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Activation of the Inducible Orphan Receptor Gene *nur77* by Serum Growth Factors: Dissociation of Immediate-Early and Delayed-Early Responses

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We have characterized the genetic elements that mediate the transcriptional activation of *nur77*, a growth factor-inducible gene encoding a member of the steroid/thyroid hormone receptor superfamily. Although initially identified as a serum-inducible immediate-early gene with expression kinetics similar to those of c-*fos*, we found that transcriptional activation of *nur77* by serum growth factors in fibroblasts is in fact composed of two components: an immediate-early component, which can occur in the absence of de novo protein synthesis, and a delayed-early component, which is dependent on de novo protein synthesis. The expression of *nur77* following serum stimulation reflects the superimposition of immediate-early and delayed-early expression. Immediate-early and delayed-early expression can be dissociated from one another by deletion or base substitution mutations of the *nur77* promoter. Immediate-early expression of *nur77* is mediated primarily by sequences located between nucleotides −86 and −126 upstream of the transcription start site. This region includes a sequence that resembles but differs from the CArG element found in other serum-inducible promoters. Upstream of the CArG-like element is a potential binding site for a transcription factor of the Ets family; the presence of this site is required for significant transcriptional induction. Delayed-early expression of *nur77* is mediated by multiple AP-1-like and GC-rich elements, which can interact with products of immediate-early genes such as Fos/Jun and Zif268, respectively. Furthermore, we show that Zif268 can activate transcription of the *nur77* promoter, suggesting that it may play a role in the delayed-early expression of *nur77*.

A variety of extracellular signalling agents, including growth and differentiation factors, act at least in part through the activation of specific genes. For example, when quiescent mouse 3T3 fibroblasts are stimulated to reenter the cell cycle by serum or growth factors, such as platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF), a set of immediate-early genes is rapidly induced without requiring de novo protein synthesis (3, 16, 26). Many immediate-early genes encode transcription factors that are thought to propagate the cellular responses to the stimulating growth factors by activating other genes needed for growth (26). Thus, a group of delayed-early genes, whose expression occurs after that of the immediate-early genes and prior to DNA synthesis, are thought to be activated by transcription factors encoded by immediate-early genes (22).

The mechanisms that mediate rapid gene activation by growth factors have been a subject of much interest. Among immediate-early genes activated by serum growth factors, the most extensively studied with regard to transcriptional regulation is the proto-oncogene c-*fos*. Central to the activation of c-*fos* is a short promoter sequence of some 20 bp known as the serum response element (SRE), which interacts with a complex of proteins (36, 44). The SRE contains at its center core the CArG box, defined by the consensus sequence $CC(A/T)_6GG$, flanked by palindromic arms. The CArG box binds to the serum response factor (SRF), and the interaction of SRF with the CArG box is essential for the transcriptional activation of c-*fos* (36, 44). Within the SRE, the 5′ arm flanking the CArG box interacts with a protein named $p62^{TCF}$, which binds the SRE only in the presence of SRF, forming a ternary complex important for transcription activation by serum growth factors (17, 41). The identity of $p62^{TCF}$ might be Elk-1 (18, 28) or SAP-1 (9), two related proteins of the Ets/GA-binding protein family of transcription factors. Recent experiments have shown that the binding of SRF to the CArG box recruits Elk-1 to the SRE (19, 45); however, the distance between the Elk/SAP and SRF-binding sites or their relative orientation does not appear critical (45).

Several other immediate-early genes also contain CArG boxes and might be regulated through an SRF-mediated mechanism. Among these genes, *zif268* (*egr-1*) (6), *krox20* (4), and β-actin (21) have Ets-like DNA-binding motifs in the vicinity of the CArG boxes, suggesting that SRF might also recruit an Elk/SAP-like protein to their promoters. However, other CArG box-containing immediate-early genes, including *cyr61* (23), the *Xenopus laevis* type 5 actin (30), and *SRF* (45), do not appear to have associated Ets-like motifs. Still another class of immediate-early genes, including c-*jun* (13), *junB* (11), and *JE* (12) do not have CArG boxes in their promoters and might be regulated through different mechanisms.

To understand the diversity of regulatory mechanisms that contribute to the coordinate activation of a large number of immediate-early genes by growth factors, we have studied the transcriptional regulation of *nur77* (15). *nur77* (also known as NGFI-B and N10 [29, 38]) encodes a member of the steroid/thyroid hormone receptor superfamily. Nur77 can act as a potent transcription activator and may function in mediating the cellular responses to growth factors by regulating the expression of specific genes (10, 49).

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Although *nur77* has expression kinetics following serum stimulation in fibroblasts that are similar to those of c-*fos* (24, 25), we found that its expression is actually composed of two components: an immediate-early component that does not require de novo protein synthesis and a delayed-early component that does. The sequence elements that mediate the immediate-early and delayed-early responses of *nur77* are readily separable from one another. Immediate-early transcription of *nur77* is regulated by sequences that are similar to but distinct from those that regulate c-*fos* expression. We also present evidence that an immediate-early gene product, Zif268, might play a role in regulating the delayed-early expression of *nur77*.

MATERIALS AND METHODS

Cell culture and transient transfections. NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (DME-10) at 37°C under 10% CO_2. Cells were washed twice with DME-0 16 to 24 h after transfection, refed with DME-10, and grown to confluence. The culture medium was replaced with DME-0.5 for another 2 days to bring cells to quiescence. Serum stimulation was carried out by changing the medium to DME-20. When indicated, cycloheximide, FGF, PDGF, and TPA (12-*O*-tetradecanoylphorbol-13-acetate) were added to final concentrations of 10 μg/ml and 180, 15, and 100 ng/ml, respectively.

Transfections were carried out as described previously (48) with modifications. Just prior to each transfection, a 1.5-ml suspension containing 125 mM $CaCl_2$, 36 μg of the test plasmid, and 24 μg of the pSV2*neo* reference plasmid (42) in 1× HBS {25 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid)], 140 mM NaCl, 0.75 mM Na_2HPO_4, pH 7.15} was allowed to form a precipitate (2). These amounts were tripled for the experiment shown in Fig. 5. Cells were plated at 2×10^6 cells per 10-cm dish 16 to 24 h before transfection. For each transfection, two plates of cells were trypsinized, pooled, gently pelleted by centrifugation, and resuspended in the $CaCl_2$-DNA-HBS mixture. Following a 15-min incubation with occasional swirling to avoid clumping, the resulting mixture was split into three plates (10 cm) containing DME-10 supplemented with 6.25 mM $CaCl_2$ and 0.05× HBS to maintain the precipitate. All media for transfected cells contained 50 U of penicillin and 50 μg of streptomycin sulfate per ml.

Construction of *nur77* minigenes. We previously constructed a *nur77* minigene, called 1148/77TR, which contains 1,148 bp of *nur77* genomic sequence upstream of the transcription start site linked to the *nur77* cDNA, followed by a *nur77* genomic DNA fragment that includes the polyadenylation signal and sequences further downstream (50). An 11-bp *Bam*HI linker was inserted between nucleotides (nt) +122 and +123 of the cDNA, relative to the major transcription start site. This minigene was modified by inserting a 91-bp fragment of irrelevant sequence into the 11-bp linker, creating the marked minigene -1148_M (M, marked). The larger insert makes the minigene-derived transcript more easily distinguishable from the endogenous transcript. A series of large 5' deletions of the *nur77* promoter spanning nt −1148 to −278 was created by *Bal* 31 exonuclease digestion of 1148/77TR. One of these had a 5' deletion endpoint at nt −278; it was placed in the marked background and named -278_M. All *nur77* marked minigenes, including those with 5' and 3' deletions as well as linker-scanning mutants (see below), are identical except for the differences noted in the *nur77* promoter. Direct sequence analyses were carried out to confirm the accuracy of each construct.

TABLE 1. 5' primers[a]

Mutant	Primer
-246_M	5'TGTCTCTAGAG-ACAATCCGCGCTCCCTGCGT
-226_M	5'TGTCTCTAGAG-TGGAACCCCGCGTGCGTCAC
-206_M	5'TGTCTCTAGAG-GCGCGCAGACATTCCAGGCC
-186_M	5'TGTCTCTAGAG-CCCCCTCTTCGCCCCGCCCC
-166_M	5'TGTCTCTAGAG-CTCGGGCTCCCCGTCCCCGC
-146_M	5'TGTCTCTAGAG-GCCTCCTCCTGGCCGCCTCC
-126_M	5'TGTCTCTAGAG-CGCCGGAACCGCGCCGCCCC
-116_M	5'ACTCTAGAGTA-GCGCCGCCCCCCGCGCCCTT
-106_M	5'TGTCTCTAGAG-CCGCGCCCTTGTATGGCCAA
-94_M	5'ACACTAGTGTA-ATGGCCAAAGCTCGCCGGGC
-86_M	5'TGTCTCTAGAG-AGCTCGCCGGGCCGTGTGCG
-74_M	5'ACTCTAGAGTA-CGTGTGCGTCAGTGGCGCCC
-64_M	5'TGTCTCTAGAG-AGTGGCGCCCCCGCCCCTCT
-52_M	5'TGTCTCTAGAG-GCCCCTCTCCATGCGTCACG
-33_M	5'TGTCTCTAGAG-GGAGCGCTTAAGATGTGGGT

[a] The hyphens in the primers listed separate the linker from *nur77* sequences.

5' and 3' deletions. A series of finer 5' deletions were derived from the -278_M minigene by a polymerase chain reaction (PCR) procedure (2). The 5' primers (listed in Table 1), which defined the deletion endpoints, were used with the 3' primer 240*Nco* (CCAGGTCCATGGTAGGCTTG), which established a common 3' endpoint at the *Nco*I site (see Fig. 1). The resulting PCR products were digested with either *Xba*I or *Spe*I (depending on the linker sequence of the PCR primer) and *Nco*I and were used to replace the corresponding promoter fragment in -278_M. The numeric designations of each mutant in Table 1 refer to the 5' deletion endpoints defined by the primers listed therein.

