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Regulation of c-*fos* transcription in mouse fibroblasts: identification of DNase I-hypersensitive sites and regulatory upstream sequences

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In quiescent mouse fibroblasts, the c-*fos* gene is expressed at very low levels, but is rapidly and transiently inducible by peptide growth factors. In this study, we have identified in quiescent cells five DNase I-hypersensitive sites located −1700, −290, +10, +240 and +700 bp relative to the 5′ cap site. After serum stimulation, the distinct nuclease hypersensitive site at position +10 rapidly disappeared, and instead a broad region of DNase I accessibility between positions 0 and +250 occurred. Nucleotide sequence analysis of the 5′-flanking region of the mouse c-*fos* gene showed that the hypersensitive site around position −290 is located in a region that is highly conserved between mouse and human, and that contains an enhancer-like structure. When the mouse c-*fos* promoter and 351 bp of 5′-flanking sequences were linked to the bacterial chloramphenicol acetyl transferase (CAT) gene and transfected into NIH3T3 cells efficient, constitutive expression of CAT activity was observed, even in unstimulated, quiescent cells. However, removal of a 256-bp stretch upstream from position −95 completely abolished CAT expression, indicating that sequences within a region of ~350 bp upstream from the cap site are indispensable for c-*fos* transcription. In addition, our findings point to the existence of other sequence elements that exert negative regulation in the absence of growth factor stimulation. Such sites may be found around the growth factor-responsive nuclease hypersensitive sites in the vicinity of the cap site.

Key words: DNase I-hypersensitive sites/c-*fos* gene/transcription regulation/cap sites

Introduction

The cellular homologue c-*fos* of the oncogene of FBJ and FBR mouse osteosarcoma viruses (Curran *et al.*, 1982; Curran and Verma, 1984) is expressed in a highly tissue-, cell type- and stage-specific fashion. High levels of c-*fos* mRNA and protein have been detected specifically in bone marrow, fetal liver, amnion and yolk sac (Müller *et al.*, 1982, 1983a, 1983b, 1984a; Curran *et al.*, 1984; Adamson *et al.*, 1985). Expression in cells of the monocytic phagocyte lineage appears to be correlated with cellular differentiation (Gonda and Metcalf, 1984; Müller *et al.*, 1984a, 1985; Mitchell *et al.*, 1985) and is dependent on the presence of macrophage-colony stimulating factor (M-CSF; CSF-1) (Müller *et al.*, 1985). In other cell types, such as fibroblasts or the epitheloid A431 cell line, c-*fos* is normally expressed at extremely low levels, but rapidly inducible by peptide growth factors (Greenberg and Ziff, 1984; Müller *et al.*, 1984b; Kruijer *et al.*, 1984; Cochran *et al.*, 1984; Bravo *et al.*, 1985). In these cells, however, expression occurs only transiently after induction, almost all c-*fos* mRNA disappearing ~2 h after stimulation.

Analysis of the structure of the mouse c-*fos* gene has defined several elements required for expression, such as a 'TATA-box' 26−31 bp upstream from the 5′ cap site and a polyadenylation signal 794−799 bp downstream from the translation termination codon (Van Beveren *et al.*, 1983). A region in the 3′-non-translated region of the gene prevents efficient expression of c-*fos* in fibroblasts (Miller *et al.*, 1984). To activate the transforming potential of c-*fos*, i.e., to achieve a high, constitutive expression of its gene product in fibroblasts, it is required to remove these 3′-inhibitory sequences (Miller *et al.*, 1984; Meijlink *et al.*, 1985). However, no other sequences that may be involved in the expression, regulation or induction of the c-*fos* gene have been described to date.

