The EMBO Journal vol.13 no.6 pp.1467–1475, 1994

An autonomous N-terminal transactivation domain in Fos protein plays a crucial role in transformation

Karin U.Jooss, Martin Funk and Rolf Müller[1]

Institut für Molekularbiologie und Tumorforschung (IMT), Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, D-35037 Marburg, Germany
[1]Correspondence

Communicated by R.Müller

To date, three functional domains have been defined in c-Fos and v-Fos proteins and have been shown to play a role in transactivation: the leucine zipper mediating hetero-dimerization, the basic DNA contact site, and a C-terminally located transactivation domain (C-TA) harbouring the HOB1 and HOB2 motifs. While the bZip region, consisting of the leucine zipper and the DNA contact site, is indispensable for transformation, the C-TA domain is not required and is actually altered by internal deletions in the FBR-MuSV. We now show that the N-terminal regions of c-Fos and v-Fos contain a second transactivation domain (N-TA). A functionally crucial motif within the N-TA domain, termed NTM, was pinpointed to a ~25 amino acid stretch around positions 60–84 which is highly conserved in FosB. Analysis of LexA fusion proteins showed that the N-TA domains of both c-Fos and FosB function in an autonomous fashion in both fibroblasts and yeast. Most importantly, deletion of the NTM motif impairs the transforming properties of v-Fos. Apart from the bZip region, the N-TA domain is the only functional domain required for transformation by v-Fos, at least when its expression is driven by the strong FBR-MuSV-LTR promoter.

Key words: fos oncogene/LexA fusion/*Saccharomyces cerevisiae*/transactivation/transformation

Introduction

The *fos* oncogene was originally discovered in the Finkel–Biskis–Jinkins mouse osteosarcoma virus (FBJ-MuSV), isolated from a spontaneous osteosarcoma-like tumour, and later also detected in the Gag fusion protein of the Finkel–Biskis–Reilly mouse osteosarcoma virus (FBR-MuSV), isolated from a radiation-induced osteosarcoma (for reviews see Müller, 1986; Verma, 1986; Cohen and Curran, 1990). Both FBJ-MuSV and FBR-MuSV cause chondro-osseous sarcomas in newborn mice and induce morphological transformation in non-established and immortalized murine fibroblast cell lines. *fos* oncogenes transform not only fibroblasts but also other connective tissue cells, including cartilage, bone and muscle cells (Jenuwein *et al.*, 1985). The transformation of muscle cells by Fos leads to a block of myogenesis, apparently by an inhibition of *myo*D expression (Lassar *et al.*, 1989). In agreement with the connective tissue specificity of Fos-induced transformation, transgenic mice expressing an ectopic c-*fos* gene have been shown to develop specifically osteogenic and chondrogenic sarcomas (Rüther *et al.*, 1989). Among the other *fos*-related genes (Cohen and Curran, 1988; Zerial *et al.*, 1989; Matsui *et al.*, 1990; Nishina *et al.*, 1990) that have been identified on the basis of their structural relatedness to c-*fos*, oncogenic properties have been shown for *fos*B (Kovary *et al.*, 1991; Mumberg *et al.*, 1991; Yen *et al.*, 1991). The *fos*B proto-oncogene is of particular interest because of the alternative splicing of its transcript which results in the synthesis of two proteins of different length and apparently antagonistic properties with respect to transregulation and transformation (Mumberg *et al.*, 1991; Nakabeppu and Nathans, 1991; Yen *et al.*, 1991).

Although both Fos-expressing retroviruses induce transformation of connective tissue cells *in vivo* and *in vitro*, the FBR-MuSV *gag-fos-fox* fusion gene seems to be the more potent oncogene (Curran and Verma, 1984; Jenuwein and Müller, 1987). In addition, the FBR-MuSV gene product has been shown to trigger the establishment of non-established fetal mouse fibroblasts, a property that is not shared by the FBJ-MuSV (Jenuwein *et al.*, 1985). A detailed structure–function analysis showed that the immortalizing potential of the FBR-MuSV *fos* oncogene is due to a point mutation close to the basic amino acid stretch encompassing the DNA contact site (Jenuwein and Müller, 1987). In contrast, the molecular basis for the enhanced transforming potential of FBR-MuSV is largely unknown.

The most crucial region of Fos for the induction of transformation lies between amino acids 111 and 220 (Jenuwein and Müller, 1987). Comprehensive mutagenesis experiments showed that this region harbours the leucine zipper and the DNA contact site (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; Neuberg *et al.*, 1989; Schuermann *et al.*, 1989; Turner and Tjian, 1989) and demonstrated that both of these functional domains are indispensable for transformation (Schuermann *et al.*, 1989; Neuberg *et al.*, 1991). These correlations suggest that transactivation of AP-1-regulated genes may be a key mechanism in Fos-induced transformation. This hypothesis is supported by the observation that transfected *fos* and *jun* genes cooperate in the induction of transformation *in vitro* (Schütte *et al.*, 1989; Neuberg *et al.*, 1991), and that the fusion of heterologous transactivation domains rescues the transforming potential of FosB-S and c-Fos (Wisdom *et al.*, 1992; Schuermann *et al.*, 1993). On the other hand, there is some evidence that there is no straight correlation between transformation and the activation of AP-1 dependent transcription. Thus, the N-terminal one-third of Fos has been shown to be required for transformation, but not for transactivation of a TPA-responsive element (TRE) reporter construct, at least when assayed in the presence of the FBR-MuSV derived C-terminus, including the *fox* sequence (Lucibello *et al.*, 1991). In another study addressing this question, the level of cellular AP-1 activity was elevated by the ectopic expression of GCN4 in rat embryo fibroblasts (Oliviero

et al., 1992). This increase in AP-1 activity, monitored by the transactivation of a TRE reporter construct, was, however, insufficient to induce transformation in cooperation with a *ras* oncogene, in contrast to combinations of *ras* plus *fos* or *ras* plus *jun*. From these results one might conclude that TREs may not be the most critical targets in *fos*-induced transformation, or that other additional transformation relevant targets might exist. In this context the transrepression of the glucocorticoid receptor or serum response elements (SREs) may be worth mentioning (for a review see Lucibello and Müller, 1991). It has, however, to be pointed out that DNA binding, which is an absolute prerequisite for transformation, is not required for either type of transrepression (Gius *et al.*, 1990; Lucibello *et al.*, 1989, 1990). In addition, viral Fos proteins are unable to repress SRE dependent transcription, pointing to a total lack of correlation between repression of the types described above and transformation.

In addition to the above mentioned well defined functional domains required for heterodimer formation and DNA binding, collectively referred to as the bZip domain, C-terminally located sequences have been shown to contain a transactivation domain (Lucibello *et al.*, 1991). Recently, a detailed structure–function analysis of this region led to the identification of two conserved motifs, HOB1 and HOB2, that were shown to play a major role in the transactivation induced by this domain (Sutherland *et al.*, 1992). The fact that HOB motifs were found not only in Fos proteins but also in one of the known transactivation domains of c-Jun, points to a more general importance of this type of transactivation domain. Identification of the HOB domain has, however, not advanced our understanding of Fos-induced transformation. The HOB1 and HOB2 motifs are impaired by internal in-frame deletions in the potent FBR-MuSV oncoprotein (van Beveren *et al.*, 1984), and terminal truncations removing the HOB domain altogether have no significant influence on the transforming properties of the protein, at least in the context of the FBR-MuSV transcriptional control elements (Lucibello *et al.*, 1991). On the other hand, previous studies had shown that the N-terminal 110 amino acids of v-Fos must contain domains that are crucial for transformation (Jenuwein and Müller, 1987). In this study, we have identified in the N-terminus of v-Fos a novel, autonomous transactivation domain (N-TA). This domain contains a conserved motif, termed NTM, that is crucial for both transformation and transactivation. The N-TA domains in v-Fos and FosB seem to be functionally distinct, in that transformation by FosB depends on the presence of both the N-TA and C-TA domains (Mumberg *et al.*, 1991; Yen *et al.*, 1991; Wisdom and Verma, 1993; Wisdom *et al.*, 1992), while the N-TA in v-Fos is sufficient for the induction of transformation (Jenuwein *et al.*, 1987; Lucibello *et al.*, 1991).

