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Functional Serum Response Elements Upstream of the Growth Factor-Inducible Gene *zif*268

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The *zif*268 gene, which encodes a protein with three typical zinc finger sequences, is induced in mouse 3T3 cells by serum, phorbol 12-myristate 13-acetate platelet-derived growth factor, and fibroblast growth factor. The induction is coordinate with that of c-*fos*. The 5′-flanking region of *zif*268 contains sequences that resemble known regulatory elements, including four CC(A or T)$_6$GG sequences similar to the core serum response elements (SREs) found upstream of c-*fos* and actin genes. To determine whether the *zif*268 SRE-like elements mediate induction, CAT (chloramphenicol acetyltransferase) plasmids with different lengths of *zif*268 upstream sequences were tested for inducibility in 3T3 cells by serum, platelet-derived growth factor, or phorbol 12-myristate 13-acetate. In addition, double-stranded oligonucleotides corresponding to each of the four *zif*268 putative SREs were tested individually for responsiveness when placed upstream of a thymidine kinase gene promoter. Each of the four SREs conferred inducibility by the agents tested, and multiple SREs resulted in greater inducibility than did a single element. Each of the *zif*268 SREs also competed with the c-*fos* SRE for binding by serum response factor present in HeLa cell nuclear extract. We conclude that the *zif*268 SRE-like sequences are functional and probably account for the coordinate induction of *zif*268 and c-*fos*.

Growth factors and other extracellular ligands rapidly induce the expression of genes that appear to mediate the cellular response to the ligand. Some of these immediate-early or early-response genes encode known or probable regulators of transcription. Among the induced genes of this type in murine 3T3 cells are *fos* (15), c-*jun* (20, 43), *junB* (42), *Krox-20* (6), *nur*77 (*NGFI-B*), (17, 30), *zif*268 (7) (also designated *NGFI-A* [29], *egr-1* [46], and *Krox-24* [24]), *fra*-1 (8), and *fosB* (51). Many of these genes are coordinately activated by serum or platelet-derived growth factor (PDGF) in mouse 3T3 cells (21, 22) and by epidermal growth factor or nerve growth factor in rat PC12 cells (2). Several also appear to be coordinately activated in hippocampal neurons of the rat during pharmacologically induced seizures (44).

The activation of *fos* by serum, PDGF, or epidermal growth factor has been shown to be mediated at least in part by an upstream *cis* element called the serum response element (SRE) (10–12, 14, 39, 47, 48). This 22-base-pair DNA sequence has dyad symmetry and is the binding site for a 67-kilodalton protein called the serum response factor (SRF) (40, 49). We and others (4, 7, 50) have found that the 5′ DNA sequence preceding the transcription start site of *zif*268 contains a number of potential binding sites for known transcription factors, including core Sp1 sites, an AP-2-like site, an AP-1-like site, two CCAAT sequences, a probable cyclic AMP response element, and four sequences that resemble the core sequence of the *fos* and actin SREs (4, 7, 50). All four of the *zif*268 SRE-like sequences contain a core of 10 nucleotides (CC[A or T]$_6$GG) present in the *fos* and actin SREs (34), but they differ in flanking sequence from the *fos* and actin signals and from each other. In view of the possibility that the coordinate induction of *fos* and *zif*268 by serum or growth factors is mediated by SRF binding to SREs, we determined whether the *zif*268 SRE-like sequences actually function as SREs and whether they bind SRF.

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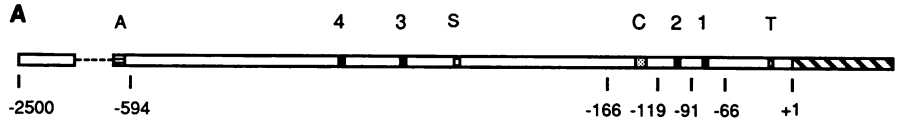
MATERIALS AND METHODS