One approach to identify sequence elements that may be implicated in transcriptional control mechanisms is to screen for nuclease-hypersensitive sites in the chromatin encompassing the gene to be investigated. Involvement of DNA regions containing DNase I-hypersensitive sites in transcription control mechanisms is strongly suggested by many investigations (e.g., Shermoen and Beckendorf, 1982; McGhee *et al.*, 1981; Fritton *et al.*, 1984; Schubach and Groudine, 1984). Nuclease-hypersensitive sites are generally found within the 1000 bp flanking the 5′ end of genes that are transcriptionally active or are inducible (for review, see Elgin, 1981, 1984) but developmentally or hormonally controlled genes often display complex patterns of nuclease-hypersensitive sites (Fritton *et al.*, 1984). DNase I-hypersensitive sites are free of nucleosomes and presumably represent binding sites for regulatory proteins and enzymes involved in gene transcription (Elgin, 1984). To identify sequence elements that may be involved in controlling the expression and induction of the c-*fos* gene in fibroblasts we searched for DNase I-hypersensitive sites in the chromatin region of the c-*fos* gene in quiescent NIH3T3 cells prior to and after stimulation with fetal calf serum (FCS). In addition, we determined the nucleotide sequence of the 5′-flanking region of the mouse c-*fos* gene to search for homologies with transcriptional regulatory elements identified in other systems. Finally, we analyzed the effect c-*fos* 5′-flanking sequences on the expression of a c-*fos* promoter-driven bacterial chloramphenicol acetyl transferase (CAT) gene to obtain direct

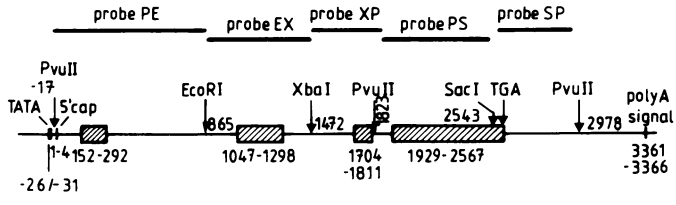

Fig. 1. Schematic representation of the structure of the mouse c-*fos* gene, showing the positions of 'TATA-box', 5′ cap site, coding sequences (boxed) and polyadenylation site (Van Beveren *et al.*, 1983). The subcloned probes used in this study are indicated by bold lines at the top of the figure: an 885-bp *Pvu*II-*Eco*RI fragment (probe PE), a 607-bp *Eco*RI-*Xba*I fragment (probe EX), a 351-bp *Xba*I-*Pvu*II fragment (probe XP), a 720-bp *Pvu*II-*Sac*I fragment (probe PS) and a 435-bp *Sac*I-*Pvu*II fragment (probe SP).

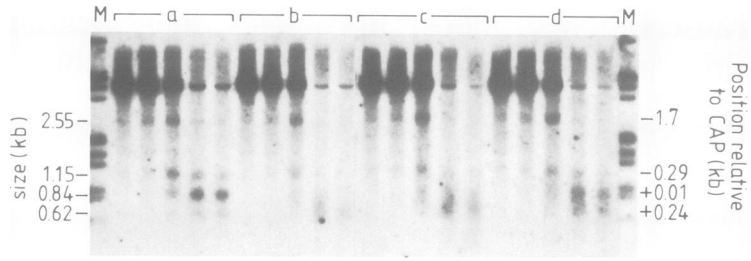

Fig. 2. Mapping of DNase I-hypersensitive sites at the region of transcription initiation and in the 5′-flanking sequences of the mouse c-*fos* gene using probe PE. Southern blot analysis of *Eco*RI-digested DNA from DNase I-treated nuclei from quiescent NIH3T3 cells **(a)** and from serum-stimulated cells after 15 min **(b)**, 30 min **(c)** or 45 min **(d)** exposure to 10% FCS. The lanes in each panel represent nuclei digested with increasing concentrations of DNase I (from left to right; for details see Materials and methods). Sizes of DNA fragments appearing after DNase I digestion of nuclei are indicated at the left-hand margin, the locations of the corresponding DNase I-hypersensitive sites relative to the 5′ cap site (see map in Figure 1) are given at the right-hand margin. The left- and right-most lines are size markers (λDNA digested with *Hind*III and *Eco*RI).

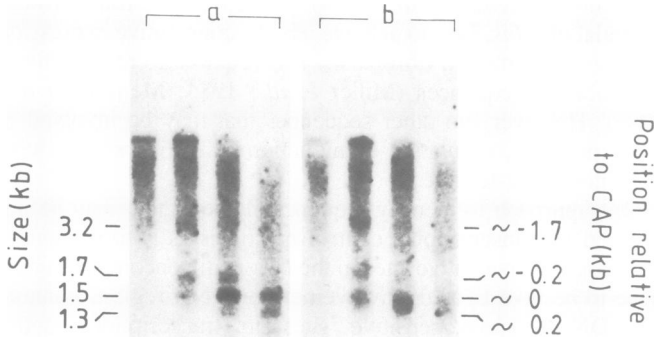

Fig. 3. Mapping of DNase I-hypersensitive sites around the cap site and in the 5′-flanking region of the mouse c-*fos* gene using probe EX. Southern blot analysis of *Xba*I-digested DNA from DNase I-treated nuclei from quiescent NIH3T3 cells **(a)** and after 10 min of serum stimulation **(b)**. See legend to Figure 2 for further details.

evidence for an involvement of upstream sequences in transcriptional control mechanisms.

