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Point mutational analysis of the human c-*fos* serum response factor binding site

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ABSTRACT

A series of point mutants were generated in the human c-fos dyad symmetry element (DSE), found within the c-fos serum response element, to study the sequence requirements for its interaction with the human HeLa cell serum response factor (SRF). Plasmids that contain base substitutions within a core $CC(A/T)_6GG$ motif in the center of the DSE did not compete, or competed very poorly, with the wild-type c-fos DSE for formation of a specific SRF-DSE complex in vitro. The $CC(A/T)_6GG$ motif is not sufficient for maximal binding of SRF, as several plasmids that contain base substitutions in the sequences flanking this core motif competed either poorer or better than the wild-type c-fos DSE for SRF binding. Evidence is presented that supports the idea that SRF binds in a symmetrical fashion. Results of in vivo transient expression assays in HeLa cells suggest that negative regulation of c-fos transcription observed in serum-deprived cells is mediated through SRF binding to the DSE.

INTRODUCTION

Transcription of the proto-oncogene, c-fos (see refs. 1-2 for review), is rapidly and transiently induced by serum growth factors in many cell types (3-5). The serum-inducibility of c-fos transcription correlates with the presence of an element, known as the serum response element (SRE), located 300 bp upstream of the c-fos mRNA start site (6). The SRE has enhancer-like properties (6-7), and a dyad symmetry element (DSE) sequence within the SRE has been identified which alone confers serum-inducibility (8-9). Deletion of the SRE (6), or deletion of one bp within the DSE (9), abolishes serum-inducible transcription from the human c-fos promoter in transiently transfected mouse NIH-3T3 cells in vivo. The DSE is a binding site for a specific protein, known as the serum response factor (SRF), present in mammalian and amphibian nuclei (8-15). SRF has been extensively purified from human HeLa cell nuclei by DNA affinity column chromatography (13-15).

Sequences homologous to the c-fos DSE have been identified in the Xenopus laevis cytoskeletal actin (12-13), and the human cytoskeletal β- (16-18) and γ-actin (19) genes. The c-fos and Xenopus actin DSE sequences both confer

serum-inducibility to heterologous promoters *in vivo* (12-13) and are thought to contain constitutive transcription stimulatory activity (7, 12-13). The *Xenopus* actin DSE sequences differ at a number of nucleotide positions from the human c-*fos* DSE (12-13). This sequence variation allows the HeLa SRF to bind the *Xenopus* actin DSE with a detectably greater affinity than the c-*fos* site. It would be of interest, therefore, to investigate the effect of specific base changes in the human c-*fos* DSE on its binding to the HeLa SRF.

In this paper we describe the generation of random point mutations in the human c-*fos* DSE, and the analysis of the *in vitro* binding of SRF, present in crude HeLa cell nuclear extracts, to these mutants by gel mobility shift (20-21) assays. Point mutations in the centre of the DSE, within the $CC(A/T)_6GG$ motif, which is identical in human and mouse c-*fos* (22-23) and is highly conserved between the mammalian and chicken cytoskeletal β-actin (16-18, 24-25) genes, adversely affects SRF binding. In addition, certain point mutations flanking the $CC(A/T)_6GG$ motif also reduce or enhance SRF binding, suggesting that these adjacent nucleotides play a role in the SRF-c-*fos* DSE interaction. The similar effects of complementary pairs of base substitutions about the centre of the DSE suggests that SRF binds to the c-*fos* DSE in a symmetrical fashion. We also describe the analysis of transcription from the wild-type and DSE point mutant c-*fos* promoters by *in vivo* transient transfection assays in HeLa cells. DSE mutations that reduce SRF binding *in vitro* increase c-*fos* promoter activity in serum-deprived cells *in vivo*, suggesting that SRF may mediate the negative regulation of c-*fos* transcription in HeLa cells.

MATERIALS AND METHODS

Preparation of HeLa Cell Nuclear Extracts.

Nuclear extracts were prepared from suspension culture HeLa S-3 cells according to the modified Dignam procedure (26) described in Wildeman *et al.* (27), except that several protease inhibitors were added to all the buffers (5 μg/ml each of leupeptin, antipain and pepstatin A, and 0.5 mM PMSF).

In Vitro Mutagenesis.

The templates used for *in vitro* mutagenesis were mp19fos (Fig. 2), which contains the human c-*fos* *Xho*I-*Nae*I fragment from -711 to +42 (23) inserted into the *Sal*I-*Sma*I sites of M13mp19 (28), and pAdSV (Fig. 3), which contains the adenovirus-2 major late promoter (MLP) from -54 to +33 inserted into the *Sma*I-*Bam*HI sites of pUC12 (29-30).

Point mutations were generated in the human c-*fos* DSE by oligonucleotide-directed mutagenesis using two methods: (a) A 28-mer oligonucleotide (oligo),