Construction of *zif*-CAT fusions and deletions. A bacteriophage containing the *zif*268 mRNA sequences and genomic flanking sequences on both the 5′ and 3′ sides was isolated from a BALB/c embryonic DNA library (obtained from P. Leder) by using a cDNA probe. A *Bgl*II fragment of approximately 2.8 kilobase pairs (kb) containing the 5′ end of the cDNA and approximately 2.5 kb of 5′-flanking sequence was subcloned upstream of the chloramphenicol acetyltransferase (CAT)-coding sequence in plasmid pBLUECAT-2 (constructed by L. Sanders). The construct contains the 5′ end of the long open reading frame of the *zif*268 mRNA fused in frame with the CAT gene open reading frame. (However, it is not known whether this part of the open reading frame is actually translated in vivo or whether another AUG, not present in this fragment, is used.) This construct was previously shown to be inducible by serum in transient assays (7). 5′ deletions were made by using exonuclease III and S1 nuclease as previously described (7). The deletion endpoints were determined by dideoxynucleotide sequencing. The 5′ endpoint of the −2500 construct was estimated from the electrophoretic mobility of the cloned fragment. For analysis of individual SRE-like elements, oligonucleotides corresponding to both strands of each putative SRE and to the c-*fos* SRE were synthesized with *Bam*HI and *Bgl*II ends. Each pair of oligonucleotides was annealed and cloned into the *Bam*HI site of plasmid pBLCAT-2, which contains the thymidine kinase (TK) gene promoter upstream of the CAT-coding sequences (28). The sequences of the insertion sites were determined, and for purposes of comparison, clones were selected that contained a single copy of each oligonucleotide in the naturally occurring orientation.

Cell culture and transfections. NIH 3T3 cells were cotransfected with the *zif*-CAT plasmid and pCH110, a plasmid containing the β-galactosidase-coding sequences under the control of the simian virus 40 early promoter (16), as previously described (7). After transfection, cells were starved for 2 days in the presence of 0.5% fetal bovine serum



B

TGCTTCCCATATATGGCCATGT	268-1
GTCCTTCCATATTAGGGCTTCC	268-2
CCAGCGCCTTATATGGAGTGGC	268-3
GAACGCCATATAAGGAGCAGG	268-4
GGATGTCCATATTAGGACATCT	fos

FIG. 1. (A) Physical map of the upstream region of *zif*268 and sequences of SRE-like elements. Symbols: ☐, sequences 5′ to the start of transcription; ▨, sequences corresponding to transcribed sequences; ---, region between −600 and approximately −2500, which is not shown on this map. Numbers below the line indicate locations of 5′ endpoints of CAT deletion constructs. Sequences related to known regulatory elements are indicated by boxes and symbols above the line: T, TATA box; C, cyclic AMP response element-like element; S, Sp1 core sequences; A, AP-1-like site. Numbers 1 to 4 indicate the positions of four SRE-like elements located in the *zif*268 promoter, with the most proximal element designated as 1. (B) Sequences of the SRE-like elements in comparison with the *fos* SRE sequence.

(FBS). At appropriate times before harvest, cells were stimulated by addition of 20% FBS, 10 ng of PDGF (BB homodimer; Genzyme Corp.) per ml, or 2 ng of phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co.) per ml. After harvest, the CAT and β-galactosidase activities were determined (13, 31). In Results, CAT activity is expressed as percent conversion per unit of β-galactosidase to correct for differences in the efficiency of transfection and protein content in the various extracts.

DNA-binding assay. Double-stranded oligonucleotides corresponding to the c-*fos* SRE were labeled with [^{32}P]dATP and [^{32}P]dCTP, using the Klenow fragment of DNA polymerase I, as described previously (36). HeLa nuclear extract, prepared by the method of Dignam et al. (9), was generously provided by E. Perez-Albuerne. Binding was carried out in 25 μl of 10 mM Tris (pH 7.5)–50 mM NaCl–1 mM dithiothreitol–1 mM EDTA–5% glycerol containing 50 μg of sonicated salmon sperm DNA per ml. HeLa cell nuclear extract (9.7 μg per reaction) was incubated without ^{32}P-labeled DNA at room temperature for 10 min; ^{32}P-labeled fragment was added to a final probe concentration of 0.5 nM, and the reaction mixtures were incubated 20 min longer at room temperature. Competitors used were double-stranded oligonucleotides corresponding to the various SREs and were included in the preincubation step (before addition of labeled fragment). After incubation, 5 μl of 0.1% bromphenol blue–0.1% xylene cyanol was added, and the entire reaction mixture was loaded onto a 6% polyacrylamide gel (30 parts of acrylamide/0.8 parts of bisacrylamide) and run in 0.25× TBE (TBE is 89 mM boric acid, 89 mM Tris base, and 2 mM EDTA). Electrophoresis was carried out at 175 V at room temperature, with recirculation of the buffer.