Results

Effect of N-terminal truncations and mutations in Fos on transformation

The E300 v-*fos* constructs and the various N-terminal truncations generated by oligonucleotide-directed mutagenesis were transfected into 208F rat fibroblasts and analysed for the induction of foci consisting of morphologically transformed cells lacking density dependent growth arrest. The results of this study are shown in the top part of Figure 1. While removal of the first 39 amino acids (E300ΔN39) had no detectable effect, deletion of a further 20 or 32 amino acids (E300ΔN59 and E300ΔN71 respectively) showed a mild but clearly visible effect on focus formation (~30% and ~60% reduction, respectively). A dramatic effect was, however, observed with construct E300ΔN83 which lacks the first 83 amino acids. In this case, the elimination of just another 12 amino acids led to a >90% reduced focus forming potential relative to E300ΔN71. Similar results were obtained with E300Δ95, lacking the N-terminal 95 residues, whereas removal of the N-terminal 110 amino acids completely abolished transformation in the focus assay. These results suggest that the region encompassing amino acids ~60 to ~100 is involved in the induction of transformation and that sequences around amino acids 72–84 play a pivotal role.

To verify this conclusion, we generated a set of E300 based constructs with seven to 11 amino acid substitutions in four different N-terminal regions, E300m1 to E300m4 (see Figure 1). The analysis of these constructs showed that mutations within the regions 40–60, 84–96 and 96–111 (constructs E300m1, E300m3 and E300m4, respectively) had no detectable effect on focus formation, while mutations in the region of amino acids 60–84 (E300m2) led to a ~85% reduction, supporting our conclusion that this sequence is located within a domain that plays the most

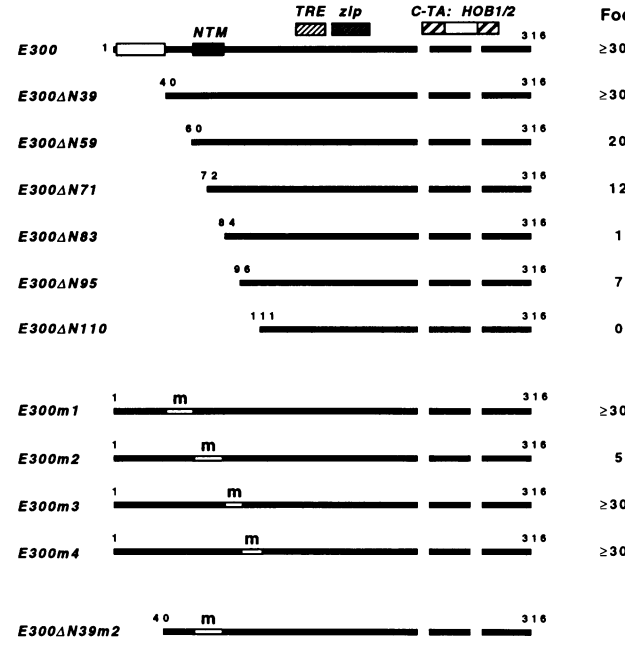

Fig. 1. Effect of N-terminal truncations and mutations on the transforming potential (focus formation) of E300 v-Fos protein in 208F rat fibroblasts. Values represent the mean of four independent experiments with standard deviations of <10% for all values. m: mutated regions; TRE: DNA contact site; zip: leucine zipper; C-TA: C-terminal transactivation domain with HOB1 and HOB2 motifs; gaps indicate the internal deletions in the FBR-MuSV derived C-terminal sequences of the E300 protein. The boxes at the N-terminus corroborate the results of this experiment: the shaded box indicates a conserved sequence motif, the NTM, which is required for transformation, but can be partially replaced by a region at the very N-terminus shown as an open box. The mutants have the following alterations: m1, MGSPVNTQDFCADLSVSSRNF → MG**AGVKT-KK**FC**AKLAVAATMF**; m2, NFIPTVTAISTSPDLQWLVQPTLV → NTL**GTVTAIATAGK**LKWLV**KG**TLV; m3, LVSSVAPSQTRAPHPY → LV**AAVAGAKTEAGLVY**; m4, PHPYGLPTQSAGAYARA → PHP**ARVGTKAAGAGARA**.

important role in transformation. Since the E300m2 construct was, however, still functional to some extent in this assay, we sought to identify the reason for this observation. One possibility was that the very N-terminal sequences might harbour another domain that can, at least in part, substitute for the function of the region mutated in E300m2. The last construct shown in Figure 1 confirms this notion: while E300ΔN39 showed wild-type-like transformation properties, the introduction of the m2 mutation completely abolished transformation. Taken together, these findings unequivocally identify the region around amino acids 72−84 as the most crucial sequence in the N-terminal third of Fos, and at the same time assign a function to the N-terminal 40 amino acids.

In order to confirm that the differences in the transforming potential described above were indeed due to an altered function of the protein rather than to a weaker expression of the mutant proteins, we performed both immunoprecipitation and immunostaining experiments with stably transfected cells. While the former analysis mainly analyses the rate of protein synthesis, immunostaining also provides information about the steady-state level and the fraction of cells expressing the exogenous protein. The data of these analyses clearly showed that several of the non-functional mutants (E300ΔN83 and E300Δ95 in Figure 2A; E300m2 and E300ΔN39m2 in Figure 2B) were indeed synthesized and expressed at levels similar to the parental E300 or the other mutated but functional proteins (E300ΔN39, E300m1, E300m3 and E300m4 in Figure 2A). We therefore conclude that the inability of certain Fos mutants to induce transformation, as discussed above, is the result of functional alterations to the mutant proteins.

