The EMBO Journal Vol.17 No.20 pp.6010–6019, 1998

Novel mutants of NAB corepressors enhance activation by Egr transactivators

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The NGFI-A binding corepressors NAB1 and NAB2 interact with a conserved domain (R1 domain) within the Egr1/NGFI-A and Egr2/Krox20 transactivators, and repress the transcription of Egr target promoters. Using a novel adaptation of the yeast two-hybrid screen, we have identified several point mutations in NAB corepressors that interfere with their ability to bind to the Egr1 R1 domain. Surprisingly, NAB proteins bearing some of these mutations increased Egr1 activity dramatically. The mechanism underlying the unexpected behavior of these mutants was elucidated by the discovery that NAB conserved domain 1 (NCD1) not only binds to Egr proteins but also mediates multimerization of NAB molecules. The activating mutants exert a dominant negative effect on NAB repression by multimerizing with native NAB proteins and preventing binding of endogenous NAB proteins with Egr transactivators. To examine NAB repression of a native Egr target gene, we show that NAB2 represses Egr2/Krox20-mediated activation of the bFGF/FGF-2 promoter, and that repression is reversed by coexpression of dominant negative NAB2. Because of their specific ability to alleviate NAB repression of Egr target genes, the dominant negative NAB mutants will be useful in elucidating the mechanism and function of NAB corepressors.

Keywords: corepressor/dominant negative/Egr/FGF/NAB

Introduction

The Egr family of zinc finger transactivators consists of immediate early genes that are induced by a variety of extracellular stimuli, such as growth factors, ionizing radiation and hypoxia. Members of the Egr family have been implicated in a diverse array of processes in a variety of cell types, including commitment to mitogenic, differentiative and apoptotic pathways (Gashler and Sukhatme, 1995). Targeted disruption of the prototype of this family, Egr1/NGFI-A, resulted in female infertility caused by a loss of LH-β expression by pituitary gonadotropes (Lee *et al*., 1996). The knockout of another family member, Egr2/Krox20, exhibits defects in hindbrain patterning, peripheral nerve myelination and bone formation (Schneider-Maunoury *et al*., 1993; Swiatek and Gridley, 1993; Topilko *et al*., 1994; Levi *et al*., 1996).

Like other transactivators such as E2F, Myc/Max and nuclear receptors, Egr proteins also associate with corepressor proteins that can repress transcription of Egr target genes. The NAB1 and NAB2 proteins potently repress transactivation by Egr1/NGFI-A and Egr2/Krox20 (Russo *et al*., 1995; Svaren *et al*., 1996). Repression by NAB corepressors depends on a direct protein interaction with a conserved region within Egr1/NGFI-A, Egr2/Krox20 and Egr3, called the R1 domain. A point mutation within the Egr1 R1 domain (I293F), which abrogates interaction with NAB proteins, renders Egr1 immune to NAB repression in transfection assays. More recently, our work has shown that NAB1 does not interfere with DNA binding by Egr1, but rather forms a complex with Egr1 that binds to Egr1 binding sites (Swirnoff *et al*., 1998). Furthermore, tethering experiments demonstrated that NAB recruitment in an Egr-independent manner results in active repression of a variety of promoters. Therefore, the formation of Egr1/NAB complexes may play a critical role in regulation of many Egr target genes, such as bFGF, PDGF (A and B chains), TGF-β1, Id1, Tissue Factor, LH-β, EphA4 and several Hox genes (Sham *et al*., 1993; Kim *et al*., 1994; Khachigian *et al*., 1995; Biesiada *et al*., 1996; Cui *et al*., 1996; Khachigian *et al*., 1996; Lee *et al*., 1996; Liu *et al*., 1996; Tournay and Benezra, 1996; Seitanidou *et al*., 1997; Wang *et al*., 1997; Theil *et al*., 1998).

The regulation of NAB expression has provided important clues regarding the physiological role of the NAB corepressors. NAB2 (also known as Mader) was initially identified as a protein that is overexpressed in a variety of melanomas (Kirsch *et al*., 1996). NAB2 induction is observed in two of the model systems that were originally used to identify Egr family members as immediate early genes, NGF-induced differentiation of PC12 cells and serum stimulation of fibroblasts (Kirsch *et al*., 1996; Svaren *et al*., 1996). Therefore, the induction of NAB2 might be involved in downregulating genes that are transiently activated by the rapid stimulation of Egr proteins in such systems. Although NAB1 is not significantly upregulated in these paradigms, work by Fan *et al*. (1997) has shown that NAB1 is induced by glucocorticoids in a leiomyosarcoma cell line, and that induction of NAB1 may play a critical role in the glucocorticoid-mediated cell-cycle arrest in this cell line.

Two individual NAB domains are highly conserved in mammalian NAB1 and NAB2 proteins as well as in a

Caenorhabditis elegans NAB homolog. NAB conserved domain 1 (NCD1) mediates a specific interaction with the Egr1 R1 domain (Svaren *et al*., 1996), and NCD2 has been shown to be critical for the repression function of NAB1 (Swirnoff *et al*., 1998). In the present study, a mutational analysis of NCD1 unexpectedly identified NAB mutants that greatly stimulate Egr1 transactivation. Further analysis revealed that NCD1 mediates self-association of NAB proteins, and that the mutants exhibit a dominant negative effect on NAB repression that is dependent on their ability to multimerize with native NAB proteins. These observations shed light on the mechanism of NAB repression and provide valuable tools for uncovering the physiological role of NAB corepressors.

Results

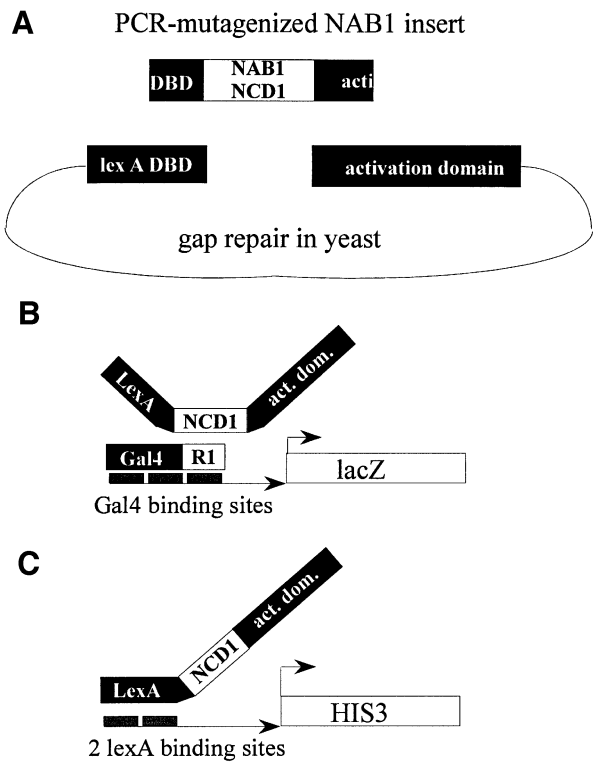
Mutagenesis of the NAB1 and NAB2 interactive domains

NAB1, NAB2 and a *C.elegans* NAB homolog each contain two domains that are highly conserved in each family member. NCD1 was contained within the relatively small NAB1 fragment (coding for 134 amino acids) that was found to interact with the R1 repression domain of Egr1 in a yeast two-hybrid screen (Russo *et al*., 1995). Furthermore, minimal NCD1 domains of both NAB2 and *C.elegans* NAB also bind specifically to the R1 domain of Egr1 (Svaren *et al*., 1996).

