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Mutual transrepression of Fos and the glucocorticoid receptor: involvement of a functional domain in Fos which is absent in FosB

Frances C.Lucibello, Emily P.Slater, Karin U.Jooss, Miguel Beato and Rolf Müller

Institut für Molekularbiologie und Tumorforschung (IMT), Philipps-Universität Marburg, Emil-Mannkopff Strasse 2, 3550 Marburg, FRG

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In this study, we show that Fos protein can repress transactivation by the glucocorticoid receptor (GR). In addition, we demonstrate that GR is capable of inhibiting, in a hormone-dependent fashion, Fos-mediated transactivation of AP-1 dependent transcription. Moreover, repression of the serum response element by Fos is abolished by the GR in the presence of hormone. Transrepression of glucocorticoid mediated induction involves a region of Fos, located between amino acids 40 and 111, to which no function has been previously assigned, and which is poorly conserved among Fos, FosB and Fra-1. In agreement with this finding, FosB is not capable of transrepressing GR activation of transcription, representing the first functional difference between Fos and FosB. We have mapped the domain of the GR which is required for repression of AP-1 dependent transcription, to the region of central DNA binding domain. Our results suggest that Fos and the GR may form transcriptionally inactive complexes and point to a regulatory interrelationship between different signal transduction pathways.

Key words: c-*fos*/FosB/glucocorticoid receptor/transregulation

Introduction

The product of the proto-oncogene c-*fos* is a transregulatory protein playing a crucial role in the control of gene expression (for a review see Curran and Franza, 1988). It has been shown that Fos forms a stable complex with the product of another oncogene, c-*jun* (Chiu *et al.*, 1988; Franza *et al.*, 1988; Lucibello *et al.*, 1988; Sassone-Corsi *et al.*, 1988a; Schönthal *et al.*, 1989) and that this complex, the transcription factor AP-1 (Angel *et al.*, 1987, 1988; Bohmann *et al.*, 1987; Lee *et al.*, 1987) binds with high affinity to an AP-1 binding site (TPA response element; TRE). This interaction and the subsequent transactivation of AP-1 dependent transcription (Chiu *et al.*, 1988; Lucibello *et al.*, 1988; Sassone-Corsi *et al.*, 1988a; Schönthal *et al.*, 1989) requires both an intact leucine repeat and a functional DNA binding domain in Fos (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; Neuberg *et al.*, 1989a,b; Schuermann *et al.*, 1989; Turner and Tjian, 1989). One function of Fos is therefore to increase the affinity of Jun for the TRE through cooperative binding.

Apart from its transactivating properties, Fos is also capable of transrepression. It has a negative regulatory effect on c-*fos* and heat shock protein 70 (HSP70) promoters (Sassone-Corsi *et al.*, 1988b; Schönthal *et al.*, 1988, 1989; Lucibello *et al.*, 1989). Transrepression of the mouse c-*fos* promoter occurs mainly through the serum response element (SRE), which is the major mediator of growth factor stimulation (König *et al.*, 1989; Lucibello *et al.*, 1989; Schönthal *et al.*, 1989; Shaw *et al.*, 1989). The mechanism involved in transrepression via the SRE is however different from that of transactivation of the TRE. Mutations in the DNA binding domain of Fos do not affect its ability to repress the *fos* promoter during serum induction, although an intact leucine repeat is still required (Lucibello *et al.*, 1989).

In the course of studies pertaining to the identification of new DNA elements which mediate regulation by Fos, we discovered that the hormone dependent activation through glucocorticoid responsive elements (GRE) is transrepressed by cotransfected Fos. The converse was also found to be true, i.e. the hormone dependent repression by the glucocorticoid receptor (GR) of Fos-mediated transactivation of AP-1 dependent transcription. Moreover, we found that the transrepression by Fos of the SRE is blocked by the GR. In order to identify the domains in the two proteins that are responsible for these transregulatory properties and to gain some insight into the molecular mechanisms involved, we analysed the transrepressing potential of both mutant Fos and the GR.

Fos is composed of a number of domains which are required for dimerization, DNA binding, transactivation of AP-1 dependent transcription, transrepression of the SRE, transformation and immortalization. Several functionally indispensable domains have been identified and shown to be located within a short evolutionarily conserved region comprising ~28% of the protein. We demonstrate here that a region of the Fos protein, for which no function has previously been assigned, is in fact crucial for transrepressing the GR dependent transcription. This domain lies between amino acids 40 and 111, a region which is not conserved among Fos related proteins, such as c-Fos, Fra-1 and FosB.

The *fos*-B gene was isolated by virtue of its homology to the DNA binding domain of the c-Fos protein (Zerial *et al.*, 1989). It encodes a nuclear protein which shares 70% homology with c-Fos. In addition, it possesses many features in common with c-Fos including: induction by serum, the ability to form a complex with Jun and related proteins, interaction of such complexes with the TRE and induction of transformation (Zerial *et al.*, 1989; M.Schuermann and R.Müller, in preparation), but no functional differences have been described to date. In this study, we report that unlike Fos, the FosB protein is not capable of transrepressing the glucocorticoid dependent transcription. Analysis of hybrid Fos-FosB proteins revealed that the amino-terminal part of the FosB protein, which is structurally different to c-Fos, is responsible for the lack of transrepressing activities in the FosB protein.