RESULTS

Effect of progressive deletion of *zif*268 SRE-like elements on gene activation. In the first series of experiments to be described, a recombinant *zif*-CAT fusion gene was used for analyzing the effect of deleting SRE-like sequences on serum or growth factor induction of the downstream CAT gene. To construct the test plasmid, a genomic *zif*268 clone containing the entire gene and 5′- and 3′-flanking sequences was first isolated from a BALB/c mouse embryo library. A 2.8-kb *Bgl*II fragment derived from this clone and containing approximately 2.5 kb of sequence upstream of the transcription start site and 339 base pairs corresponding to the 5′ end of the mRNA was ligated into the *Bgl*II site of the vector pBLCAT-3 (28) such that the coding sequence of *zif*268 was fused in frame to the first codon of the CAT gene. We have shown previously that when a plasmid containing this construct was used to transfect NIH 3T3 cells, CAT activity was induced by serum (7). 5′ deletions of the *zif*-CAT recombinant were made by using exonuclease III, and the endpoint of each deletion was determined by DNA sequencing (Fig. 1). Each construct was transfected into NIH 3T3 cells and tested for inducibility of CAT activity by 20% fetal bovine serum, PMA, or PDGF as described in Materials and Methods.

Both the full-length plasmid and the −594 plasmid, which have all four SRE-like elements (Fig. 1), showed marked stimulation of CAT expression by serum, PMA, and PDGF (Fig. 2). The −166 and −119 constructs, which had lost the two distal SRE-like sequences (Fig. 1), retained responsiveness to serum, PMA, and PDGF, but both the basal level and the stimulated level of CAT were considerably lower than was seen with the full-length plasmid or the −594 construct. The −91 construct, with one remaining SRE-like sequence (Fig. 1), showed a very low basal of CAT expression and slight or no response to the three stimulants, whereas the −61 construct, with no SRE-like sequences, had no detectable basal or stimulated expression. In sum, in the construct containing the most proximal element (SRE 1) alone, there was little activity, the construct with SREs 1 and 2 was more active, and the construct with SREs 1 to 4 was most active. Since sequences other than SREs had been deleted and the basal level of CAT expression was affected by deletion, it is not clear from these results whether SRE 1 is active or what the relative activity of each SRE is. However, the results suggest that all four SREs together are more active than the two proximal SREs.

Activity of individual SRE-like elements. To determine whether the single *zif*268 SREs are functional, oligonucleotides corresponding to each *zif*268 SRE and to the c-*fos* SRE