To probe the structure and function of the NCD1 domain, we devised a system to obtain NCD1 mutants that were defective in binding to Egr1. To this end, the yeast two-hybrid system was modified by incorporating a yeast technique that was developed originally to mutagenize critical residues within DNA-binding domains (Liu *et al*., 1993; Wilson *et al*., 1993). The NAB1–NCD1 interactive domain was cloned into a fusion of the LexA DNA-binding domain (DBD) with the activation domain of Gal4 (Liu *et al*., 1993), resulting in a trifunctional protein, LN1G (shown in Figure 1). When this construct is expressed in yeast together with the Gal4 DBD–Egr1 R1 fusion protein, the Egr and NAB domains interact, linking the Gal4 DBD and activation domains, and thereby activating the GAL1/LacZ reporter (Figure 1B).

The NAB1 interactive domain was mutated by PCR mutagenesis (Muhlrad *et al*., 1992) and then cotransformed into yeast along with the gapped plasmid (Figure 1A). Gap repair of the LN1G plasmid with the PCR fragment containing NCD1 resulted in the transformation of a library of mutations into the receptor strain containing the Gal4 DBD–Egr1 R1 fusion. More than 90% of the transformed colonies were lacZ+, indicating that the two-hybrid interaction between NAB1 NCD1 and the Egr1 R1 domain activated the lacZ reporter in these colonies. White colonies (*lacZ–*) were picked as potentially harboring mutations that disrupt interaction of NCD1 with the R1 domain, thereby preventing expression of the GAL1/lacZ reporter.

Although the screen for *lacZ–* colonies was designed to obtain mutations that disrupt interaction of NCD1 with the Egr1 R1 domain, loss of lacZ expression could also result from either nonsense or frameshift mutations that prevent translation through the Gal4 activation domain, or mutations which decrease the stability of the LN1G



D

NAB NCD1 domain	lacZ	HIS3
wild type	+	+
nonsense and frameshift mutations	-	-
mutations preventing interaction with Egr1 R1 domain	-	+

Fig. 1. Yeast two-hybrid mutagenesis scheme. (**A**) NAB1 NCD1 (residues 7–105) was cloned between the LexA DBD and the Gal4 activation domain to make the LN1G construct. NCD1 was amplified by PCR using primers in the LexA and Gal4 sequences to introduce random mutations. Cotransformation of this fragment along with the gapped LN1G plasmid into yeast and subsequent gap repair incorporates the PCR-mutagenized NCD1 insert within the LN1G plasmid. (**B**) Wild-type inserts give rise to a two-hybrid interaction with the Gal4 DBD/R1 domain plasmid and thereby activate the lacZ reporter gene. (**C**) The LN1G fusion protein also activates transcription through LexA binding sites that are cloned upstream of the *HIS3* gene, which allows growth in the absence of histidine. (**D**) Mutants that disrupt the interaction between NAB1 NCD1 and the R1 domain of Egr1 will be *lacZ–*. Frameshift or nonsense mutations in NCD1 prevent translation through the Gal4 activation domain, and interfere with activation of the *HIS3* gene. By selecting only yeast colonies that are *lacZ–* (white) and *HIS3+*, the screen identifies missense mutations that specifically disrupt the interaction between NAB1 and the R1 domain of Egr1.

fusion protein. To eliminate such colonies from the screen, the *lacZ–* colonies were subsequently plated on medium lacking histidine to test for expression of a second reporter (Figure 1C) in which two LexA operator sequence elements are cloned upstream of a minimal promoter fused to the *HIS3* coding region (Liu *et al*., 1993). The NAB inserts from colonies that were *lacZ–*, *HIS3+* were amplified by PCR, and the resulting products were sequenced to identify the NCD1 mutations.

From this analysis, we obtained >30 point mutations (Figure 2) which were distributed over a 50 amino acid stretch of NCD1. The most frequently mutated residues