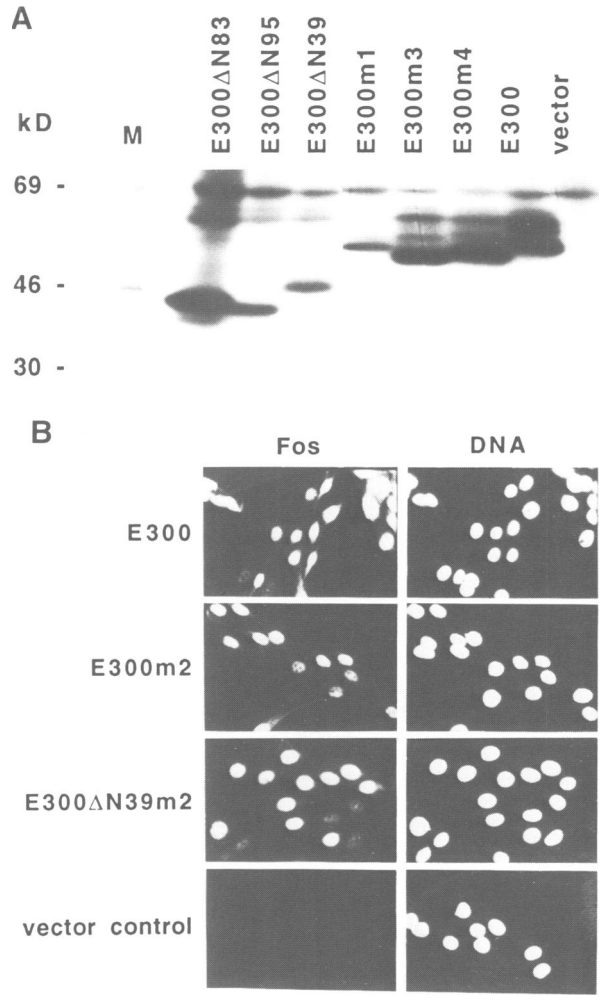

Fig. 2. (**A**) Synthesis of E300 and mutant Fos proteins in stably transfected 208F cells. G418^r mass cultures were metabolically labelled with [^{35}S]methionine and Fos proteins were immunoprecipitated with Fos-specific 455 antibodies. The slightly increased electrophoretic mobility of the E300m3 and m4 mutants might be due to the loss of serine residues which represent potential phosphorylation sites. vector: cells transfected with the empty expression vector. M: molecular mass markers. (**B**) Detection of E300 and mutant Fos proteins in stably transfected 208F cells by indirect immunofluorescence (left panel) and staining for nuclear DNA with Hoechst 33258 (right panel). 'vector control': cells transfected with the empty expression vector.

Identification of a novel transactivation domain near the N-terminus of Fos

We next investigated whether the N-terminus of v-Fos might contain a transactivation domain. E300 and several N-terminal truncations were tested in 208F cells for the activation of a 5×TRE-CAT reporter construct (Figure 3). The results of this experiment clearly showed that truncations of amino acids 1−59 had no noticeable effect, but further N-terminal deletions either significantly decreased (E300ΔN71) or impaired (E300ΔN83 and E300ΔN95) the transactivation properties of E300. To avoid the background of endogenous AP-1, subsequent experiments were carried out with fusions of E300 protein, or fragments thereof, and the C-terminus of the bacterial transcription factor LexA. This 202 amino acid protein (Lex202) contains the dimerization and DNA-binding domains and, when fused to a heterologous transactivation domain, can transactivate reporter gene constructs containing a LexA binding site in their promoter. Figure 4 shows that this approach indeed works with the E300 v-Fos protein: Lex−E300 gave an 18-fold transactivation relative to LexA202, and the known C-terminal transactivation domain (C-TA), including the HOB motifs, transactivated the reporter 9-fold in NIH3T3 cells. Furthermore, the analysis of N-terminal E300 fragments in this assay showed that the region between amino acids 40 and 110 indeed harbours a transactivation function: only those constructs containing this domain (Lex−F111, Lex−F171 and Lex−F40/171) gave a 4- to 5-fold transactivation, while Lex−F111/171 did not show any

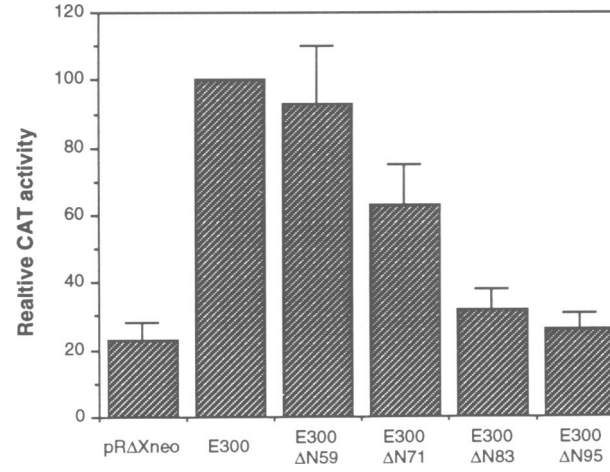

Fig. 3. Transactivation of a 5×TRE-CAT reporter gene by E300 v-Fos protein and N-terminal truncations (see Figure 1) in 208F cells. Values represent the mean of two independent experiments with standard deviations ≤15% for all values.

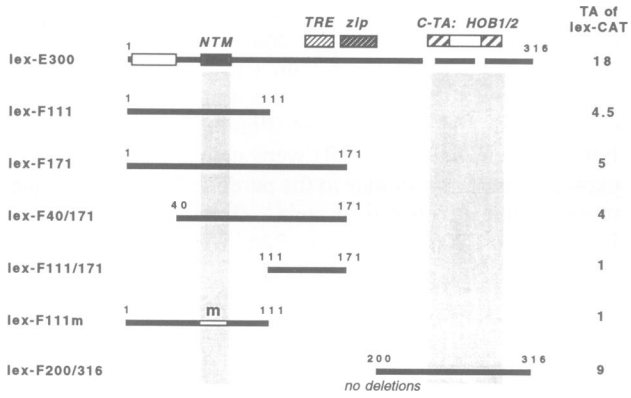

Fig. 4. Transactivation properties of E300 v-Fos protein and Fos fragments fused to the C-terminus of the 202 amino acid product of the *lexA* gene (LexA202) in NIH3T3 cells using a *lexA−CAT* reporter gene. Only the Fos moieties of the fusion constructs are shown. Results are given relative to the value obtained with native LexA202. Values represent the mean of four independent experiments with standard deviations in the range 10−20% for all values. NTM: N-TA motif within the N-terminal transactivation domain identified in the present study. The shaded areas indicate the two independent transactivation domains in Fos. See legend to Figure 1 for further details.

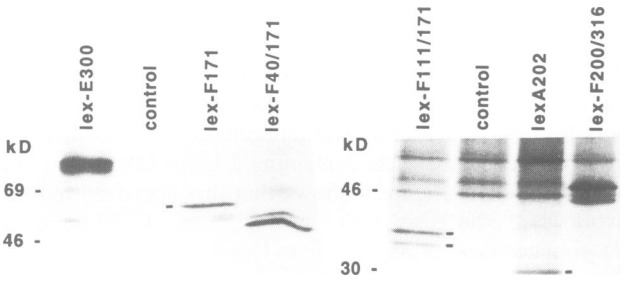

Fig. 5. Synthesis of LexA202−Fos fusion proteins in stably transfected NIH3T3 cells. G418^r mass cultures were metabolically labelled with [^{35}S]methionine and LexA proteins were immunoprecipitated with an antibody raised against a GST−LexA fusion protein. LexA202: non-fused LexA fragment; control: untransfected cells. Lines indicate the position of the immunoprecipitated LexA proteins. In some cases, more than one band appears. The reason for this is unclear, but could be due to the usage of internal start or stop codons.

transactivation, and was therefore termed N-TA. Most importantly, in perfect agreement with the transformation study (Figure 1), the introduction of the m2 mutation into Lex−F111 (Lex−F111m) completely abolished transactivation. These results were seen reproducibly in three independent experiments. Immunoprecipitation analyses of stably transfected cells showed that all LexA proteins investigated, including the non-functional Lex−F111/171, were expressed at similar levels (Figure 5). This confirms our conclusion that the N-terminal half of Fos contains only one transactivation domain that is detectable by the approach used in the present study, and that this domain coincides with the N-terminal region required for transformation.