To generate linker-scanning mutants and heterologous promoter fusion constructs, we created a series of 3' deletion mutants containing *nur77* promoter sequences extending from a common 5' endpoint at nt −278 to 3' endpoints at nt −174, −154, −126, −114, −106, −94, −86, −72, −60, and −41 by using a PCR protocol (2). The minigene construct -278_M was used as the template for PCR, and the resulting PCR products were digested with *Hin*dIII and either *Spe*I or *Xba*I, depending on the linker sequence. The 5' endpoint was defined by the primer pGEM2d3T (TAGGGAGACCG GAAGCTTGG). The numeric designation of each mutant listed in Table 2 refers to the nucleotide of the 3' endpoints defined by the primers also listed in Table 2.

Linker-scanning mutants. Linker-scanning mutants in the 278-bp promoter background (LS series) were generated by insertion of the appropriate 3' deletion fragment upstream of

TABLE 2. Primers to generate linker-scanning mutants

Deletion	Primer
−174B	5'TGTCTCTAGAG-GCGAAGAGGGGGGGCCTGGA
−154B	5'TGTCTCTAGAG-GGGGAGCCCGAGGGGGCGGG
−126B	5'TATCTCTAGAG-GGAGGCGGCCAGGAGGAGGC
−114B	5'TGTCACTAGTG-GCGGTTCCGGCGGGAGGCGG
−106B	5'ACACTAGTGTA-GGGGCGGCGCGGTTCCGGCG
−94B	5'TGTCACTAGTG-ACAAGGGCGCGGGGGGCGGC
−86B	5'ACACTAGTGTA-TTGGCCATACAAGGGCGCGG
−72B	5'TGTCTCTAGAG-CGGCCCGGCGAGCTTTGGCC
−60B	5'TGTCTCTAGAG-CACTGACGCACACGGCCCGG
−41B	5'TGTCTCTAGAG-TGGAGAGGGGCGGGGGCGCC

the corresponding 5′ deletion, preserving wild-type spacing. Numeric designations refer to the nucleotide replaced by the linker. Linker-scanning mutants in the 126-bp promoter context (LSP series) were generated by PCR with the -126_M primer and 240*Nco* by using the corresponding LS linker-scanning mutants as templates. For LSP 114/106, a special top-strand primer (−126TSpe [5′ TGTCTCTAGAG-CGCC GGAACCGCCACTAGAG]) was used.

Heterologous promoter fusions. Portions of the *nur77* promoter were placed upstream of the herpes simplex virus thymidine kinase (tk) promoter linked to the chloramphenicol acetyltransferase (CAT) gene (see Fig. 6 to 9). These constructs were made by modifying the 17MX2tkCAT plasmid (47; a gift from P. Chambon), which contains two copies of the Gal4 transcription factor-binding site upstream of the tk promoter linked to the CAT gene. *nur77* promoter fragments used were generated by PCR, resulting in DNA fragments including the nucleotide indicated flanked by *Hin*dIII and *Xba*I sites. These fragments were cloned into 17MX2tkCAT digested with *Hin*dIII and *Xba*I, which removed the Gal4-binding sites. All fragments generated for promoter-proximal fusion constructs (see Fig. 6, 8, and 9) have *Xba*I ends and were inserted into the *Xba*I site of tkCAT, which has the Gal4-binding sites removed from 17MX2tkCAT. The 106-82 tkCAT and c-*fos* SRE tkCAT fusions (see Fig. 8) were made by utilizing the following oligonucleotides with *Xba*I site ends: 106-82**T** (5′ CTAGCC GCGCCCTTGTATGGCCAAAGCT) with 106-82**B** (5′ CTA GAGCTTTGGCCATACAAGGGCGCGG) and SREXBAT (5′ CTAGGGATGTCCATATTAGGACATCTGC) with SR EXBAB (5′ CTAGGCAGATGTCCTAATATGGACATCC).

S1 nuclease protection assay. Total RNA was isolated as described elsewhere (5) and analyzed by an S1 nuclease protection assay (39, 50). The *nur77* probe, 5′ end labeled at the *Nco*I site in -278_M, contains sequences extending to the *Hin*dIII site at nt −278. This 625-nt probe protects 340 nt of correctly initiated transcripts derived from the minigene and 116 nt of the endogenous transcript. The *neo* probe, 5′ end labeled at the *Bgl*II site in pSV2*neo*, contains sequences extending to the *Acc*I site at nt −461. This 847-nt probe protects 386 nt of correctly initiated *neo* transcript. The tkCAT probe, 5′ end labeled at a *Bsu*36I site engineered in the CAT gene 14 bp upstream of the *Eco*RI site, contains tk sequences extending to nt −105. This probe protects 307 nt of correctly initiated tkCAT transcript.

Hybridization for S1 nuclease protection assays was performed with a volume of less than 100 μl containing 400 mM NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4], 2 mM EDTA, and 80% formamide with 20 μg of total cellular RNA and approximately 20 ng of probe at 2×10^5 cpm. Following a 15-min incubation at 85 to 95°C to denature the double-stranded probes, samples were transferred to either 55°C for *nur77* protection assays or 51°C for *neo* or tkCAT protection assays and incubated for 16 to 24 h. Samples were then digested with 300 μl of a buffer containing 400 U S1 nuclease (Boehringer Mannheim), 0.3 M NaCl, 66 mM sodium acetate (pH 4.5), and 4 mM $ZnSO_4$ for 1 h at 37°C. The reaction was terminated with the addition of 80 μl of 4 M ammonium acetate–20 mM EDTA (pH 8.0) and 5 μg of yeast tRNA. Reaction products were phenol extracted, ethanol precipitated, and analyzed on 8.3 M urea–4% polyacrylamide gels, visualized by autoradiography, and quantified by using the Betagen Betascope 603. The levels of activities of the transfected test genes were normalized to *neo* expression for transfection efficiencies and expressed as a percentage of a designated construct. Samples with and without cycloheximide treatment were quantified separately.

Generation of Zif268-specific antisera. The pET vector system (43) was used to express Zif268 (7) in *Escherichia coli*. The *zif268* cDNA was cloned into pET8c by using *Nco*I and *Bgl*II. Plasmids were transformed in *E. coli* BL21 (DE3) containing the pLysS plasmid (43). Expression of foreign proteins was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 1 to 2 h at 37°C. Total cell lysate was separated on sodium dodecyl sulfate–10% polyacrylamide gels, and the Zif268 protein was eluted from the appropriate gel slice and used as an antigen to immunize New Zealand White rabbits by conventional protocols (14). The specificity of the antiserum was demonstrated by its ability to immunoprecipitate Zif268 translated in vitro but not the closely related protein Krox20 (1) (data not shown).

Gel shift analysis. Whole-cell extracts used for DNA binding were prepared as described elsewhere (34). Binding assays were carried out as described elsewhere (7). The Zif268-binding site was formed by annealing two oligonucleotides, 5′-GATCCTATGCGGGGGCGTATG and 5′-GTCC ATACGCCCCCGCATAG. When indicated, an antiserum was added to binding-reaction mixtures and allowed to incubate for an additional 20 min prior to gel electrophoresis.

RESULTS

Inducible expression of *nur77* minigenes in fibroblasts. *nur77* was identified as an immediate-early gene inducible by serum growth factors in mouse 3T3 fibroblasts. The kinetics of its induction are similar to those of c-*fos* (24). Upon serum stimulation, transcription of *nur77* is detectable within 5 min, reaches a peak level by 10 to 20 min, and is then repressed by 1 to 2 h (25, 38). Stimulation of cells in the presence of a protein synthesis inhibitor prolongs *nur77* transcription and stabilizes its mRNA, resulting in superinduction (25). To study its regulation, we cloned the *nur77* gene and sequenced its promoter (50). The results of our sequence analysis agree with those reported previously (38), and the *nur77* sequence is closely homologous to that of its rat homolog (46).

The sequence of the *nur77* promoter region is shown in Fig. 1. Although transcription of *nur77* initiates heterogeneously over several nucleotides, we refer to the major transcription start site as nt +1 (38, 50). A number of potential binding sites for known transcription factors can be recognized and are noted in Fig. 1.

To analyze the transcriptional regulation of *nur77*, we constructed a series of *nur77* minigenes with various promoter mutations and designed an S1 nuclease protection assay in which transcripts correctly initiated from the transfected minigenes can be quantitatively assessed. The minigenes contain *nur77* promoter sequences linked to the *nur77* cDNA, followed by a fragment of *nur77* 3′ genomic DNA containing the polyadenylation signal (Fig. 1C). An insert of some 100 bp of irrelevant sequence was placed between nt +122 and +123 of the cDNA. The minigene-derived transcript is virtually identical to that of the endogenous *nur77* mRNA except for this insert, and it contains several AUUUA sequences that may mediate the short half-life of the mRNA (40). Both the endogenous *nur77* mRNA (77_{END}) and the minigene-derived transcript (77_M) can be detected in the same S1 nuclease protection assay, allowing quantitation of both transcripts in the same cell populations. To control for transfection efficiencies, a parallel S1 nuclease protection assay was carried out to detect the transcript derived from a