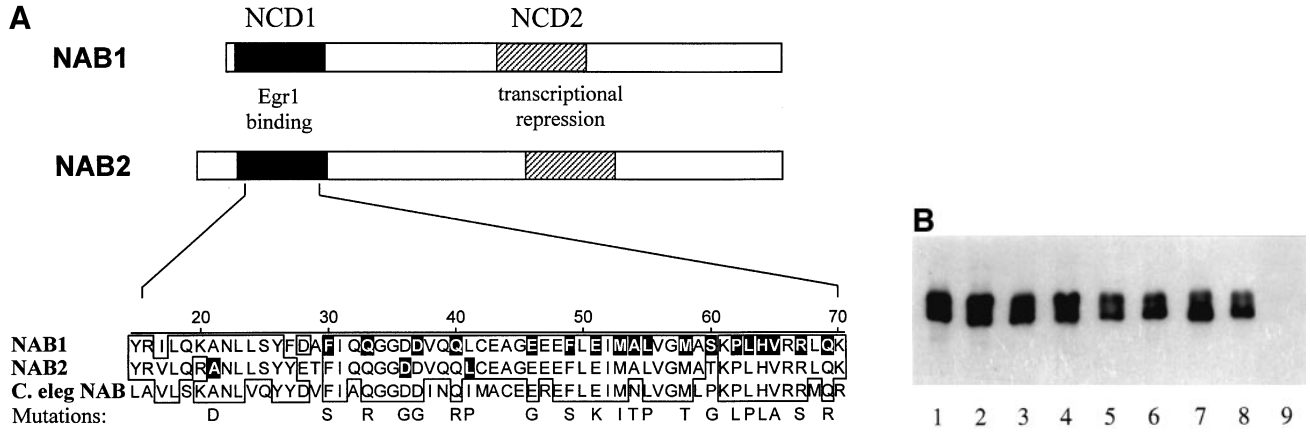


Fig. 2. NAB mutations prevent interaction with Egr1. (**A**) The positions of the two conserved domains within NAB1 and NAB2 are shown. Identical residues are boxed in the sequence alignment of a portion of NCD1 from mouse NAB1, mouse NAB2 and *C.elegans* NAB. Residues that were mutated in the screen are shaded, and the identities of the mutations are listed below the sequence alignment. Mutations obtained in a screen of NAB2 NCD1 are also indicated. Numbering is derived from the mouse NAB1 protein sequence (DDBJ/EMBL/GenBank accession No. 1197671). For simplicity, residue numbers in this paper are always derived from the NAB1 numbering system, even for mutations that reside in NAB2. For example, the A21D mutation in NAB2 actually affects residue 52 of the mouse NAB2 sequence (DDBJ/EMBL/GenBank accession No. 1197731). (**B**) CV-1 cells were transfected with CMV expression constructs for wild-type and mutant versions of NAB2. Equal amounts of lysates from these cells were examined by Western blotting using a monoclonal antibody directed against NAB2. The samples were obtained from cells transfected with the following versions of NAB2: (i) wild type; (ii) E51K; (iii) H64L; (iv) Q33R,H64Q double point mutant; (v) F30S; (vi) A21D; (vii) Δ15-23; and (viii) Δ48-53. Lane 9 indicates CV-1 cells transfected with a control CMV expression vector.

clustered in the latter half of this subdomain. With only three exceptions, all residues that were mutated in the screen are ones that are identical in NAB1, NAB2 and *C.elegans* NAB. One exception is L41, which is quite similar to the isoleucine in the corresponding position of *C.elegans* NAB. The A54T mutation changes a residue that is conserved in both NAB1 and NAB2, but not in *C.elegans* NAB. Changing the S60 residue might be expected not to interfere with the interaction, since *C.elegans* NAB is quite capable of binding the R1 domain of Egr1 despite the presence of a proline at this position (Svaren *et al*., 1996); however, it is possible that the mutation to glycine may disrupt an important secondary structure.

NAB mutants that activate Egr1 activity

The NCD1 domains of NAB1 and NAB2 are highly homologous, and both NAB1 and NAB2 interact with the R1 domain of Egr family members with equal specificity. However, NAB2 seems to repress somewhat more efficiently than NAB1 in transfection assays (unpublished observations), and the expression of NAB2, in contrast to NAB1, is induced by stimuli that also induce Egr1 expression (Svaren *et al*., 1996). Therefore, we decided to introduce several NCD1 point mutations into a full-length NAB2 expression construct for further analysis. In addition, NAB2 constructs containing a double mutant (Q33R,H64Q, obtained in the same mutagenesis screen) and two small deletions (Δ15-23, Δ48-53) within the NCD1 domain were also created. All of the constructs were transfected into CV-1 cells, and lysates of the cells were examined for NAB2 expression by blotting with an anti-NAB2 monoclonal antibody (Kirsch *et al*., 1996). As shown in Figure 2B, the expression levels of all the point mutant versions of NAB proteins, as well as the two small deletions, were quite similar, which provided independent confirmation that the mutations obtained in the mutagenesis screen did not adversely affect protein stability.

The NAB2 constructs were then tested for their ability to repress Egr1 activity in mammalian cells (Figure 3A). In this assay, a version of Egr1 containing the Gal4 DBD substituted for the native DBD (Egr1/Gal4; Russo *et al*., 1993) was used to activate a luciferase reporter gene fused to a minimal promoter containing Gal4 binding sites. Wild-type and mutant versions of NAB2 were tested for their ability to repress transactivation by the Egr1/Gal4 hybrid. For some mutants in the N-terminal half of NCD1, such as F30S and A21D (Figure 3A), weak repression was observed at higher levels (>10 ng), but all the point mutants were clearly inferior to wild-type NAB2 in repression of Egr1/Gal4 activity. The two small deletions also gave little or no repression in this assay.

Surprisingly, a subset of the mutants increased the activity of Egr1/Gal4 dramatically (Figure 3). The NAB2 E51K mutant, as well as a double mutant obtained in our initial screen, NAB2 (Q33R/H64Q), activated Egr1/Gal4 activity >20-fold. Significant activation occurred when these mutants were expressed at levels similar to those required for repression by native NAB2 (<5 ng). The effect is not unique to NAB2 because the same E51K mutation in NAB1, as well as a M53I mutation, behaved quite similarly in this assay (Figure 3B).

NCD1 mediates dimerization

The unexpected enhancement of Egr1 activity by NAB mutants suggests that NAB proteins possess an uncharacterized activity that was unmasked by these point mutations. A possible mechanism for this was suggested by observations obtained in parallel experiments, in which we sought to identify other proteins besides Egr1 that might interact with the NAB1 NCD1 domain. Using a yeast two-hybrid screen of a rat brain library, we unexpectedly identified partial clones of NAB1 and NAB2 that interacted specifically with NAB1 NCD1. The NAB1 and NAB2 clones contained all of NCD1 but only part of NCD2. The association appeared to be specific, because no