The N-TA domain is functional in yeast

LexA−Fos has previously been shown to transactivate a *lexA−lacZ* reporter construct in *Saccharomyces cerevisiae* (Lech *et al.*, 1988). It was therefore of interest to investigate

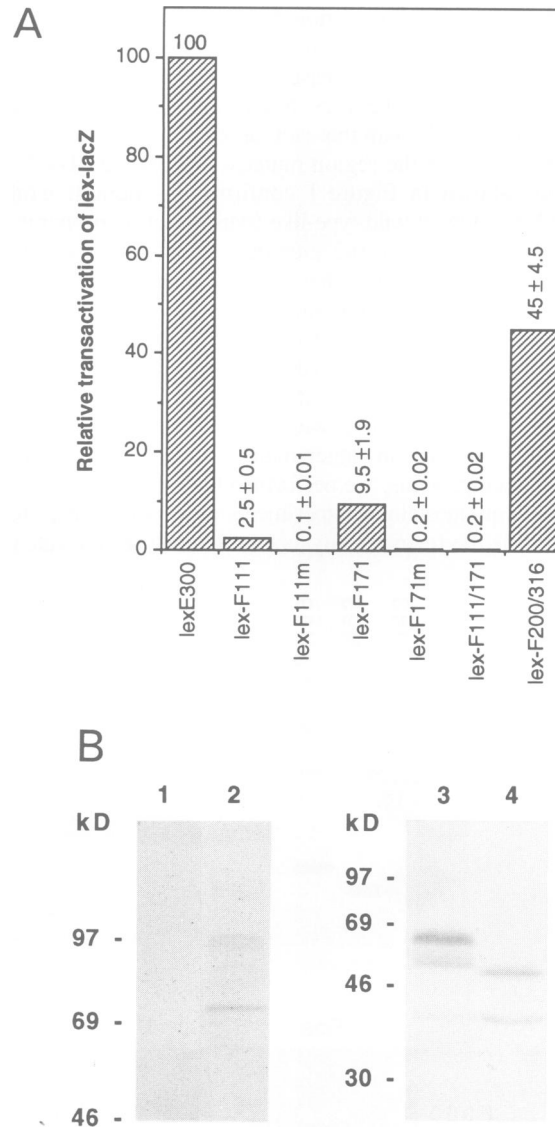

Fig. 6. (**A**) Transactivation properties of E300 v-Fos protein and Fos fragments fused to the C-terminus of LexA in *S.cerevisiae* cells using a *lexA−lacZ* reporter gene. Results (β-galactosidase activities) are given relative to the value obtained with native LexE300. See legend to Figure 4 for further details. Values represent the mean of measurements carried out with four independent transformants. The standard deviation was in the range 3−10% for all values. (**B**) Detection of Fos proteins in transformed *S.cerevisiae* cells by immunoblotting using an antiserum directed against LexA. Lane 1: untransformed cells (control); lane 2: E300; lane 3: Lex−F111; lane 4: Lex−F111m. The lower bands represent degradation products of the exogenous Fos proteins.

whether the N-TA domain might also function as an independent transactivation domain in yeast. Figure 6A shows that this is indeed the case. Both Lex−F111 and Lex−F171 gave transactivation values that were ~25-fold and 50-fold higher than the corresponding constructs containing the m2 mutation (Lex−F111m and Lex−F171m) or the Lex−F111/171 construct. The overall transactivation by the Lex−F111 and Lex−F171 proteins was considerably weaker than that by the E300 wild-type and Lex−F200/316 constructs, but this may be due to the nature of the fusion proteins or the availability of interacting factors in yeast, as discussed in further detail below (see Discussion). Also in this case, we analysed expression of the exogenous

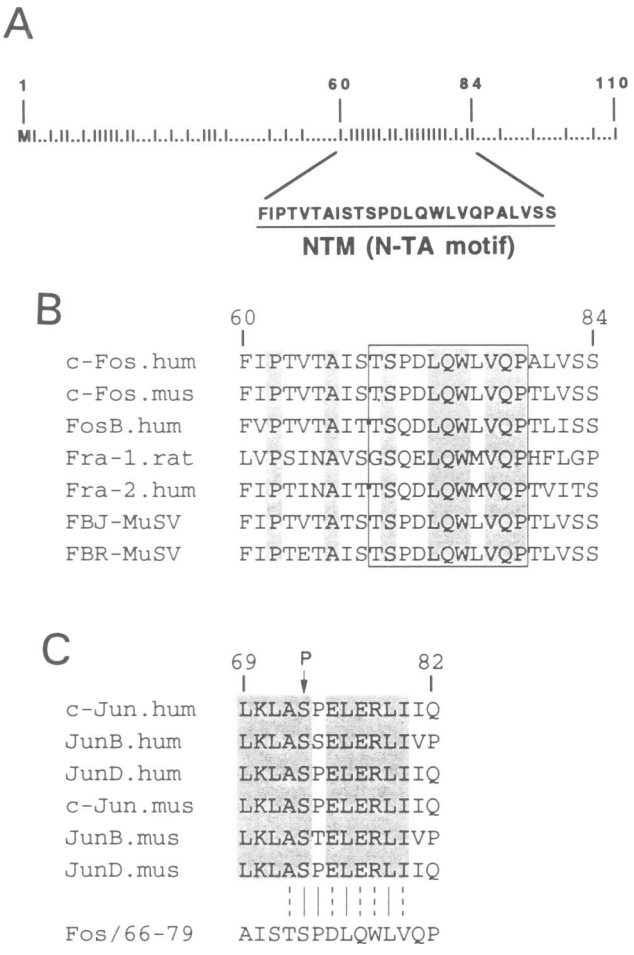

Fig. 7. (**A**) Homologies in the N-termini of c-Fos/v-Fos (van Beveren *et al.*, 1983) and FosB (Zerial *et al.*, 1989). Vertical lines indicate identities, dots represent mismatches. The amino acid sequence of the region of highest homology (positions 60–84; NTM) is shown below. (**B**) Alignment of the NTM motifs in different members of the Fos family. Identities in all proteins are marked by shading. The box indicates the region of greatest homology. (**C**) Alignment of the c-Fos NTM core with different members of the Jun family. Identities in all proteins are marked by shading. The regulatory phosphorylation site, Ser73, in c-Jun is indicated by an arrow. A consensus sequence is shown at the bottom.

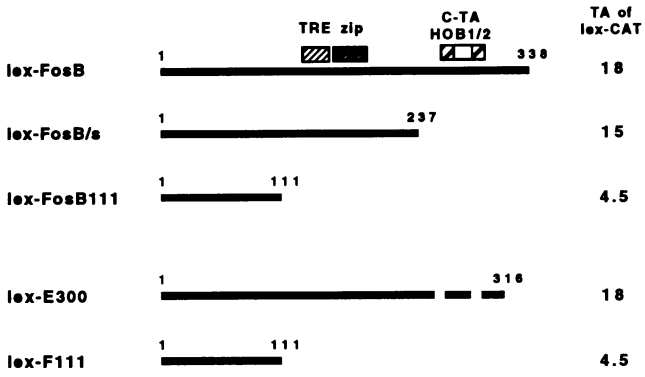

Fig. 8. Transactivation properties of FosB proteins and FosB fragments fused to the C-terminus of LexA in NIH3T3 cells using a *lexA–CAT* reporter gene. The Lex–Fos constructs are included for comparison. Only the FosB and Fos moieties of the fusion constructs are shown. Results are given relative to the value obtained with native LexA202. Values represent the mean of three independent experiments with standard deviations of ≤10% for all values. See legend to Figure 1 for further details.

proteins. Immunoblot analyses showed that all the fusion proteins were expressed at similar levels, irrespective of the inactivating m2 mutation (Figure 6B and data not shown).