CGCAGATGTCCTAATATGGACATCCTGT, complementary to positions -323 to -296 of the human c-fos promoter (23), was synthesized using an Applied Biosystems DNA synthesizer. This oligo was synthesized containing random mutations by the cross-contamination of each of the four nucleoside phosphoramidites with approximately 1.7% of each of the other three, as described in Hutchison et al. (31). This "doped" oligo was hybridized to a uracil-containing mp19fos single-stranded DNA template prepared by propagation of the mp19fos phage in dut$^-$ ung$^-$ BW313 (32). Second strand synthesis was performed as described in Grundstrom et al. (33), followed by transformation of JM107. Individual plaques were picked, and single-stranded DNA was isolated and sequenced by the dideoxy chain termination technique (34) using an oligo primer, GGCTCCCCCCAGGGCTA, complementary to -264 to -248 of the human c-fos promoter (23). Approximately 30% of the templates sequenced contained a mutated DSE. (b) Two "doped" oligos, CAGGATGTCCATATTAGGACATCTGC (26-mer), consisting of positions -322 to -297 of the human c-fos DSE (23) plus a C residue at the 3' end, and CCGGGCAGATGTCCTAATATGGACATCCTGAGCT (34-mer), complementary to the former oligo and including the cohesive ends of the SstI and XmaI, were synthesized as described above. 650 pmol of each of the purified oligos were annealed according to the method described in Hutchison et al. (31), and an aliquot of the annealing reaction was then ligated to SstI- and XmaI-digested (XmaI and SmaI are isoschizomers) pAdSV, followed by transformation of DH-1 cells. Individual colonies were picked, and the plasmid DNA isolated was analyzed by restriction enzyme digestions. Plasmids containing a single DSE insert were sequenced as above (34), following a procedure (35) modified for double-stranded plasmids using the Boehringer Mannheim reverse sequencing primer. Approximately 25% of the sequenced plasmids contained a mutated DSE.

Preparation of Plasmid and Template DNA.

Plasmid DNA was purified by ultra-centrifugation through CsCl gradients twice as described in Maniatis et al. (36). The concentration of DNA was measured by optical density at 260 nm and the quality of plasmid DNA was determined by visualization of EthBr-stained DNA electrophoresed through 1% agarose gels. Greater than 90% of the plasmid DNA was in a supercoiled form.

pAdSF, containing the wild-type human c-fos DSE from -322 to -297 (23) in the SstI-XmaI sites of pAdSV (see Fig. 3), was digested with EcoRI and SmaI. The wild-type DSE fragment was purified by polyacrylamide gel electrophoresis and was radio-labelled at the EcoRI site by 3'-end repair using the Klenow fragment of DNA polymerase I in the presence of α-[^{32}P]-dATP (36).

Gel Mobility Shift Assay.

The gel mobility shift assay using HeLa nuclear extracts was as described previously (20-21), with a few modifications. For experiments involving unlabelled competitors of pAdSF and its mutants, 0.33 ng (26 fmol) of the ^{32}P-labelled template was used per reaction. The wild-type plasmid pAdSF, or its mutants, were added such that each reaction contained a 5-, 20-, or 80-fold molar excess of the unlabelled plasmid competitor relative to the labelled template; pAdSV was added to each reaction to bring the total amount of unlabelled plasmid to 2 μg. The labelled template was mixed with 1 μg each of poly(dI:dC)-poly(dI:dC) and poly(dA:dT)-poly(dA:dT) (Pharmacia), 1 μg MspI-digested pUC18 (37) and 2 μg of the unlabelled plasmid competitors in a 10 μl volume containing 80 mM KCl and 2 mM EDTA. A 10 μl volume of nuclear extract (5-30 μg) and nuclear extract buffer (20 mM Hepes (pH 7.9), 1 mM $MgCl_2$, 20 mM KCl, 2 mM DTT, 17% glycerol and protease inhibitors) was added; the reaction mixture was incubated at room temperature (RT) for 25 min and put on ice for 5 min. The reaction mixtures were loaded directly onto a 4% polyacrylamide (30:1::acrylamide:bis-acrylamide) gel that had been pre-electrophoresed in 0.25 X TBE (36) buffer for 2 h at 150 V at RT. After electrophoresis for 2 h at 150 V at RT, the gel was dried and exposed to Kodak XAR X-ray film with a DuPont Cronex intensifying screen at -70°C.

For experiments involving unlabelled competitors of mp19fos and its mutants, the procedure was as described above, except that 0.11 ng (9 fmol) of ^{32}P-labelled template was used, and M13mp19 or M13mp18 was used to bring the amount of unlabelled plasmid in each reaction up to 2 μg.

In Vivo Transfection Assay.

The pGβ promoter-less in vivo expression vector (see Fig. 6) contains the rabbit β-globin gene from -9 to +1650 present in the HindIII-AatII fragment of $pSEG_0$ (38) inserted into the HindIII-AatII sites of pUC18 (modified by the insertion of a ClaI linker at the repaired EcoRI site). pRGβ was constructed by inserting the repaired NdeI-HindIII fragment of pRSVCAT (39), containing the RSV LTR from -487 to +38, into the SmaI-HindIII sites of pGβ. pfGβ was constructed by inserting the SacI-NaeI fragment of pF4 (6), containing the human c-fos promoter from -732 to +44, into the SacI-SmaI sites of pGβ.

1 X 10^6 Hela cells cultured in α medium plus 10% fetal bovine serum (FBS) were transfected with 20 μg pfGβ or mutants derived thereof, 1-5 μg pRGβ plus salmon sperm DNA to a total of 30 μg by calcium phosphate-DNA coprecipitation as previously described (40). The medium was decanted after 24 h; the cells were washed once with PBS and once with serum-free α medium, and incubated a

further 24 h in α medium plus 0.5% FBS. Transfected, serum-deprived cells were serum-stimulated by addition of FBS to 15% for 30 min. Total cell RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction (41), and treated with DNase I as described in Frederickson et al. (42). 20 μg RNA was analysed by primer extension mapping exactly as described recently (42).

RESULTS

A specific factor in HeLa nuclear extracts interacts with the human c-fos DSE in vitro.

A sensitive gel mobility shift assay (20-21) was used to confirm that SRF (13-15), a protein present in human HeLa cell nuclei, binds specifically to the human c-fos DSE in vitro. It had been shown previously that a 29 bp oligonucleotide spanning the c-fos DSE (from -322 to -297) is sufficient to form a specific complex with the HeLa SRF in a gel mobility shift assay (9). Therefore, we prepared a similar ^{32}P-labelled c-fos DSE probe (EcoRI-SmaI fragment of pAdSF, see Materials and Methods) and assayed its ability to bind the HeLa SRF in the presence of unlabelled plasmid DNA competitors (Fig. 1). With increasing amounts of nuclear extract, two major shifted bands (C_s and C_{ns1}), representing two protein-DNA complexes, were observed. The appearance of a third shifted band, C_{ns2}, which is a non-specific protein-DNA complex, is not reproducibly observed. The fact that C_{ns1} cannot be competed out by unlabelled plasmid competitors containing or lacking the human c-fos DSE suggests that this band also represents a non-specific protein-DNA complex.