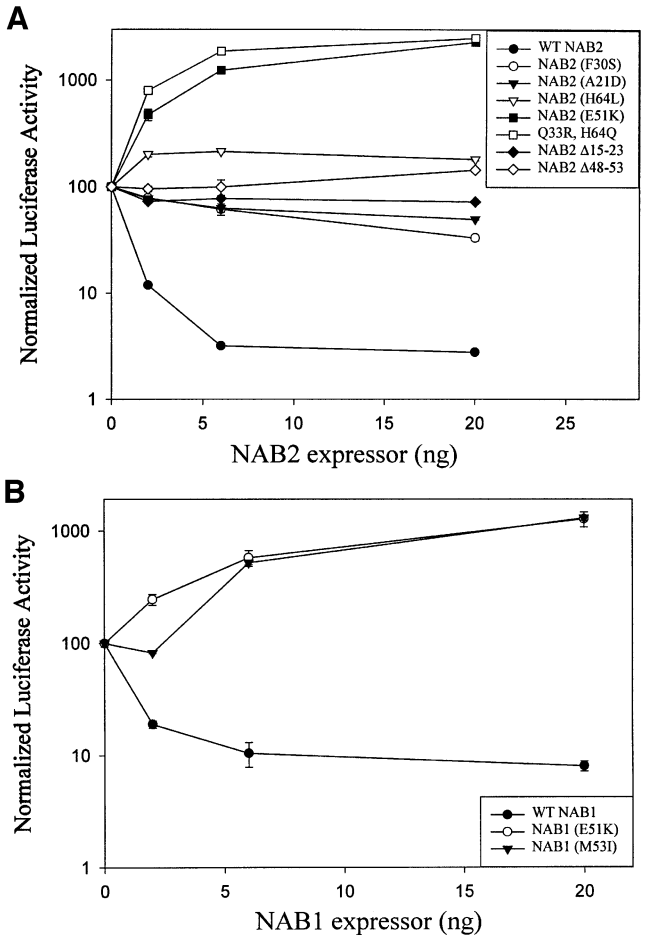


Fig. 3. NCD1 mutations in NAB1 and NAB2 fail to repress Egr1 activity. CV-1 cells were transfected with a luciferase reporter containing five Gal4 binding sites upstream of an E1B minimal promoter and a plasmid expressing Egr1/Gal4 (20 ng), along with the indicated amounts (ng) of a CMV expression plasmid for wild-type or mutant NAB2 (**A**), or a CMV expression plasmid for wild-type or mutant NAB1 (**B**). Normalized luciferase activity is defined as the activity of Egr1/Gal4 in the absence of NAB2, which is set at 100%. The *y*-axis is a log scale of normalized luciferase activity.

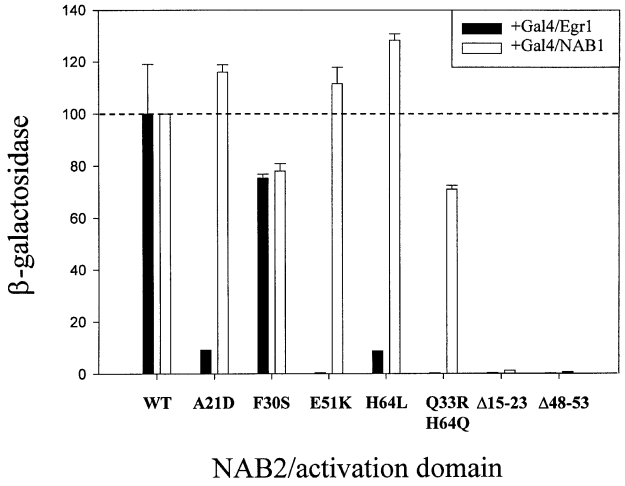


Fig. 4. NAB multimerization and Egr1 binding are separable functions of NCD1. The yeast two-hybrid system was used to test several NCD1 mutants for their ability to interact with the R1 domain of Egr1, and to multimerize with a wild-type NCD1 domain. The indicated mutations were introduced into pRS4525LN2G, a multicopy LEU2 plasmid containing NCD1 of NAB2 fused between the LexA and Gal4 domains depicted in Figure 1. The wild-type and mutant versions of pRS425 LN2G were transformed into yeast strain Y190 containing either the Egr1 R1 domain (Gal4/Egr1, filled bars), or the NAB1 NCD1 domain (Gal4/NAB1, open bars). The measurements of β-galactosidase activity were all normalized to the activity of wild-type pRS4525LN2G with either Gal4/Egr1 or Gal4/NAB1, respectively, which was set at 100 (indicated by the dotted line). The error bars indicate the standard deviation of duplicate measurements from independent cultures. The activity of Y190 containing either pRS425LN2G alone, or either of the Gal4 fusions alone (Gal4/NAB1 or Gal4/Egr1), was not significantly higher than the background of the assay.

interaction was observed between NAB1 NCD2 and either of these clones (data not shown).

To confirm these results, we fused a minimal NCD1 domain of NAB2 with the Gal4 activation domain to create LN2G (as described for NAB1 in Figure 1). Pairing this construct with the Gal4 DBD–NAB1 NCD1 fusion yielded a strong positive signal in the yeast two-hybrid assay (Figure 4), showing that minimal NCD1 domains of NAB1 and NAB2 are sufficient to mediate an interaction between NAB molecules. Therefore, we conclude that NCD1 can mediate formation of dimers (or higher order multimers) of NAB proteins.

The NCD1 mutants disrupt binding with Egr1, but do not affect multimerization

To test further the specificity of the NAB–NAB interaction, the NAB2 NCD1 mutations (see Figure 3) were introduced into the LN2G construct, and these were tested for their ability to interact with NAB1 NCD1 in the yeast two-hybrid assay. Although all of the NAB2 point mutants were defective in binding to Egr1, they all retained the ability to multimerize with NAB1 (Figure 4). However, the two small deletion mutations were defective in dimerization when compared with the wild-type and point mutant versions of NAB2 NCD1.

The NCD1 mutants were also tested for interaction with the Egr1 R1 domain in the yeast two-hybrid assay. Although the point mutations were identified originally because they disrupted interaction with Egr1, the LN1G fusion used in the screen was on a single copy (CEN-ARS) plasmid. In this experiment, the mutations were inserted into a NAB2 NCD1 fusion construct (LN2G) on a multicopy (2 micron) yeast plasmid in order to test whether overexpression of the mutant would reveal a detectable interaction with Egr1.

Consistent with the results of the initial screen, all of the point mutants of NAB2 NCD1 were defective in binding to Egr1, although the A21D, F30S and H64L mutations allowed for low levels of interaction with Egr1 when they were expressed on the high copy plasmid (Figure 4). Significantly, both the E51K point mutant and a double point mutation of NAB2 NCD1 (Q33R,H64Q) were fully capable of dimerizing with NAB1 but showed no interaction with Egr1, indicating that these interactions can be uncoupled.

Coimmunoprecipitation of NAB1 and NAB2

The multimerization of NAB proteins was confirmed using a coimmunoprecipitation assay (Figure 5A). The indicated native or mutant versions of NAB2 were expressed along with wild-type NAB1 in QT-6 cells, and NAB1 was