We also demonstrate that the effects of the GR on the TRE and SRE are hormone dependent. The GR is composed of at least three structural and functional domains: a carboxy-terminal domain responsible for hormone binding, a central domain responsible for DNA binding and an amino-terminal domain that modulates transactivation, though transactivation functions have also been mapped to the hormone and DNA binding domains (Beato, 1989). Activation of transcription requires the binding of the receptor to GREs in the vicinity of hormonally induced genes (Beato, 1989). Glucocorticoids are also able to repress transcription of certain genes by a poorly understood mechanism that involves binding of the GR to variant GREs (Akerblom *et al.*, 1988; Drouin *et al.*, 1989), and only requires the DNA binding domain of the receptor (Oro *et al.*, 1988). The analysis of a number of receptor mutants indicates that whilst most of the amino and carboxy termini can be deleted with no adverse effect, a region encompassing the DNA binding domain of GR is necessary for its transregulatory effects on the TRE and SRE.

Results

Transrepression of the GR binding site by Fos

Promoters containing a GRE linked to a reporter gene, chloramphenicol acetyl transferase (CAT), are transactivated in HeLa cells by the GR in the presence of the synthetic glucocorticoid, dexamethasone (Chalepakis *et al.*, 1990). In the present study we discovered that this transactivation was repressed ~5-fold by the Fos protein E300. In order to determine which functional domain in Fos is required for the transrepression of the GRE, we analysed a series of Fos protein mutants which had substitutions in the acidic, basic and leucine repeat between amino acid positions 134 and 193, a region of the protein previously shown to be crucial for DNA binding and dimerization with Jun (Neuberg *et al.*, 1989a,b, 1990; Schuermann *et al.*, 1989). Some of the mutants used are defective in TRE DNA binding, in transactivation of AP-1 dependent transcription and/or are unable to form a complex with Jun (Lucibello *et al.*, 1988, 1989; Schuermann *et al.*, 1989; M.Neuberg, M. Schuermann, F.C.Lucibello and R.Müller, in preparation). The ability of the mutants to transactivate the TRE is given at the top of Figure 1, which shows the results obtained with the Fos mutants. All the mutants were found to be able to transrepress glucocorticoid dependent transcription. Mutations in the acidic/basic region of the Fos proteins actually showed an increase in transrepression potential compared with the parent construct E300, which was independent of the net charge of this domain. This phenomenon was not investigated further in this study although possible explanations are presented in the Discussion. The results clearly indicate that an intact leucine repeat, the ability to bind DNA (TRE binding) and the ability to transactivate AP-1 dependent transcription are not a prerequisite for transrepression of the GRE by Fos.

A second group of Fos mutants was therefore used in order to locate the region of Fos protein required for transrepression of the GRE. We analysed deletion constructs which either lacked part of the amino and/or carboxy terminus (see Figure 2). The truncation of the carboxy terminus up to position 171, which lies within the leucine repeat, was found not to have any effect on the transrepression potential, indicating that the region between amino acids 171 and 316 has no crucial role, if any, in the repression of the GRE. In addition, the deletion of the first 40 amino acids was also found not to interfere with the transrepressing potential of Fos. Two independently made constructs were however found not to be able to transrepress the GRE: BR800 and M110s. Both proteins lack the first 110 amino acids although they differ in their carboxy termini. Taken together, the results indicate that the amino terminus of the Fos protein, between amino acids 40 and 111, is required for the protein to repress transactivation of the GRE by the GR.

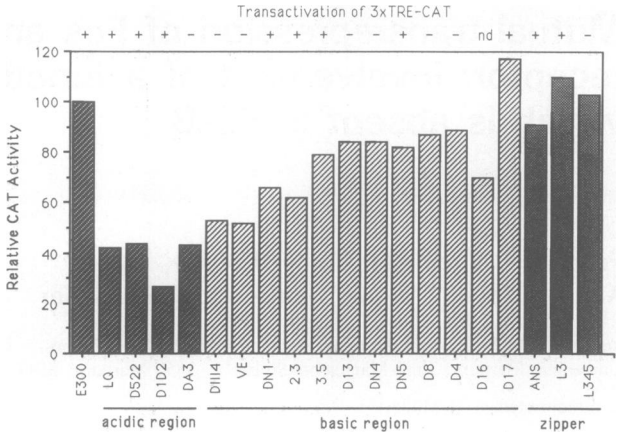


Fig. 1. GRE transrepressing properties of mutant Fos proteins. Fos mutants were cotransfected with TGT2.1cat (containing two copies of a canonical GRE; G.Chalepakis, M.Truss, E.P.Slater, S.Mader and M.Beato, submitted) and the CAT activity was measured after incubation with dexamethasone. The relative CAT activity is given as an average of three experiments taking the parent Fos construct E300 as 100. At the top of the figure the potential of the Fos mutants to transactivate AP-1 dependent transcription is given. The Fos mutants are arranged approximately according to the site of the mutation in the protein.