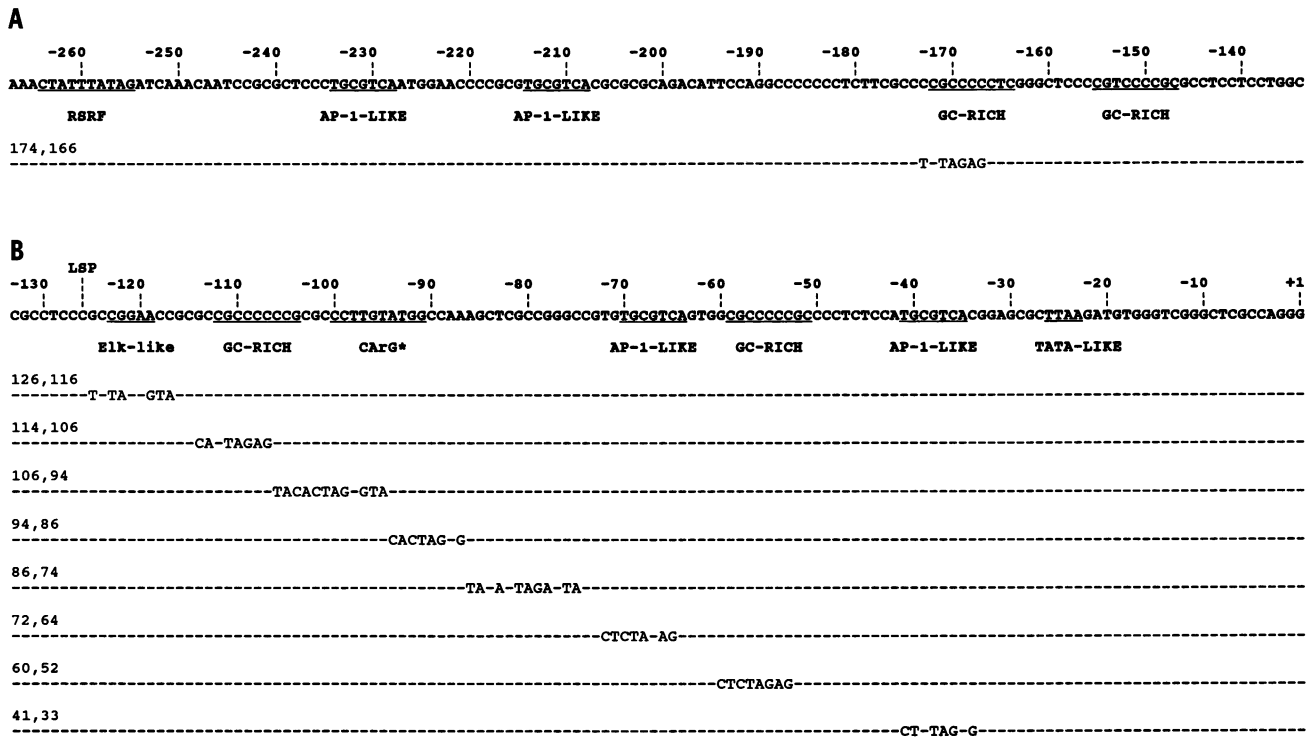

FIG. 1. Structure of the *nur77* promoter, site-specific mutations, and a representative *nur77* minigene. *nur77* promoter sequences from nt −267 to −134 (A) and from −133 to +1 (B) are shown. Sequences of linker-scanning mutations are indicated below the corresponding promoter sequences; identity to the wild-type sequence is denoted by a dash. Sequences similar to those of the binding sites for RSRF, AP-1, Zif268 (GC-rich), and Ets transcription factors are underlined. Elk refers to the binding site for the Ets family of transcription factors, including Elk (18) and SAP (9). A weak match to the TATA homology and an imperfect CArG box (CArG*) are also underlined. The boundary for the LSP construct series at −126 is indicated. (C) Structure of a representative *nur77* minigene for transfection studies. Shown in the diagram is the -126_M minigene (see Materials and Methods). Arrow shows the transcription start site. The 91-bp insert of irrelevant sequence marking the minigene is indicated by the filled box. The radioactively labeled end of the probe for S1 protection assays is indicated by an asterisk. The 340-nt protected product derived from the transfected minigene and the 116-nt protected product from the endogenous *nur77* mRNA are shown.

cotransfected pSV2*neo* plasmid (42), which codes for the neomycin (*neo*) resistance gene driven by the simian virus 40 early promoter.

During the course of our studies, we discovered that activation of *nur77* by serum growth factors in fibroblasts is composed of two components: an immediate-early phase and a delayed-early phase. The immediate-early phase does not require de novo protein synthesis and suggests activation through posttranslation modification of existing transcription factors. The delayed-early phase requires de novo protein synthesis and suggests the involvement of newly synthesized transcription factors, possibly encoded by immediate-early genes. The addition of cycloheximide, a protein synthesis inhibitor, separates these two components. Expression in the presence of cycloheximide indicates immediate-early expression. On the other hand, expression only in the absence of cycloheximide reflects delayed-early expression.

The observation that *nur77* expression is a superimposition of an immediate-early response and a delayed-early response made clear to us that our experiments must be designed to distinguish between immediate-early and delayed-early activation. Accordingly, rapidly growing 3T3 cells were trypsinized and transfected in suspension with a test minigene and plated onto three dishes, thus ensuring that each plate of cells was equivalently transfected. Cells were brought to quiescence, and then one plate was stimulated for 2 h with serum only, allowing both immediate-early and delayed-early expression; the second plate was stimulated with serum and cycloheximide, permitting only immediate-early expression, and the third was kept quiescent as a control.

We began our analysis with 1.2 kb of sequences upstream of the transcription start site; this DNA fragment was capable of mediating accurate serum-inducible expression of *nur77* (data not shown). We then created a series of 5′ deletion mutants that removed successively more sequences from the promoter-distal region. This analysis showed that a promoter fragment containing up to nt −278 mediated virtually the same inducibility as the 1.2-kb promoter fragment

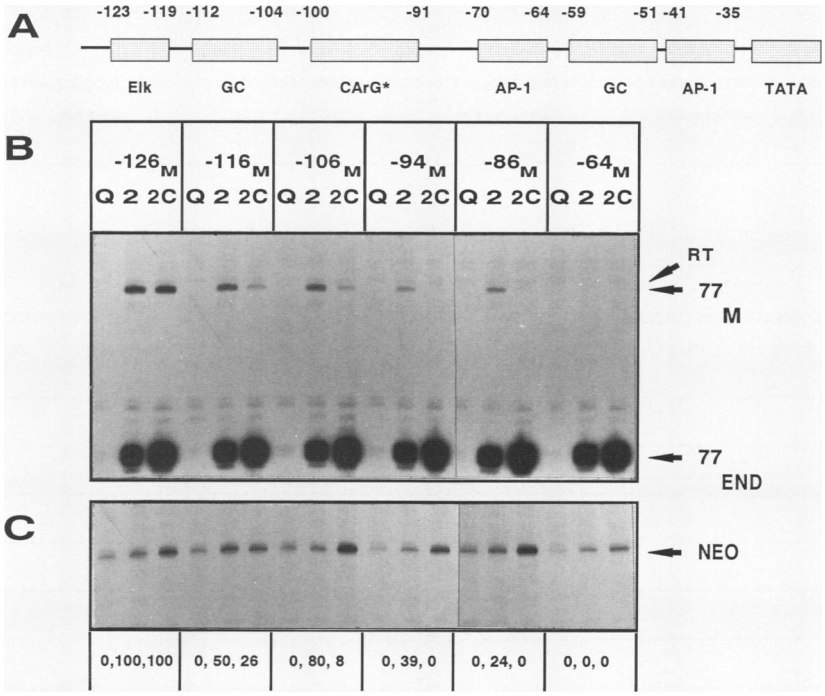

FIG. 2. 5′ deletion analysis of the *nur77* promoter. (A) A schematic representation of the *nur77* promoter, showing the boundaries of various recognizable sequence elements. (B) S1 nuclease protection assay of expression from various *nur77* minigenes with 5′ promoter deletions. The numeric designation of each mutant indicates the nucleotide to which the deletion extends. The data from each minigene are aligned under the corresponding promoter region deleted. For each construct, cells from a single transfection were separated into three portions: one maintained as quiescent cells (Q), one stimulated with serum for 2 h (2), and another stimulated with serum in the presence of cycloheximide for 2 h (2C). The positions of the 340-nt minigene-derived S1-protected product (77_M) and the 116-nt protected product of the endogenous *nur77* mRNA (77_{END}) are indicated. RT, S1 digestion artifact or product derived from read-through transcripts initiated from upstream vector sequences. (C) Parallel S1 protection assay to detect the neomycin message (NEO) derived from the cotransfected pSV40*neo* reference plasmid as a measure of transfection efficiency. Quantitation was performed as described in Materials and Methods, and the results are presented below each lane. The data in this figure are quantified relative to those for -126_M.

(data not shown). Further analysis showed that deletion up to nt −126 resulted in a promoter that was still serum inducible, although the level of induction was reduced (data not shown). Therefore, in this report we focused on a detailed analysis of the *nur77* promoter in the context of both the 278- and the 126-bp promoter fragments.

5′ deletion analysis defines minimal promoter regions for immediate-early and delayed-early expression. Since we determined that a minigene construct containing 126 bp of upstream sequence is serum inducible, we analyzed 5′ deletions of the 126-bp promoter to define the sequences necessary for serum induction (Fig. 2). When 5′ deletions removed an Elk-1-like sequence (-116_M), the levels of immediate-early expression were significantly reduced but still detectable (see lanes 2C in Fig. 2). No effect on immediate-early expression was observed with deletion of a GC-rich sequence (-106_M). Delayed-early expression in these deletion mutants remained unaltered, since stimulation in the absence of cycloheximide resulted in expression levels close to that of the 126-bp promoter. Further deletions to nt −94 and −86, however, completely eliminated immediate-early expression and severely reduced delayed-early expression. In these mutants, transcripts were detectable only when protein synthesis was allowed to occur, while none was detectable when cycloheximide was added (Fig. 2). Further deletion to nt −64 and beyond rendered the promoter completely uninducible. These data place the 5′ boundaries of the minimal promoter for strong immediate-early expression at nt −126, for weak immediate-early expression at nt −106, and for delayed-early expression at nt −86.