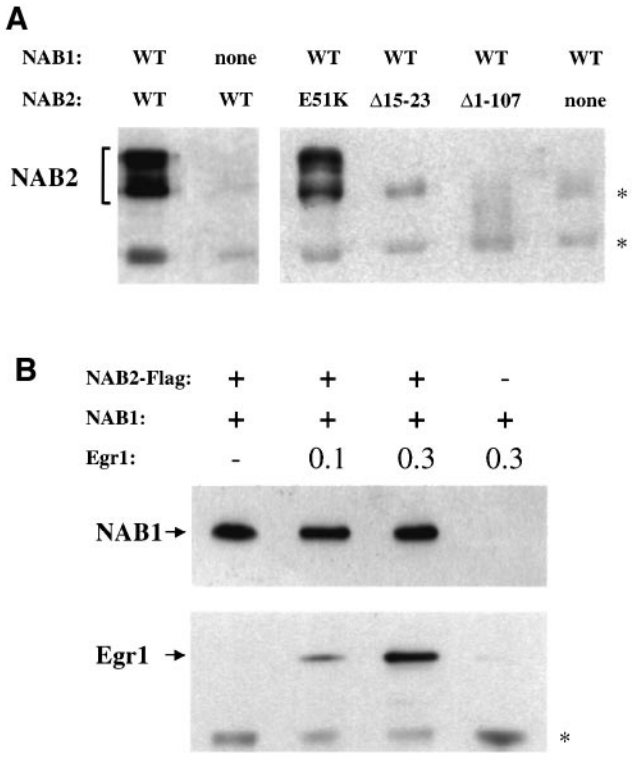
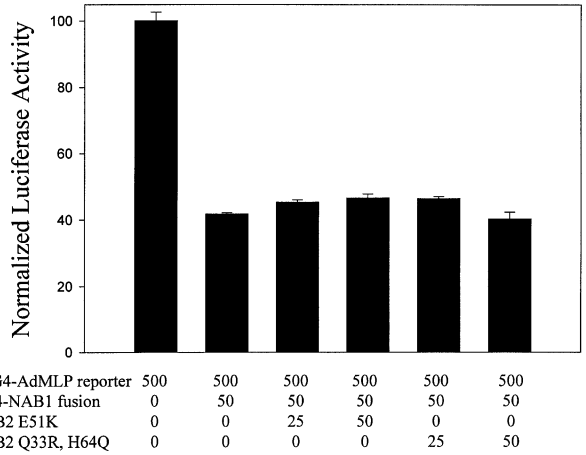


Fig. 5. NAB2 coimmunoprecipitates with NAB1. (**A**) QT-6 cells were transfected with the indicated NAB2 expression constructs along with wild-type NAB1. Lysates of transfected cells were immunoprecipitated using an anti-NAB1 polyclonal antibody. The immunoprecipitated samples were resolved using SDS–PAGE, blotted to nitrocellulose and probed using an anti-NAB2 monoclonal antibody. The NAB2 protein runs as a doublet. Western blot analysis of the crude lysates showed that the mutant versions of NAB2 were expressed at an equivalent level to that of native NAB2 (data not shown). The fainter bands indicated by the asterisks are background bands detected by the anti-NAB2 antibody in lysates from cells not transfected with NAB2. (**B**) QT-6 cells were transfected with a NAB1 expression construct (6 μg), and where indicated, a construct expressing NAB2 fused with a C-terminal Flag tag (2 μg). Lysates of transfected cells were incubated with 0, 0.1 or 0.3 μg of recombinant Egr1 and then immunoprecipitated using an anti-Flag antibody. The immunoprecipitates were resolved by SDS–PAGE, and probed with an anti-NAB1 polyclonal antibody (top panel). The same blot was reprobed with an anti-Egr1 monoclonal antibody (bottom panel). Western blot analysis demonstrated that equal amounts of NAB1 protein were present in all lysates prior to immunoprecipitation (data not shown). The faint band indicated by the asterisk is a background band detected by the anti-Egr1 antibody.

precipitated from cell lysates using an anti-NAB1 polyclonal antiserum. The presence of NAB2 in the immunoprecipitates was assessed by Western blot analysis using an anti-NAB2 monoclonal antibody. When the two proteins were expressed together, NAB2 was efficiently coimmunoprecipitated by the NAB1 antibody. The NAB1 antibody is directed against a peptide epitope that is not conserved in NAB2 (Swirnoff *et al*., 1998), and does not immunoprecipitate NAB2 in the absence of NAB1 (Figure 5A). The interaction between NAB1 and NAB2 is dependent on NCD1, as a derivative of NAB2 lacking NCD1 (Δ1-107) was unable to interact with NAB1 in this assay. Furthermore, a NAB2 deletion mutant (Δ15-23) which abrogates NAB multimerization in the yeast two-hybrid assay (Figure 4) also did not interact with NAB1. In contrast, the NAB2 (E51K) mutant, which disrupts Egr1



5XG4-AdMLP reporter	500	500	500	500	500	500
Gal4-NAB1 fusion	0	50	50	50	50	50
NAB2 E51K	0	0	25	50	0	0
NAB2 Q33R, H64Q	0	0	0	0	25	50

Fig. 6. Dominant negative mutants do not interfere with the NAB repression mechanism. CV-1 cells were transfected with a luciferase reporter containing AdMLP with five Gal4 binding sites upstream and a NAB1–Gal4 DBD expression construct, along with the indicated amounts (ng) of NAB2 expression constructs bearing dominant negative mutations (E51K or Q33R,H64Q). Normalized luciferase activity is the activity of the AdMLP luciferase reporter alone, which is set at 100%.

binding yet retains NAB multimerization capability in the yeast two-hybrid assay, was clearly able to interact with NAB1 in the coimmunoprecipitation assay as well.

The fact that NCD1 mediates both Egr binding and NAB multimerization raises the question of whether NAB multimerization excludes NAB–Egr interactions. To address this issue, we introduced recombinant Egr1 into a lysate of transfected QT-6 cells expressing both NAB1 and NAB2 (tagged with the Flag epitope, NAB2-Flag). Immunoprecipitation with an anti-Flag antibody, followed by Western blot analysis with an anti-NAB1 antibody, confirmed the association of NAB1 with NAB2 (Figure 5B). When lysates were preincubated with up to 300 ng of recombinant Egr1 protein, no displacement of NAB1 from the complex was observed. Furthermore, an anti-Egr1 antibody confirmed that Egr1 was also present in the immunoprecipitated complex, indicating that NAB multimerization does not preclude Egr1 binding.

NCD1 mutants interfere with Egr1–NAB interaction

The superactivation of Egr1 was observed only with point mutants (E51K and Q33R,H64Q) that were totally incapable of interaction with Egr1 but retained wild-type ability to multimerize with NAB proteins. This suggested that these mutants do not directly affect Egr1 itself, but rather prevent repression by endogenous NAB proteins and thereby derepress Egr1 activity. The activating point mutants could exert a dominant negative effect on NAB activity either at the level of Egr–NAB interaction or at a subsequent step of the repression mechanism.

