Transcriptional repression mediated by the serum response factor

Wolfram H. Ernst, Ralf Janknecht, Michael A. Cahill, Alfred Nordheim*

Institut für Molekularbiologie, Medizinische Hochschule Hannover, D-30623 Hannover, Germany

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Abstract The serum response element (SRE) contributes to transcriptional repression of the c-*fos* proto-oncogene. We show that the transcription factor SRF is able to repress SRE-dependent transcription, apparently by sequestering a co-activator. Only the DNA-binding core region is required for this SRE-dependent repression. Furthermore the phosphorylation status at potential casein kinase II sites within an N-terminal repression domain affects SRE-independent transcription. SRF may thus pleiotropically influence cellular transcription, representing a novel aspect of SRF function.

Key words: c-*fos*; Serum response factor; Squelching; TATA-box binding protein; Transcription regulation

1. Introduction

The transmission of extracellular signals often ends in the cell nucleus with the rapid and transient transcriptional activation of immediate early genes. One regulatory sequence mediating this transcriptional activation is the serum response element (SRE), which contributes to transcriptional regulation of a variety of genes, including the c-*fos* proto-onocogene [1]. In addition, the c-*fos* SRE is critical for basal repression of c-*fos* transcription [2–4].

The first SRE-binding protein identified was the serum response factor (SRF), a ubiquitously expressed nuclear phosphoprotein capable of dimerization [5,6]. The c-*fos* SRE–SRF complex has the potential to recruit ternary complex factors which belong to the Ets transcription factor family [7,8]. Mitogen-activated protein kinases phosphorylate ternary complex factors, thereby enhancing their transactivation potential and thus leading to c-*fos* induction [9–11].

Functional analyses of SRF have indicated that it acts as a transcriptional activator in vitro [5]. The C-terminal region of SRF may be responsible for this transcriptional activation since it functions as a transactivation domain when fused to the DNA-binding domain of the yeast protein GAL4 [12,13]. Contrarily, the N-terminus of SRF mediates repression of transcription in GAL4-SRF fusions [12]. In vitro studies revealed that the core region of SRF (amino acids 133–222) is sufficient for DNA binding, dimerization and ternary complex factor recruitment [14]. However, modulation of the DNA-binding properties of SRF can be achieved by phosphorylation in the N-terminal region, where five phosphorylation sites have been mapped in vivo [15,16]. Additional sites might be phosphorylated in vivo in the C-terminal region of SRF [13,17]. Since an increase of SRF phosphorylation occurs after growth factor stimulation in some cell lines, induced phosphorylation of SRF might affect c-*fos* stimulation [6,18].

In this report, it is demonstrated that SRF can repress transcription in vivo both SRE-specifically and SRE-independently. SRF could thereby contribute to c-*fos* down-regulation. Furthermore, SRF may interfere with the function of many other transcription factors by regulatory squelching.

2. Materials and methods

The coding sequence of human *SRF* cDNA was fused to an artificial Kozak sequence and cloned into SG-new, a derivative of pSG5 [19], thereby generating a eukaryotic expression plasmid (SG-SRF) directing the production of full-length SRF. CMV-SRF was generated by cloning human *SRF* cDNA into the eukaryotic expression vector pEV3S [20] such that the first 9 amino acids of SRF were replaced by the sequence MASWGSELPI. Truncated versions of SRF were constructed by cloning parts of the *SRF* coding sequence into the eukaryotic pEV expression plasmids [20]. The C-terminal truncation ΔC9 was identical to CMV-SRF at its N-terminus, but displayed the following amino acids at its C-terminus (the last authentic SRF amino acid is numbered): E_{242}ARAAGYLAS. N-terminal truncations possessed the following amino acids at their N-termini (the first authentic SRF amino acid is numbered): ΔN2 (MASWGSGYPG$_{73}$) and ΔN4 (MASWGSGYPD-KLG$_{113}$). ΔN2/ΔC9 and ΔN4/ΔC9 were composites of ΔC9 and either ΔN2 or ΔN4.