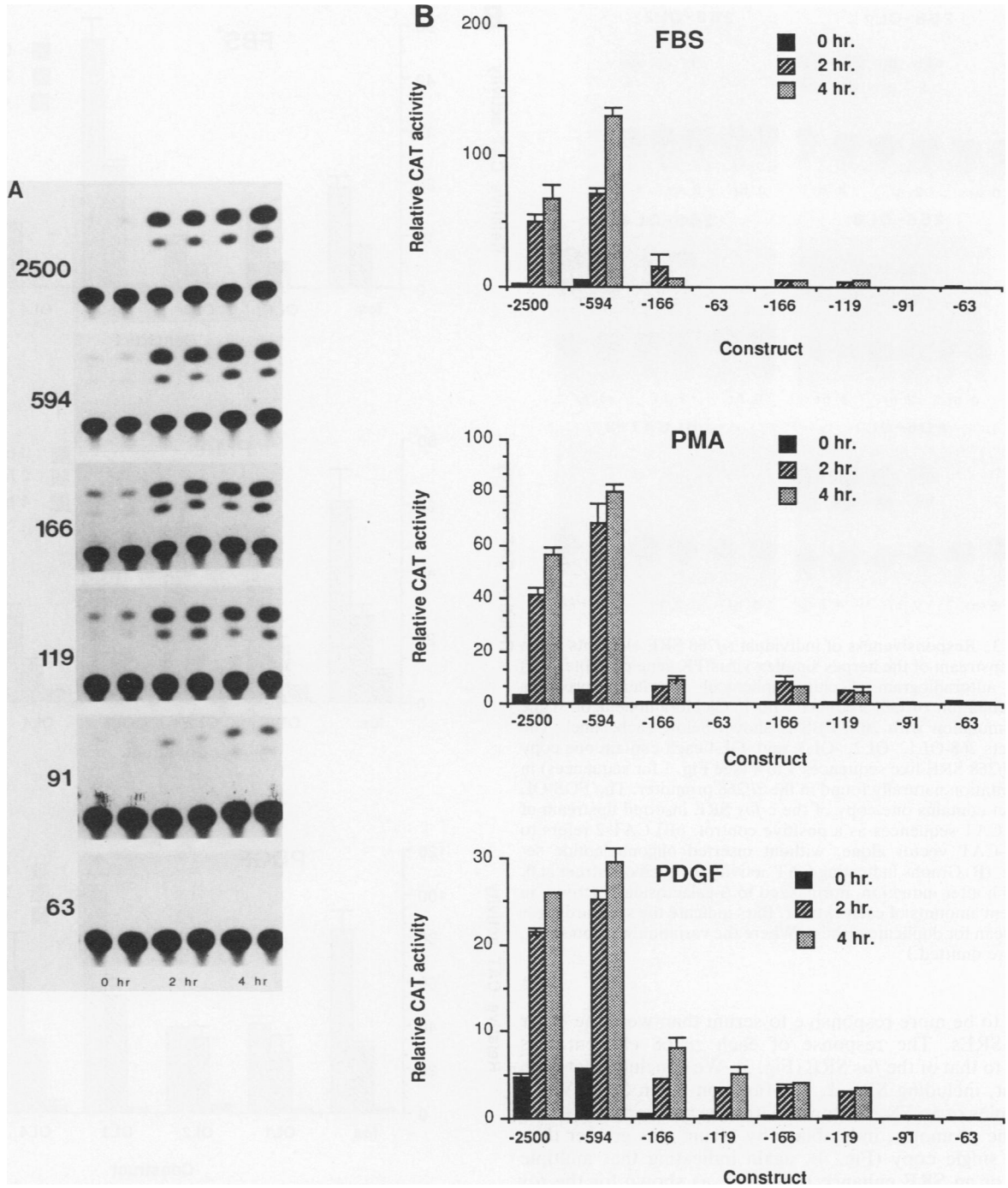
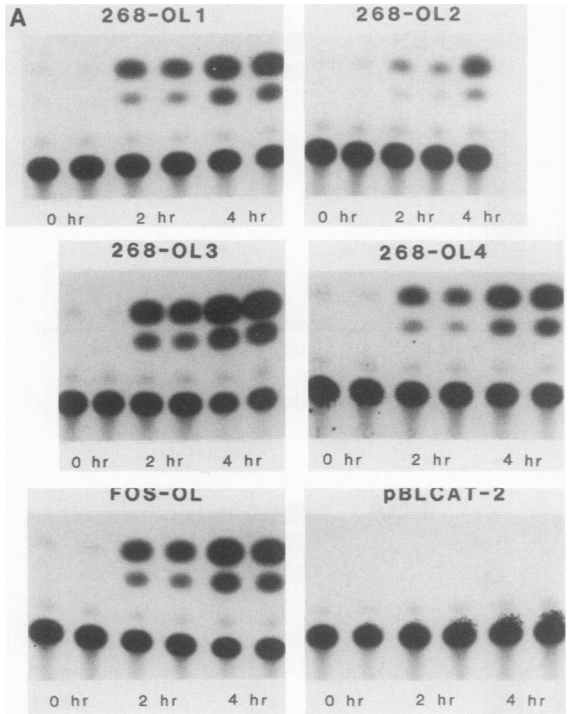


FIG. 2. Induction of CAT activity in cells transfected with *zif*-CAT plasmids after treatment with 20% FBS, 2 ng of PMA per ml, or 10 ng of PDGF per ml. NIH 3T3 cells were cotransfected with *zif*-CAT plasmids and the β-galactosidase plasmid pCH110 and were then serum-starved and stimulated as described in the text. (A) Typical autoradiogram of chloramphenicol acetylation products after incubation of extracts of serum-stimulated cells with [^{14}C]chloramphenicol. The times after serum addition are given below the panels; numbers to the left indicate the amount of 5′-flanking sequence retained in each construct (Fig. 1). Similar autoradiograms were obtained after PMA and PDGF induction. (B) Graphs indicating CAT activity for each construct at 0, 2, and 4 h after induction, normalized to the β-galactosidase activity in equivalent amounts of each cell extract. Bars indicate the standard error of the mean for duplicate dishes. (Where the variation was too small, bars were omitted). In each panel, two separate experiments have been combined. In the case of FBS and PMA, experiment 1 included constructs −2500 to −63.