A conserved motif within the N-TA domain

An alignment of the N-terminal 111 amino acids of human c-Fos and FosB (DNAStar MegAlign, Clustal method) showed two areas of significant homology (Figure 7A). The strongest homology was found with amino acids 60–84 (22/25 residues = 88% identity; 100% similarity), a region that overlaps with the N-TA domain identified above, and is also conserved, albeit to a slightly lesser extent, in Fra-1 and Fra-2 (Figure 7B). This sequence motif was termed NTM (*N-TA motif*). A second region of homology was detected within the N-terminal 36 amino acids (19/36 residues = 52.8% identity) which may be significant in view of the fact that this domain can partially suppress the m2 mutation (Figure 1; see Discussion).

A comparison of the NTM with the N-termini of different members of the Jun family also revealed significant homology (Figure 7C), but this homology was confined to the NTM core boxed in Figure 7B. Interestingly, this homology occurs in a region of Jun that is part of a transactivation domain (Baichwal and Tjian, 1990) and shows a very high degree of intra-family conservation (see Figure 7C). These observations suggest that the NTM might play a role in proteins other than Fos, but this hypothesis remains to be examined.

The high degree of homology in the NTM suggested that the N-terminus of FosB might also contain an autonomous transactivation domain. We therefore investigated this question by fusing FosB, the splice variant FosB/s and an N-terminal 111 amino acid fragment of FosB to LexA202 and assayed the fusion proteins for transactivation in NIH3T3 cells. The results depicted in Figure 8 clearly show that both Lex–FosB and Lex–FosB/s were able to transactivate the *lexA–CAT* reporter as efficiently as the LexE300 construct (15- to 18-fold) and, likewise, that the N-terminal fragments of v-Fos and FosB, Lex–F111 and Lex–B111, possess a very similar transactivation potential (4.5-fold). As described for the Lex–Fos fusion proteins, immunoprecipitation analyses using the LexA-specific antibodies were performed and showed similar expression levels for all the proteins analysed in this experiment (data not shown).

Discussion

The induction of transformation by Fos proteins is a poorly understood process. Although it is clear that transformation is dependent on a functional bZip domain consisting of the leucine zipper as the dimerization interface and the basic DNA contact site (Schuermann *et al.*, 1989; Neuberg *et al.*, 1991), other transformation-relevant domains in v-Fos/c-Fos were poorly defined prior to the present study. In particular, transregulatory domains that are also crucial for transformation had not been identified (see Introduction). In addition, apart from the C-terminal domain (C-TA) containing the HOB1 and HOB2 motifs (Sutherland *et al.*, 1992), transactivation domains within v-Fos or c-Fos protein were not known. Previous work had shown that apart from the bZip domain, the N-terminal 110 amino acids were crucial for transformation (Jenuwein and Müller, 1987;

Lucibello *et al.*, 1991), but the molecular function of this region was unknown and a more closely defined functional domain in this portion of the protein had not been delineated. Although this region of Fos had been shown to play a role in repression of the glucocorticoid receptor (GR) (Lucibello *et al.*, 1990), this finding on its own could not explain the relevance of this domain for the induction of transformation. As described in the Introduction, repression of the GR is independent of a functional DNA contact site in Fos, while transformation is strictly dependent on DNA binding. It had also been shown in previous studies that the sequences C-terminal to the bZip structure only play a marginal role, if any, in transformation, at least when Fos is expressed at high levels from a FBR-MuSV based vector (Jenuwein and Müller, 1987; Lucibello *et al.*, 1991). This finding indicated that the C-TA domain referred to above is not crucial for transformation, a conclusion that is supported by the fact that both HOB motifs are deleted to a large extent from the most potent viral Fos oncoprotein, the FBR-MuSV gene product (van Beveren *et al.*, 1984). In the present study, we describe a novel functional domain (N-TA) near the N-terminus of Fos that fulfils the criteria of both a transformation-mediating and independent transactivation domain.

The data shown in Figures 1–3 unequivocally demonstrate that, with respect to transformation, the sequences around amino acids 72–84 represent the most crucial region in the N-terminus of Fos. It is very likely that the entire functional N-TA domain in this region is larger than the core 12 amino acids (72–84) since adjacent truncations also have some effect. In addition, a motif within the N-TA domain, the NTM, which is highly conserved in FosB and other members of the Fos family, encompasses amino acids 60–84 (see Figure 7A and B), suggesting that the N-TA domain spans at least this region of the two proteins. A closer inspection of this amino acid sequence reveals no close resemblance to any of the common features of known transactivation domains, but a partial homology of the NTM core sequence to a functionally crucial region with the N-terminal activation domain of c-Jun (Baichwal and Tjian, 1990) is detectable (Figure 7C), suggesting that the NTM motif may also be involved in transactivation by Jun proteins. In addition, the high content of serine and threonine within the NTM is striking (7/25 residues in c-Fos = 28%). Some of these residues occur in close proximity to proline residues, suggesting that the NTM may be a substrate for protein kinases such as cdc2 or casein kinase II. In addition, *in vitro* phosphorylation experiments suggested that the N-terminus of c-Fos can be phosphorylated by several protein kinases, including PKC and DNA-dependent kinase (Abate *et al.*, 1991). Further studies will have to address this question, especially with respect to a possible regulatory function of phosphorylation as in the case of c-Jun (Boyle *et al.*, 1991; Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Lin *et al.*, 1992). In this context, the conservation of Ser73 in c-Jun and Ser70 in c-Fos is intriguing in light of the fact that transactivation by c-Jun is regulated through the phosphorylation of Ser73 (Pulverer *et al.*, 1991; Smeal *et al.*, 1991).

The analysis of fusion proteins consisting of LexA and portions of E300 v-Fos clearly demonstrate that N-TA represents a transactivation domain that functions autonomously, i.e. independently of its natural context, both in NIH3T3 fibroblasts (see Figures 4 and 5) and in *S.cerevisiae*, as does the C-TA domain. A comparison of the transactivation potential of the full-length E300 protein, the C-TA domain and the N-TA domain suggests that the values obtained with both domains separately attached to LexA do not add up to the full E300 value. This may, however, have a simple explanation. With E300, but not the N- and C-terminal fragments lacking the leucine zipper, a potential contribution of interacting Jun proteins has to be taken into account. In addition, in their normal context in Fos protein, the N- and C-terminal transactivation domains may be in a more favourable overall conformation. Especially in the case of the N-TA domain such a conclusion is conceivable: in Fos N-TA is located at the N-terminus, while in the LexA fusion it is at the C-terminus of the protein, thus very close to the LexA moiety and therefore subject to possible structural hindrance. We therefore believe that the absolute transactivation values obtained with LexA fusion proteins have to be considered with caution. The major benefit of this assay is the low background activity, thus allowing the identification of transactivation domains irrespective of their strength. An even greater difference between the transactivation potential of the N-TA domain and the E300 protein or the C-TA domain was seen in yeast. Here, as discussed above, the same problems regarding the N-terminal fusion may exist. In addition, it is possible that yeast cells contain lower levels, or even lack, factors that have to interact with the N-TA domain for maximum transactivation. A clarification of this question has to await the identification of these factors and the cloning of the respective genes, a project that is currently being pursued in our laboratory. Furthermore, at present it cannot be ruled out that N-TA and C-TA interact in a synergistic fashion.

