptor recognize different isoforms of PDGF. Science **240**:1529–1531.
26. **Hazel, T. G., D. Nathans, and L. F. Lau.** 1988. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. Proc. Natl. Acad. Sci. USA **85**:8444–8448.
27. **Kawasaki, E. S., M. B. Ladner, A. M. Wang, J. VanArsdell, M. K. Warren, M. Y. Coyne, V. L. Schweickart, M. Lee, K. J. Wilson, A. Boosman, E. R. Stanley, P. Ralph, and D. F. Mark.** 1985. Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). Science **230**:291–296.
28. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
29. **Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein. Nature (London) **312**:711–716.
30. **Lemaire, P., O. Revelant, R. Bravo, and P. Charnay.** 1988. Two

mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc. Natl. Acad. Sci. USA **85**:4691–4695.
31. **Lenardo, M. J., C. Fan, T. Maniatis, and D. Baltimore.** 1989. The involvement of NF-kB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. Cell **57**:287–294.
32. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage Sp6 promoter. Nucleic Acids Res. **12**:7035–7056.
33. **Misra, R. P., V. M. Rivera, J. M. Wang, P. Fan, and M. E. Greenberg.** 1991. The serum response factor is extensively modified by phosphorylation following its synthesis in serum-stimulated fibroblasts. Mol. Cell. Biol. **11**:4545–4554.
34. **Muller, R., R. Bravo, J. Burckhardt, and T. Curran.** 1984. Induction of c-fos gene and protein by growth factors precedes activation of c-myc. Nature (London) **312**:716–720.
35. **Norman, C., M. Runswick, R. Pollock, and R. Treisman.** 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell **55**:989–1003.
36. **Olashaw, N. E., W. Kusmik, T. O. Daniel, and W. J. Pledger.** 1991. Biochemical and functional discrimination of platelet-derived growth factor α and β receptors in Balb/c-3T3 cells. J. Biol. Chem. **266**:10234–10240.
37. **Oquendo, P., J. Alberta, D. Wen, J. L. Graycar, R. Derynck, and C. D. Stiles.** 1989. The platelet-derived growth factor-inducible KC gene encodes a secretory protein related to platelet a-granule proteins. J. Biol. Chem. **264**:4133–4137.
38. **Pledger, W. J., C. D. Stiles, H. N. Antoniades, and C. D. Scher.** 1977. Induction of DNA synthesis in Balb/c-3T3 cells by serum components: reevaluation of the commitment process. Proc. Natl. Acad. Sci. USA **74**:4481–4485.
39. **Pollock, R., and R. Treisman.** 1991. Human SRF-related proteins: DNA-binding properties and potential regulatory targets. Genes Dev. **5**:2327–2341.
40. **Prywes, R., and R. G. Roeder.** 1986. Inducible binding of a factor to the c-fos enhancer. Cell **47**:777–784.
41. **Prywes, R., and R. G. Roeder.** 1987. Purification of the *c-fos* enhancer-binding protein. Mol. Cell. Biol. **7**:3482–3489.
42. **Qureshi, S. A., X. Cao, V. P. Sukhatme, and D. A. Foster.** 1991. v-src activates mitogen-responsive transcription factor egr-1 via serum response elements. J. Biol. Chem. **266**:10802–10806.
43. **Rollins, B. J., E. D. Morrison, and C. D. Stiles.** 1988. Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. Proc. Natl. Acad. Sci. USA **85**:3738–3742.
44. **Rollins, B. J., P. Stier, T. Ernst, and G. G. Wong.** 1989. The human homolog of the JE gene encodes a monocyte secretory protein. Mol. Cell. Biol. **9**:4687–4695.
45. **Ryder, K., L. F. Lau, and D. Nathans.** 1988. A gene activated by growth factors is related to the oncogene v-jun. Proc. Natl. Acad. Sci. USA **85**:1487–1491.
46. **Sacca, R., and B. H. Cochran.** 1990. Identification of a PDGF-responsive element in the murine c-myc gene. Oncogene **5**:1499–1505.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
49. **Sassone-Corsi, P., J. Visvader, L. Ferland, P. L. Mellon, and I. M. Verma.** 1988. Induction of proto-oncogene fos transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. Genes Dev. **2**:1529–1538.
50. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**:705–716.
51. **Shaw, G., and R. Kamen.** 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46**:659–667.
52. **Shyu, A., M. E. Greenberg, and J. G. Belasco.** 1989. The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes Dev. **3**:60–72.
53. **Spencer, C. A., and M. Groudine.** 1991. Control of c-myc regulation in normal and neoplastic cells. Adv. Cancer Res. **56**:1–48.
54. **Treisman, R.** 1985. Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5′ element and c-fos 3′ sequences. Cell **42**:889–902.
55. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcriptional response of the c-fos gene to serum factors. Cell **46**:567–574.
56. **Treisman, R.** 1987. Identification and purification of a polypeptide that binds to the c-fos serum response element. EMBO J. **6**:2711–2717.
57. **Treisman, R.** 1990. The SRE: a growth factor responsive transcriptional regulator. Semin. Cancer Biol. **1**:47–58.
58. **Wagner, B. J., T. E. Hayes, C. J. Hoban, and B. H. Cochran.** 1990. The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. EMBO J. **9**:4477–4484.
59. **Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin.** 1990. The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature (London) **346**:191–193.
60. **Williams, T. M., D. Moolten, J. Burlein, J. Romano, R. Bhaerman, A. Godillot, M. Mellon, F. J. Rauscher, and J. A. Kant.** 1991. Identification of a zinc finger protein that inhibits IL-2 gene expression. Science **254**:1791–1794.
61. **Wolf, F. W., R. M. Marks, V. Sarma, M. G. Byers, R. W. Katz, T. B. Shows, and V. M. Dixit.** 1992. Characterization of a novel tumor necrosis factor-α-induced endothelial primary response gene. J. Biol. Chem. **267**:1317–1326.
62. **Zerial, M., L. Toschi, R. Ryseck, M. Schuermann, R. Muller, and R. Bravo.** 1989. The products of a novel growth factor activated gene, fos B, interacts with JUN proteins enhancing their DNA binding activity. EMBO J. **8**:805–813.
63. **Zinn, K., D. DiMaio, and T. Maniatis.** 1983. Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell **34**:865–879.
64. **Zullo, J. N., B. H. Cochran, A. S. Huang, and C. D. Stiles.** 1985. Platelet-derived growth factor and double-stranded ribonucleic acids stimulate expression of the same genes in 3T3 cells. Cell **43**:793–800.