were synthesized with *Bam*HI and *Bgl*II ends and ligated into the *Bam*HI site of the vector pBLCAT-2 (28) upstream of the TK gene promoter of the vector (−105 to +51), which controls the CAT gene. Cells were then transfected with each construct, and induction of CAT by serum, PDGF, or PMA was determined (Fig. 3). (Less extensive experiments with fibroblast growth factor gave similar results [data not shown].) All oligonucleotides were active, and SRE 3 ap-



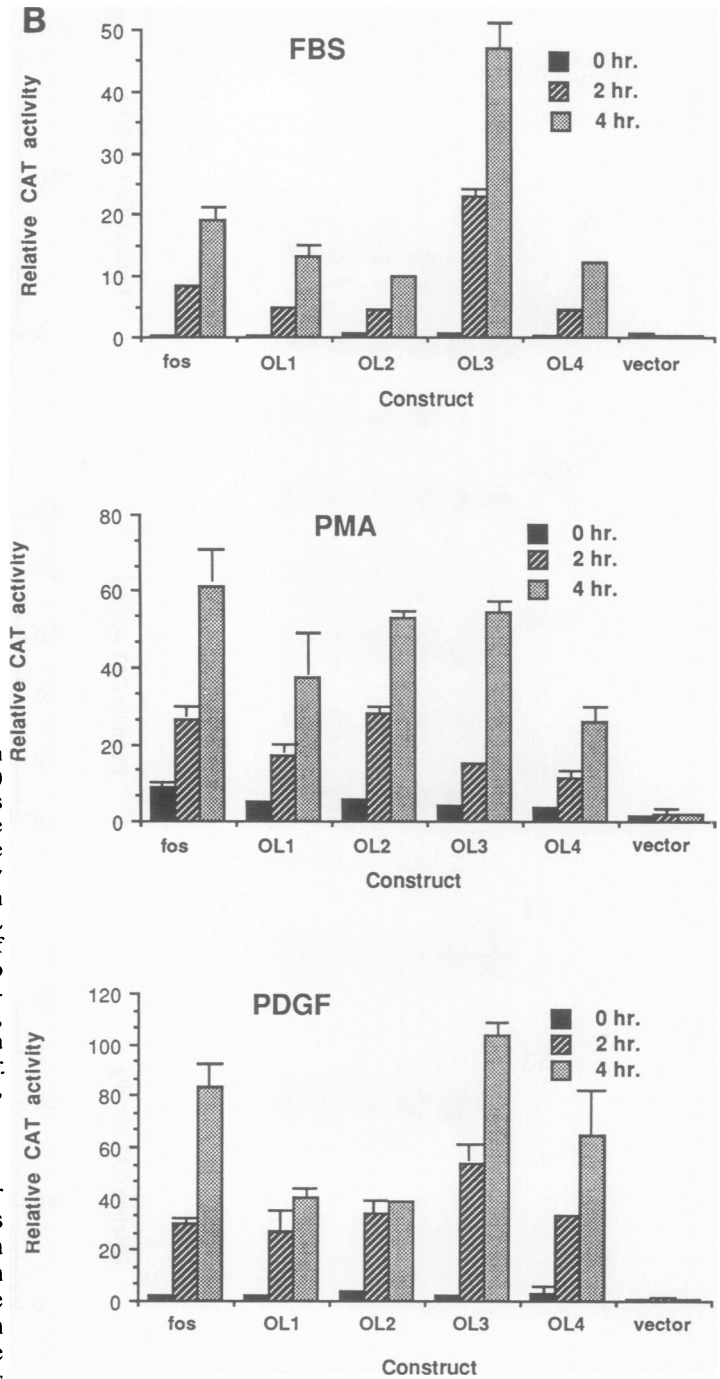


FIG. 3. Responsiveness of individual *zif*268 SRE elements when cloned upstream of the herpes simplex virus TK gene promoter. (A) Typical autoradiogram of chloramphenicol acetylation products after incubation of cell extracts with [^{14}C]chloramphenicol. Time after stimulation with 20% FBS is shown below each panel. The constructs 268-OL1, -OL2, -OL3, and -OL4 each contain one copy of the *zif*268 SRE-like sequences 1 to 4 (see Fig. 1 for sequences) in the orientation naturally found in the *zif*268 promoter. The FOS-OL construct contains one copy of the c-*fos* SRE inserted upstream of the TK-CAT sequences as a positive control. pBLCAT-2 refers to the TK-CAT vector alone, without inserted oligonucleotide sequences. (B) Graphs indicating CAT activity for each construct at 0, 2, and 4 h after induction, normalized to β-galactosidase activity in equivalent amounts of each extract. Bars indicate the standard error of the mean for duplicate dishes. (Where the variation was too small, bars were omitted.)

peared to be more responsive to serum than were the other *zif*268 SREs. The response of each *zif*268 element was similar to that of the *fos* SRE (Fig. 3). We conclude that each element, including SRE 1, can function as an SRE. When two copies of an SRE oligonucleotide were inserted 5′ to the TK gene promoter, inducibility by serum was greater than with a single copy (Fig. 4), again indicating that multiple copies of an SRE enhance the effect, as shown for the *fos* SRE (48). Also shown in Fig. 4 is the importance of the spacing between the C · G pairs in the core SRE sequence. When one A · T pair was deleted in the OL2 core sequence, giving the sequence (GTCCTTCCATATAGGGCTTCC), the oligonucleotide did not confer serum responsiveness. In contrast, deletion of a base pair in the sequence flanking the core had no effect (Fig. 4).

