Cell-type specific multiprotein complex formation over the c-fos serum response element in vivo: ternary complex formation is not required for the induction of c-fos

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ABSTRACT
At the serum response element (SRE) of the c-fos proto-oncogene a ternary complex with two proteins, ternary complex factor (p62TCF) and serum response factor (SRF) can be formed. Its formation has previously been suggested to be necessary for efficient induction of c-fos transcription by serum in mouse NIH 3T3 fibroblasts (1) and by phorbol esters, but not by serum, in mouse BALB/c 3T3 fibroblasts (2). It is shown here by genomic dimethyl sulfate (DMS) footprinting that this ternary complex is indeed formed in NIH 3T3 cells in vivo. However, cells were found, murine F9 teratocarcinoma stem cells, in which the pattern of protection and hyperreactivity is consistent with the absence of p62TCF in the ternary complex in vivo, although inducibility of the endogenous c-fos gene is not impaired. Both in NIH 3T3 cells and in F9 cells a mutated c-fos promoter that binds SRF but fails to form a ternary complex, was inducible by serum and phorbol esters to the same extent as the wild-type promoter. The data suggest that ternary complex formation is not an absolute prerequisite for the transient induction of c-fos. Ternary complex formation rather appears to enhance overall promoter efficiency. A cell type specific component determines the formation of the multicomponent transcription factor complex in vivo.

INTRODUCTION
The expression of an increasing number of eukaryotic genes seems to be controlled by DNA-binding multiprotein complexes (3). The c-fos proto-oncogene (4) encodes a transcription factor itself involved in the formation of such multiprotein complexes, largely by association of Fos protein with members of the Jun family (5). Fos has been implicated in the control of cell growth (6, 7), cell differentiation (8) and neuronal signal processing (9, 10). The expression of c-fos is controlled by another multiprotein complex. The rapid and transient induction of c-fos after stimulation of cells by a variety of agents, e.g. serum growth factors (11), phorbol esters (12, 13) or UV irradiation (13) depends on proteins binding to the serum response element (SRE) (14) which mediates also the negative autoregulation of c-fos transcription (15, 16). The SRE region can bind a multiprotein complex, both in vitro and in vivo (1, 17), involving ternary complex factor (p62TCF) (1, 18), serum response factor (SRF) (19) and at least one other protein (17) 3′ of SRF. In previous studies it has been suggested that SRE-mutants in c-fos promoter constructs which still bind SRF but are unable to form a ternary complex with SRF and p62TCF are only poorly serum-inducible upon transfection into mouse NIH 3T3 fibroblasts (1) and are severely impaired in their inducibility by the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA), but not by serum in mouse BALB/c 3T3 fibroblasts (2). The occupation of the SRE by proteins in vivo was previously shown to be unaltered in the course of transient c-fos induction by epidermal growth factor (EGF) in the human epidermal carcinoma cell line A431 (17) which suggests that regulation of c-fos transcription is affected by modifications or protein-protein interactions of proteins that are always bound to DNA. To be certain that the SRE is protected also at a very low level of basal transcription, I performed genomic dimethyl sulfate (DMS) footprinting analysis in murine NIH 3T3 and F9 teratocarcinoma stem cells. The latter are known to show no detectable basal level of c-fos expression (20). In both cell types, the SRE and the adjacent API-like binding site were occupied in unstimulated cells and the occupation was not altered during transient induction of c-fos by the phorbol ester TPA. There was, however, a difference between NIH 3T3 and F9 cells in the extent of the in vivo DMS footprint on the SRE which is consistent with the lack of p62TCF in the complex in F9 cells. In both cell types, ternary complex formation was not necessary for induction of c-fos. The role of the cell-type specific difference in multiprotein complex formation is not clear yet but it may be associated with promoter efficiency rather than inducibility.

MATERIALS AND METHODS
Plasmid constructs
The mouse c-fos CAT promoter fusion constructs -356wt, -356pm18, the α-globin internal control plasmid and the plasmids used for probe synthesis for the RNease protection assay have been described elsewhere (2, 26). All plasmids were kindly provided by Dr. Michael Gilman (Cold Spring Harbor Laboratory).
Cell culture
NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). F9 teratocarcinoma stem cells were grown in a 1:1 mixture of DMEM and F12 medium supplemented with 2mM glutamate, 170μM β-mercaptoethanol and 10% FCS (DMEM/F12/10% FCS). Before treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (100 ng/ml), NIH 3T3 cells were grown for 36–40h in DMEM/0.5% FCS.