Linker-scanning mutations in the 126-bp promoter context define sequences required for serum inducibility. To define the sequences required for serum inducibility more precisely, we carried out linker-scanning mutagenesis in the context of the 126-bp promoter, which mediates both immediate-early and delayed-early expression. A series of mutants were generated (LSP series; Fig. 1B), and their expressions were analyzed (Fig. 3). Three classes of mutant phenotypes were observed. The first class of mutations abolished immediate-early expression but affected delayed-early expression minimally (LSP 106/94 and LSP 94/86; Fig. 3). The second class of mutations severely reduced but did not abolish immediate-early expression (LSP 114/106 and LSP 86/74). A third class of mutations dramatically reduced both immediate-early and delayed-early expression, as seen in those altering the promoter-proximal AP-1-like elements (LSP 72/64 and LSP 41/33) or in the GC-rich element (LSP 60/52). Thus, in the 126-bp promoter context, sequence elements in the regions from nt −106 to −86 are required for immediate-early expression, whereas nt −72 to −33 are required for both immediate-early and delayed-early expression.

Linker-scanning mutations in the 278-bp promoter context identify primary and accessory elements. Redundant sequence elements might functionally compensate for one

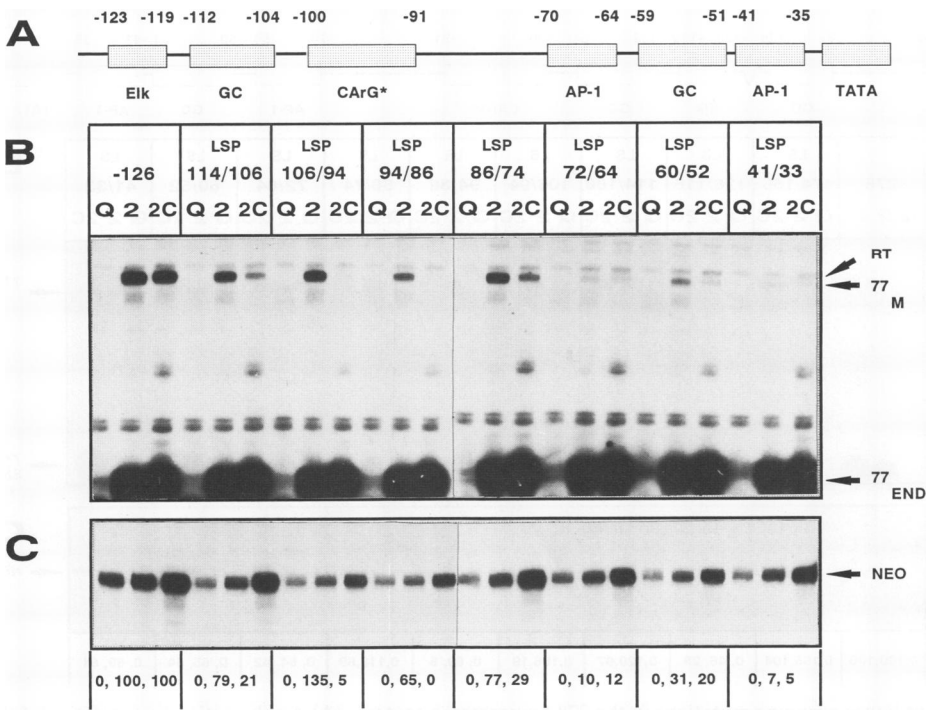

FIG. 3. Analyses of linker-scanning mutations in the 126-bp promoter context. (A) A schematic representation of the *nur77* promoter. (B and C) S1 nuclease protection assay of transcripts derived from the -126_M minigene (−126) and various linker-scanning mutants in the 126-bp promoter context (LSP series). The numeric designation of each mutant denotes the 5′ and 3′ nt boundaries of sequences substituted by the linker (see Fig. 2), and the data of each mutant are aligned under the corresponding region shown in panel A where the sequence was mutated. Data are presented as described in the legend to Fig. 2.

another. For example, the promoter-proximal AP-1-like elements that are required for both immediate-early and delayed-early expression in the 126-bp promoter context (Fig. 3) could be functionally replaced by other AP-1-like sequences occurring further upstream. We therefore analyzed linker-scanning mutations in the context of 278-bp promoter (LS series). Indeed, mutations in the two promoter-proximal AP-1-like (LS 72/64 and LS 41/33) and GC-rich (LS 60/52) elements that had severe effects in the 126-bp promoter did not affect expression in the 278-bp promoter (Fig. 4). This result argues that mutations within nt −72 to −33 did not affect the function of a necessary basal promoter (Fig. 3).

In contrast, severe effects on immediate-early expression were observed with LS 106/94 and LS 94/86, which alter the distal and proximal halves of the CArG-like element, respectively. These mutations did not affect delayed-early expression significantly. The effects of LS 94/86, which nearly abolished all immediate-early transcription, were reproducibly more severe than those of LS 106/94. Since mutations in the distal half of a CArG box should have affected interactions with SRF more severely (27, 44), this result suggests that the role of the CArG-like element does not involve a simple interaction with the SRF. Significant reduction in inducibility was also observed with mutations that alter the Elk/SAP-like sequence (LS 126/116); however, mutations altering the distal GC-rich elements (LS 114/106 and LS 174/166) and the mutations in LS 86/74 had little effect on expression.

These data allow us to distinguish between primary and accessory elements. For the immediate-early response, the AP-1-like and GC-rich elements play an accessory role. In the 126-bp promoter context, they are necessary but not sufficient for the immediate-early response (Fig. 2 and 3). The functions of these elements can be compensated by the addition of upstream sequences containing similar elements (Fig. 4). By contrast, effects of mutations in the CArG-like element and the Elk/SAP element cannot be offset by other elements. These sequences may play a primary role in *nur77* immediate-early activation.

Kinetics of immediate-early and delayed-early expression. As described above, linker-scanning mutants LSP 106/94 and LSP 94/86 lost cycloheximide-resistant (immediate-early) expression but retained cycloheximide-sensitive (delayed-early) expression (Fig. 3). This observation predicts that these mutants should have delayed expression kinetics relative to those of the wild type. We tested this by comparing the time course of expression of LSP 94/86 upon serum stimulation with that of its wild-type parent, -126_M (Fig. 5). Transcripts from -126_M can be detected 30 min after serum addition, increasing to a peak level at 120 min and then declining thereafter, similar to expression of the endogenous *nur77*. As predicted, LSP 94/86 displayed more-delayed expression kinetics, with transcripts detectable only after 90 min of stimulation and increasing to a peak level at 120 min. These data are consistent with the interpretation that LSP 94/86 has lost immediate-early expression while retaining delayed-early expression, whereas the wild type has both components superimposed.

Another feature of *nur77* regulation is that transcription is repressed soon after activation (25). The sequences contained within -126_M are sufficient to mediate transcriptional repression with kinetics virtually identical to those of the endogenous gene (Fig. 5 and data not shown). We conclude that none of the sequences required for repression of imme-

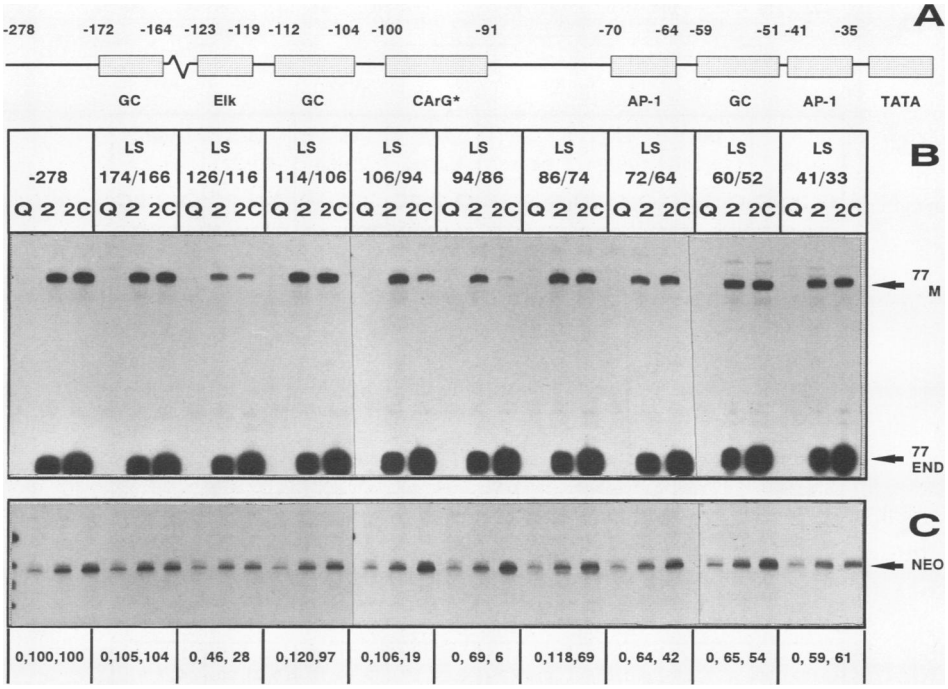

FIG. 4. Analyses of linker-scanning mutations in the 278-bp promoter context. (A) A schematic representation of the *nur77* promoter. (B and C) S1 nuclease protection analysis of transfected minigenes carrying linker-scanning mutations in the 278-bp promoter context (LS series). The numeric designation of each mutant refers to the 5′ and 3′ boundaries of sequences replaced by the linker. Data are presented as described in the legends to Fig. 2 and 3, except that the data were quantified relative to those of -278_M.

diate-early or delayed-early response are located upstream of nt −126.