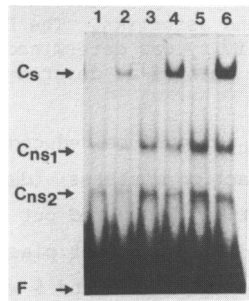

Fig. 1. Titration of a HeLa nuclear extract for SRF binding activity. Approximately 0.33 ng of the 38 bp ^{32}P-labelled EcoRI-SmaI fragment of pAdSF was incubated with 8 (lanes 1, 2), 16 (lanes 3, 4) or 24 (lanes 5, 6) μg HeLa nuclear extract and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. The competitors used were pAdSF (lanes 1, 3, 5) and pAdSV (lanes 2, 4, 6). Symbols: F, free probe; C_s, specific SRF-DSE complex; C_{ns1} and C_{ns2}, major and minor non-specific complex.

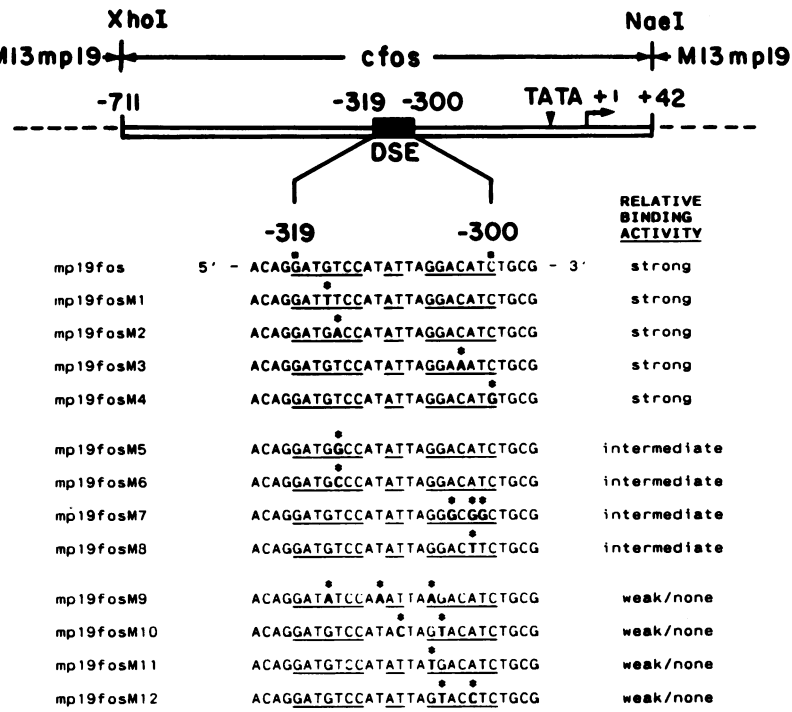

Fig. 2. Structure and SRF binding activity of human c-fos DSE point mutants in an M13mp19 vector. The human c-fos promoter sequence is represented by boxed lines. The position of the DSE is indicated by the solid box. M13mp19 vector sequences are represented by dotted lines. The nucleotide sequences from -323 to -296 are listed for the wild-type c-fos (mp19fos) promoter and for each of the DSE point mutants (mp19fosM1 to M12). Base substitutions are indicated by bold lettering and asterisks. The DSE inverted repeat is underlined. Relative binding activity was determined as described in Materials and Methods, and mutants were defined as described in the legend to Figure 4.

Moreover, the intensity of C_{ns1} could be reduced by increasing the amount of MspI-digested pUC18 in the reaction mixtures (data not shown). In contrast, the upper band, C_s, was effectively competed out by the c-fos DSE-containing plasmid, pAdSF, but not by pAdSV, the parent plasmid into which the c-fos DSE was inserted (Fig. 1, compare lanes 1, 3 and 5 with lanes 2, 4 and 6), thus demonstrating that C_s represents the specific SRF-c-fos DSE complex.

Generation of specific base substitutions in the human c-fos DSE.

To study the nucleotide sequence requirements for efficient in vitro DSE binding by the HeLa SRF, random point mutations were generated in the human c-fos DSE by oligonucleotide-directed mutagenesis. To facilitate functional studies in vivo (see below), a series of point mutations, the mp19fos series,