Genomic footprinting
Genomic footprinting was done by the ligation mediated, single sided polymerase chain reaction (PCR) technique (21). For DMS treatment the medium was removed, the cells were washed with phosphate buffered saline (PBS) and 0.5% DMS in DMEM/10 mM HEPES pH 7.5 was added at room temperature (RT) for 2 min. After washing the cells two times with ice-cold PBS genomic DNA was prepared as described (22). In vitro methylation of protein-free DNA (1 min at RT) and piperidine cleavage were performed according to ref. 23. The cleavage products were specifically amplified and radioactively labeled as described elsewhere (21). Specific amplification of the cleavage products by PCR was done in a Coy TempCycler and the amplification products were resolved on a standard sequencing gel (23). The primers used for the coding strand were: 1, 5'CTCTGTCGTCAACTCTACGCC; 2, 5'CTCTACGCCAAGCCTTCAACCC; 3, 5'ACGCCCAGGCTTTCAACCCTCCGCTT; for the non-coding strand: 1, 5'CCTGCTGGCCTGGGCGCTGCACC; 2, 5'CTGCACGCCCTCAGTGCTG; 3, 5'GTGGAGTGTCTAGTGCTGACG-CGG. For the coding strand and non-coding strand (values in brackets) 19 cycles were performed each consisting of 1 min at 94°C, 2 min at 63 (65)°C and 3 min at 76°C. For the last primer extension step using endlabeled primer 3 the segments consisted of 2 min at 94°C, 2 min at 66 (68)°C and 10 min at 76°C.

Nuclear run-on analysis
Preparation of nuclei and nuclear run-on analysis were performed as described previously (16). The probes used for hybridization were as in ref. 16.

Gel retardation analysis
Nuclear extract preparation and gel retardation analysis were carried out according to ref. 24.

Transfections
Transfection of plasmid DNA was performed using the calcium phosphate co-precipitation technique (25). 14–16h prior to transfections cells were seeded at a density of 2.5 x 10^4 per 15 cm tissue culture plate and 25 μg of either the wild-type c-fos

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Fig. 1. In vivo DMS-reactivity of the c-fos SRE and its flanking sequences in NIH 3T3 and F9 cells. a. coding strand; n, naked DNA: 0', 15', 60', DNA from cells treated with TPA for the times indicated. Protection and hyperreactivity of guanosines are indicated by filled circles and by asterisks, respectively. b. non-coding strand. c. Summary showing positions of guanosines with altered in vivo DMS-reactivity in NIH 3T3 and F9 cells. The SRE (solid line) and the API-like binding site (dashed line) are boxed; symbols as in a.; numbers indicate nucleotide positions with respect to the transcriptional start site.
promoter construct or of the mutant promoter construct were transfected together with 5 μg of the α-globin internal control plasmid per plate. NIH 3T3 cells were incubated with the precipitate for 12–14h, F9 cells for 7h at 37°C. The cells were washed twice with warm PBS and DMEM/0.5% FCS was added to NIH 3T3 cells for 36h, and DMEM/F12/10% FCS was added to F9 cells for 14–16h prior to treatment of the cells with either TPA (100ng/ml) or FCS (20% final concentration). RNA for RNase protection analysis was prepared 45 min later.

**RESULTS AND DISCUSSION**

In comparison to protein-free (naked) genomic DNA methylated in vitro (Fig. 1a, lane 1 and 5) the DMS-reactivity pattern in the coding strand of the c-fos 5'-flanking region at the SRE and the adjacent API-like binding is altered at several guanosine residues, both in NIH 3T3 cells (lanes 2–4) and F9 cells (lanes 6–8). The pattern of protection and hypersensitivity against DMS-methylation is summarized in Fig. 1c. In NIH 3T3 cells the DMS-reactivity pattern is identical to that described for human A431 cells (17) indicating the binding of murine proteins similar to those in A431 cells. In F9 cells, the API-like binding site adjacent to the SRE shows a DMS-reactivity pattern identical to that in NIH 3T3 and A431 cells. In the SRE, however, two guanosine residues at positions −319 and −318 becomes detectable in vivo upon induction of c-fos transcription in F9 cells. Because F9 cells cannot be starved efficiently for serum, the phorbol ester TPA was used as inducing agent. In NIH 3T3 cells, the DMS-reactivity pattern did not differ between non-treated and TPA treated cells (Fig. 1a, lanes 2–4) suggesting constitutive binding of the multiprotein complex including p62 TCIF. This corresponds to the situation in A431 cells during EGF stimulation (17) and in NIH 3T3 cells stimulated by serum (my own unpublished results). In F9 cells, however, neither at 15 minutes after TPA application when transcriptional activity is maximal (Fig. 1a, lane 7, and Fig. 2), nor at 60 minutes after TPA treatment when transcription is already down-regulated (Fig. 1a, lane 8, and Fig. 2), was an enhanced protection of the guanosines at positions −319 and −318 detectable.