Results

DNase I-hypersensitive sites in c-fos chromatin

The chromatin conformation of the c-*fos* gene and its flanking sequences were screened for DNase I sensitivity using an indirect end-labelling technique (Wu, 1980) with a battery of c-*fos* DNA probes before and after stimulation with FCS. For this purpose, quiescent NIH3T3 cells were harvested before or various times after serum stimulation. Cell nuclei were digested with increasing amounts of DNase I, the isolated DNA was cut with restriction endonucleases, size separated by agarose gel electrophoresis, blotted and hybridized to appropriate probes.

Figure 1 shows the map of the mouse c-*fos* gene with the relevant restriction sites and the five subcloned fragments that were used as probes. We first anlayzed the gene at the region of transcription initiation and its 5′-flanking sequences with probe PE using *Eco*RI to digest the isolated DNA. Four bands of different intensities in addition to the unbroken restriction fragment were detected on the Southern blot autoradiogram after hybridization to probe PE, approximately corresponding to positions −1700, −290, +10 and +240 bp, respectively, in the map of the c-*fos* gene (Figures 1 and 2a). When a similar experiment was performed with nuclei of cells which had been stimulated with FCS for 15 min, DNase I-hypersensitive sites were observed at positions −1700 and −290 bp (Figure 2b), similar to quiescent cells. The distinct sites around positions +10 and +240 bp found in quiescent cells; however, could not be identified after 15 min of serum stimulation, and instead a broad region between positions 0 and +250 became accessible to DNase I (compare Figure 2a and b). At 45 min after serum stimulation, the nuclease-hypersensitive sites reappeared and showed a pattern of DNase I-hypersensitive sites similar to quiescent cells (Figure 2d).

To confirm these results nuclei from quiescent and stimulated cells were analyzed for DNase I-hypersensitive sites using probe EX (Figure 1). Digestion of nuclei from quiescent cells with increasing amounts of DNase I prior to cleavage of the purified DNA with *Xba*I revealed four hypersensitive sites (Figure 3a), approximately at positions −1700, −290, +10 and +240 bp, respectively, in the map of the c-*fos* gene, closely resembling the pattern found with probe PE. Experiments with nuclei of cells stimulated with FCS for 10 min (Figure 3b) also showed nearly unchanged DNase I hypersensitivity of the sites approximately at position −1700 and −290, and a disappearance of the distinct sites at positions +10 and +240.

To examine whether other regions of the c-*fos* gene may also contain nuclease-hypersensitive sites, we analyzed the DNase I sensitivity of nuclei from quiescent and stimulated cells with probe XP (DNA digested with *Pvu*II). In addition to the previously observed sites at positions −290, +10 and +240 bp, which in this experiment are compressed in a broad region beneath the unbroken restriction fragment, another site around position +700 was detected (Figure 4). Interestingly, the DNase I hypersensitivity of this site appeared to be increased after 30 and 45 min of FCS stimulation (Figure 4c, d). The significance of this finding, however, cannot be assessed at present.

We also analyzed the chromatin region of the 5′ half of the c-*fos* gene using probe PS and *Sac*I digestion. No sub-bands beneath the unbroken restriction fragments other than those corresponding to the sites described above (i.e., Figures 2, 3 and 4) could be detected on the autoradiograms of the blot indicating that there are no DNase I-sensitive sites between positions +700 and +2900 bp (data not shown). A further search for DNase I-sensitive sites in the 3′ direction of the gene using probe SP (Figure 1) and *Sac*I-digested DNA failed to reveal preferential cleavage sites between the *Sac*I site at position 2543 bp in the c-*fos* gene and another *Sac*I site in the 3′-flanking sequence ~5.2 kbp downstream from the 5′ cap site (data not shown). The results of the DNase I mapping experiments are summarized in Figure 5.