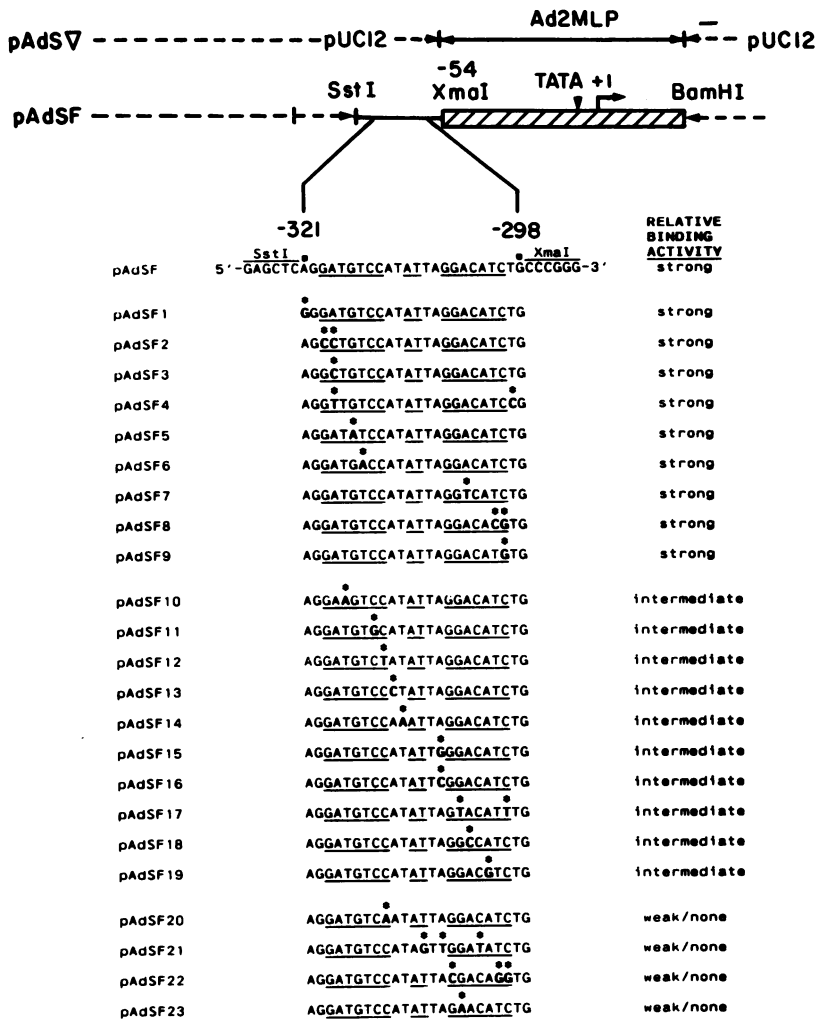

Fig. 3. Structure and SRF binding activity of human c-fos DSE point mutants in a chimaeric DSE-MLP in vitro expression vector. The MLP sequence from -54 to +33 (in pAdSV, see Materials and Methods) is represented by the hatched box. pUC12 vector sequences are represented by dotted lines. The nucleotide sequences of the wild-type human c-fos DSE (pAdSF) and mutants (pAdSF1 to F23) from -321 to -298 inserted between the SstI and XmaI sites are listed. Base substitutions and the DSE inverted repeat are as indicated in Figure 2. Relative binding activity was as described in the legend to Figure 4.

was created between positions -319 and -300 in the natural c-fos promoter (see Materials and Methods and Fig. 2). Another series of point mutations was generated in a chimaeric construction in which the c-fos DSE from -321 to

-298 was inserted directly upstream of the TATA box of the MLP (see Materials and Methods and Fig. 3). These chimaeric promoter constructs, the pAdSF series, were generated to facilitate the development of a functional in vitro assay for SRF activity. We analyzed 12 point mutants in the natural c-fos promoter (mp19fos series) and 23 point mutants in the chimaeric c-fos DSE/MLP (pAdSF series). The nucleotide sequence of one strand of each of these mutants was confirmed by the dideoxy chain termination technique (34-35) prior to the analyses described below.

Gel mobility shift competition assays indicate varying competitive strengths for different human c-fos DSE point mutants.

We tested the ability of the mp19fos and pAdSF series of mutants (Figs. 2 and 3) to compete for DSE binding in vitro. The 38 bp EcoRI-SmaI fragment of pAdSF was ^{32}P-labelled as before and used as probe in the gel mobility shift assay. A panel of representative gel mobility shift competition experiments is shown in Fig. 4. Unlabelled competitor plasmids were diluted to 5-, 20-, and 80-fold molar excess relative to the ^{32}P-labelled DSE probe; the total amount of competitor DNA was adjusted to 2 μg for each reaction by addition of pAdSV or M13mp19. The competitive strength of a mutant, as a function of concentration, was compared to that of pAdSF (Fig. 4A, pAdSF) or mp19fos (Fig. 4B, mp19fos). The parent plasmids, pAdSV and M13mp19, were used as the negative competitor controls in Figures 4A and 4B, respectively. We observed that different mutants had varying competitive strengths in the gel mobility shift assays. To facilitate analysis, we separated the DSE point mutations into three classes according to their ability to compete for SRF binding: (1) strong - competitors that compete as well as (Figs. 4A, pAdSF5, and 4B, mp19fosM1), or better than (Figs. 4A, pAdSF7, and 4B, mp19fosM2) pAdSF or mp19fos, (2) weak - competitors that compete as poorly as pAdSV (Fig. 4A, pAdSF21 and F22) or M13mp19 (Fig. 4B, mp19fosM9 and M10), and (3) intermediate - competitors that compete poorer than pAdSF or mp19fos but better than pAdSV or M13mp19 (Figs. 4A, pAdSF10 and F17, and 4B, mp19fosM5 and M7). The results of several gel mobility shift competition assays is summarized for both the mp19fos (Fig. 2) and pAdSF (Fig. 3) series. Some mutations in one series also appear in the other (mp19fosM2 and pAdSF6, and mp19fosM4 and pAdSF9, in Figs. 2 and 3): the two identical DSE point mutants in the different vectors had similar effects, suggesting that the competitive strength of a particular point mutant, relative to its wild-type parent, is similar in the two vector systems.