Transient transfection of mouse fibroblast NIH 3T3 cells, induction of quiescent cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), measurement of luciferase activity, and normalization of transfections were performed as described [17]. Relative luciferase activity is given as the mean (± S.D.) of at least three independent experiments.

3. Results

c-*fos* transcription is down-regulated to a low basal level in continuously growing NIH 3T3 cells [4]. Under these growth conditions we transfected an SRF expression plasmid (SG-SRF) or its parental plasmid SG-new into NIH 3T3 cells and assessed their impact on transcription with a luciferase reporter construct driven by two c-*fos* SREs (SRE_2-tk80-luc) [10]. At the highest amount of expression plasmid used (3 μg), transcription from the SRE_2-tk80-luc reporter construct was approximately 2-fold repressed in the presence of SG-new (Fig. 1A). This suggests that eukaryotic promoter elements within SG-new titrate general components of the transcriptional machinery and thus lead to unspecific repression of transcription. Accordingly, transcription from the SRE-less reporter construct tk80-luc [10] was also repressed 2-fold at 3 μg of transfected SG-new (Fig. 1B). However, upon transfection of 3 μg SG-SRF, transcription from the SRE_2-tk80-luc reporter was approximately 19-fold repressed (Fig. 1A). The difference in the repression between SG-SRF and SG-new, which is a factor of approximately 9, is SRF-specific. This SRF-mediated repression was primarily SRE-dependent, since transcription from the SRE-less tk80-luc construct was nearly unaffected by over-

*Corresponding author. Fax: (49) (511) 532 4283.

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expression of SG-SRF when compared to SG-new (Fig. 1B). These results demonstrate that over-expressed SRF is able to repress transcription via the c-*fos* SRE.

Previously, it has been shown that SRF affects preinitiation complex formation by TFIID in vitro [21]. Thus, we wondered whether SRF may also interfere in vivo with the function of TFIID, which consists of the TATA-box binding protein (TBP) and the TBP-associated factors (TAFs). Therefore the SG-TBP expression plasmid was co-transfected with either 1.5 μg SG-new or 1.5 μg SRF. Increasing amounts of TBP resulted in the repression of SRE-dependent transcription in the presence of 1.5 μg SG-new (Fig. 2A). This repression was not due to eukaryotic promoter sequences within SG-TBP titrating general components of the transcriptional machinery, as suggested above for SG-new, since this type of unspecific repression only marginally increased above 1.5 μg of SG-new or SG-SRF (see Fig. 1). Rather, this effect was very likely due to the titration of TAFs by a surplus of TBP or of general transcription factors by a surplus of TFIID, which would deplete the TAF pool available to TATA-box bound TBP molecules or the pool of general transcription factors available to interact with TATA-box bound TFIID, respectively. Surprisingly, this squelching effect of TBP was not observable with the SRE_2-tk80-luc reporter in the presence of 1.5 μg SG-SRF (Fig. 2A). These results suggest that exogenous SRF already sequesters a factor required for SRE-mediated transcription and therefore TBP over-expression would have no additional effect. This factor appears not to be TBP itself or another general transcription factor, since the solely TATA-box dependent transcription of the tk80-luc reporter was comparably repressed by TBP over-expression in the presence of 1.5 μg SG-new and of 1.5 μg SG-SRF (Fig. 2B). Thus, the SRE-specific repression of transcription by over-expressed SRF is probably due to the sequestration of a co-activator.