To test whether the mutants might interfere with the repression function of NAB1 rather than Egr1 binding, we employed a system in which NAB1 is tethered directly to an active promoter (Figure 6). Previous work had shown that fusion of full-length NAB1 to the Gal4 DBD can actively repress a variety of active promoters in which Gal4 binding sites have been inserted (Swirnoff *et al*., 1998). As shown in Figure 6, expression of the NAB1–Gal4 DBD fusion represses the Adenovirus major late

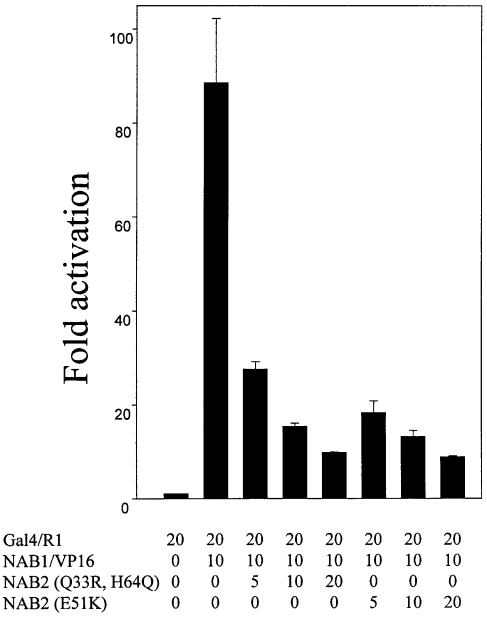


Fig. 7. Dominant negative mutants specifically target NAB–Egr interaction. CV-1 cells were transfected with a luciferase reporter containing five Gal4 binding sites, and expression constructs for a fusion of the Gal4 DBD with the Egr1 R1 domain (Gal4–R1), and a fusion of NAB1 with the VP16 activation domain (NAB1–VP16). The luciferase activity of the reporter in the presence of Gal4–R1 alone was set as 1. The fold activation of the mammalian two-hybrid assay caused by coexpression of Gal4–R1 (20 ng) with the NAB1–VP16 fusion protein (10 ng) was measured in the absence or presence of the indicated NAB2 mutants.

promoter (AdMLP) by ~60%. The Gal4 DBD alone does not repress the same promoter, and NAB1–Gal4 DBD has no effect on the AdMLP if no upstream Gal4 binding sites are present (Swirnoff *et al*., 1998). Coexpression of the NAB2 (E51K) or NAB2 (Q33R,H64Q) mutants does not interfere with the ability of NAB1–Gal4 DBD to repress the Gal4-AdMLP reporter, although expression of the same mutants has a profound effect on Egr1 activity (Figure 3). Therefore, the NAB2 (E51K) or NAB2 (Q33R,H64Q) mutants do not appear to affect NAB repression function directly, since the mutations have no effect when NAB1 is tethered directly to a promoter in an Egr1-independent manner. This is consistent with previous observations showing that NCD1 is not required for the repressive function of NAB1 (Swirnoff *et al*., 1998).

Next we tested whether the NAB2 mutants interfered with binding of NAB proteins to Egr1 by examining the effect of the NAB2 (E51K) and NAB2 (Q33R,H64Q) mutants on a mammalian two-hybrid assay of interaction between NAB1 and the R1 domain of Egr1 (Figure 7). A fusion protein consisting of the VP16 activation domain linked to the C-terminus of NAB1 (NAB1–VP16) can activate transcription via an interaction with Egr1 (Swirnoff *et al*., 1998). A similar two-hybrid interaction can be reconstituted using a fusion of the Egr1 R1 domain with the Gal4 DBD (Gal4–R1). As shown in Figure 7, expression of Gal4–R1 together with NAB1–VP16 activates the Gal4-luciferase reporter >80-fold. However, coexpression of NAB2 (E51K) or NAB2 (Q33R,H64Q) significantly inhibits binding of NAB1–VP16 to Gal4–R1, as measured by the mammalian two-hybrid assay. Therefore, these results confirm that these mutants interfere

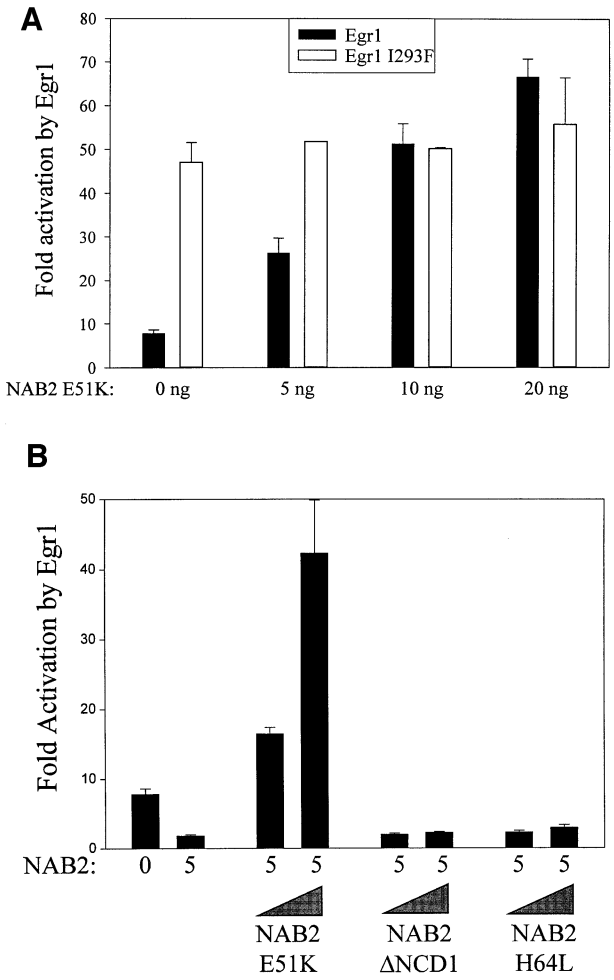


Fig. 8. Native Egr1 is derepressed by dominant negative NAB2. (**A**) CV-1 cells were transfected with a luciferase reporter construct containing two Egr1 binding sites upstream of the prolactin minimal promoter and 20 ng of either wild-type or point mutant (I293F) Egr1, in the presence of increasing amounts (0, 5, 10 and 20 ng) of NAB2 (E51K). The scale indicates the fold activation of this reporter by Egr1. (**B**) CV-1 cells were transfected with the same reporter as in (A), 20 ng of wild-type Egr1, and where indicated, 5 ng of a NAB2 expression construct, and 10 or 20 ng of the indicated NAB2 mutants.

with interactions between Egr1 and a NAB1 protein that contains a wild-type NCD1 domain.

Repression of native Egr1 is neutralized by dominant negative NAB2

To show that native Egr1, as well as Egr1/Gal4 chimeras, can also be derepressed by a dominant negative NAB mutant, we utilized a luciferase reporter construct that contains two Egr1 binding sites upstream of a prolactin minimal promoter. As shown in Figure 8A, activation of this reporter by native Egr1 is significantly enhanced in a dose-dependent manner by inclusion of NAB2 (E51K). In contrast, the elevated activity of Egr1 (I293F), which is immune to repression by NAB proteins by virtue of a point mutation in the R1 domain (Russo *et al*., 1995; Svaren *et al*., 1996), is not affected by addition of NAB2 (E51K).