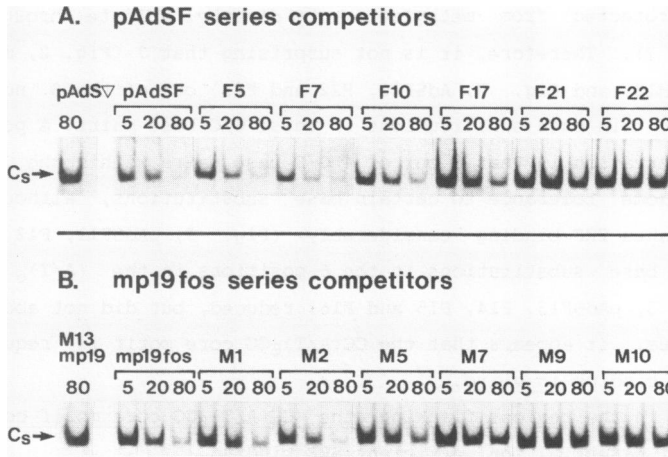

Fig. 4. Competition of SRF binding to the human c-fos DSE by DSE mutants in the (A) pAdSF and (B) mp19fos series. (A) Competition with pAdSF and its mutants. Approximately 0.33 ng of the ^{32}P-labelled EcoRI-SmaI fragment of pAdSF (see Fig. 3) was incubated with 15 μg HeLa nuclear extract and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. The name and molar excess of plasmid competitor are indicated on top of each lane. Three classes of DSE mutants were defined by their ability to compete with the wild-type c-fos DSE for SRF binding in vitro: strong (pAdSF5, F7) competitive ability equal to or greater than pAdSF; weak/none (pAdSF21, F22), competitive ability as poor as pAdS▽; and intermediate (pAdSF10, F17), competitive ability greater than pAdS▽ but poorer than pAdSF. (B) Competition with mp19fos and its mutants. Binding conditions were as described above, except that 0.11 ng of radiolabelled template was used per reaction. Three classes of DSE mutants were defined as described above, except that mutants are compared to mp19fos and M13mp19, rather than pAdSF and pAdS▽, respectively. Examples of strong (mp19fosM1, M2), intermediate (mp19fosM5, M7) and weak (mp19fosM9, M10) competitors are shown. For clarity, only the relevant portion of the autoradiogram is shown. Symbols are as in Figure 1.

The CC(A/T)$_6$GG motif in the c-fos DSE is necessary for efficient SRF binding.

The presence of strong competitors clearly indicates that changes in nucleotides at particular positions do not affect in vitro binding of SRF to the DSE, as detected by this assay. The observation that all of the strong competitors retain the 10 nucleotides in the centre of the DSE (CCATATTAGG, spanning from -314 to -305) suggests that this CC(A/T)$_6$GG motif is crucial for efficient SRF binding in vitro (strong competitors, Figs. 2 and 3). Moreover, each of the weak competitors contain at least one base substitution in the CC(A/T)$_6$GG motif (weak competitors, Figs. 2 and 3). The G residues at positions -313, -314, -306 and -305 in the human c-fos DSE have been shown to

be strongly protected from methylation by dimethyl sulfate through binding SRF (see Fig. 7). Therefore, it is not surprising that 7 (Fig. 2, mp19fosM9, M10, M11 and M12, and Fig. 3, AdSF20, F22 and F23) out of the 8 non-binding competitors contain base substitutions in these G/C base pairs. A potentially important observation is that 3 out of 4 G/C base pairs within the $CC(A/T)_6GG$ motif show some tolerance to certain base substitutions, although these changes do weaken SRF binding considerably (Fig. 3, pAdSF11, F12 and F17). Four single base substitutions in the 6 positions in the $(A/T)_6$ "spacer" region (Fig. 3, pAdSF13, F14, F15 and F16) reduced, but did not abolish, SRF binding. Thus, it appears that the $CC(A/T)_6GG$ core motif is required for efficient SRF binding in vitro.

Dyad symmetry in the regions flanking the $CC(A/T)_6GG$ core motif contributes to, but is not essential for, efficient SRF binding.

It should be noted here that the c-fos DSE has complete dyad symmetry for 7 base pairs flanking the $(A/T)_6$ "spacer", and it has been suggested (8) that the SRF binding site is the dyad itself. If SRF requires a symmetrical binding site, then one might predict that the effect of a mutation on one side of the DSE would have the same effect as its complement on the other side of the DSE. Indeed, such a result was found for all complementary pairs of single base substitution mutants: pAdSF6 and pAdSF7 are strong competitors; mp19fosM5 and pAdSF18, mp19fosM8 and pAdSF10, and pAdSF13 and pAdSF15 are intermediate competitors; and mp19fosM11 and pAdSF20 are weak competitors (Figs. 2 and 3). We confirmed this conclusion quantitatively by scanning appropriate exposures of the autoradiograms by densitometry (data not shown). However, three lines of evidence suggest that it is not simply the dyad symmetry of the c-fos SRF binding site per se that is imparting the efficient nature of its binding. First, no single point substitution outside the $CC(A/T)_6GG$ motif obliterates SRF binding in vitro. Second, several positions in the region flanking the $CC(A/T)_6GG$ motif could be changed without a detectable effect on SRF binding (strong competitors, Figs. 2 and 3). There are, however, are several base substitutions which reduced SRF binding (Fig. 2, mp19M5, M6, M7 and M8, and Fig. 3, pAdSF10, F18 and F19), suggesting that certain positions are important for the SRF-DSE interaction. Third, two of the point mutations (Fig. 3, pAdSF6 and F7) in the flanking regions detectably increase SRF binding in vitro. Therefore, dyad symmetry in the c-fos DSE may be disrupted while still retaining efficient in vitro SRF binding activity.