![Fig. 2. Nuclear run-on analysis of gene transcription in NIH 3T3 and F9 cells at various times after TPA treatment. fos, c-fos proto-oncogene; jun, c-jun proto-oncogene; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase gene; λ, hybridization to Lambda phage DNA as control for unspecific hybridization. Note that in the F9 experiment 10 times more c.p.m. of labeled transcripts were used than in the case of the NIH 3T3 cells.](image)

Since it has been shown previously that only c-fos promoter constructs capable of binding p62 TCIF, and, therefore, of forming a ternary complex, are induced efficiently by serum in NIH 3T3 cells (1) and by phorbol esters in BALB/c 3T3 cells (2), I examined whether the p62 TCIF-dependent DMS-protection at positions −319 and −318 becomes detectable in vivo upon induction of c-fos transcription in F9 cells. Because F9 cells cannot be starved efficiently for serum, the phorbol ester TPA was used as inducing agent. In NIH 3T3 cells, the DMS-reactivity pattern did not differ between non-treated and TPA treated cells (Fig. 1a, lanes 2–4) suggesting constitutive binding of the multiprotein complex including p62 TCIF. This corresponds to the situation in A431 cells during EGF stimulation (17) and in NIH 3T3 cells stimulated by serum (my own unpublished results). In F9 cells, however, neither at 15 minutes after TPA application when transcriptional activity is maximal (Fig. 1a, lane 7, and Fig. 2), nor at 60 minutes after TPA treatment when transcription is already down-regulated (Fig. 1a, lane 8, and Fig. 2), was an enhanced protection of the guanosines at positions −319 and −318 detectable.

Although F9 cells show little or no p62 TCIF binding in vivo, transient transcriptional activation of c-fos by TPA occurs
efficiently in both cell types as shown by nuclear run-on analysis (Fig. 2) using nuclei from cells treated in parallel with the cells used for footprinting analysis. However, to detect basal c-fos transcription in F9 cells, 10 times more c.p.m. of labeled transcripts had to be used than in the case of NIH 3T3 cells. Although the fold induction of c-fos was identical in F9 and NIH 3T3 cells, by normalizing for total c.p.m. and GAPDH, the overall level of c-fos transcription was markedly lower in F9 cells.

In order to exclude that a possible TPA-dependent protection of the guanosines at positions -319 and -318 would be missed in F9 cells because c-fos might have been induced by TPA in only a minority of cells, c-fos expression was examined in untreated cells and in cells treated with TPA for 90 minutes by indirect immunofluorescence analysis using anti-Fos antibodies. TPA treatment induced expression of Fos-protein in virtually all cells (not shown).

Lack of ternary complex formation in F9 cells in vivo is not due to the absence of p62TCF. This was demonstrated in gel retardation experiments using an SRE oligonucleotide probe (see Fig. 3a) and nuclear extracts from NIH 3T3 and F9 cells, respectively. Extracts from both NIH 3T3 (Fig. 3b, lane 1) and from F9 cell nuclei (lane 4) gave rise to two complexes, called complex I and II (1). The complexes are specific because they can be competed efficiently by an excess of unlabeled SRE oligonucleotide (lanes 2 and 5) but not by an unrelated oligonucleotide (lanes 3 and 6). Complex II indeed corresponds to the ternary complex described previously (1): A mutated SRE oligonucleotide, SREel (see Fig. 3a) carrying a mutation that abolishes ternary complex formation (1) showed no complex II formation (lanes 7 and 8).