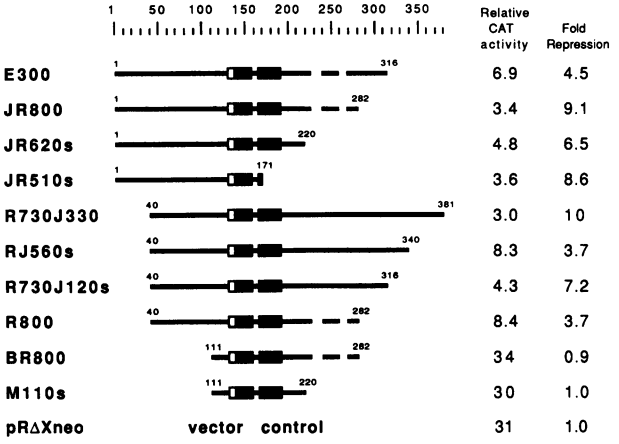


Fig. 2. Schematic representation of mutant Fos proteins and their ability to repress a GRE. The open box represents the acidic domain; the hatched box the basic DNA binding domain and the black box the leucine repeat. Numbers indicate amino acid positions. The ability of the Fos construct to repress GR dependent transcription is given as an average of three experiments. Relative CAT activity: per cent conversion of [^{14}C]chloramphenicol; 31% conversion in the control corresponds to a CAT activity of 1200 pmol/mg of protein/h.

Functional difference between Fos and FosB

Since we have shown that an evolutionarily non-conserved part of the Fos protein was involved in the repression of GR dependent transcription, we decided to analyse the transcriptional potential of the *fos* related gene FosB, which differs greatly from Fos in this domain (Zerial *et al.*, 1989). The experiment, displayed in Figure 3, indeed showed that FosB was unable to inhibit GR transactivation of the GRE. We therefore analysed the effects of Fos−FosB hybrid constructs for their ability to transrepress (see Figure 3). The results showed that construct CB, which has the amino terminus of Fos and the carboxy terminus from FosB, was able to transrepress nearly as well as E300 (3.0- and 4.5-fold respectively). On the other hand, the two constructs with the amino terminus from FosB (BC and BE300) behave like FosB and were not able to transrepress glucocorticoid dependent transcription. These results not only confirm that the amino terminus of Fos is required for transrepression of the GRE but also demonstrates the first functional difference between the two proteins Fos and FosB.

Transrepression of AP-1 dependent transcription by the GR

The transfection of Fos into HeLa cells results in transactivation of AP-1 dependent transcription of a reporter construct carrying multiple copies of the TRE linked to a CAT gene (Chiu *et al.*, 1988; Lucibello *et al.*, 1988; Sassone-Corsi *et al.*, 1988a; Schönthal *et al.*, 1989). Since we had shown that Fos represses glucocorticoid dependent transcription we decided to investigate whether the reverse was also true: does the GR interfere with AP-1 dependent transcription? We found that cotransfection of a wild-type GR (GR11) repressed both basal level activity and Fos-induced transactivation of the TRE in the presence of dexamethasone by 3- and 9-fold respectively. In the absence of hormone, no significant effect of the GR was observed (see Figures 4 and 5).

In order to localize the region of the receptor which is required for this repression, we tested a number of GR mutants for their potential to interfere with both basal level and Fos-induced AP-1 dependent transcription. The results, shown in Figure 4, indicate that the deletion of the amino terminus (GR10) up to amino acid position 406 reduced the repressing potential of the GR, but nevertheless in the presence of hormone GR10 inhibited Fos TRE transactivation by 4.5-fold and basal level activity of the TRE by 2.6-fold. Deletion of the carboxy terminus from amino acid 557 to 795 (GR13) had a similar effect as GR10, but the effect was largely independent of hormone (the ligand binding domain is deleted in this construct).

However, two constructs with mutations in the DNA binding domain of the GR (GR20 and G442) were found to have no inhibitory effect on either basal level activity or Fos-induced expression of the TRE. In contrast, these constructs seem to have a slightly stimulatory effect, although the significance of this observation is hard to judge at present. One of these mutants, GR20, has a small internal deletion of amino acids 493 to 496 in the DNA binding domain which prevents the receptor protein from binding DNA (Rusconi and Yamamoto, 1987; S.Rusconi, personal communication). The second receptor mutant, G442, has a single base substitution Lys-442 to Gly and, although still capable of binding DNA with reduced affinity, the protein is not able