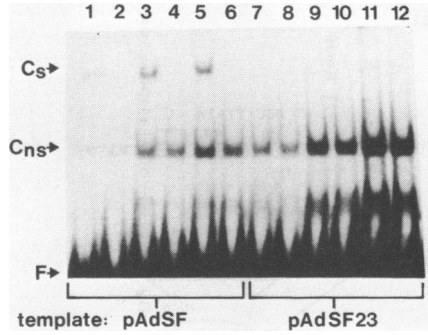

Fig. 5. Titration of a HeLa nuclear extract with radio-labelled templates pAdSF and pAdSF23. Equivalent quantities (0.33 ng) of ^{32}P-labelled EcoRI -SmaI fragments of pAdSF (lanes 1-6) and pAdSF23 (lanes 7-12) were used as templates; the labelling efficiency for the two templates was approximately the same. The templates were incubated with 15 (lanes 1, 2, 7, 8), 30 (lanes 3, 4, 9, 10), or 60 (lanes 5, 6, 11, 12) μg HeLa nuclear extract and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. 2 μg of either pAdSV (odd numbered lanes) or pAdSF (even numbered lanes) were used as plasmid competitors. Symbols are as in Figure 1.

A radiolabelled template of the weak competitor pAdSF23 binds SRF very poorly in vitro.

To confirm that the apparent competitive abilities of the mutant c-fos DSE plasmids observed above is due directly to the sequence of the mutated DSE itself, we radio-labelled the EcoRI-SmaI fragment of pAdSF and the weak mutant, pAdSF23 (Fig. 3), and used them directly as templates in gel mobility shift assays (Fig. 5). As expected, the ability of the mutant pAdSF23 DSE probe to form a specific complex with the SRF was much lower than the wild-type DSE probe (compare lanes 7, 9 and 11 with lanes 1, 3 and 5). Since the in vitro SRF binding of pAdSF23 directly correlates with its competitive strength, it is likely that the "phenotype" of a DSE mutant is mostly, if not solely, due to its DSE sequence.

Mutations in the DSE alter c-fos promoter activity in serum-deprived, but not in serum-stimulated, HeLa cells in vivo.

It has been reported that binding of SRF to the DSE is required for the activation of transcription from the c-fos promoter by serum in mouse NIH-3T3 cells (6, 9) and by EGF or TPA in HeLa cells (43). To determine whether the DSE is required for serum-stimulated transcription from the c-fos promoter in HeLa cells, we constructed in vivo expression vectors with the wild-type and

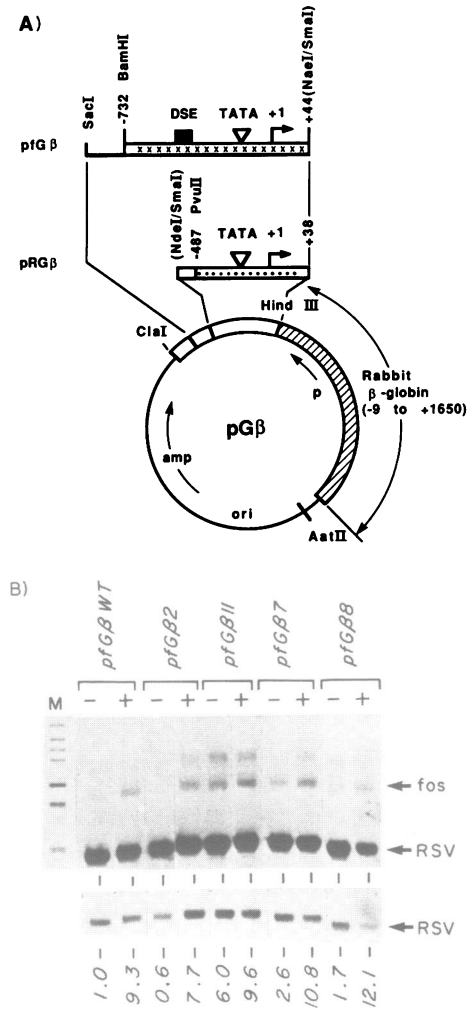

Fig. 6. Expression of the wild-type and DSE point mutant c-fos promoters in serum-deprived and serum-stimulated HeLa cells in vivo. (A) Structure of the pGβ, pfGβ and pRGβ in vivo expression vectors. Construction of pGβ, pfGβ and pRGβ were as described in Materials and Methods. Salient features of these vectors are indicated in the figure. DSE point mutants in the mp19fos series (M2, M7, M8 and M11) were transferred into the pfGβ vector by replacement of the SacII-ApaI fragment spanning the DSE in pfGβ with those of the mutants, resulting in pfGβ2, 7, 8 and 11, respectively. p: the oligonucleotide used for primer extension mapping is complementary to positions +70 to +37 of the rabbit β-globin mRNA. (B) Effect of DSE mutations on c-fos promoter activity in vivo. RNA was isolated from serum-deprived (-) or serum-stimulated (+) HeLa cells transfected with 20 μg of the plasmids indicated at the top of the figure plus 5 μg each of pRGβ and salmon sperm DNA, and 20 μg total RNA was

analyzed as described in Materials and Methods. Primer extension products corresponding to RNA transcripts initiated from the c-fos promoter (fos) or RSV LTR (RSV) are indicated at the right. [A band migrating more slowly than the fos band was not reproducibly observed]. Autoradiograms of appropriate exposures were scanned using a Bio-Rad Video 620 Densitometer. The ratio of fos to RSV (a lighter exposure is also shown) signal intensity was calculated and normalized to 1 for the pfGβWT serum-deprived lane, as shown at the bottom of the figure. M: ^{32}P-labelled MspI-digested pBB322 as size markers.