Nucleotide sequence of c-fos upstream region

To investigate whether c-*fos* upstream sequences including the DNase I-hypersensitive site around position −290 bp may con-

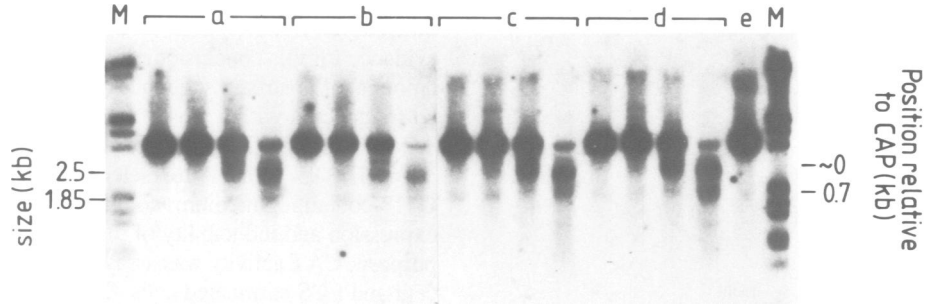

Fig. 4. Mapping of DNase I-hypersensitive sites in the 5′ region of the mouse c-*fos* gene using probe XP. Southern blot analysis of *Pvu*II-digested DNA from DNase I-treated nuclei from quiescent NIH3T3 cells **(a)** and after 15 min **(b)**, 30 min **(c)** and 45 min **(d)** of serum stimulation. **(e)** *Pvu*II-digested DNA directly isolated from cells. See legend to Figure 2 for further details.

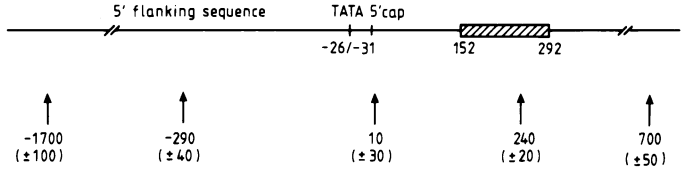

Fig. 5. Positions of DNase I-hypersensitive sites in the chromatin region of the c-*fos* gene in quiescent NIH3T3 cells. Arrows indicate locations of hypersensitive sites. Values in parentheses indicate absolute variations obtained in different, independent experiments.

tain sequences that show homology with regulatory elements identified in other systems, we determined the nucleotide sequence of the 550 nucleotides flanking the 5′ region of the mouse c-*fos* gene, as described in Materials and methods. Two intriguing features emerged from this analysis. (i) When compared with the published sequence flanking the human c-*fos* gene (van Straaten *et al.*, 1983), a marked homology was detectable (Figure 6a). Although this homology was found throughout the 550-bp flanking sequence (~70% homology), the region between positions −270 and −423 bp was particularly well conserved (~92% homology), containing an uninterrupted 51-bp stretch of complete homology (between positions −270 and −320 bp). Taken together with our finding of a DNase I-hypersensitive site approximately at position −290 bp, these pronounced sequence homologies between the mouse and human sequences suggest an important, probably regulatory function for these regions flanking the c-*fos* gene. (ii) When the c-*fos* flanking region was compared with other published nucleotide sequences, an enhancer-like structure in the 51-bp region of uninterrupted homology was detected. The sequence CTTTACACA at position 314−322 closely resembles the core sequence of the adenovirus E1a enhancer (in the opposite orientation; Figure 6a, b), and is very similar to the core sequences of several other enhancer elements as well as to the consensus core sequence for transcriptional enhancers (Figure 6b; Yaniv, 1984). Homology to several enhancer sequences was also detectable in other regions close to the consensus sequence at positions 314−322, e.g., a homology to the MSV enhancer at positions −288 to −300 bp (Figure 6a). Another potentially interesting sequence (GTTCCGCCCA) motif was identified at positions −66 to −75 bp which corresponds to the consensus sequence (opposite orientation) of a binding site for the transcription factor Sp1 ($^{G}_{T}$GGGCGG$^{GGC}_{AAT}$; Dynan and Tjian, 1985). The significance of this homology, however, remains unclear, since the human sequence in this region does not correspond to the consensus sequence for a Sp-1 binding site (Figure 6a).