Our findings demonstrate that the N-TA region has a similar importance for the induction of transformation as the leucine zipper and the DNA contact site, and is in fact the only region in Fos that is required for transformation apart from the bZip domain. This conclusion has several important implications. First, the question arises what the biological function of the second well-defined transactivation domain, C-TA, might be. It is unlikely that with respect to transformation both domains act additively, since the C-TA, but not N-TA domain is dispensable (see above). An alternative explanation could be that both domains are required for the transactivation of different targets, and that only the N-TA associated targets are relevant in terms of transformation. If this is indeed the case, the corresponding Fos mutants would provide a convenient tool to classify Fos target genes according to their relevance for transformation, a problem that is unresolved at present. In this context it is worth mentioning that target sites activated by Fos protein complexes other than the TRE have been identified, for example an NF-κB binding site in the HTLV promoter (Stein *et al.*, 1993). With the help of the mutants described in this study it is now possible to address this question.

While this study was in progress, Wisdom and Verma (1993) reported that the N-terminus of FosB plays a role in transactivation and transformation, and identified a functionally important region of 33 amino acids that partly overlaps with the homologous region identified in v-Fos in the present study. These authors concluded, however, that the N-terminal region of the FosB protein does not contain an independent transactivation domain. This result is in clear contrast to our observations, which demonstrate that both c-Fos/v-Fos and FosB harbour autonomous transactivation domains in their N-terminal sequences. The reason for this

discrepancy might be associated with the problems arising with fusion proteins discussed above. Wisdom and Verma (1993) used GAL4 fusions, while our study was carried out with LexA fusion proteins. In both cases the fusions were established at the N-termini of the Fos/FosB moieties. It cannot be ruled out that the GAL4–FosB conformation obstructs access to the N-TA domain, leading to a non-functional construct. For this reason, we feel that 'negative' results obtained with fusion proteins, especially if the junction is close to the domain to be investigated, cannot be conclusively interpreted. Based on our results, we conclude that c-Fos/v-Fos do not differ from FosB with respect to the context-independence of their N-TA domains. There are, however, other major differences between the functions of the N-terminal and C-terminal transactivation domains in c-Fos/v-Fos and FosB, suggesting different strategies for the induction of transformation and transactivation by these proteins. First, domain swapping experiments clearly demonstrated that the N-terminal portions of v-Fos and FosB are functionally not equivalent (Schuermann *et al.*, 1991). The replacement of the N-terminal half in E300 with the corresponding region from FosB (B-E300) led to a 5-fold reduction in the number of foci, while the reverse construct (CB-Fos) exhibited a 10-fold higher transforming potential. Second, truncation of the C-terminus in FosB, as in the case of FosB/s, abolishes transformation (Mumberg *et al.*, 1991; Yen *et al.*, 1991; Wisdom and Verma, 1992; Wisdom *et al.*, 1993). In contrast, the corresponding alteration in v-Fos has only a marginal effect on the induction of morphological transformation, i.e. the N-terminal transactivation domain in v-Fos (see Figure 1; Jenuwein and Müller, 1987; Lucibello *et al.*, 1991), but not in FosB is sufficient for transformation (Mumberg *et al.*, 1991; Yen *et al.*, 1991; Wisdom and Verma, 1992; Wisdom *et al.*, 1993). This suggests that the N-terminal regions in both proteins fulfil different functions with respect to transformation. It is possible that these differences are due to the amino acid substitutions that occur in the NTM of FosB relative to c-Fos/v-Fos which have altered the transactivation properties of the N-TA domain. It will be interesting to see whether this hypothesis is correct, and whether these structural differences have an impact on target specificity and/or the strength of transactivation of transformation relevant genes.

Finally, we have identified another region in v-Fos that seems to represent a functional domain. This domain is located within the first 39 amino acids of the protein and was identified by virtue of its ability to rescue partially the transforming potential of a NTM mutant (see Figure 1; E300m2 versus E300ΔN39m2). It might be argued that this region contains a cryptic transactivation domain that normally has no function. Two facts speak against such a conclusion. First, the N-terminal 36 amino acids show a conservation of 52.8% among c-Fos and FosB, while other sequences N-terminal to the bZip domain are not conserved to any significant extent, apart from the NTM. This suggests that a function worth preserving is associated with the very N-terminal region. Second, in certain mutant configurations other than the E300m2 construct, positive effects of the first 39 amino acids on transformation have been observed (Jenuwein *et al.*, 1987). It is therefore possible that this region indeed harbours a transactivation domain that plays an accessory role in the normal function of Fos but which, with respect to transformation, is dispensable unless other functional domains of the protein are impaired. The fact that no transactivating properties could be seen in the context of LexA fusion proteins is largely meaningless because of the potential difficulties arising from its close proximity to the LexA moiety (see above). As mentioned above we are currently engaged in the identification of proteins that interact with the defined functional domains in Fos. It is hoped that this study will provide us with the tools that are now needed to resolve the remaining open questions.

Materials and methods

Cell culture

208F 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/ml) and streptomycin (100 μg/ml).

Focus assay

DNA transfection experiments were essentially carried out as described (Müller and Müller, 1984). After trypsinization, 7×10^5 cells were seeded per 6 cm dish and transfected 24 h later with 3 μg of *fos* expression plasmid purified by Quiagen columns (Diagen), plus 8 μg of 208F carrier DNA. Ten hours later the calcium phosphate-precipitated DNA was washed off the cells and the cultures were split in a 1:3 ratio. Subsequently the medium was changed every 3–4 days. Foci of morphologically altered cells were counted 3 weeks after transfection.

CAT assay

CAT assays were carried out according to Pothier *et al.* (1992). 10^5 NIH3T3 or 208F cells were seeded and transfected 12 h later with 3 μg of expression plasmid and 4 μg of reporter plasmid. Twelve hours later the precipitate was washed off and the cells were harvested 36 h after transfection in 200 μl of buffer A (Pothier *et al.*, 1992). Cells were lysed by three freeze–thaw cycles. After a 10 min incubation at 70°C, the supernatant was transferred to a fresh tube. To assay the lysate, 15 μl of extract were incubated with 1 μl 200 μCi/ml [^{14}C]chloramphenicol, 5 μl 5 mg/ml butyryl CoA, 10 μl 1 M Tris, pH 8.0 and 69 μl H_2O for 30 min. The butyrylated chloramphenicol was separated from the unmodified form by a simple phase extraction using 210 μl xylene–trimethylpentadecan (1:2). CAT activity was determined by counting 200 μl of the top organic phase in 3–5 ml scintillation fluid. CAT activity was standardized as described by Jooss and Müller (1992) and Abken and Reifenrath (1992). The 5×TRE-CAT plasmid (Angel *et al.*, 1987) was kindly provided by D.P.Herrlich (Karlsruhe, Germany).

LexA constructs

In yeast, LexA fusion proteins were expressed from the plasmid pRS416Met25LexN0. This plasmid is based on the expression vector pRS416MET25 (Sewing *et al.*, 1994) in which the complete *lexA* repressor gene (Horii *et al.*, 1981) plus a spacer of three glycine residues was inserted as a PCR-generated *Xba*I–*Bam*HI fragment. The E300 v-*fos* gene was fused in-frame to the *lexA* repressor using a PCR fragment with a 5′ *Bam*HI site followed by natural ATG codon and a 3′ *Sal*I site followed by a synthetic stop codon and a *Xho*I site. For expression in mammalian cells the *Xba*I–*Xho*I fragment from resulting plasmid including the *lexA* repressor was cloned into the expression vector pMSE (Schuermann, 1990) in which a synthetic *Xba*I–*Sal*I linker had been introduced (pMSEKJ). The different *fos* genes, or fragments thereof, were each cloned as a PCR-generated *Bam*HI–*Sal*I fragment into the yeast or human expression vectors. The reporter plasmid was generated by cloning a single *lexA* operator (oligonucleotide lexA) into the *Bam*HI and *Sal*I sites of ptkCATFL. Nucleotide sequences were verified by DNA sequencing.