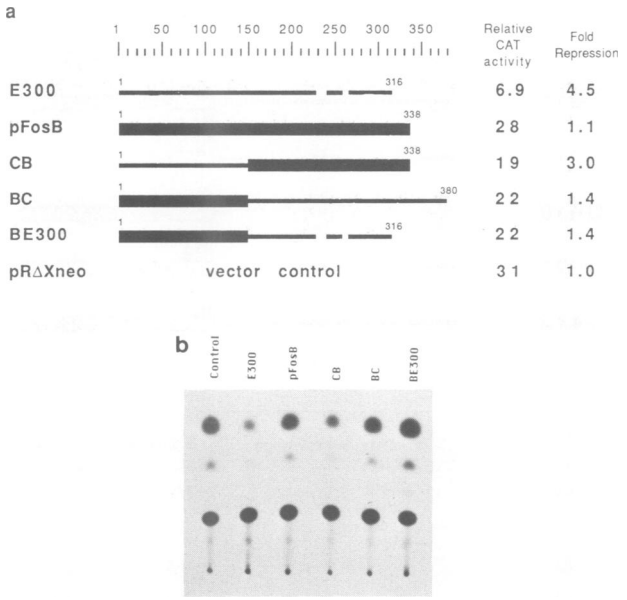


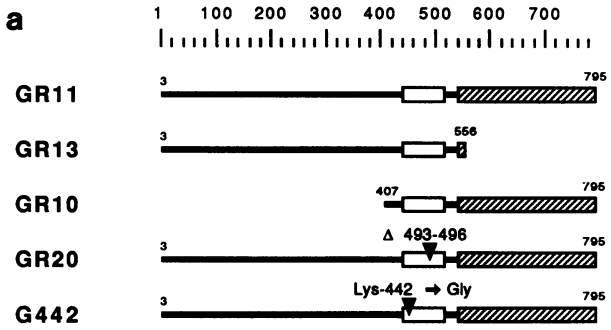
Fig. 3. Schematic representation of mutant Fos−FosB hybrid proteins and their ability to transrepress glucocorticoid dependent transcription. (**a**) Black lines represent Fos, the hatched boxes FosB sequences. Numbers indicate amino acid positions. The potential of the constructs to repress glucocorticoid dependent transcription of TGT2.1cat is shown. (**b**) Representative CAT assay analysing the ability of various Fos−FosB constructs to inhibit TGT2.1cat in the presence of hormone. Control: pRΔXneo.

to transactivate GR dependent transcription (Hollenberg and Evans, 1988).

As shown above, the GR constructs GR11, GR10 and GR13 all repressed the TRE−CAT construct in the absence of cotransfected Fos. Since cotransfections of GR11, GR10 and GR13 had no effect on the RSV-LTR used as a control (data not shown), the most likely explanation is that the transfected GR protein interacts with the endogenous Fos in HeLa cells, which is then no longer able to transactivate AP-1 dependent transcription. The results obtained using the GR mutants clearly indicate that the region of the DNA binding domain of the receptor is required in order to bring about a repression of AP-1 dependent transcription. The repression of the wild-type GR requires the presence of hormone.

GR inhibits Fos repression of the c-fos promoter

Fos represses its own promoter during serum stimulation, the major target being the SRE (König *et al.*, 1989; Lucibello *et al.*, 1989; Schönthal *et al.*, 1989). Since we have shown that the GR will inhibit Fos transactivation of the TRE, it was of great interest to determine whether the receptor would also affect the potential of Fos to transrepress the c-*fos* promoter. Since the c-*fos* promoter is composed of many regulatory elements, a simple construct, pSREΔ8 (the SRE is linked directly to the TATA box and the CAT gene) which can be readily induced by serum (Lucibello *et al.*, 1989), was used. Cotransfection of pJM (the encoded protein is identical with mouse c-Fos except for the presence of the five FBJ-MSV-specific point mutations; Van Beveren *et al.*, 1983) with pSREΔ8 resulted in a 5-fold repression during serum stimulation. In the absence of dexamethasone, cotransfected receptor, GR11 and GR20, did not affect Fos repression of SRE dependent transcription (see Figure 6). However, as depicted in Figure 6, in the presence of



b

Construct	Basal level expression		Fos-induced expression	
	-dex	+dex	-dex	+dex
vector control	3.1	2.1	12.4	11.1
GR11	3.6	1.2	10.3	1.3
GR13	1.1	1.4	3.5	3.0
GR10	3.7	1.4	10.3	4.3
GR20	2.8	2.8	11.4	19.0
G442	3.1	6.6	11.4	18.0

Fig. 4. Schematic representation of the GR mutants and their ability to transrepress both basal level and Fos-induced AP-1 dependent transcription. (**a**) The empty box represents the DNA binding domain; the hatched box indicates the steroid binding domain; the black arrows show the positions of the mutations within the DNA binding domain. Numbers represent amino acid positions. (**b**) Summary of the results obtained in three independent experiments. The GR was transfected with p3xTREtkcat3 ± E300 in the presence or absence of dexamethasone and the CAT activity was measured 48 h post transfection. The results are given as the fold repression by the GR as compared with the empty expression vector under the same conditions.

hormone, the wild-type receptor GR11 partially inhibited the repression of the pSREΔ8 by Fos (2-fold as opposed to 5-fold in the absence of transfected receptor). The mutant receptor, GR20, with the internal deletion in the DNA binding domain, did not affect Fos ability to repress the SRE. Similar results were also obtained with receptor mutant G442 (data not shown). The data obtained indicate that, as with the transrepression of AP-1 dependent transcription by the receptor, the GR requires a functional DNA binding domain in order to inhibit, in a hormone dependent fashion, the serum induced repression of the SRE by Fos.