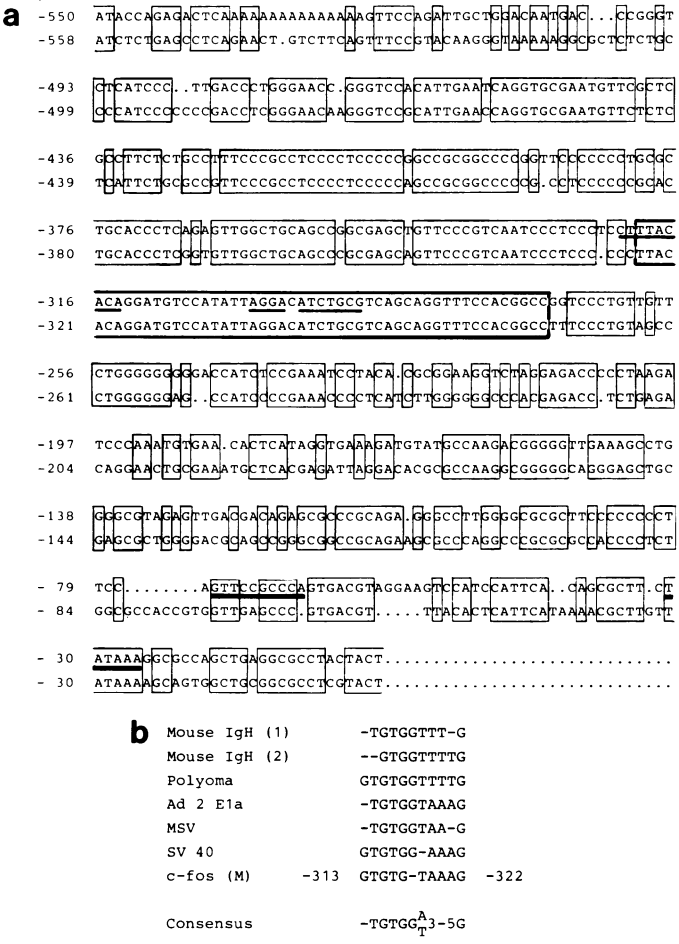

Fig. 6. (a) Nucleotide sequence of the 5′ region of the mouse c-*fos* gene (upper sequence) compared with the corresponding human sequence (lower sequence; van Straaten *et al.*, 1983). The sequences start with nucleotide −1 (T) which is the first nucleotide upstream from the putative cap site (CCAA; Van Beveren *et al.*, 1983). Underlined sequences (thin lines) depict homologies with the adenovirus E1a enhancer core sequence (−314 to −322) and with the MSV enhancer (−288 to −300 bp). Bold lines indicate homology with the Sp1 binding site consensus sequence (−66 to −75) and 'TATA-box' (−26 to −31). Boxes indicate regions of homology between mouse and human sequences. The fold-lined box depicts the 50-bp region of uninterrupted homology. **(b)** Alignment of c-*fos* flanking sequences with various transcriptional enhancer core sequences (Yaniv, 1984).

c-fos upstream sequences are required for transcription

The mapping of a DNase I-hypersensitive site around position −290 and the identification of an enhancer-like structure in the