Recently, a C-terminal transactivation and an N-terminal repression domain have been identified in fusion proteins of SRF and the yeast protein GAL4 [12,13]. The C-terminal transactivation domain has been localised to SRF amino acids 406–476 [13] and the repression domain extends from amino acids 45–141 (R.J. and W.H.E., unpublished results). In order to investigate whether these domains mapped in artificial GAL4-SRF fusion proteins have a function in the context of the proper SRF molecule, we constructed several mutations (Fig. 3A). All of these molecules can bind to the c-*fos* SRE by virtue of the core region, and their expression was confirmed and quantified in comparative terms by gel retardation assays (data not shown). Once again, SRF repressed SRE-driven transcription whereas transcription from the tk80-luc reporter construct was nearly unaffected (compare pEV3S to CMV-SRF in Fig. 3B). Deletion of the C-terminal transactivation domain in ΔC9 did not abolish the repression of SRE-driven transcription, but rather led to a 2-fold higher degree of SRE-dependent repression relative to CMV-SRF without affecting transcription from the tk80-luc reporter. In contrast, the N-terminal truncations ΔN2 and ΔN4 behaved as CMV-SRF. Similarly, the ΔN2 and ΔN4 mutants did not significantly differ from CMV-SRF in their ability to stimulate SRE-dependent transcription upon TPA treatment of starved cells, whereas the loss of the transactivation domain in ΔC9 did cause a significant reduction (Fig. 3C). Thus, the N-terminal repression domain seemingly does not affect SRF's transcriptional competence, while the C-terminal transactivation domain contributes to both basal and TPA-stimulated transcription, yet is not required for repression of SRE-dependent transcription by SRF. Therefore, the core region of SRF is most likely required and sufficient for the SRE-dependent repression of transcription.

We speculated that the function of the N-terminus was obstructed in the presence of the C-terminus. Thus, we constructed the N-terminal truncations ΔN2 and ΔN4 in the context of ΔC9 (Fig. 3A). Consistent with a partial loss of the N-terminal repression domain, the respective mutant ΔN2/ΔC9 displayed a 2-fold higher level of relative luciferase activity with

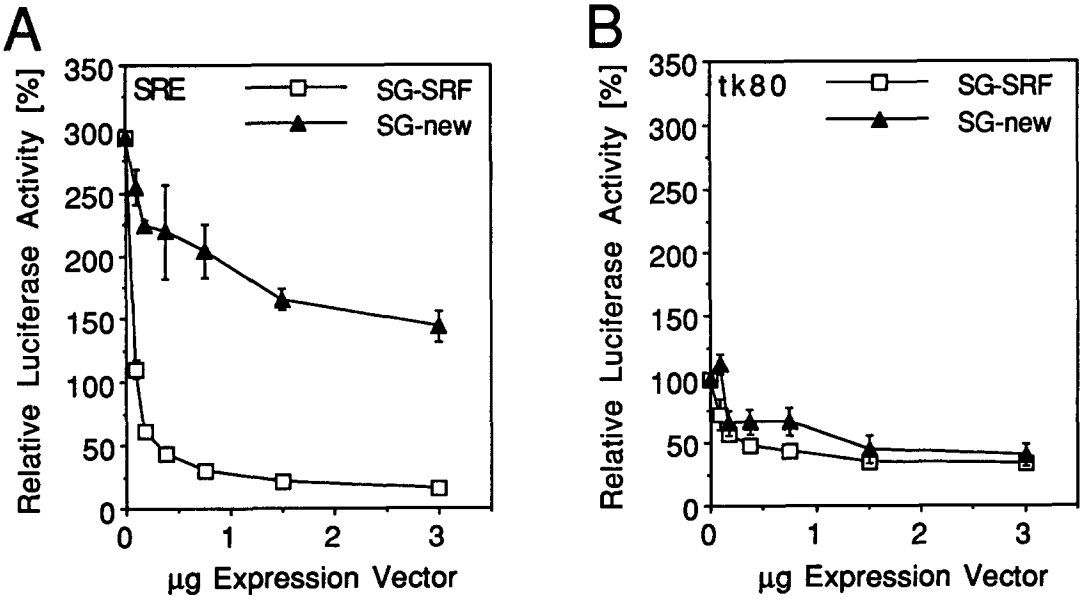

Fig. 1. SRF-mediated repression of transcription. The SRE_2-tk80-luc reporter plasmid (A) or the SRE-less tk80-luc reporter plasmid (B) were co-transfected with different amounts of either the SG-SRF expression plasmid or the parental vector SG-new. Transfected NIH 3T3 cells were then kept in medium containing 10% fetal calf serum for 36 h. Luciferase activity of cells transfected with solely tk80-luc was arbitrarily set to 100%.