***nur77* promoter fragments confer inducibility on a heterologous promoter.** Although the experiments described above show that *nur77* minigenes with short 5′ flanking sequences are serum inducible, it remains possible that sequences within the *nur77* cDNA help to mediate induction. We therefore tested the activities of various *nur77* promoter

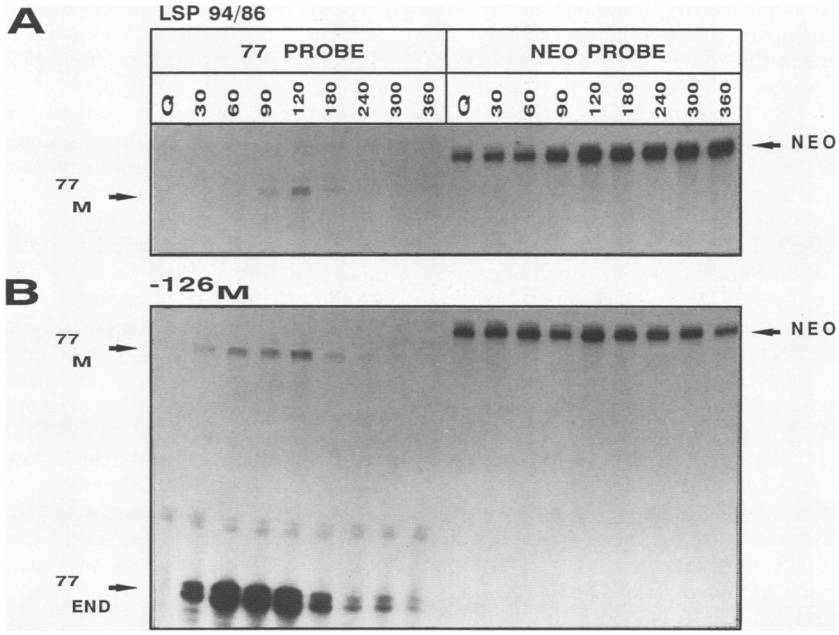

FIG. 5. Dissociation of immediate-early and delayed-early expression. The time courses of expression of the linker-scanning mutant LSP 94/86 (A) and the minigene -126_M (B) upon serum stimulation were examined by S1 nuclease protection assay. Transfected cells were either kept quiescent (Q) or stimulated with serum for the indicated durations (in minutes), and RNA was isolated and analyzed with the *nur77* and *neo* probes. The protected products of the endogenous transcripts (77_{END}) from the samples in panel A (data not shown) were identical to those shown in panel B.

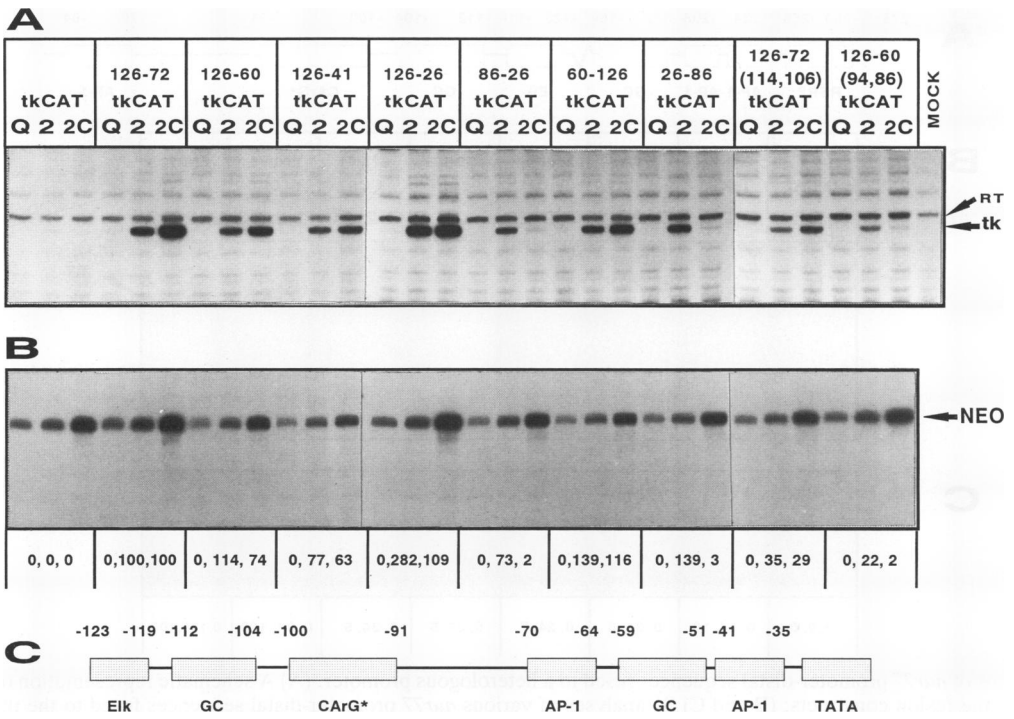

FIG. 6. Analyses of *nur77* promoter-proximal sequences fused to a heterologous promoter. (A and B) S1 nuclease protection assay of transfected minigenes with various *nur77* promoter-proximal sequences fused to a heterologous promoter (tk) driving expression of the CAT message. The numeric designation of each construct refers to the 5′ and 3′ boundaries of promoter sequences fused to the heterologous promoter. Cells transfected with either the parental plasmid tkCAT or various promoter fusions were separated into three portions: one kept quiescent (Q), one serum stimulated for 2 h, and one serum stimulated in the presence of cycloheximide for 2 h (2C). The positions for the S1-protected products corresponding to initiation from the tk promoter fusions and the neomycin reference plasmid (NEO) are shown. RT, S1 digestion artifact or read-through transcript initiated from upstream sequences in the vector. At the top right, the numbers in parentheses refer to a linker-scanning mutation contained in the designated constructs. Mock, S1 protection product from mock transfected cells. Data are presented as described in the legend to Fig. 2, except that the data were quantified relative to those of the 126-72 tkCAT construct. (C) A schematic representation of the promoter sequences used in the fusion constructs.

sequences by fusing them upstream of a heterologous reporter gene, namely, the herpes simplex virus tk promoter linked to the bacterial CAT gene (tkCAT). Expression of these chimeric promoters was analyzed after transfection by an S1 nuclease protection assay similar to that of the marked *nur77* minigenes (Fig. 6).

A fragment containing nt −126 to −72 of *nur77* was able to confer strong immediate-early expression on tkCAT (126-72 tkCAT; Fig. 6). Addition of an AP1-like element (126-60 tkCAT), a GC-rich sequence (126-41 tkCAT), and a second AP1-like element (126-26 tkCAT) did not alter immediate-early expression significantly. In contrast, nt −86 to −26 conferred only delayed-early expression (86-26 tkCAT). Reversing the orientation of these fragments has no effect (60-126 tkCAT and 26-86 tkCAT), indicating that they can function in either orientation within the promoter. Similar results were obtained when the same fragments were inserted upstream of a β-globin promoter driving expression of CAT (data not shown). We thus conclude that sequences within nt −126 to −72 are sufficient for immediate-early expression and that sequences within nt −86 to −26 are sufficient for delayed-early expression.

We also examined linker-scanning mutations in the context of heterologous promoter fusions. When the CArG-like element was mutated in a fragment containing nt −126 to −60 fused to tkCAT [126-60 (94,86) tkCAT], immediate-early expression was abolished (Fig. 6). When the GC-rich sequences were mutated in a fragment containing nt −126 to −72 fused to tkCAT [126-72 (114,106) tkCAT], immediate-early expression was reduced threefold. However, a similar linker-scanning mutation in the 126-bp *nur77* promoter context affected immediate-early expression severely (LSP 114/106; Fig. 3). These results are consistent with the idea that the GC-rich element plays an accessory role for which elements in the tk promoter can sufficiently compensate.

3′ boundary of the sequence required for immediate-early expression. To determine the 3′ boundary of the sequence required for growth factor-regulated expression, we analyzed 3′ deletions of the 278-bp *nur77* promoter fused to tkCAT (Fig. 7). Whereas 278-86 tkCAT and 278-72 tkCAT displayed strong immediate-early expression, 278-94 tkCAT showed only residual expression, suggesting that sequences between nt −86 and −94 are critical for immediate-early expression. Further 3′ deletion through nt −106, −114, −154, and −174 showed little change. 278-174 tkCAT still showed residual immediate-early expression, but the level of expression is only 5 to 10% of that observed with 278-86 tkCAT. Thus, the contribution of the upstream sequences to total immediate-early expression is minimal. We have not mapped precisely the upstream sequences mediating this weak induction, although the binding site for RSRF (33) and two AP-1-like elements could be recognized (Fig. 1). Taken together with the 5′ deletion analysis (Fig. 2), the sequence required for strong immediate-early induction resides be-