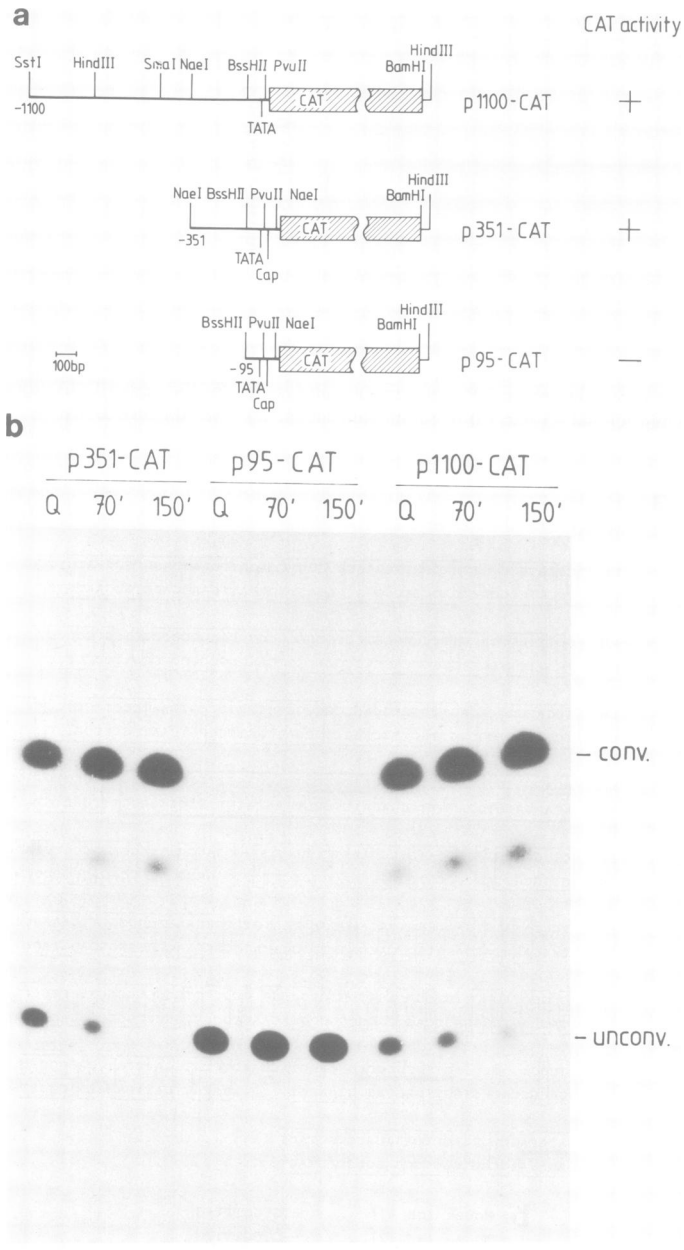

Fig. 7. **(a)** c-*fos*-CAT constructs. A *Bgl*II-*Bam*HI fragment containing the CAT gene was subcloned in pUC19 (Norrander *et al.*, 1983), excised with *Sma*I and *Bam*HI and ligated to fragments containing the c-*fos* promoter plus variable amounts of upstream sequences: an 1100-bp *Sst*I-*Pvu*II fragment (the *Pvu*II site at position −17 was ligated with the *Sma*I site of the excised CAT fragment; construct p1100-CAT), and a *Nae*I-*Nae*I fragment containing 351 bp of flanking sequence (the *Nae*I site at position +41 was ligated to the *Sma*I of the CAT fragment; p351-CAT). The latter construct was used to generate p95-CAT by *Bss*HI digestion and removal of 256 bp of upstream sequences. All plasmids were constructed in pUR19. Bold lines, c-*fos* 5′ and flanking sequences; hatched boxes, CAT gene; thin lines, plasmid sequences. The *Nae*I sites at position +41 and the *Pvu* site at position −17 shown in this figure are destroyed due to blunt end ligation with the *Sma*I site of the CAT fragment. **(b)** CAT assay of extracts from pooled G418-resistant NIH3T3 colonies after transfection with constructs p351-CAT, p95-CAT or p1100-CAT. Q: serum-deprived cells (kept in 0.5% FCS for 24 h); 70 and 150: serum-deprived cells 70 and 150 min after stimulation with 10% FCS. Equal amounts of protein were incubated with ^{14}C-labeled chloramphenicol in each lane and the reaction products were separated by t.l.c. and made visible by autoradiography. Exposure time was 17 h.

same region strongly suggest an important role for these sequences in transcription of the c-*fos* gene. To obtain direct evidence for this conclusion, the c-*fos* promoter including various amounts of upstream sequences was linked to the CAT gene and co-transfected with a plasmid carrying the TK-promoter driven *neo* gene (pHS 272; Brady *et al.*, 1984; Southern and Berg, 1982) into NIH3T3 cells. After selection of the transfected cells in G418-containing medium, colonies were pooled and analyzed for expression and inducibility of the transfected CAT gene. For this purpose, CAT activity was assayed *in vitro* in extracts from quiescent and FCS-stimulated cells. Three different constructs (Figure 7a) were tested, containing, in addition to the c-*fos* promoter, either 1100 bp (p1100-CAT), 351 b (p351-CAT) or 95 bp (p95-CAT) of 5′-flanking sequence. As shown in Figure 7b, efficient expression of CAT activity was observed with constructs p1100-CAT and p351-CAT. In contrast no activity was detectable after transfer of construct p95-CAT, although the cells had taken up and expressed the transfected DNA (data not shown). Interestingly, expression of CAT activity from plasmids p1100-CAT and p351-CAT was equally efficient in quiescent cells and in stimulated cells 70 or 150 min after addition of FCS (Figure 7b). This result indicates that a 256-bp stretch between the *Nae*I site at position −351 and the *Bss*HI site at position −95 is required for transcription driven by the c-*fos* promoter. This region, however, does not appear to be regulated by growth factor signals.