As described above, derepression of Egr1 activity by dominant negative NAB mutants presumably reflects neutralization of endogenous NAB protein(s). To demonstrate

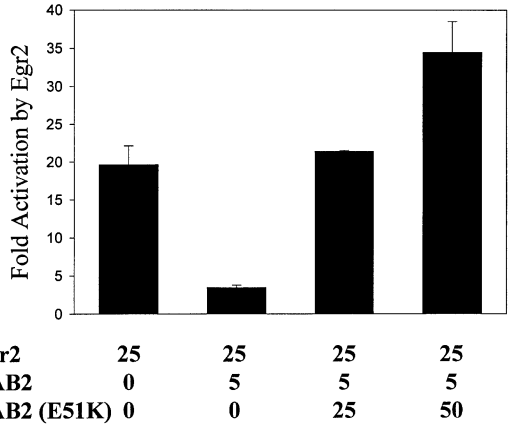


Fig. 9. Dominant negative NAB2 reverses repression by NAB2 of the bFGF promoter. JEG-3 cells were transfected with a reporter construct consisting of the human bFGF promoter (–500 to +160) and the indicated amounts (in ng) of CMV expression plasmids for Egr2/Krox20, wild-type NAB2, and NAB2 (E51K). The *y*-axis indicates the fold activation of this reporter by Egr2. Similar results were obtained in CV-1 cells, although the basal activity of the bFGF promoter was significantly elevated in this cell line (data not shown).

that dominant negative NAB can specifically reverse the repression by a defined NAB molecule, we tested the effect of NAB2 (E51K) on repression of Egr1 activity by cotransfected NAB2. Consistent with previous results (Svaren *et al*., 1996), introduction of only 5 ng of wild-type NAB2 efficiently repressed Egr1 activity (Figure 8B), but 20 ng of NAB2 (E51K) reverses the repression by wild-type NAB2 effectively. Addition of a NAB2 protein with a deletion of the NCD1 domain, or one with a point mutation in NCD1 (H64L) that retains partial binding to Egr1, does not interfere with repression by wild-type NAB2 in this assay.

Dominant negative NAB2 reverses NAB repression of the bFGF promoter

The bFGF promoter has been shown to be activated by Egr1 in transfection experiments (Biesiada *et al*., 1996; Wang *et al*., 1997). We found that another member of the Egr family, Egr2/Krox20, also activates the bFGF promoter very efficiently (Figure 9). NAB2 repressed Egr2 transactivation of this promoter, consistent with our previous demonstration that Egr2 activation of a synthetic promoter is repressed by both NAB1 and NAB2 (Russo *et al*., 1995; Svaren *et al*., 1996). Furthermore, NAB2 (E51K) neutralized the repression exerted by NAB2, demonstrating that NAB repression of a native promoter can also be reversed by a dominant negative NAB mutant.

Discussion

Structural and functional analyses of protein–protein interactions are facilitated greatly by identification of point mutations that abrogate such interactions, and several groups have employed distinct versions of the yeast two-hybrid system for this purpose (Li and Fields, 1993; White *et al*., 1995; Brachmann *et al*., 1996; Shan *et al*., 1996; Vidal *et al*., 1996). To identify point mutations within the NAB corepressors that disrupt binding to the R1 domain of Egr1, we developed a novel version of the yeast two-hybrid system by incorporating a technique that was developed originally to identify point mutations in mammalian DNA-binding domains (Liu *et al*., 1993; Wilson *et al*., 1993). This method yielded a number of point mutations which, when inserted into full-length NAB1 or NAB2 constructs, reduced or eliminated NAB repression of Egr1 in mammalian cells.

Surprisingly, a subset of the NAB mutants enhanced Egr1 activity >20-fold in transfection experiments. This superactivation phenomenon is similar to that previously observed when Egr1 was rendered incapable of binding NAB proteins due to a deletion or point mutation of its R1 domain (I293F) (Russo *et al*., 1993, 1995). In fact, the NAB2 (E51K) mutant elevates the activity of wild-type Egr1 to a level comparable with that of Egr1 (I293F), but does not alter the activity of Egr1 (I293F) itself (Figure 8). Therefore, some of the NCD1 mutations appear to interfere with the ability of endogenous NAB proteins to repress Egr1, and are designated dominant negative mutations.

Use of the yeast two-hybrid screen revealed that NCD1 not only binds to the Egr1 R1 domain, but also mediates a multimerization of NAB proteins. The most effective dominant negative mutants of NAB1 and NAB2 were found to be capable of dimerization, but were absolutely deficient in binding to Egr1. In contrast, some other NCD1 mutants (H64L, A21D, F30S) weakly repress Egr1 activity when expressed at higher levels. Yeast interaction assays revealed that these mutant versions of NCD1 possessed a weak but measurable affinity for Egr1, which accounts for their ability to repress Egr1 weakly in transfection experiments.

The isolation of dominant negative mutants on the basis of their inability to interact with Egr1 strongly suggests that they exert their effect at the level of Egr1–NAB interaction. This hypothesis was confirmed by showing that dominant negative NAB mutants disrupt the Egr1–NAB interaction in a mammalian two-hybrid assay (Figure 7). Therefore, our current model is that dominant negative NAB mutants are able to multimerize with wild-type NAB partners, and the resulting complex is unable to bind to Egr proteins. These mutants are therefore analogous to dominant negative alleles of p53, which mediate oligomerization, but prevent DNA-binding and/or transcriptional activation (Kern *et al*., 1992; Shaulian *et al*., 1992; Brachmann *et al*., 1996).

The fact that NCD1, a domain of ~100 amino acids, mediates both Egr1 binding and NAB multimerization raises the issue of whether both interactions occur simultaneously. One possible model is that NAB multimerization would exclude Egr1 binding. However, one would then expect that all of the NAB point mutants would behave as dominant negative mutants, because they are all fully competent to dimerize, but are deficient in Egr1 binding to some degree (Figure 4). We tested this model more directly by investigating whether Egr1 could displace NAB1 from a NAB1–NAB2 complex in a coimmunoprecipitation assay (Figure 5B). No displacement was observed, and Egr1 was detected in the immunoprecipitated complex, indicating that NAB proteins bind Egr1 as a multimer.