several DSE point mutant c-fos promoters inserted upstream of a promoter-less rabbit β-globin gene (Fig. 6A). The wild-type construct pfGβ and DSE mutants pfGβ2, 7, 8 and 11 (which contain the DSE point mutations in mp19fosM2, M7, M8 and M11, respectively) were transfected into HeLa cells, and analyzed for c-fos promoter activity, as determined by the level of β-globin transcripts that initiate from the c-fos mRNA start sites. As an internal control, cells were co-transfected with pRGβ, which has the RSV LTR inserted upstream of the promoter-less rabbit β-globin gene (Fig. 6A). Fisch et al. (1987) recently showed that the RSV LTR is not responsive to EGF or TPA in transfected HeLa or human A431 cells (43). As shown in Fig. 6B, the amount of β-globin transcripts initiated from the RSV LTR mRNA start sites did not change upon stimulation of the transfected, serum-deprived HeLa cells with 15% FBS for 30 min, demonstrating that the RSV LTR is also not responsive to serum in HeLa cells. In contrast, transcripts initiated from the wild-type c-fos promoter were almost 10-fold greater in amount in serum-stimulated as compared to serum-deprived HeLa cells (Fig. 6B, pfGβWT). However, mutation of the DSE did not significantly alter c-fos promoter activity in serum-stimulated cells but, rather, appeared to modulate the level of c-fos promoter activity in serum-deprived cells. For those DSE mutants tested, an inverse correlation was observed between SRF binding activity in crude Hela nuclear extracts in vitro (mp19fosM2>wt>M8>M7>M11, Figs. 2 and 4 and data not shown) and c-fos promoter activity in serum-deprived, transfected Hela cells in vivo (pfGβ2<wt<8<7<11, Fig. 6B). Thus, the binding of SRF to the DSE appears to be involved in the repression of c-fos transcription in serum-deprived HeLa cells.

DISCUSSION

Specific point mutations were introduced into the natural c-fos promoter by oligonucleotide-directed mutagenesis using a "doped" oligo as the primer and a uracil-containing M13 phage DNA as the single-stranded template. The utilization of these two techniques allows for the generation of random point mutations, with reasonable frequency (about 30%), in specific regions of DNA where no convenient restriction enzyme sites flank the sequences of interest.

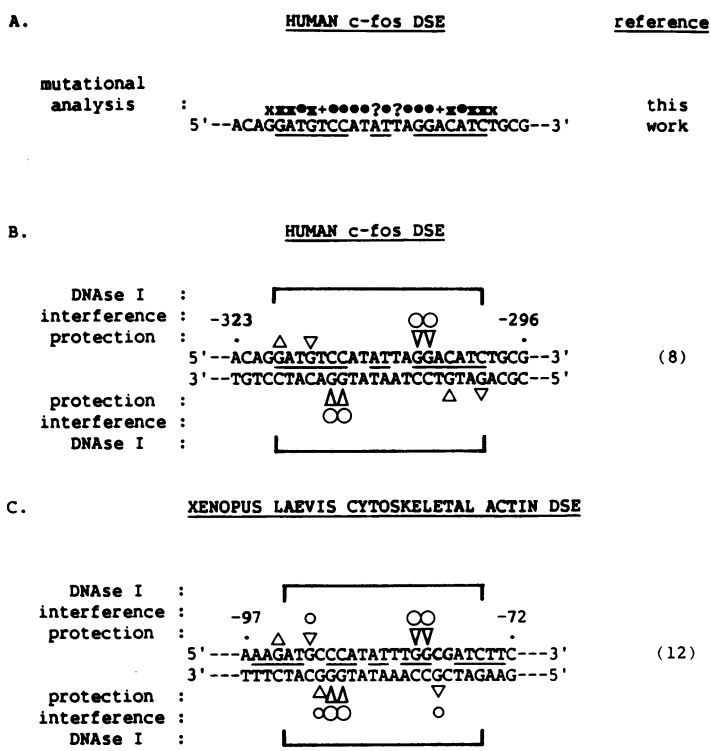

Fig. 7. Summary of experimental data on the sequence requirements for SRF binding. Summary of (A) our mutational analysis and (B) published protection and interference data on the human c-fos DSE. (C) Summary of the published protection and interference data on the Xenopus actin DSE. References for the experiments summarized are listed on the right of the figure. Numbers on top of the sequences represent the nucleotide position relative to the mRNA start site. The type of experiment is indicated on the left of the figure. Symbols: X, unimportant nucleotides for SRF binding; •, specific nucleotides required for efficient SRF-DSE interaction; +, specific nucleotides that increase the strength of the SRF-DSE interaction, when appropriately mutated; ?, specificity of nucleotide unknown due to the lack of a mutation. O, ∇, and Δ, refer to methylation interference, protection, and enhancement, respectively; the size of these three symbols represents the magnitude of the observed effects. The limit of DNase I protection is indicated by brackets.

For the chimaeric promoter, we synthesized the complementary strands of the DSE with "doped" oligos, and inserted this discrete enhancer element, via flanking restriction sites, upstream of the MLP TATA box and mRNA start site. This experimental design offers the flexibility of studying specific promoter elements in different contexts, such as inserting the DSE into a heterologous promoter in vivo expression vector to investigate its constitutive or serum-responsive transcription regulatory activity.

The gel mobility shift competition results (Figs. 2 and 3) demonstrate that base changes in the c-fos DSE affect the binding of SRF to different degrees, depending upon the position of the point substitution. Dimethyl sulphate (DMS) interference and protection experiments have been utilized to examine interactions at specific G and A residues throughout the c-fos DSE (8) and the conserved DSE-like motif in the Xenopus laevis cytoskeletal actin promoter (12) (herein called Xenopus actin DSE). Figure 7 summarizes our mutational analysis, together with the published DNase I protection and DMS interference and protection experiments (8, 12), on the SRF-DSE interactions.

The G residues in the $CC(A/T)_6GG$ motif in the c-fos and the Xenopus actin DSE have been shown to be (1) strongly protected against methylation by DMS when bound to SRF and (2) required for binding since methylation of these residues prevents binding (8, 10, 12 and Fig. 7). The nature of the SRF-DSE interactions that may be occurring at these positions is, however, not clear. Our point mutational analysis supports the DMS interference and protection data, which indicates that the most critical base pairs in the c-fos DSE for SRF binding are the G/C base pairs in the $CC(A/T)_6GG$ core motif. However, not all the single point mutations in these G/C base pairs completely abolish SRF binding in vitro (Fig. 3, pAdSF11, F12, and F17). Therefore, in contrast to the prediction that mutations at positions -305, -306, -313, and -314 should prevent SRF binding (8), we observe a certain flexibility in the G/C base pairs in the highly conserved $CC(A/T)_6GG$ motif within the c-fos DSE for SRF binding in vitro.