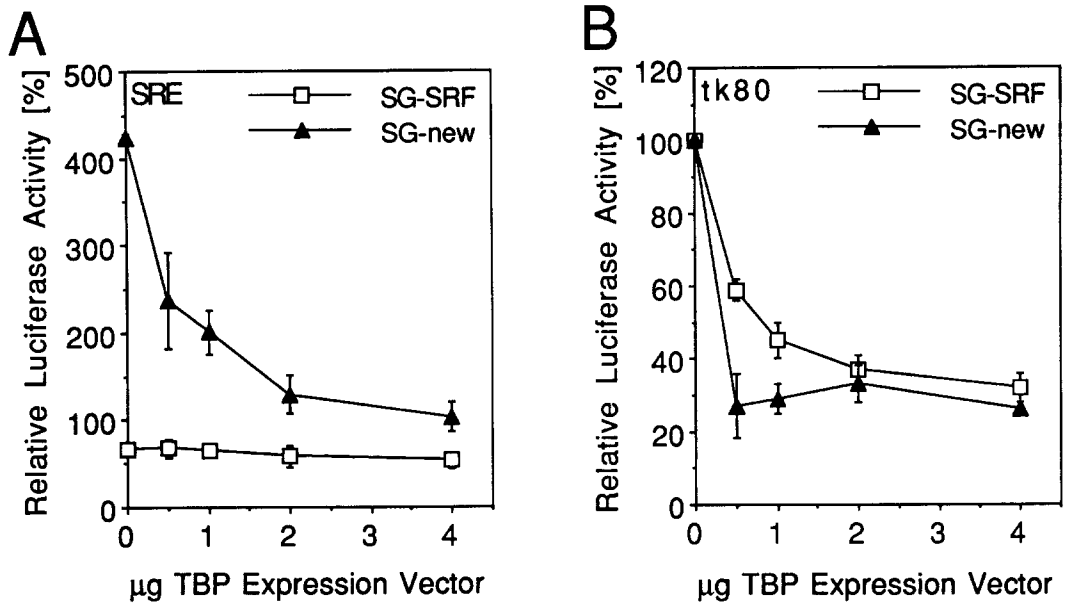

Fig. 2. Effects of TBP on transcription. The SRE_2-tk80-luc (A) or the tk80-luc reporter plasmid (B) were co-transfected with increasing amounts of human TBP expression plasmid and either 1.5 µg SG-SRF expression plasmid or 1.5 µg of the parental vector SG-new. Luciferase activity derived from NIH 3T3 cells transfected with the expression vector SG-new in the absence of TBP was arbitrarily set to 100%.

the SRE_2-tk80-luc reporter compared to ΔC9, which was not observed with the tk80-luc reporter (Fig. 3B). Thus, the N-terminal repression domain of SRF contributes to SRE-specific transcriptional repression mediated by ΔC9. Although the N-terminal repression domain is apparently functionally impeded by the C-terminus in full-length SRF, conformational changes within SRF could alter this situation at binding sites other than the c-*fos* SRE or upon interaction with different affiliated proteins. Precedent for such a mechanism is provided by the yeast protein MCM1, which shares with SRF a homologous core region. MCM1 undergoes conformational changes at selected promoter sites and thereby exposes a previously shielded transactivation domain [22]. Further deleting parts of the N-terminal repression domain in ΔN4/ΔC9 drastically increased relative luciferase activity from both the SRE_2-tk80-luc and the tk80-luc reporter (Fig. 3A,B), indicating that these effects were binding-site unspecific.

We questioned whether phosphorylation was involved in the effects on binding site independent transcription mediated by the N-terminus. For this purpose, the four potential casein kinase II sites S^{77}, S^{79}, S^{83} and S^{85} [15,16,23] were mutated to alanine in the M4/ΔC9 mutant (Fig. 3A). Both SRE-driven and SRE-independent relative luciferase levels were elevated in comparison to ΔC9 (Fig. 3B), indicating that this effect is not SRE-specific. Further mutating the phosphoacceptor S^{103} [15,18] to alanine in the mutant M9/ΔC9 had no additional effect. The M6/ΔC9 mutant, differing from ΔC9 only by the mutation of S^{103} to alanine, displayed a phenotype comparable to ΔC9. Thus, the presence of S^{103} does not affect SRE-independent transcription. Taken together, these results demonstrate that the presence of the potential casein kinase II sites in the region 77–85 influences ΔC9's impact on SRE-independent transcription.