Fos constructs

To facilitate the production of mutants in the N-terminal region of E300Δ2 (Schuermann *et al.*, 1989), we first created unique restriction sites in this region, using oligonucleotide-directed mutagenesis according to the gapped duplex DNA method based on the pMa/c5-8 vector system (Schuermann *et al.*, 1989). Three unique restriction sites were generated into E300Δ2, i.e. *Cla*I(118), *Spe*I(245) and *Bss*HII(325), yielding E300CSB (position 1 represents the first base in the start codon of the FBJ-MuSV *fos* gene; van Beveren *et al.*, 1983). Mutants were created by introducing double-stranded oligonucleotides into this parent construct. E300ΔN39: a filled-in *Ava*I fragment of E300Δ2 was cloned into the *Sma*I site of pUC19. E300ΔN59: oligonucleotide N59 was cloned into the *Eco*RI and *Cla*I sites of E300Δm1; E300ΔN71: oligonucleotide N71 was cloned into the *Eco*RI and *Spe*I sites

of E300 CSB; E300ΔN83: oligonucleotide N83 was cloned into the *Eco*RI and *Spe*I sites of E300Δm2; E300ΔN95: oligonucleotide N83 was cloned into the *Eco*RI and *Spe*I sites of E300Δm3; E300ΔN110: a *Bgl*II−*Hin*dIII fragment of E300Δ2 was cloned into pUC19; E300Δm1: oligonucleotide Δm1 was cloned into the *Cla*I and *Spe*I sites of E300 CSB; E300Δm2: oligonucleotide Δm2 was cloned into the *Cla*I and *Spe*I sites of E300 CSB; E300Δm3: oligonucleotide Δm3 was cloned into the *Spe*I and *Bss*HII sites of E300 CSB; E300Δm4: oligonucleotide Δm4 was cloned into the *Spe*I and BssHII sites of E300 CSB; E300m1: oligonucleotide m1 was cloned into the *Cla*I site of E300Δm1; E300m2: oligonucleotide m2 was cloned into the *Spe*I site of E300Δm2; E300m3: oligonucleotide m3 was cloned into the *Spe*I site of E300Δm3; E300m4: oligonucleotide m4 was cloned into the *Bss*HII site of E300Δm4; E300ΔN39m2: an *Sst*I−*Sal*I fragment amplified via PCR, using E300m2 as the template was cloned into pUC19. Primer Δ39 and primer Fos-backward were used for amplification. For expression in 208F cells and 3T3 cells, the mutants were transferred into the expression vector pRΔXneo (Jenuwein and Müller, 1987).

For the constructs E300ΔN39 and E300ΔN110 internal methionines were used as start codons (E300Δ39: aa40, E300Δ110: aa111). In the case of E300ΔN59, the ATG of the Δm1 oligonucleotide (indicated in bold below) was used as the start codon. With E300ΔN71, the start codon was introduced via the oligonucleotide N-71. In the case of E300ΔN83 and E300ΔN95 the start codon is derived from the N83 oligonucleotide.

Oligonucleotides

The oligonucleotides chosen for *in vitro* mutagenesis correspond to the non-coding strand of the E300 v-*fos* gene (Schuermann *et al.*, 1989). In the oligonucleotides shown below, numbers in parentheses refer to nucleotide position in E300, and bold characters indicate mutated bases. Primer Δ39: 5′-GCGGGAGCTCTCATCGATG-3′; primer Fos-backward: 5′-CCGCTCGAGTCAGTCGACATCAATCTCCAAGCTCCATGGGC-3′; *Cla*I, 5′-(137)GACAGGAGAGCCCATCGATGAGAAGGAGTCGGC(104)-3′; *Spe*I, 5′-(262)GCCACGGAGGAGACTAGTGTGGGCTGCACCAGC(230)-3′; *Bss*HII, 5′-(347)GGTCTTCACCATTCCCGCGCGCGCGTAAGCCCCAGC(311)-3′; N59, 5′-AATTCGAGCTCCCCAATGT-3′; N71, 5′-AATTCGAGCTCC*ATG*ATGGACCTGCAGTGGCTGGTGCAGCCCACA-3′ (start codon italicized); N83, 5′-AATTCGAGCTCC*ATG*ATG-3′ (start codon italicized); Δm1, 5′-CG*ATG*TTTATCCCCACGGAGGCCATCTCCACCAGCCCTGACCTGCAGTGGCTGGTGCAGCCCACA-3′ (start codon for E300ΔN59 italicized); Δm2, 5′-CGA-TGGGCTCTCCTGTCAACACACAGGACTTTTGCGCAGATCTGTCCGTCTCTAGTCGCAACACA-3′; Δm3, 5′-CTAGTCTACGGACTCCCCACCCAGTCTGCTGGGGCTTACGCG-3′; Δm4, 5′-CTAGTCTCCTCCGTGGCCCCATCGCAGACCAGAGCGCCCCATCCTGCG-3′; m1, 5′-CGATGGGCGCTGGTGTCAAGACAAAGAAGTTTTGCGCAAAGCTGGCTGTCGCTGCTA-3′; m2, 5′-CTAGGTACGGTGACAGCCATCGCTACCGCTGGTAAGCTGAAGTGGCTGGTGAAGGGTACG-3′; m3, 5′-CTAGTCGCTGCTGTGGCCGGTGCTAAGACCGAGGCGGGT-3′; m4, 5′-CGCGTGGGTACCAAGGCTGCTGGGGCTGGTGCG-3′; lexA, 5′-TCGACGTACTGTATGTACATACAGTACG-3′.

Immunoprecipitation and immunofluorescence

Immunoprecipitations were performed according to Wick *et al.* (1992). Immunostaining for Fos protein was carried out by indirect immunofluorescence as described (Jooss and Müller, 1992).

Immunoblot analysis of yeast cells

A polyclonal serum from rabbit was raised against a GST−LexA fusion protein (Pharmacia-LKB). Yeast protein extracts were prepared by boiling 2×10^7 cells in 50 mM Tris−HCl pH 6.8; 10 mM DTT; 2% SDS; 0.1% bromophenol blue and 10% glycerol. Half of the supernatant following high speed centrifugation (15 min, 20 000 *g*) was used for immunoblot analysis as described by Ausubel and Frederick (1991).

Yeast strains and media

The yeast strain expressing the *lexA* reporter construct (**a**; *lys2*-801^{amber}; *ade2*-10^{ochre}; *leu2*-Δ1; *trp*Δ63; *his3*Δ200; *ura3*::*lexA*Op/*CYC1*/*lacZ*) was generated from the strain YPH499 (Sikorski and Hieter, 1989). The *CYC1*/*lacZ* reporter construct with one *lexA* operator was isolated from the plasmid 1107 (Lech *et al.*, 1988) and integrated between the *Nco*I and the *Ppu*MI sites of the chromosomal *URA3* gene. Strains were grown on selective medium containing 2% glucose (Ausubel and Frederick, 1991). β-galactosidase assays were performed in each case for four independent transformants according to Ausubel and Frederick (1991).