Discussion

Expression of the c-*fos* gene in mouse fibroblasts is subject to efficient and rapidly acting regulatory mechanisms. Thus, c-*fos* expression in serum-deprived, quiescent cells is extremely low, but a dramatic accumulation of c-*fos* mRNA is observed within minutes after growth factor stimulation (Müller *et al.*, 1984b; Kruijer *et al.*, 1984). This is at least in part due to an increased transcriptional activity (Greenberg and Ziff, 1984). Transcription of the c-*fos* gene is the earliest known effect on gene activity induced by growth factors. However, c-*fos* expression is not only rapidly induced to high levels, but expression is shut off with similar efficiency, practically no c-*fos* mRNA being detectable ~2 h after growth factor stimulation. Although the nucleotide sequence of the entire c-*fos* gene has been determined (Van Beveren *et al.*, 1983) and a 'TATA-box' at the 5′ end has been identified, other sequence elements involved in gene transcription and induction of expression remain unknown. As a first step to elucidate these regulatory mechanisms we have screened c-*fos* chromatin in quiescent and serum-stimulated NIH3T3 cells for nuclease-hypersensitive sites.

Our analysis revealed the presence of three DNase I-hypersensitive sites clustering around the 5′ terminus of the c-*fos* gene, one site around position +700 in the c-*fos* gene, and one site ~1700 bp upstream from the 5′ cap site (Figures 2, 3, 4 and 5). In contrast to the DNase I-hypersensitive sites around positions −1700 bp, −290 bp and 700 bp, no distinct sites were detectable at positions +10 and +240 bp after 10−15 min of serum stimulation (Figures 2b and 3b). Similar results were obtained in four different experiments using different probes for the 5′ end of the mouse c-*fos* gene and different restriction enzymes to digest the DNA purified from DNase I-treated nuclei. These results suggest that the DNase I hypersensitivity of the site at position +10 changes after serum stimulation, perhaps due to the binding of proteins required for or controlling gene

transcription. It cannot be resolved at this point whether the site at position +240 also changes after growth factor stimulation, or whether it is not visible due to the broadening of the site at position +10 (Figures 2b and 3b). These nuclease-hypersensitive sites seem to reappear at later times after serum stimulation (Figure 2d), i.e., when transcription is shut-off. This observation is intriguing since it strongly suggests an involvement of these regions in the mechanism of gene induction by growth factors.

The other nuclease-hypersensitive sites found approximately at positions −1700, −290 and +700 bp may also represent regions involved in regulation of transcription, e.g., by providing binding sites for regulatory proteins. This hypothesis is lent support by the results of the nucleotide sequence analysis (Figure 6). First, we were able to demonstrate a striking homology between the mouse and human 5′-flanking sequences. This conspicuous evolutionary conservation strongly suggests an important function for this region. Second, we detected in this region sequence motifs that correspond to consensus sequences of transcription-regulating elements identified in other systems, most notably an enhancer-like structure in the region of uninterrupted homology between mouse and human between positions −270 and −320. The DNase I sensitivity site at position −290 apparently falls into this region and could therefore represent a binding site for a protein interacting with the putative enhancer sequence.

Further evidence for the presence of a functional transcription-controlling element in the upstream region of close homology between mouse and human is provided by the results of the CAT assay (Figure 7). These results clearly show that a 256-bp stretch between positions −351 and −95 is required for transcriptional activity of the c-*fos* promoter. This is exactly the region containing the enhancer-like structure and one of the DNase I-hypersensitive sites. Taken together, these results suggest that this upstream region regulates the c-*fos* promoter by displaying at least some properties of transcriptional enhancers. Further experiments are required, however, to define this transcription-controlling element more closely, both in terms of localization and function (e.g., effects of orientation and position).