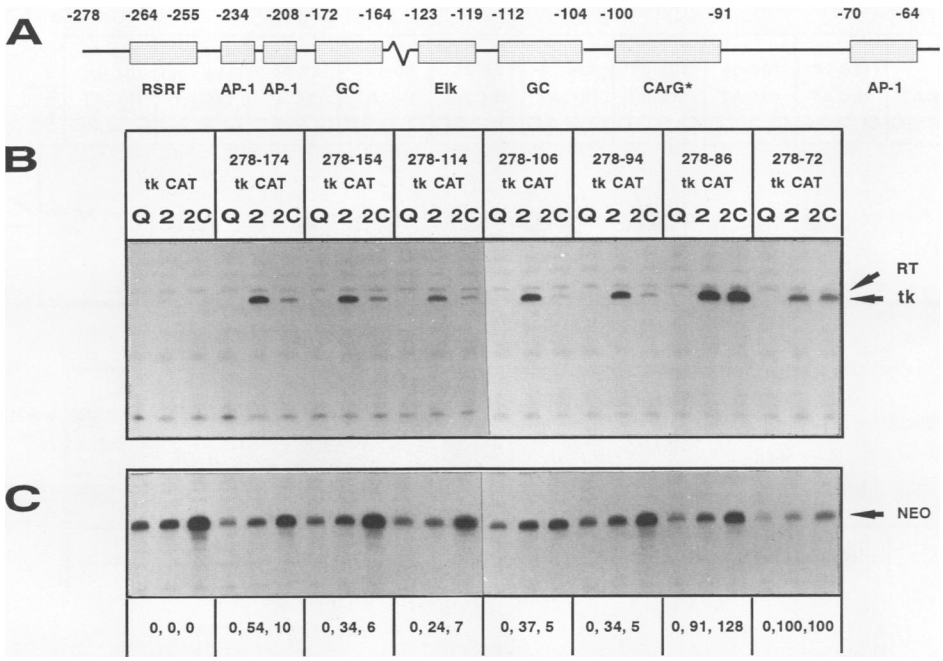

FIG. 7. Analyses of *nur77* promoter-distal sequences fused to a heterologous promoter. (A) A schematic representation of *nur77* promoter sequences used in the fusion constructs; (B and C) S1 analyses of various *nur77* promoter-distal sequences fused to the tk promoter linked to the CAT coding sequence. Data are presented as described in the legend to Fig. 6 and are quantified relative to the 278-72 tkCAT construct.

tween nt −126 and −86 and that for weak expression resides between nt −106 and −86. All fusion constructs tested were also capable of mediating delayed-early expression, consistent with the presence of redundant sequence elements, as suggested by the results shown in Fig. 4.

Activation by purified growth factors and phorbol ester. We tested whether the sequence that mediates serum induction of *nur77* can also mediate induction by purified FGF or PDGF or by the phorbol ester TPA. The 126-72 tkCAT construct, which was responsive to serum-induced immediate-early expression, was also responsive to immediate-early activation by TPA, FGF, and PDGF (Fig. 8). The 86-26 tkCAT construct, which was responsive to serum-induced delayed-early expression, was likewise responsive to delayed-early activation by TPA, FGF, and PDGF (Fig. 8). Thus, the sequences that mediate induction of *nur77* by serum also mediate activation by purified growth factors and activators of protein kinase C.

The Elk/SAP-like element is required for high-level induction. Since we have identified the sequences between nt −126 and −86 as being critical for immediate-early activation of *nur77*, we tested the requirement for specific sequences within this region. Specific sequences corresponding to various portions of this fragment were inserted upstream of tkCAT, and the inducibility of these chimeric constructs was assessed. The imperfect CArG box conferred little inducibility to tkCAT expression (106-82 tkCAT; Fig. 9). Extending the *nur77* sequences 10 bp upstream and 10 bp downstream (116-72 tkCAT) of the CArG box had no effect. In contrast, the tkCAT promoter including the c-*fos* SRE conferred a high degree of inducible expression. When the Elk/SAP-like site was added to the *nur77* CArG-like sequence, however, a high level of inducible expression was observed (126-72 tkCAT). Thus, the imperfect CArG box confers little inducibility by itself. The imperfect CArG box must function in conjunction with the Elk/SAP-like element for significant induction.

Zif268 can activate *nur77* delayed-early transcription. Since expression of delayed-early genes requires de novo protein synthesis, proteins encoded by immediate-early genes have been hypothesized to play a role in their regulation. Our data show that the promoter-proximal AP-1-like and GC-rich elements are necessary and sufficient for delayed-early transcription of *nur77* (Fig. 3 and 7). Known transcription factors

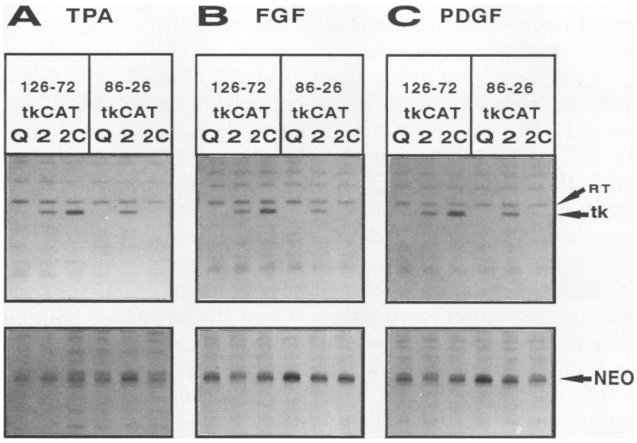

FIG. 8. Activation by purified growth factors and phorbol ester. The 126-72 tkCAT and 86-26 tkCAT constructs were transfected into cells, and the cells were either kept quiescent (Q) or stimulated with the phorbol ester TPA (A), purified FGF (B), or purified PDGF (C) for 2 h in the presence (2C) or absence (2) of cycloheximide. S1 nuclease protection analyses were carried out and data are presented as described in the legends to Fig. 6 and 7.

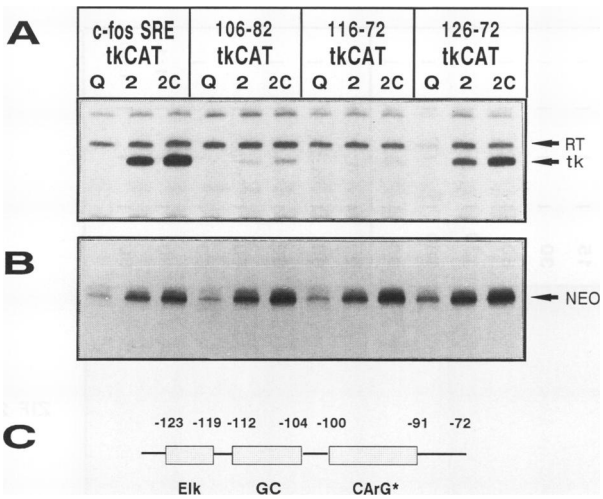

FIG. 9. Analyses of oligonucleotides fused to a heterologous promoter. (A) S1 nuclease protection assay for expression from a tkCAT promoter fused to various oligonucleotides, including the c-*fos* SRE sequence, or nt −106 to −82, nt −116 to −72, and nt −126 to −72 of the *nur77* promoter. The protected product of the CAT transcript (tk) is indicated, as is that of an artifactual S1 product (RT). (B) Parallel S1 protection analysis of the *neo* transcript derived from the cotransfected pSV40*neo* reference plasmid. (C) A schematic representation of *nur77* promoter sequences used in the fusion constructs.

that might interact with the AP-1-like sequences include members of the Fos and Jun families (37) and CREB (31). The GC-rich sequence might interact with SP1 (20) or members of a family of zinc finger proteins including Zif268 (7, 8, 35), encoded by an immediate-early gene. We have begun to examine the possibility that these proteins regulate *nur77* delayed-early expression.

To test whether Zif268 can transactivate *nur77*, we cotransfected a Zif268 expression plasmid with the -126_M minigene into 3T3 cells. When Zif268 was present, the *nur77* minigene was active even when cells were quiescent, whereas in its absence the minigene is not transcribed (Fig. 10A; also see Fig. 2 to 4). These results show that Zif268 can transactivate *nur77*.

To examine whether Zif268 interacts with the *nur77* promoter directly, we carried out in vitro footprinting studies with recombinant Zif268 protein produced in bacteria. These experiments confirmed that Zif268 can bind to the GC-rich region centered at nt −55 with high affinity (data not shown). Furthermore, Zif268 appears to be the predominant protein binding to this site soon after serum stimulation. Cell extracts prepared at various times after serum stimulation were incubated with labeled oligonucleotides containing the GC-rich sequence and separated by nondenaturing electrophoresis (Fig. 10B). While several protein-DNA complexes were formed upon the oligonucleotide, the predominant complex was most evident from 60 to 120 min after serum stimulation. Competition with an excess of unlabeled probe abolished all complexes (Fig. 10B, lane 8). The addition of Zif268-specific antiserum, but not the preimmune serum or antiserum to a different immediate-early protein, supershifted only this major DNA-protein complex (Fig. 10B). Moreover, the timing of the appearance of the major complex is consistent with the period of Zif268 synthesis (7). Taken together, these data indicate that Zif268 synthesized during the immediate-early phase of stimulation can bind to the *nur77* promoter and transactivate it.

DISCUSSION

***nur77* is both an immediate-early gene and a delayed-early gene.** Stimulation of quiescent fibroblasts by serum growth factors activates a specific genetic program thought to mediate cellular mitogenic responses (26). The first participants of this genetic program are the immediate-early genes, which are rapidly activated without requiring de novo protein synthesis. The proteins encoded by some of these genes are hypothesized to activate the delayed-early genes, whose expression does require de novo protein synthesis. By analogy with the developmental program of complex viruses, some of the immediate-early genes are expected to encode regulatory proteins, and the delayed-early genes are expected to encode proteins that are directly required for the duplication of cellular components (25).

The first unexpected finding in the present study is that *nur77* is expressed with the overlapping kinetics of both an immediate-early gene and a delayed-early gene (Fig. 3 to 5). Functionally defined, the immediate-early component of *nur77* expression is insensitive to protein synthesis inhibitors, whereas the delayed-early component is blocked by such inhibitors. The immediate-early and delayed-early expressions of *nur77* are regulated by distinct and separable sequence elements (Fig. 6). Despite having a delayed-early component, the expression of *nur77* is rapid and transient (24, 25). In fact, *nur77* was first recognized as an immediate-early gene with expression kinetics similar to those of c-*fos* (24, 25, 38). Thus, the primary function of delayed-early expression is more likely to increase the diversity of regulatory pathways that can control *nur77* expression, rather than to extend the duration of *nur77* expression after stimulation.