GR does not bind to the TRE and Fos does not bind to the GRE

To exclude the possibility that the observed inhibition of Fos-dependent transactivation by GR could be due to competition for the TRE sites, we performed DNA binding experiments with Fos and Jun protein expressed in *Baculo* virus, *in vitro* translated Fos and purified GR from rat liver (Chalepakis *et al.*, 1988). An example is shown in Figure 7. As expected, *Baculo* virus expressed Fos did not bind to a significant extent to the TRE probe, Jun showed weak binding and both proteins bound cooperatively with high affinity (left panel of Figure 7). Under the same condition, the Fos–Jun complex did not show any specific binding to the GRE (right panel). The lower bands are due to unspecific binding as shown in competition experiments (data not

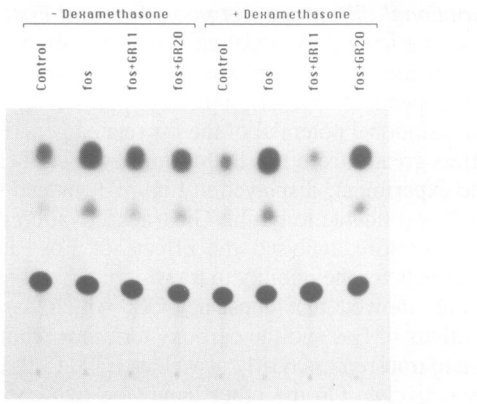


Fig. 5. Effect of GR11 and GR20 on p3xTREtkcat3 in the presence of cotransfected *fos* ± dexamethasone. CAT activity was measured 48 h post transfection. Control: empty expression vectors. DNA concentration was kept constant during transfection by the addition of the corresponding expression vector.

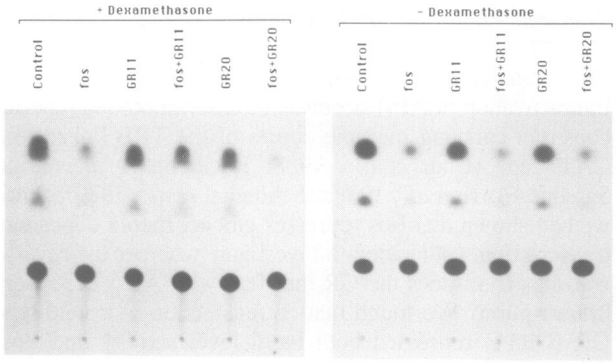


Fig. 6. The effect of GR on Fos repression of SRE dependent transcription during serum stimulation. pSREΔ8 was cotransfected with *fos* (JM or empty vector) and plus glucocorticoid mutant (or empty vector) into NIH3T3 cells. After transfection the cells were placed in serum free medium ± dexamethasone and 36 h later were stimulated with 10% FCS ± dexamethasone for a further 12 h before being harvested and CAT activity determined. Control: empty expression vectors.

shown). In the reverse experiment, no binding of purified rat liver GR to the TRE oligo was detected, at concentrations of receptor sufficient to bind quantitatively to a GRE oligo (data not shown). Thus, competition of Fos and the GR for DNA binding sites cannot be the explanation for the observed effects.

Discussion

The c-*fos* gene plays a crucial role, not only in the intracellular transduction of numerous extracellular stimuli by converting short-term signals into a long-term genomic response, but also in the process of transformation by other oncogenes. The latter is suggested by the observation that revertants of Fos-transformed cells are resistant to transformation by other oncogenes such as *ras* (Zarbl *et al.*, 1987) and the regulation of c-Fos by certain transforming proteins (ras; Angel *et al.*, 1988; Wasylyk *et al.*, 1988). According to the prevailing hypothesis, Fos acts at a central position in intracellular signal transduction by initiating the

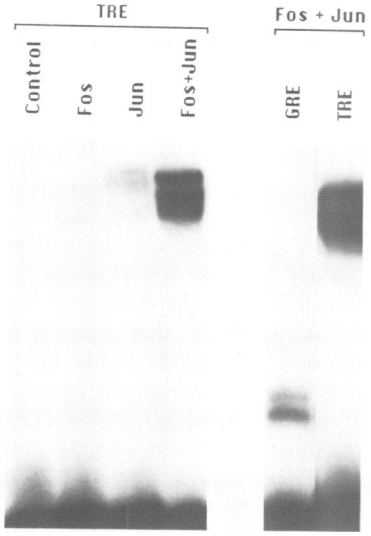


Fig. 7. Binding of Fos and Jun to the TRE and GRE. Extracts from the insect cell line SF infected with Baculovirus recombinants expressing either Fos or Jun were analysed in gel retardation assays for their ability to bind to a consensus TRE (AAGCATGAGTCAGACAC) or the GRE (AGCTTAGTTTATTGGGACACAGTGTCCTTACCACAAGGATGG) probe. **Left panel**: cooperative binding of Fos and Jun to the TRE; **right panel**: binding of the Fos−Jun complex to the TRE but not to the GRE. The lower bands represent unspecific complexes as shown in competition experiments (not shown). Control: no cell extract added.