Binding of *zif*268 SREs by SRF. The *fos* and *zif*268 SREs share the core CC(A or T)$_6$GG sequence, but the *zif*268 SREs lack the flanking symmetrical sequences present in the *fos* SRE (47). To determine whether the various SREs are bound by the same protein, we used a nuclear extract from Hela cells as a source of SRF (48) and determined whether each of the *zif*268 SREs inhibits the binding of *fos* SRE by SRF. Each of the *zif*268 SRE oligonucleotides competed with the *fos* SRE for binding by the nuclear extract, although SRE 2 was less active than the others (FIG. 5a). (Note that the SRE 2 core sequence is identical to that of the *fos* SRE.) We conclude that all four *zif*268 SREs are binding sites for the SRF, but either the precise AT sequence of the core or the flanking sequences affect the efficiency of binding. When a modified SRE sequence containing five A · T pairs as spacer was tested, it competed very poorly with the *fos* SRE for binding by HeLa cell SRE (Fig. 5b). A naturally occurring SRE-related sequence containing five A · T base pairs from the *zif*268 promoter (nucleotides −341 to −320) was also tested in this assay (core sequences

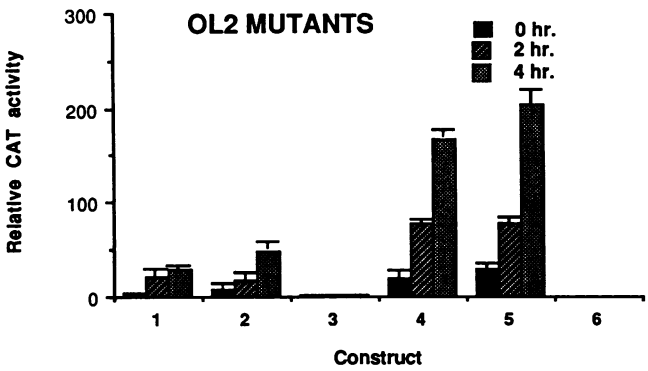


FIG. 4. Serum responsiveness of plasmids containing mutations in the OL2 SRE sequences. NIH 3T3 cells were cotransfected with pCH110 and with the OL2 oligonucleotide (which contains the wild-type *zif*268 SRE2 sequences 5′ of the TK-CAT sequences in pBLCAT-2) or with plasmids carrying mutations in the SRE sequences. After serum deprivation and stimulation by 20% FBS, the amount of CAT activity of cell extracts was determined and normalized to the amount of β-galactosidase activity. Columns: 1, OL2 wild-type plasmid; 2, a derivative of OL2 that contains a deletion of one nucleotide in the flanking sequences of the SRE (GTCCTTC CATATTAGGGCTTCC); 3, a derivative of OL2 that contains a deletion in the core A and T residues (GTCCTTCCATATAGGGCT TCC); 4 and 5, two separate constructs containing two tandem copies of the OL2 SRE sequences; 6, pBLCAT-2 vector alone.