The fact that constructs p1100-CAT and p351-CAT are expressed constitutively, even in the absence of growth factor stimulation, points to the existence of further transcription-controlling regions. Such regulatory elements should be expected to exert a negative effect on transcription in quiescent cells that is abolished after growth factor stimulation. One possible candidate for such a regulatory element is the 3′-non-translated region of c-*fos* which has been shown to inhibit transformation of fibroblasts (Miller *et al.*, 1984; Meijlink *et al.*, 1985). However, this region probably affects RNA stability or translation rather than gene transcription, so that possibly other sequences in the c-*fos* gene play a role in regulation of transcription. Such sites may be associated with the intragenic DNase I-hypersensitive sites identified in this study, in particular the growth factor-responsive nuclease-hypersensitive region in the vicinity of the cap site. The analysis of various additional c-*fos* gene constructs as well as the performance of protein binding studies are now required to unravel what appears to be a complex network of transcriptional and post-transcriptional regulatory mechanisms.

Materials and methods

Cell culture

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. For chromatin structure analyses, cells were made quiescent by keeping sub-confluent cultures for 3 days in 0.5% FCS.

Isolation of nuclei

NIH3T3 cell monolayers were washed three times with phosphate-buffered saline (PBS), scraped in chilled PBS, and pelleted at 800 *g* and 4°C for 5 min. Cell pellets (~2×10^8 cells each) were resuspended in 2ml of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, incubated for 3 min on ice, homogenized in a Dounce homogenizer and centrifuged at 800 *g* and 4°C for 5 min. The nuclear pellets were washed twice with 10 ml of 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 3 mM $MgCl_2$ and centrifuged as above. The nuclei were resuspended in 200 μl of 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 87% glycerol and stored at −80°C.

Analysis for DNase I-hypersensitive sites

Aliquots of nuclei (60 μg DNA each) were digested in 150 μl of 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$ with increasing amounts of DNase I (0, 2, 6, 20 and in some experiments 40 units, respectively) for 10 min at 0°C. The reactions were stopped by addition of 15 μl of 0.5 M EDTA, 0.5% sarcosyl. After RNase and the proteinase K treatment, the DNA was purified by organic solvent extraction, and aliquots (6 μg each) were digested with appropriate restriction endonucleases, size separated by electrophoresis on 1% agarose gels and transferred to nitrocellulose papers (Southern, 1975). The blots were hybridized for 12 h at 42°C in the presence of 6% polyethylene glycol (Renz and Kurz, 1984). c-*fos* gene fragments were subcloned from c-*fos* (mouse)-2 (Curran *et al.*, 1983) in pUC18 (Norrander *et al.*, 1983), released from the plasmid by appropriate restriction enzymes, purified from vector sequences by agarose gel electrophoresis and ^{32}P-labeled by nick translation (Rigby *et al.*, 1977) to a specific activity of 8×10^8 c.p.m./μg DNA.

Nucleotide sequence analysis

Two overlapping fragments of the 5′-flanking c-*fos* region (*Sac*I-*Nae*I and *Hind*III-*Pvu*II) [from c-*fos* (mouse)-2 (Curran *et al.*, 1983)] were subcloned in plasmid pUC19 (Norrander *et al.*, 1983). These subclones and the genomic mouse c-*fos* clone were used to determine the nucleotide sequence of both strands of the 550 bp flanking the c-*fos* gene according to the procedure of Maxam and Gilbert (1980). The first 130 nucleotides of this sequence (−1 to −130) are identical to those previously published by Van Beveren *et al.* (1983).

Transfection and CAT assay

2×10^6 NIH3T3 cells were co-transfected with 10 μg of CAT constructs, 1 μg of TK-neo plasmid (pHS 272; Brady *et al.*, 1984) and 20 μg of NIH3T3 carrier DNA, essentially as described (Graham and van der Eb, 1973; Corsaro and Pearson, 1981). After selection in 0.7 mg G418/ml (Southern and Berg, 1982), colonies were pooled and grown up for analysis. Trypsinized cells from one confluent 150 mm dish were lysed in 500 μl buffer containing 10 mM Tris pH 7.6; 1 mM EDTA; 100 mM NaCl; 5 mM $MgCl_2$ and 0.5% Nonidet P-40. Homogenates were vortexed for 10 s and centrifuged for 5 min at 3000 *g*. Supernatants were withdrawn and 30 μl (25 μg protein) were used for determination of CAT activity according to published procedures (Gorman *et al.*, 1982).

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