The function of the A-T rich centre of the $CC(A/T)_6GG$ core motif on the binding of SRF is not known. Single base substitutions resulting in G/C base pairs within the A-T rich region (Fig. 3, pAdSF13, F15 and F16) of the c-fos DSE may have either reduced the flexibility of DNA bending upon SRF binding, disrupted specific contacts between SRF and the DSE, or both. Interestingly, a T→A transversion at -311 (Fig. 3, pAdSF14), maintains an A-T rich center, but has only an intermediate competitive strength. The effects of the above point mutations suggest that for optimal SRF binding in vitro, the $(A/T)_6$ spacer region must be retained and the arrangement of A and T bases in this spacer is important. It is noteworthy that in the case of nuclear factor I (NFI) and its binding site, $TGG(N)_{6-7}GCCAA$, base changes in the $(N)_{6-7}$ spacer can also reduce the efficiency of NFI binding in vitro (44). However, we cannot exclude the possibility that certain other base changes in the $(A/T)_6$ spacer region, presumably A→T transversions, may have no detectable effect on SRF binding. Support for this idea is the variability found in the

nucleotide sequences in the $(A/T)_6$ spacer regions of the Xenopus actin DSE (12-13) and human cytoskeletal β-actin in vitro SRF binding sites (42).

The sequences flanking the $CC(A/T)_6GG$ motif contribute to the specificity of binding between SRF and the c-fos DSE. Our data indicates that base substitutions at positions -304 and -315 of the c-fos DSE may either strengthen (e.g., Fig. 2, mp19fosM2; Fig. 3, pAdSF6 and F7) or weaken (Fig. 2, mp19fosM5 and M6; Fig. 3, pAdSF18) its interaction with SRF. However, base substitutions at positions -316 or -303 (Fig. 2, pAdSF5; Fig. 3, mp19fosM1 and M4) result in mutants with wild-type competitive strengths, indicating that certain positions in the flanking region are less important than others. Methylation interference effects observed at these latter two positions (8, 12 and Fig. 7) are probably due more to steric hindrance than to destruction of specific protein-DNA interactions. Moreover, in the human cytoskeletal β-actin gene, two of the in vitro SRF binding sites contain identical $CC(A/T)_6GG$ motifs, yet demonstrate an ~ 10-fold difference in their binding affinities for SRF (42), suggesting that these flanking sequences can be quite important in determining the strength of the SRF-DSE interaction.

We note here that the human c-fos DSE is completely symmetrical; the DMS methylation protection pattern of the c-fos DSE is also symmetrical about its central axis. Mutational analysis suggests that, indeed, the binding of SRF to the DSE is symmetrical due to the observation that complementary single point mutations tend to have similar competitive strengths. In addition, the recent data on the binding of the HeLa SRF to the Xenopus actin DSE (12-13) suggests that the affinity of SRF for the DSE can be increased by making the element more dyad symmetrical. Duplication of either the 5' or 3' half of the Xenopus actin DSE results in greater or poorer SRF binding, respectively (13). Taken together, these observations suggest that the SRF binding site may consist of two "half-sites" cooperating to facilitate the formation and/or stability of the SRF-DSE complex. It would be interesting to create artificial binding sites that duplicate the 5' half of the pAdSF6, or the 3' half of F7, both of which bind SRF better than the c-fos DSE, such that the new sites contain the mutation symmetrically on both halves of the DSE. We would predict that these sites should interact with SRF more strongly than either the c-fos DSE or their singly mutated counterparts (pAdSF6 and F7).

The SRF present in the nuclei of HeLa cells that were either rapidly growing, serum-deprived, or serum-deprived and then serum-stimulated prior to harvesting, binds with similar affinity to the human c-fos DSE in vitro (8). The contribution of this consistent in vitro binding of SRF to the DSE to the

in vivo transcriptional regulation of c-*fos* in HeLa cells is not known. Of potential importance is the recent observation (45) that SRF phosphorylation is required for DNA binding activity *in vitro*. It will be of obvious interest to use purified HeLa SRF (13-15), with the c-*fos* DSE point mutants described here, to study the SRF-DSE interaction in finer detail.

We constructed *in vivo* expression vectors to test the biological activity of the c-*fos* DSE mutants generated in this study by transient expression assays in HeLa cells. We (S.L., unpublished results) and others (8-9) have observed that the kinetics of serum induction of c-*fos* transcription in HeLa cells are similar to that in NIH3T3 cells. Sassone-Corsi and Verma (46) have shown that the human c-*fos* promoter is negatively regulated by 5' flanking sequences in serum-deprived NIH-3T3 cells. Our results (Fig. 6) demonstrate that the c-*fos* promoter is also negatively regulated in serum-deprived HeLa cells, and further, this repression appears to be mediated by SRF. However, unlike previous reports (6, 9), we did not observe a requirement for the DSE for activation of c-*fos* transcription in serum-stimulated HeLa cells. It has been shown that the responsiveness to various external stimuli of the c-*fos* promoter requires 5' flanking sequences other than the DSE (2). In fact, the human c-*fos* promoter from -277 to -223 also confers serum-responsiveness in NIH-3T3 cells (47). It will be of interest to determine whether the DSE is necessary for induction of the c-*fos* promoter by specific mitogens, and which sequences in the c-*fos* 5' flanking region are required for serum-inducible expression in the absence of a functional DSE, in serum-deprived HeLa cells.

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