..CCAATATGG..) and failed to compete with the *fos* SRE for binding (data not shown).

DISCUSSION

In 3T3 cells, the *zif*268 promoter appears to be activated by serum, PDGF, and PMA, at least in part, via its four SREs. Deletion of upstream sequences that include all four SREs abolished induction of a downstream gene, and the presence of multiple SREs led to greater induction than did the presence of a single SRE. Our deletion experiments, however, do not exclude a role for other upstream sequences in induction by serum or the other inducers tested. When assayed separately in a TK-CAT construct, each *zif*268 SRE was about as active as the *fos* SRE. This result correlated with the ability of the *zif*268 SREs to compete with the *fos* SRE for binding to SRF present in a nuclear extract of HeLa cells, although the poorer competition by SRE2 was not reflected in the expression assay. Taken together, the results strongly support the hypothesis that in 3T3 cells the coordinate activation of *fos* and *zif*268 by the ligands tested is mediated, at least in part, by the binding of SRF to the respective SREs.

Previous studies of serum inducibility of *Krox-20*, another immediate-early gene related to *zif*268, also implicate SREs in the coordinate responsiveness of immediate-early genes (5). Why *zif*268 has four functional SREs, *Krox-20* has two, and *fos* has only one is not clear. One would expect that at a limiting concentration of SRF, *zif*268 would be induced to a greater extent than *fos*. In 3T3 cells, *zif*268 appears to be