Introducing the M4-, M6- or M9-mutations into the context of full-length CMV-SRF paralleled the trends observed with the corresponding ΔC9 mutants, although the effects were much less pronounced (Fig. 3A,B). This again implies that the presence of the C-terminus of SRF impedes the function of the N-terminal domain. Finally, mutation of the phosphorylation sites did not significantly affect the ability to mediate TPA induction, since the M4, M6 and M9 mutants were comparable to CMV-SRF and the M4/ΔC9, M6/ΔC9 and M9/ΔC9 mutants resembled the respective ΔC9 molecule (Fig. 3C).

4. Discussion

Although it has been shown that basal repression of the c-*fos* gene is dependent on a functional SRE [2–4], none of the many SRE-binding proteins [1] has been demonstrated to mediate c-*fos* repression. Our results show that over-expressed SRF can repress SRE-dependent transcription in NIH 3T3 cells. Neither the N-terminal repression domain nor the C-terminal transactivation domain were required for this function. Endogenous SRF is also 'over-expressed' after serum stimulation with a delayed kinetics relative to c-*fos* [5,24]. Thereby endogenous SRF may contribute to post-induction repression of c-*fos*, possibly by sequestering a co-activator required for SRE-mediated transcription. Two lines of evidence argue for such an indirect mechanism: (i) introduction of two c-*fos* SREs into tk80-luc led to an approximately 3-fold enhanced level of luciferase activity in the absence of any exogenous SRF (see Fig. 1), indicating that the SRE, and thus very likely its binding protein SRF, actually activates transcription. Since even small doses of exogenous SRF led to a reduction of transcription, this would imply that the concentration of endogenous SRF in NIH 3T3 cells is close to, or already in, the squelching region of its dose–response curve. (ii) Over-expression of TBP, which results in squelching of TATA-box mediated transcription in the presence and absence of over-expressed SRF, squelched SRE-dependent transcription only in the absence of exogenous