How many other immediate-early genes identified heretofore also exhibit this biphasic transcriptional regulation? Since stimulation in the presence of protein synthesis inhibitors results in superinduction of many immediate-early genes due to prolonged transcription and mRNA stabilization (25), any effect on delayed-early expression would be masked. The delayed-early component of *nur77* expression was not discovered until mutations that dissociated the immediate-early response from the delayed-early response were generated and analyzed, both in the presence and in the absence of de novo protein synthesis. Thus, it is possible that other immediate-early genes also have similarly complex regulation, since many of them have AP-1-like and GC-rich sequences in their promoters. However, their promoters must be carefully analyzed to uncover the possibility of delayed-early expression overlapping with immediate-early regulation.

Regulation of immediate-early expression. Several lines of evidence support the conclusion that nt −126 to −86, which include an imperfect CArG element, a GC-rich element, and an Elk/SAP element, constitute the primary sequence requirements for the immediate-early component of expression. (i) Deletion analyses placed the 5′ and 3′ boundaries for the essential sequence for strong immediate-early expression at nt −126 and −86, respectively (Fig. 2 and 7). (ii) Linker-scanning mutations in each of these sequence elements severely affected immediate-early expression (Fig. 3). (iii) A DNA fragment containing this sequence confers inducibility on a heterologous promoter by serum (Fig. 6 and 8) and purified growth factors (Fig. 9). The GC-rich element probably plays an accessory role, since a linker-scanning muta-

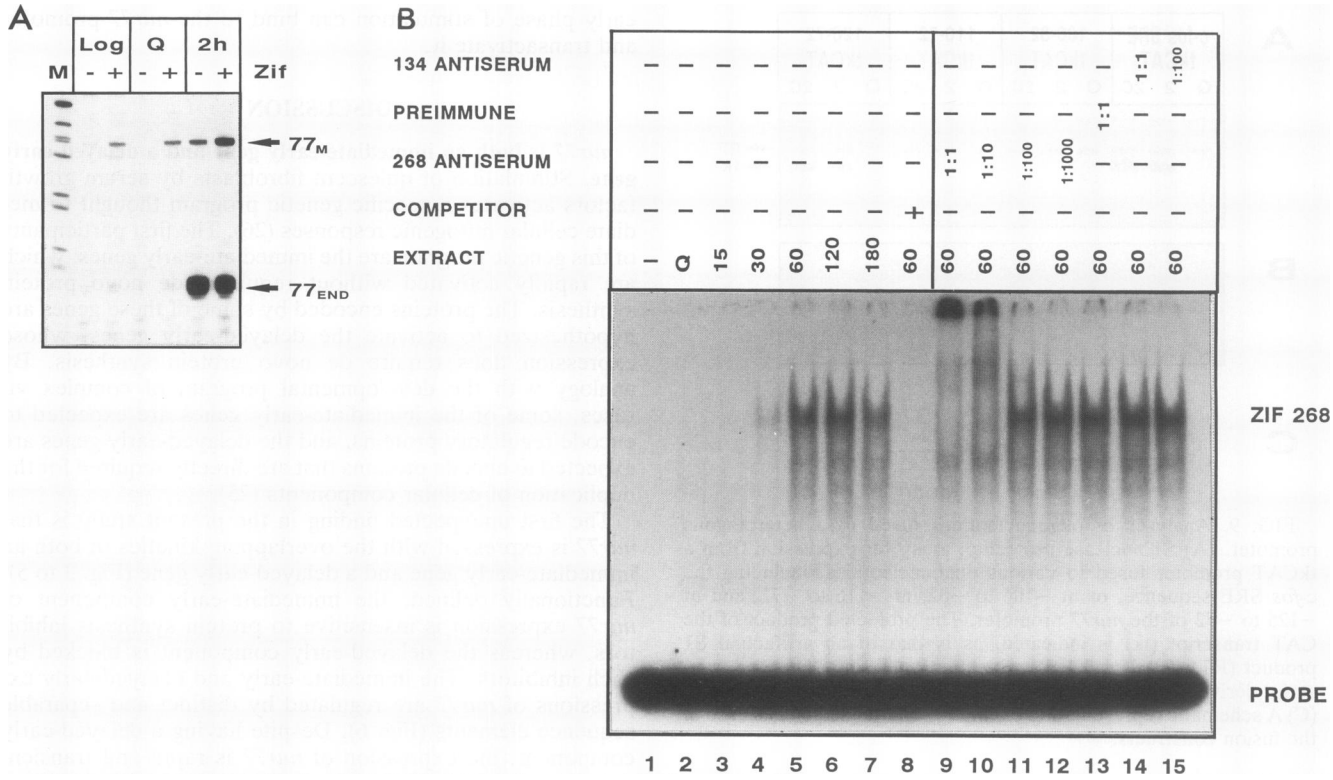

FIG. 10. Zif268 regulation of *nur77* delayed-early expression. (A) Zif268 can transactivate *nur77*. NIH 3T3 cells were cotransfected with either a plasmid vector expressing Zif268 under the cytomegalovirus promoter (pCMV5*zif* [5 μg])(+) or the vector alone (−) and the *nur77* -126_M minigene (30 μg), and the expression of *nur77* was assayed when transfected cells were logarithmically growing (Log), kept quiescent (Q), or kept quiescent and then serum stimulated for 2 h (2h). (B) Gel shift analysis of whole-cell extracts from serum-stimulated NIH 3T3 cells. Lanes 1 to 8 contain the data for a gel shift analysis in which labeled oligonucleotide containing the Zif268 consensus binding site was incubated with either no extract (lane 1) or extracts from cells serum stimulated for the indicated times (in minutes). The reaction mixture in lane 8 also contains a 1,000-fold excess of the same oligonucleotide unlabeled. Lanes 9 to 15 contain the data for a supershift analysis in which the binding reaction mixture used in lane 5 was incubated with the indicated dilutions of either a Zif268-specific antiserum, preimmune serum, or an antiserum to another immediate-early protein that has no homology to Zif268. The positions of unbound probe and the Zif268-containing complex are indicated on the right.

tion altering it seriously affected immediate-early expression in the 126-bp promoter context (LSP 114/106; Fig. 3) but had little effect in the 278-bp promoter context (LS 114/106; Fig. 4). In contrast, mutations in either the CArG-like element or the Elk-like element had severe effects in both promoter contexts, suggesting that these two elements work in concert to play primary roles in immediate-early activation of *nur77*.

No mutation or configuration of *nur77* promoter sequences tested in this study relieved repression in the quiescent state. It is possible that in the *nur77* promoter, there is no genetic element specifically committed to transcriptional repression in the quiescent state. Rather, the inactivity of *nur77* during quiescence might be achieved by the absence or the posttranslational inactivation of positively acting transcription factors.

A number of immediate-early genes analyzed to date appear to be regulated by sequence elements that include the CArG box, defined by the consensus sequence CC(A/T)$_6$GG, which binds to SRF (36, 44). The imperfect CArG box present in the *nur77* promoter deviates from the consensus sequence by interrupting the central A/T core with a GC base pair. Such mutations are thought to severely impair binding to SRF (27), thus preventing SRF to act. Indeed, the imperfect CArG of *nur77* binds poorly to SRF in vitro (data not shown) and by itself is unable to mediate significant serum induction (Fig. 9).

Recent work demonstrated that serum induction of c-*fos* is dependent on SRF, which recruits another protein factor, p62^{TCF}, to the c-*fos* SRE, forming a ternary complex (19, 45). SRF binds strongly to the c-*fos* CArG box, which conforms to the consensus sequence. p62^{TCF} (Elk/SAP) does not bind to SRE unless SRF is already bound (41). Furthermore, the distance between the SRF- and Elk-binding sites does not appear critical (45). In the case of *nur77*, since SRF binds poorly to the imperfect CArG box, SRF may not be able to act as a recruiting agent for an Elk-like protein. On the other hand, the Elk-like site present in *nur77* is expected to have a much higher affinity for Elk or SAP than that found in the c-*fos* SRE (32). Therefore, it is possible to speculate that the binding of an Elk/SAP-like protein to the Elk-like site of *nur77* may facilitate the binding of SRF to the imperfect CArG box, and the resulting ternary complex may be important for activation. According to this hypothesis, the order of assembly of transcription factors upon the inducible promoters would be different for *fos* and *nur77*.

While mutations in the two promoter-proximal AP-1-like (LS 72/64 and LS 41/33) and GC-rich (LS 60/52) elements

had severe effects on immediate-early expression in the 126-bp promoter, they did not affect expression in the 278-bp promoter (Fig. 3 and 4). Therefore, the function of these elements can be replaced by sequences upstream. These elements do not constitute a requisite basal promoter in association with the TATA box upon which enhancer elements might be added; rather, they may serve only to enhance and amplify the effects of the inducible elements.

Delayed-early expression and multiple mechanisms of activation. Our data indicate that nt −26 to −74 mediate the delayed-early component of *nur77* expression, although sequences further upstream can compensate for mutations in these sequences. Several lines of evidence support this conclusion. (i) A fragment containing nt −26 to −86 confers delayed-early inducibility on a heterologous promoter in either orientation (Fig. 6). (ii) Linker-scanning mutations in the context of the 126-bp promoter affecting nt −33 through −72 severely affected delayed-early expression, whereas linker-scanning mutations affecting nt −74 to −86 had no effect (Fig. 3). (iii) Linker-scanning mutations that abolish immediate-early expression exhibit delayed expression kinetics upon serum stimulation (Fig. 4). Taken together, these data indicate that sequences that lie between nt −26 and −74 mediate delayed-early expression.

Within this region lie two AP-1-like sequences and a GC-rich element that contains the binding sites for SP1 and the Zif268 family of transcription factors. Both AP-1 and Zif268 are encoded by immediate-early genes; thus, it is reasonable that these proteins might mediate the delayed-early response. We show here that Zif268 can indeed transactivate the *nur77* minigene in a cotransfection assay (Fig. 10). Although we also showed that Zif268 binds to the *nur77* promoter, our experiments do not prove that Zif268 acts directly to transactivate *nur77*. Moreover, it is clear that delayed-early expression requires more than just the presence of Zif268, since linker-scanning mutations affecting either of the two AP-1-like elements flanking the Zif268-binding site abolish delayed-early response. It is likely that interactions among the protein factors binding to these sites may be important for correct regulation.