regulation of gene expression in response to external or oncoprotein-mediated signals and is therefore considered a 'master switch' (for a review see Curran and Franza, 1988). To elucidate the molecular mechanism by which Fos acts in such signal transduction cascades it is of paramount importance to understand how its expression is regulated, and how the Fos protein exerts its transforming function. We have therefore initiated a study pertaining to the identification of proteins (such as other oncogene products and transcription factors) which might be regulators of c-*fos* transcription. Here we report that in addition to the two known transregulating properties of Fos, i.e. its role in transactivating AP-1 dependent transcription and in transrepressing the c-*fos* promoter via the SRE, Fos can also repress GR mediated transactivation. In addition, we show that both Fos-mediated transactivation of AP-1 dependent transcription and transrepression of the SRE is repressed by the GR.

Repression of GR-dependent transcription by Fos

The molecular mechanism(s) involved in the repression of the GR by Fos seems to be fundamentally different from the other known Fos-mediated effects on gene expression. Transactivation of AP-1 dependent transcription requires complex formation with Jun via an intact leucine zipper (Kouzarides and Ziff, 1977; Gentz *et al.*, 1989; Schuermann *et al.*, 1989; Turner and Tjian, 1989), the interaction of this complex with the TRE via a bipartite DNA-binding site formed by Fos and Jun (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; Neuberg *et al.*, 1989a,b; Turner and Tjian, 1989) and the presence of transactivation domains which are at present not well defined (M.Neuberg, unpublished observations). Transrepression of the SRE also requires an intact leucine zipper in Fos, although the putative protein Fos interacts with in this case is not known, but does not involve DNA binding (Lucibello *et al.*, 1989). Repression of the GR, as shown in this study, does not seem to involve any of these functional domains. Mutations which impair the leucine zipper, destroy the DNA binding site or abolish transactivation do not interfere with transrepression of the GR dependent transcription. Rather, a region in the protein, which had had very little attention paid to it previously, was found to be crucial for this function of Fos. This region between amino acids 40 and 111, not conserved among different Fos family members, mediates repression of the GR. It can therefore be concluded that neither the formation of a complex via the leucine zipper nor binding to DNA via the basic region contacting the TRE play a role in transrepression of GR dependent transcription. Interestingly, mutations in the region around amino acids 134−140 increase the transrepressing potential of Fos (Figure 1). We could recently show that these mutations also decrease the transactivating properties of Fos (F.C.Lucibello, unpublished observations). One might therefore speculate that, if Fos and the receptor form a complex (see below), Fos may contribute its transactivation domain. Impairment of this region could thus enhance its transrepressing potential.

The fact that the region between amino acids 40 and 111 is functionally crucial is interesting because of its divergence in other Fos-related proteins such as FosB and Fra-1 (Zerial *et al.*, 1989). Analysis of the transrepressing potential of FosB indeed showed its inability to transrepress the GR. By using hybrid constructs of Fos and FosB the region responsible for the failure of FosB to transrepress could be pinpointed to its amino-terminal half which is in perfect agreement with the results obtained with the Fos deletion mutants. This result is of particular relevance since it shows the first functional difference between two members of the Fos family.

Inhibition by the GR of AP-1 dependent transactivation and SRE-mediated transrepression by Fos

The observation that GR is able to inhibit transactivation and transrepression by Fos suggests that the negative interaction between the two signal transduction pathways is reciprocal. Using deletion mutants of GR the inhibitory function of Fos transactivation could be mapped to a short region of the GR (amino acids 407−566) encompassing the DNA binding domain and a few flanking amino acids including one of the two mapped nuclear localization signals (Picard and Yamamoto, 1987). What particular function within this region is important for the observed effect is unknown, but DNA binding itself could be important as the two mutant GRs that exhibit defective binding to the GRE were also inactive in repressing Fos mediated transactivation. However, the obvious possibility that repression is mediated by the GR competing with Fos for binding to the AP-1 site, seems improbable in view of the lack of affinity of GR for the consensus TRE. Thus, the situation is different from the reported glucocorticoid inhibition of the TPA-induced expression of the proliferin gene (Mordacq and Linzer, 1989). In this case, GR acts through its binding to a DNA region including AP-1 which is responsible for induction of the gene by TPA, whereas no GR binding to the TRE used in our experiments could be detected.

The inhibitory effect of the GR on Fos transactivation

reported here also seems different from the repression of the prolactin gene by the oestrogen receptor (Adler *et al.*, 1988). In this case, the DNA binding domain of the receptor can be deleted without influencing repression, whereas a 63 amino acid region adjacent to the DNA binding domain seems to be essential (Adler *et al.*, 1988). This is clearly different from the GR repression effect reported in this study which is abolished by a single amino acid exchange within the DNA binding domain.

Complex formation between Fos and the GR?