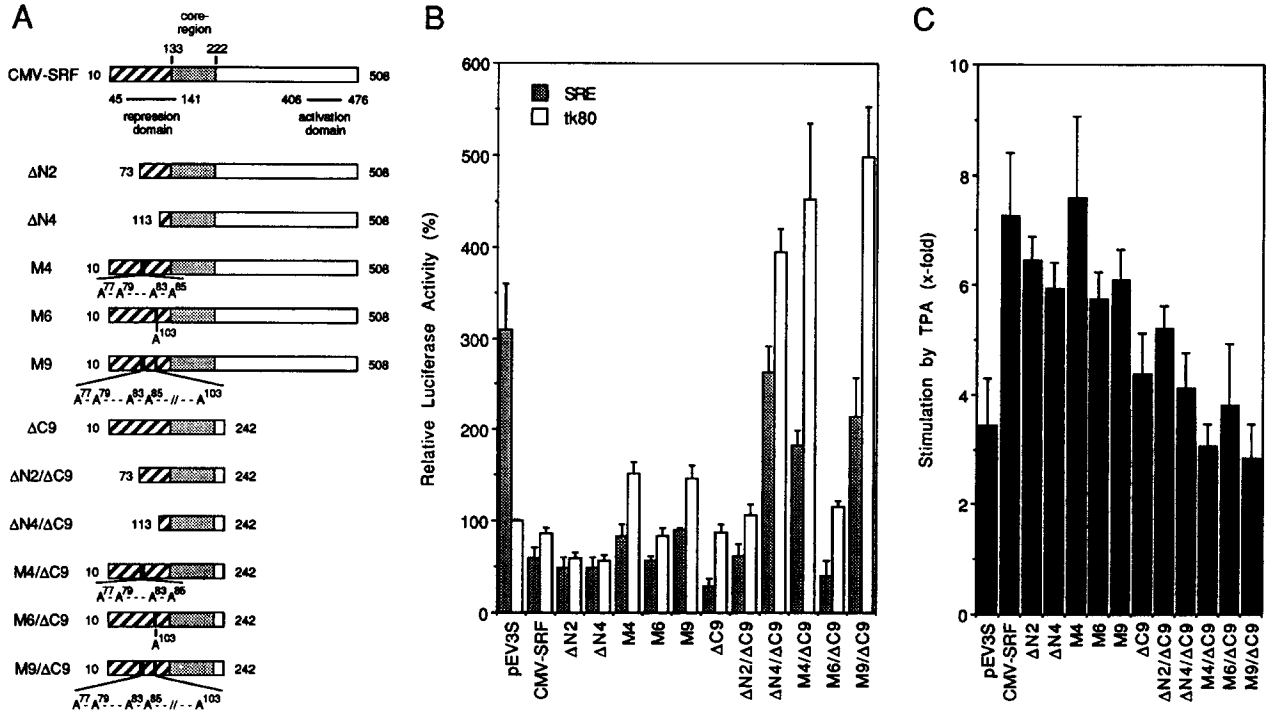

Fig. 3. Transcriptional activity of SRF truncations and phosphorylation mutants. (A) Schematic presentation of SRF molecules utilized. The positions of serine-to-alanine exchanges are indicated. (B) 1.5 μg of the indicated expression plasmid was co-transfected with SRE_2-tk80-luc (stippled bars) or tk80-luc (white bars) into NIH 3T3 cells that were kept in medium containing 10% fetal calf serum for 36 h after transfection. Luciferase activity obtained from cells transfected with the empty expression vector pEV3S and the tk80-luc reporter construct was set to 100%. (C) Starved NIH 3T3 cells, which had been transfected with 1.5 μg of the indicated expression plasmid and with the SRE_2-tk80-luc reporter plasmid, were induced for 3 h with TPA. The enhancement of luciferase activity over starved cells is depicted.

SRF. This strongly suggests that exogenous SRF already sequesters a co-activator indispensable for SRE-dependent transcription. This co-activator does not appear to be TBP itself, since exogenous TBP could not alleviate the SRF-mediated repression of SRE-driven transcription and similarly exogenous SRF did not inhibit TBP-mediated squelching observable with the tk80-luc reporter. The latter fact also argues against the possibility that this co-activator is a general transcription factor such as TFIIF, with which SRF reportedly interacts [25].

The sequestration of a co-activator by SRF could also affect promoters without SRF binding sites if other transcription factors rely on the same co-activator for function. Indeed, squelching of such a common co-activator has been described for SRF in in vitro transcription assays [26]. Thereby, SRF may pleiotropically influence the transcriptional status of a cell. Such a scenario of regulatory squelching may be widespread [27] and could also explain how the Fos protein represses its own expression via the SRE, independently of its own DNA-binding domain [28–30]. It is even conceivable that both SRF and Fos sequester the same co-activator and thus jointly affect c-*fos* down-regulation after c-*fos* induction.

SRF may additionally affect the general transcriptional profile of a cell by virtue of its casein kinase II target sites S^{77}, S^{79}, S^{83} and S^{85} [15,16,23]. Mutating these in vivo phosphorylation sites to alanine in the context of the ΔC9 protein raised the levels of luciferase activity SRE-independently. Presently, we cannot definitively explain this phenomenon, yet these alanine mutants may sequester a co-activator the high abundance of which normally induces some type of squelching. Alternatively, these alanine mutants may recruit a transcriptional repressor. Since ΔN4/ΔC9, which nearly matches the DNA-binding core region, also displayed such a phenotype, it is proposed that the core region interacts with this hypothetical co-activator or repressor and that this interaction is prevented by the phosphorylation of the casein kinase II sites within the N-terminus. We note that this interaction partner would not be the co-activator sequestered by full-length SRF in Fig. 1, since there the SRE-independent transcription was unaffected by SRF. The effects on SRE-independent transcription upon mutating the casein kinase II sites at S^{77}, S^{79}, S^{83} and S^{85} were much less pronounced yet also measurable with the full-length SRF molecule, suggesting that the N-terminus is functionally impeded in the presence of the C-terminus. Irrespective of the mechanism involved, our data show for the first time the modulation of transcription by the SRF casein kinase II sites in the region 77–85 in an SRE-independent fashion.

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