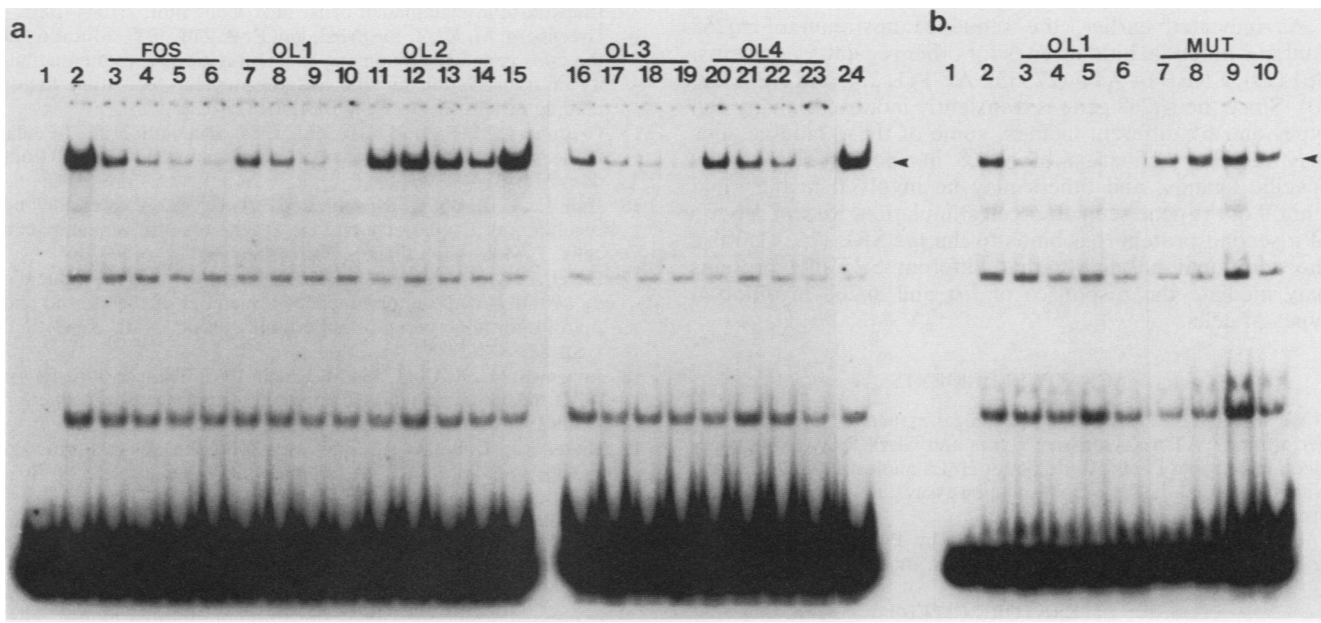


FIG. 5. (A) Competition between *zif*268 SREs and the c-*fos* SRE for HeLa cell SRF. Binding reactions were carried out as described in the text, using 9.7 μg of HeLa nuclear extract protein and 0.5 nM ^{32}P-labeled c-*fos* SRE oligonucleotide per reaction. Oligonucleotide competitor DNAs were incubated with the extracts before addition of labeled DNA. The arrowheads point to the specific complexes formed upon incubation with the c-*fos* SRE. Lanes: 1, probe alone (no extract); 2, 15, and 24, no competitor; 3 to 6, competition with the c-*fos* double-stranded oligonucleotide itself; 7 to 10, competition with OL1; 11 to 14, competition with OL2; 16 to 19, competition with OL3; 20 to 23, competition with OL4. Competitor DNA was used in 2-fold (lanes 3, 7, 11, 16, and 20), 5-fold (lanes 4, 8, 12, 17, and 21), 10-fold (lanes 5, 9, 13, 18, and 22), and 25-fold (lanes 6, 10, 14, 19, and 23) molar excesses of competitor DNA over probe DNA. (B) Effect of deletion of one of the core AT nucleotides on the ability of the OL1 oligonucleotide to compete with the c-*fos* SRE element for SRF binding. Binding reactions were carried out as described above. The arrowhead points to the complexed c-*fos* SRE. Lanes: 1, probe alone (no extract); 2, no competitor; 3 to 6, competition with the OL1 wild-type oligonucleotide; 7 to 10, competition with the OL1 mutant oligonucleotide (core sequence CCATATAGG). Competitor DNA was used in 2-fold (lanes 3 and 7), 5-fold (lanes 4 and 8), 10-fold (lanes 5 and 9), and 50-fold (lanes 6 and 10) molar excesses of DNA over probe DNA.

induced slightly earlier than *fos*, but there is little difference in the degree of induction (22). Possibly, other factors influence induction of each gene by serum growth factors. In this regard, there is evidence that a protein that binds to a sequence adjacent to the core SRE of *fos* is involved in serum responsiveness (45). This putative protein-binding site is not present in the sequence flanking the core SRE of any of the *zif*268 elements. Thus, the *fos* response, but not that of *zif*268, may be enhanced by binding of a second protein adjacent to the SRE.

Whereas the SREs of *fos* have extensive dyad symmetry outside the core sequence, the SREs of *zif*268 do not. Both methylation protection and interference experiments (34) and mutational analysis (11, 14, 25) indicated that the C · G base pairs of the *fos* core SRE are important for activity, and the *zif*268 mutant SRE missing one core A · T pair confirms that spacing of the CC-GG contact sites is important. Other functional SREs, notably four such elements upstream of the human α-cardiac actin gene (32) and two upstream or in the first intron of *Krox-20* (5), also do not show the dyad symmetry external to the core CC(A or T)$_6$GG of the *fos* SRE. In the case of the α-cardiac actin SREs, one of the core sequences contains a G · C pair in the A+T-rich sequence; hence, the α-cardiac actin elements have been called the CArG box (for CC[A + T rich]$_6$GG) (32, 38). Like the four *zif*268 SREs, both the CArG boxes upstream of the α-cardiac actin gene and the SREs found in Krox-20 also compete with the *fos* SRE for binding to SRF prepared from HeLa cell nuclei (3, 5). Therefore, the SRF can bind to DNA sequences that retain the CC(A or T rich)$_6$GG core sequence. However, the relative affinity of SRF for different sites is influenced by flanking sequences (25, 37; Fig. 5).

As indicated earlier, the sequence upstream of *zif*268 contains potential binding sites for other regulatory proteins: Sp1 (19), CREB or ATF (27, 35), AP-1 (1, 23), and AP-2 (18, 33). Since the *zif*268 gene is transiently induced in many cell types and by different ligands, some of these binding sites may mediate activation of *zif*268 in specific cells or by specific ligands, and others may be involved in the rapid shutoff of the gene seen after cell stimulation. Recent reports of a second protein that binds to the *fos* SRE (26, 41) raise the additional possibility that different SRF-like proteins may mediate the responses of *fos* and *zif*268 in different types of cells.

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