The fact that we have been able to show, in transient assays, the effect of Fos on GR mediated transcription as well as the reverse, led us to attempt to show some direct interaction between the two proteins (data not shown). A variety of techniques were used for this purpose. Initially, we attempted to immunoprecipitate the labelled *in vitro* translated receptor plus cold Fos (± Jun) as a complex with an anti-Fos antibody (see Schuermann *et al.*, 1989 for details). The reverse was also tried under different stringencies. These experiments were also performed using purified rat GR (Chalepakis *et al.*, 1988). Under the conditions used, no complex between the receptor and Fos was observed. Since the Fos–Jun complex binds readily to the TRE *in vitro*, and the GR binds to the GRE, we assessed whether the addition of receptor and Fos (± Jun) respectively altered the pattern of 'shifting' in a gel retardation assay. As well as using *in vitro* translated proteins, Fos and Jun proteins expressed in Baculovirus, purified rat GR, a small bacterially expressed receptor 15 kd protein, encompassing the DNA binding domain (Chalepakis *et al.*, 1990), was also used. Under no circumstances was the pattern of the band shift altered by the addition of receptor or by Fos (± Jun). Finally, we tried to identify complexes by immunoprecipitation of lysates from a NIH3T3 derived cell line which expresses a very high level of Fos, and which can be further induced by the addition of dexamethasone or cadmium (see Müller *et al.*, 1986 for details). Under the conditions used, we were not able to show complex formation between the GR and Fos.

These results certainly do not rule out the possibility that Fos and the GR form a protein–protein complex. The interaction may be relatively weak and unstable under the conditions of our assays. In addition, it is possible that the antibodies used in this study disrupt or weaken the interaction. Further studies using a range of different conditions for *in vitro* reconstitution and immunoprecipitation including different proteins and antibodies will have to be performed to address the question as to whether Fos and the GR directly interact.

Although it is conceivable that the DNA-binding region of the GR may interact with the amino-terminal region of Fos, thus forming an inactive complex (see below), alternative and indirect mechanisms for the mutual repression can be envisaged. There have been previous reports that expression of the v-*mos* oncogene interferes with glucocorticoid induction of hormone responsive genes (Jaggi *et al.*, 1986; Hamilton and DeFranco, 1989). Similar effects have been reported for H-*ras* and v-*src*, whereas expression of the c-*myc* oncogene does not influence glucocorticoid response (Jaggi *et al.*, 1986; Vacca *et al.*, 1989). In the case of v-*mos*, it has been shown that it shortens the nuclear retention time of GR (Qi *et al.*, 1989). Since the GR is a phosphoprotein, and phosphorylation has been claimed to influence GR activation (for a review see Auricchio, 1989), the effects of these cytoplasmic oncogenes could be mediated by modulation of the activity of kinases or phosphatases that, directly or indirectly, act on the GR. Alternatively, v-*mos* and H-*ras* could influence GR activity indirectly by virtue of their known induction of *fos* expression (Schönthal *et al.*, 1988).

A role of the GR repressing domain in transformation?

Another important function concerns the role of the GR repressing domain in Fos in the induction of transformation. We have previously shown that deletion of this region between amino acids 40 and 111 results in a dramatic drop in transforming activity although the protein retains some transforming properties (Jenuwein and Müller, 1987). In this same study, the functionally indispensable region was shown to be the central part, now known to harbour the leucine zipper, the DNA binding site and perhaps a transactivating domain (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; Neuberg *et al.*, 1989a,b; Schuermann *et al.*, 1989; Turner and Tjian, 1989; Hirai *et al.*, 1990). Impairment of any of these functions results in the total loss of the transforming potential of Fos (Schuermann *et al.*, 1989; M.Neuberg, M. Schuermann, F.C.Lucibello and R.Müller, in preparation). It therefore seems that this domain of Fos and the functions encoded therein play a primary role in transformation. The fact that the region between amino acids 40 and 111 has a strong effect on the transforming properties of Fos suggests that this domain may encode another function required for the efficient induction of transformation (it has been shown that deletion of this region does not affect protein expression; Jenuwein and Müller, 1987). This function may be the formation of complexes with the GR and perhaps other steroid receptors. These observations and conclusions could be assembled to a model of Fos-induced transformation where more than one molecular property of Fos plays a role. In addition to the transactivation of AP-1 driven genes, which may be (one of) the most crucial functions of Fos in the process of transformation, other events, like the inactivation of factors by protein–protein interaction, may be necessary

Table I. Mutant *fos* constructs

Mutant name	Position(s)	Mutation
Acidic region		
LQ	134–135	PE → LQ
D522	135–138	EEEV → EDEQ
D1D2	135–138	EEEV → DIDD
DA3	135–138	EEEV → DAAA
Basic region		
DIII4	139–143	KRRIRRERNK → KRRIQQQQQNQ
VE	139–140	KR → VE
DN1	139–141	KRR → AAC
2.3	142	I → M
3.5	143	R → C
D13	143–144	RR → KK
DN4	144	R → A
DN5	145	E → A
D8	146–148	RNK → QNQ
D4	153–159	KCRNRRR → QCQNQQQ
D16	153–155	KCR → RCK
D17	157–159	RRR → KKK
Leucine zipper		
ANS	173–175	EDE → ANS
L3	179	L → V
L345	179; 186; 193	LLL → VAV

for complete transformation. It is thus possible that Fos induced transformation involves both the direct transactivation involving DNA binding and the indirect regulation of genes by inactivating positive or negative regulators.