If p62TCF were indeed absent from the protein complex over the SRE in F9 cells in vivo, as suggested by the genomic footprinting data, a mutant c-fos promoter construct that binds SRF but fails to form the ternary complex involving p62TCF should be similarly inducible by TPA as the wild-type promoter construct upon transfection into F9 cells. In contrast, if ternary complex formation seen in NIH 3T3 were necessary for c-fos induction, the mutant promoter should be non-inducible in these cells. In order to test for such functional differences, mouse c-fos promoter CAT fusion constructs containing either wild-type promoter sequences from positions -356 to +109 (-356wt) (2) or a mutated c-fos promoter carrying a point mutation that prevents ternary complex formation (-356pm18) (2) were transiently transfected into F9 teratocarcinoma stem cells and into NIH 3T3 cells together with a plasmid expressing the human a-globin gene (26) which served as an internal control for transfection efficiency and RNA recovery. RNA amounts produced by these constructs in untreated cells and in cells treated with TPA and serum, respectively, were determined by RNase protection analysis (Fig. 4). In both types of cells, the mutated promoter construct and the wild-type promoter were equally inducible (Fig. 4a + b). However, by normalizing the transfection efficiency to the a-globin internal control, in NIH 3T3 cells (not in F9 cells) the overall level of transcription of the mutated promoter was lower than the transcriptional level of the wild-type construct. Serum inducibility in NIH 3T3 and F9 cells was also retained with constructs containing the SREel oligonucleotide (see Fig. 3a) in front of either the Herpes simplex thymidine kinase promoter or a heterologous TATA-box linked to the CAT gene (data not shown).

The most likely interpretation of the genomic footprints is that p62TCF is not involved in the complex in F9 cells in vivo. Therefore, the mutation (-356pm18) that prevents ternary complex formation does not alter function. The API1-like binding site adjacent to the SRE, however, is occupied also in F9 cells. Since F9 cells contain no detectable levels of API1 (28), this suggests the participation of a factor different from API1 in the formation of the multiprotein complex. Although both cell types posses the ability to form a ternary complex in vitro, it is not clear yet what determines the complex in vivo and what its functional implications are. In NIH 3T3 cells, p62TCF is part of the in vivo complex. Therefore, the mutation matters with respect to promoter efficiency (not inducibility). Possibly, the cell types differ in an unknown additional partner of the complex or in a modifying enzyme both of which are not relevant to in vitro DNA binding.

The genomic footprints in F9 cells, the nuclear run-on data and the transfection experiments in F9 and NIH 3T3 cells presented here suggest that ternary complex formation is not an absolute prerequisite for c-fos induction by serum or phorbol esters. The ability of ternary complex formation in vivo, however, may enhance overall promoter activity while all the signalling does not require the complex but rather operates through the SRF alone. The results are at variance with those of other laboratories. Data of Shaw et al. (1) have suggested that ternary complex formation is necessary for efficient serum induction of c-fos in NIH 3T3 cells. A recent study of Graham and Gilman (2) performed with BALB/c 3T3 cells implies that induction by phorbol esters, but not by serum depends on ternary complex formation. Since it is known that in vitro SRF/p62TCF shows an at least fifty fold higher affinity to the SRE than SRF alone (18), p62TCF might function as a stabilizing factor that helps SRF in efficient binding to the SRE, rather than to serve as target for stimuli induced modifications. This explanation implies that SRF itself could be the target for different signal transduction pathways and is consistent with the observation that in the studies in which ternary complex formation was shown to be necessary for an efficient induction by serum (1) and phorbol esters (2), inducibility of the mutants unable to form a ternary complex was only reduced but not abolished. This hypothesis does not exclude the possibility that p62TCF, just by increasing the efficiency of SRF binding, could be necessary for induction of c-fos via the SRE in cells containing lower levels of SRF or containing an
SRF that binds less efficiently to DNA due to a different modification pattern. Why in a single cell type one stimulus (TPA) but not another (serum) needs ternary complex formation (2), could be explained by different efficiencies of the signal transduction pathways used (protein kinase C-dependent versus protein kinase C-independent ones). Thus phorbol esters might be less potent in inducing SRF modifications than serum in certain cells and, in order to stimulate transcription efficiently, p62TCF would be necessary in these cells to allow the fewer molecules of modified SRF to bind with higher efficiency to the SRE.

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