Recent studies support the conclusion that there are multiple mechanisms that mediate transcriptional activation by growth factors. The activation of c-*jun* by serum, for example, is not mediated through a CArG box (13). The differences in sequence requirements for activation of *nur77* and c-*fos* suggest that although they might be activated through some common components, there may be substantial differences in the mechanism of their regulation. Recently, Freter et al. (12) found that a 7-nt sequence located in the 3′ untranslated region of the *JE* gene is able to mediate immediate-early expression in fibroblasts. Although this 7-nt sequence motif is also present in the 3′ untranslated region of *nur77*, it is not required for immediate-early expression of *nur77* by reason of two arguments. First, *nur77* promoter sequences alone can confer immediate-early inducibility on a heterologous promoter. Second, specific mutations within the *nur77* promoter sequence not affecting this 7-nt motif can abolish inducibility in the context of a *nur77* minigene.

In this study, we have focused on *nur77* expression in fibroblasts. However, *nur77* is also transcriptionally activated in other cell types by other agents, possibly through different mechanisms. In the rat pheochromocytoma cell line PC12, *nur77* is transcriptionally activated by treatment with nerve growth factor and membrane-depolarizing agents without requiring de novo protein synthesis. In PC12 cells, nt −22 to −86 of the *nur77* promoter mediate immediate-early inducibility by both nerve growth factor and membrane depolarization (50). Furthermore, there is no evidence for a delayed-early component in the induction by these two agents in PC12 cells. These findings point to a multiplicity of regulatory mechanisms that may control immediate-early gene expression in different cell types stimulated by different agents.

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REFERENCES

1. **Ackerman, S. L., A. G. Minden, G. T. Williams, C. Bobonis, and C. Y. Yeung.** 1991. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. Proc. Natl. Acad. Sci. USA **88**:7523–7527.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1990. Current protocols in molecular biology. John Wiley, New York.
3. **Bravo, R.** 1990. Genes induced during the G0/G1 transition in mouse fibroblasts. Semin. Cancer Biol. **1**:37–46.
4. **Chavrier, P., U. Janssen Timmen, M. G. Mattei, M. Zerial, R. Bravo, and P. Charnay.** 1989. Structure, chromosome location, and expression of the mouse zinc finger gene *krox-20*: multiple gene products and coregulation with the proto-oncogene c-*fos*. Mol. Cell. Biol. **9**:787–797.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
6. **Christy, B., and D. Nathans.** 1989. Functional serum response elements upstream of the growth factor-inducible gene *zif*268. Mol. Cell. Biol. **9**:4889–4895.
7. **Christy, B., and D. Nathans.** 1989. DNA binding site of the growth factor-inducible protein Zif268. Proc. Natl. Acad. Sci. USA **86**:8737–8741.
8. **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 protein family. Mol. Cell. Biol. **11**:3835–3841.
9. **Dalton, S., and R. Treisman.** 1992. Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell **68**:597–612.
10. **Davis, I. J., T. G. Hazel, and L. F. Lau.** 1991. Transcriptional activation by Nur77, a growth factor-inducible member of the steroid hormone receptor superfamily. Mol. Endocrinol. **5**:854–859.
11. **de Groot, R. P., J. Auwerx, M. Karperien, B. Staels, and W. Kruijer.** 1991. Activation of junB by PKC and PKA signal transduction through a novel cis-acting element. Nucleic Acids Res. **19**:775–781.
12. **Freter, R. R., J.-C. Irminger, J. A. Porter, S. D. Jones, and C. D. Stiles.** 1992. 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. Mol. Cell. Biol. **12**:5288–5300.
13. **Han, T.-H., W. W. Lamph, and R. Prywes.** 1992. Mapping of epidermal growth factor-, serum-, and phorbol ester-responsive sequence elements in the c-*jun* promoter. Mol. Cell. Biol. **12**:4472–4477.
14. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor, New York.
15. **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.
16. **Herschman, H. R.** 1991. Primary response genes induced by

growth factors and tumor promoters. Annu. Rev. Biochem. **60**:281–319.
17. **Hill, C. S., R. Marais, S. John, J. Wynne, S. Dalton, and R. Treisman.** 1993. Functional analysis of a growth factor-responsive transcription factor complex. Cell **73**:395–406.
18. **Hipskind, R. A., V. N. Rao, C. G. Mueller, E. S. Reddy, and A. Nordheim.** 1991. Ets-related protein Elk-1 is homologous to the c-*fos* regulatory factor $p62^{TCF}$. Nature (London) **354**:531–534.
19. **Janknecht, R., and A. Nordheim.** 1992. Elk-1 protein domains required for direct and SRF-assisted DNA-binding. Nucleic Acids Res. **20**:3317–3324.
20. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51**:1079–1090.
21. **Kawamoto, T., K. Makino, S. Orita, A. Nakata, and T. Kakunaga.** 1989. DNA bending and binding factors of the human β-actin promoter. Nucleic Acids Res. **17**:523–537.
22. **Lanahan, A., J. B. Williams, L. K. Sanders, and D. Nathans.** 1992. Growth factor-induced delayed early response genes. Mol. Cell. Biol. **12**:3919–3929.
23. **Latinkic, B. V., T. P. O'Brien, and L. F. Lau.** 1991. Promoter function and structure of the growth factor-inducible immediate early gene *cyr61*. Nucleic Acids Res. **19**:3261–3267.
24. **Lau, L. F., and D. Nathans.** 1985. Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. EMBO J. **4**:3145–3151.
25. **Lau, L. F., and D. Nathans.** 1987. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-*fos* or c-*myc*. Proc. Natl. Acad. Sci. USA **84**:1182–1186.
26. **Lau, L. F., and D. Nathans.** 1991. Genes induced by serum growth factors, p. 257–293. *In* P. Cohen and J. G. Foulkes (ed.), Molecular aspects of cellular regulation, vol. 6. Hormonal regulation of transcription. Elsevier, Amsterdam.
27. **Leung, S., and N. G. Miyamoto.** 1989. Point mutational analysis of the human c-*fos* serum response factor binding site. Nucleic Acids Res. **17**:1177–1195.
28. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell **73**:381–393.
29. **Milbrandt, J.** 1988. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron **1**:183–188.
30. **Mohun, T., N. Garrett, and R. Treisman.** 1987. Xenopus cytoskeletal actin and human c-*fos* gene promoters share a conserved protein-binding site. EMBO J. **6**:667–673.
31. **Montminy, M. R., and L. M. Bilezikjian.** 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature (London) **328**:175–178.
32. **Nye, J. A., J. M. Petersen, C. V. Gynther, M. D. Jonsen, and B. J. Graves.** 1992. Interaction of murine Ets-1 with GGA-binding sites establishes the Ets domain as a new DNA-binding motif. Genes Dev. **6**:975–990.
33. **Pollock, R., and R. Treisman.** 1991. Human SRF-related proteins: DNA-binding properties and potential regulatory targets. Genes Dev. **5**:2327–2341.
34. **Rattner, A., M. Korner, H. Rosen, P. A. Baeuerle, and Y. Citri.** 1991. Nuclear factor κB activates proenkephalin transcription in T lymphocytes. Mol. Cell. Biol. **11**:1017–1022.
35. **Rauscher, F. J., III, J. F. Morris, O. E. Tournay, D. M. Cook, and T. Curran.** 1990. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science **250**:1259–1262.
36. **Rivera, V. M., and M. E. Greenberg.** 1990. Growth factor-induced gene expression: the ups and downs of c-*fos* regulation. New Biol. **2**:751–758.
37. **Ryseck, R. P., and R. Bravo.** 1991. c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. Oncogene **6**:533–542.
38. **Ryseck, R. P., H. MacDonald Bravo, M. G. Mattei, S. Ruppert, and R. Bravo.** 1989. Structure, mapping and expression of a growth factor inducible gene encoding a putative nuclear hormonal binding receptor. EMBO J. **8**:3327–3335.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
40. **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.
41. **Shaw, P. E., H. Schroter, and A. Nordheim.** 1989. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-*fos* promoter. Cell **56**:563–572.
42. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
43. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185**:60–89.
44. **Treisman, R.** 1990. The SRE: a growth factor responsive transcriptional regulator. Semin. Cancer Biol. **1**:47–58.
45. **Treisman, R., R. Marais, and J. Wynne.** 1992. Spatial flexibility in ternary complexes between SRF and its accessory proteins. EMBO J. **12**:4631–4640.
46. **Watson, M. A., and J. Milbrandt.** 1989. The NGFI-B gene, a transcriptionally inducible member of the steroid receptor gene superfamily: genomic structure and expression in rat brain after seizure induction. Mol. Cell. Biol. **9**:4213–4219.
47. **Webster, N., J. R. Jin, S. Green, M. Hollis, and P. Chambon.** 1988. The yeast UASg is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 transactivator. Cell **52**:169–178.
48. **Williams, G. T., and R. I. Morimoto.** 1990. Maximal stress-induced transcription from the human HSP70 promoter requires interactions with the basal promoter elements independent of rotational alignment. Mol. Cell. Biol. **10**:3125–3136.
49. **Wilson, T. E., T. J. Fahrner, M. Johnston, and J. Milbrandt.** 1991. Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Science **252**:1296–1300.
50. **Yoon, J. K., and L. F. Lau.** 1993. Transcriptional activation of the inducible nuclear receptor gene *nur77* by nerve growth factor and membrane depolarization in PC12 cells. J. Biol. Chem. **268**:9148–9155.