Materials and methods

Cell culture

HeLa and NIH3T3 cells were cultured in Dulbecco–Vogt modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal calf serum, 0.5% glucose, penicillin (100 U/mol) and streptomycin (100 μg/ml).

Transfections

DNA transfections were essentially carried out according to Lucibello *et al.* (1988). After trypsinization, 4×10^5 HeLa cells were seeded per 3 cm plate and transfected 24 h later. 2.5 μg of the reporter plasmid (3xTREtkcat3) were coprecipitated either with 1.25 μg E300Δ2 wild-type Fos (Jenuwein and Müller, 1987) or with empty expression vector DNA, and with 1.25 μg GR expression constructs (or empty vector). Alternatively, 2 μg of the TGT2.1cat (G.Chalepakis, M.Truss, E.P.Slater, S.Mader and M.Beato, submitted) reporter plasmid were coprecipitated with 1 μg wild-type GR and 2 μg Fos expression vector DNA (either empty or containing Fos protein mutants). The DNA precipitate was left on the cells overnight. The cells were then washed twice with phosphate-buffered saline (PBS) and incubated with 10% FCS–DMEM ± 10^{-7} M dexamethasone for 48 h. Cells were lysed *in situ* in 125 μl lysis buffer, and 10–15 μl was used to determine CAT activity as described by Gorman *et al.* (1982).

For the analysis of the effect of transfected GR on the Fos repression of SRE dependent transcription, 1×10^5 NIH3T3 cells were seeded per plate and were later transfected with 2.5 μg of pSREΔ8 ± 1.25 μg pJM ± 1.25 μg GR11/GR20/G442 or 1.25 μg of the corresponding empty expression vector. After transfection the cells were placed in serum free DMEM ± dexamethasone for 36 h and then stimulated with 10% FCS ± dexamethasone for a further 12 h. In most experiments transfection efficiencies were controlled for by the cotransfection of 0.5 μg RSVlacZ.

Plasmids

All Fos constructs (Jenuwein and Müller, 1987; Lucibello *et al.*, 1989; Schuermann *et al.*, 1989; M.Neuberg, M.Schuermann, F.C.Lucibello and R.Müller, in preparation) were cloned into the expression vector pRΔXneo as described in Jenuwein and Müller (1987). E300 is a FBR-FBJ-MuSV hybrid construct (N terminus from FBJ-MuSV corresponding to c-*fos*; middle region from FBR-MuSV including the FBR-MuSV specific point mutations and deletions; C terminus up to amino acid 316 from FBJ-MuSV/c-*fos*). See Table I for details of Fos substitution mutants.

Rat GR mutants GR11 (amino acids 3–795); GR 10 (amino acids 407–795); GR13 (amino acids 3–556) were cloned into the expression vector pSTCMV, and GR20 (amino acids 3–795 Δ493–496) into the vector pCMV were kindly provided by Rusconi and Yamamoto (1987). The human GR mutant G442 which has a single substitution at position 442, lysine to glycine, was obtained from S.M.Hollenberg and R.Evans.

3xTREtkcat3. The *Hin*dIII–*Bam*HI fragment from pTREtkZ (Lucibello and Müller, 1989) which contains three copies of the AP-1 binding site, was cloned into the *Hin*dIII–*Bam*HI site of ptkcat3 (provided by G.Schütz).

TG'T2.1cat. An oligonucleotide (upper strand: 5'AGCTTAGTTTATTG-GGACACAGTGTCCTTACCACAAGGATGG3') was cloned into the *Hin*dIII–*Sal*I site of ptkcat3.

BC. A 750 bp *Pst*I fragment encompassing the 3' terminus of c-*fos* was cloned into the *Pst*I site of pUC19SM which contains a 700 bp 5' fragment of FosB (*Hin*dIII–*Pst*I). A *Sal*I–*Sst*I partial 1477 bp fragment was then cloned into the *Sac*I–*Xho*I site of pRΔXneo.

CB. The 1100 bp *Pst*I–*Hin*dIII fragment of FosB was cloned into the *Pst*I–*Hin*dIII site of pZ300 (E300 cloned in pTZ18) which then has 460 bp of the 3' of *fos*. The 1600 bp *Sst*I–*Sal*I fragment of *fos*–FosB was then cloned into the *Sst*I–*Xho*I site of the expression vector pRΔXneo.

BE300. The *Pst*I–*Hin*dIII fragment from E300 was cloned into the *Pst*I–*Hin*dIII (partial) site of pUC19SM. The *Sst*I–*Xho*I fragment was then cloned into the *Sst*I–*Xho*I site of pRΔXneo.

DNA binding experiments

Binding of the purified GR from rat liver to the oligonucleotides containing either consensus HRE or TRE were performed as previously described (Chalepakis *et al.*, 1990). Binding of the *in vitro* translated Fos protein or of Fos expressed in Baculovirus was performed as described in Risse *et al.* (1989).

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