Acknowledgements

We are grateful to Dr P.Herrlich for the 5×TRE-CAT plasmid, Dr F.C.Lucibello and J.Zwicker for critically reading the manuscript, and to Dr M.Krause and S.Klingelhöfer for synthesis of oligonucleotides. This work was supported by the Deutsche Forschungsgemeinschaft (Mu601/7-1 and SFB215/D8) and the Dr Mildred Scheel-Stiftung für Krebsforschung.

References

Abate,C., Marshak,D.R. and Curran,T. (1991) *Oncogene*, **6**, 2179−2185.
Abken,H. and Reifenrath,B. (1992) *Nucleic Acids Res.*, **20**, 3527.
Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.H., Jonat,C., Herrlich,P. and Karin,M. (1987) *Cell*, **49**, 729−739.
Ausubel,I. and Frederick,M. (1991) *Current Protocols in Molecular Biology*. Wiley, New York.
Baichwal,V.R. and Tjian,R. (1990) *Cell*, **63**, 815−825.
Boyle,W.J., Smeal,T., Defize,L.H.K., Angel,P., Woodgett,J.R., Karin,M. and Hunter,T. (1991) *Cell*, **64**, 573−584.
Cohen,D.R. and Curran,T. (1988) *Mol. Cell. Biol.*, **8**, 2063−2069.
Cohen,D.R. and Curran,T. (1990) *Crit. Rev. Oncogenesis*, **1**, 65−88.
Curran,T. and Verma,I.M. (1984) *Virology*, **135**, 218−228.
Gentz,R., Rauscher,F.J.,III, Abate,C. and Curran,T. (1989) *Science*, **243**, 1695−1699.
Gius,D., Cao,X., Rauscher,F.J.,III, Cohen,D.R., Curran,T. and Sukhatme,V.P. (1990) *Mol. Cell. Biol.*, **10**, 4243−4255.
Horii,T., Ogawa,T. and Ogawa,H. (1981) *Cell*, **23**, 689−697.
Jenuwein,T. and Müller,R. (1987) *Cell*, **48**, 647−657.
Jenuwein,T., Müller,D., Curran,T. and Müller,R. (1985) *Cell*, **41**, 629−637.
Jooss,K. and Müller,R. (1992) *Oncogene*, **7**, 1933−1941.
Kouzarides,T. and Ziff,E. (1988) *Nature*, **336**, 646−651.
Kovary,K., Rizzo,C.A., Ryseck,R.-P., Noguchi,T., Raynoschek,C., Pelosin,J.-M. and Bravo,R. (1991) *New Biol.*, **3**, 870−879.
Lassar,A.B., Thayer,M.J., Overell,R.W. and Weintraub,H. (1989) *Cell*, **58**, 659−667.
Lech,K., Anderson,K. and Brent,R. (1988) *Cell*, **52**, 179−184.
Lin,A., Frost,J., Deng,T., Smeal,T., Al-Alawi,N., Kikkawa,U., Hunter,T., Brenner,D. and Karin,M. (1992) *Cell*, **70**, 777−791.
Lucibello,F.C. and Müller,R. (1991) *Crit. Rev. Oncol.*, **2**, 259−276.
Lucibello,F.C., Lowag,C., Neuberg,M. and Müller,R. (1989) *Cell*, **59**, 999−1007.
Lucibello,F.C., Slater,E.P., Jooss,K.U., Beato,M. and Müller,R. (1990) *EMBO J.*, **9**, 2827−2834.
Lucibello,F.C., Neuberg,M., Jenuwein,T. and Müller,R. (1991) *New Biol.*, **3**, 671−677.
Matsui,M., Tokuhara,M., Konuma,Y., Nomura,N. and Ishizaki,R. (1990) *Oncogene*, **5**, 249−255.
Müller,R. (1986) *Biochim. Biophys. Acta*, **823**, 207−225.
Müller,R. and Müller,D. (1984) *EMBO J.*, **3**, 1121−1127.
Mumberg,D., Lucibello,F.C., Schuermann,M. and Müller,R. (1991) *Genes Dev.*, **5**, 1212−1223.
Nakabeppu,Y. and Nathans,D. (1991) *Cell*, **64**, 751−759.
Neuberg,M., Schuermann,M., Hunter,J.B. and Müller,R. (1989) *Nature*, **338**, 589−590.
Neuberg,M., Schuermann,M. and Müller,R. (1991) *Oncogene*, **6**, 1325−1333.
Nishina,H., Sato,H., Suzuki,T., Sato,M. and Iba,H. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 3619−3623.
Oliviero,S., Robinson,G.S., Struhl,K. and Spiegelman,B.M. (1992) *Genes Dev.*, **6**, 1799−1809.
Pothier,F., Ouellet,M., Julien,J.-P. and Guerin,S.L. (1992) *DNA Cell Biol.*, **11**, 83−90.
Pulverer,B.J., Kyriakis,J.M., Avruch,J., Nikolakaki,E. and Woodgett,J.R. (1991) *Nature*, **353**, 670−674.
Rüther,U., Komitowski,D., Schubert,F.R. and Wagner,E.F. (1989) *Oncogene*, **4**, 861−865.
Schuermann,M. (1990) *Nucleic Acids Res.*, **18**, 4945−4946.
Schuermann,M., Neuberg,M., Hunter,J.B., Jenuwein,T., Ryseck,R.-P., Bravo,R. and Müller,R. (1989) *Cell*, **56**, 507−516.
Schuermann,M., Jooss,K. and Müller,R. (1991) *Oncogene*, **6**, 567−576.
Schuermann,M., Hennig,G. and Müller,R. (1993) *Oncogene*, **8**, 2781−2790.
Schütte,J., Viallet,J., Nau,M., Segal,S., Fedorko,J. and Minna,J. (1989) *Cell*, **59**, 987−997.
Sewing,A., Rönicke,V., Bürger,C., Funk,M. and Müller,R. (1994) *J. Cell. Sci.*, **107**, in press.

Sikorski,R. and Hieter,P. (1989) *Genetics*, **122**, 19.
Smeal,T., Binétruy,B., Mercola,D., Birrer,M. and Karin,M. (1991) *Nature*, **354**, 494–496.
Stein,B., Baldwin,A.S.,Jr, Ballard,D.W., Greene,W.C., Angel,P. and Herrlich,P. (1993) *EMBO J.*, **12**, 3879–3891.
Sutherland,J.A., Cook,A., Bannister,A.J. and Kouzarides,T. (1992) *Genes Dev.*, **6**, 1810–1819.
Turner,R. and Tjian,R. (1989) *Science*, **243**, 1689–1694.
van Beveren,C., van Straaten,F., Curran,T., Müller,R. and Verma,I.M. (1983) *Cell*, **32**, 1241–1255.
van Beveren,C., Curran,T. and Verma,I.M. (1984) *Virology*, **135**, 229–243.
Verma,I.M. (1986) *Trends Genet.*, **2**, 93–96.
Wick,M., Lucibello,F.C. and Müller,R. (1992) *Oncogene*, **7**, 859–867.
Wisdom,R. and Verma,I.M. (1993) *Mol. Cell. Biol.*, **13**, 2635–2643.
Wisdom,R., Yen,J., Rashid,D. and Verma,I.M. (1992) *Genes Dev.*, **6**, 667–675.
Yen,J., Wisdom,R.M., Tratner,I. and Verma,I.M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5077–5081.
Zerial,M., Toschi,L., Ryseck,R.-P., Schuermann,M., Müller,R. and Bravo,R. (1989) *EMBO J.*, **8**, 805–813.

Received on November 19, 1993; revised on December 28, 1993