Although our mutagenesis screen identified many NCD1 point mutations that prevent Egr binding, examination of several point mutants dispersed throughout the NCD1

domain failed to identify any that inhibit NAB–NAB interaction (Figure 4; data not shown). Analysis of the NCD1 sequence predicts three amphipathic helices, suggesting that one or more of these may form an extensive multimerization interface, which may not be susceptible to disruption by point mutations. Although two small deletions (<10 amino acids) within NCD1 prevented NAB multimerization, it is possible that these deletions disrupt the secondary and/or tertiary structure of the domain. The fact that all of the point mutants that we tested could still mediate dimerization suggests that these mutations disrupt specific NAB–Egr1 interfaces, rather than overall folding of the NCD1 domain.

To complement our studies using synthetic reporters, we have demonstrated that NAB repression is effective on a native Egr target promoter. The bFGF/FGF-2 promoter is activated by Egr1 cotransfection (Biesiada *et al*., 1996; Wang *et al*., 1997), and we have shown that Egr2 appears also to activate the promoter very efficiently. Significantly, we found that NAB2 is capable of inhibiting the Egr-activated expression of this promoter, and this repression is reversed efficiently by expression of a dominant negative NAB2 mutant. Therefore, it is likely that NAB proteins regulate transcription of bFGF as well as other Egr target promoters such PDGF (A and B chains), TGF-β1, Id1, Tissue Factor, LH-β, EphA4 and several Hox genes (Sham *et al*., 1993; Kim *et al*., 1994; Khachigian *et al*., 1995, 1996; Biesiada *et al*., 1996; Cui *et al*., 1996; Lee *et al*., 1996; Liu *et al*., 1996; Tournay and Benezra, 1996; Seitanidou *et al*., 1997; Wang *et al*., 1997; Theil *et al*., 1998).

It is not known whether bFGF is a direct target gene of Egr2/Krox20 *in vivo*, but both Egr2 and bFGF are known to be expressed in Schwann cells and the central nervous system (Hassan *et al*., 1994; Topilko *et al*., 1994). Furthermore, bFGF has been reported to activate Egr2 expression in gastrula ectoderm, suggesting that Egr2 might be involved in autocrine/paracrine signaling by bFGF in nervous system development (Lamb and Harland, 1995). Recently, a mutation in Egr2 was found in a patient diagnosed with congenital hypomyelinating neuropathy (CHN) (Warner *et al*., 1998). The position of this mutation (I268N) within Egr2 corresponds directly to that of the Egr1 (I293F) mutation, which renders Egr1 resistant to NAB repression (Russo *et al*., 1993, 1995). Since the features of CHN are similar to the defects in peripheral nerve myelination observed in the Egr2 knockout (Topilko *et al*., 1994; Scherer, 1997), this finding suggests that NAB–Egr2 interactions play a vital role in Schwann cell development.

Dominant negative mutants of a variety of transcriptional regulators have proven to be useful tools for studying the physiological role of these proteins *in vivo*. In the case of NAB corepressors, we have so far been unable to identify a tissue or cell line that does not express NAB1 and/or NAB2, and therefore gain-of-function experiments involving NAB overexpression are problematic because endogenous levels may mask any effect. Since dominant negative NAB mutants negate function of both NAB1 and NAB2, they should facilitate investigation of the function of NAB corepression in both cell lines and transgenic model systems.

Materials and methods

Plasmids

The LN1G plasmid contains a fragment encompassing NCD1 of mouse NAB1 (residues 7–105) fused between the N-terminal LexA DBD and the C-terminal Gal4 activation domain found in plasmid pBM2462 (Liu *et al*., 1993). Plasmid pRS425LN2G contains a similar fusion of mouse NAB2 NCD1 with the same LexA and Gal4 domains, which is expressed from pRS425, a high copy LEU2 plasmid (Christianson *et al*., 1992). Expression vectors for wild-type Egr1/NGFI-A, Egr2/Krox20, Gal4/Egr1 (Russo *et al*., 1993) and fusions of full-length NAB1 with either the Gal4 DBD (NAB1–Gal4 DBD) or the VP16 activation domain (NAB1–VP16) have been described (Swirnoff *et al*., 1998). The M53I and E51K point mutations were inserted into a CMV expression vector for NAB1 (Swirnoff *et al*., 1998). The indicated point and deletion mutations were introduced into the yeast two-hybrid vector (pRS425LN2G) as well as a CMV expression vector for full-length NAB2 (pCMVNAB2, Svaren *et al*., 1996). The Flag tag was fused to the C-terminus of NAB2 to create NAB2-Flag. For mammalian two-hybrid analysis, the R1 domain of Egr1 (Russo *et al*., 1993) was fused to the Gal4 DBD in plasmid pM1 (Sadowski and Ptashne, 1989) to make Gal4–R1. The *HIS3* gene containing two LexA binding sites was subcloned from pBM2546 (Wilson *et al*., 1991; Liu *et al*., 1993) into a LYS2 plasmid, pRS317 (Sikorski and Boeke, 1991), to make pRS317–HIS3.

Mutagenesis

Primers in the LexA (ATGAAAGCGTTAACGGCCAGGCA) and Gal4 (CTTTGACCTTTGTTACTACTCTC) domains were used in a PCR reaction to amplify a fragment from the LN1G plasmid containing NAB1 (or NAB2) NCD1. The fragment was cotransformed with 5 μg of *Not*I/*Xho*I digested pBM2462 into yeast strain YM2632 (*MAT***a,** *ura3-52, his3-200, ade2-101, lys2-801, leu2-3,112, trp1-901, met2, gal4-542, gal80-538*, $can^{R,}$ *LEU2*::*GAL1*-lacZ) bearing a plasmid expressing a fusion of the Egr1 R1 domain with the Gal4 DBD (Russo *et al*., 1995) and the pRS317–HIS3 plasmid. The transformation was plated on medium lacking uracil, tryptophan and lysine. After 2–3 days, colonies were assayed for lacZ expression (Rose *et al*., 1990). White colonies were streaked out on similar medium with the additional omission of histidine to eliminate frameshift, nonsense and destabilizing mutations (Liu *et al*., 1993). Colonies were tested again for lacZ expression to confirm the phenotype, and then a small amount of a single colony was introduced into a PCR reaction using the reverse Gal4 primer listed above and a forward LexA primer (GAAGGGTTGCCGCTGGTAGGTCG). The PCR reaction was purified using Geneclean and sequenced on an Applied Biosystems model 373 automated DNA sequencer.

Yeast two-hybrid analysis

A rat brain two-hybrid library (Clontech) was transformed into yeast strain Y190 (Harper *et al*., 1993) bearing a fusion of the Gal4 DBD with residues 7–133 of mouse NAB1 NCD1. Clones coding for interacting proteins were isolated as described (Russo *et al*., 1995). To test for interaction of mutants of NAB2 NCD1 with Egr1, the pRS425LN2G plasmid containing the indicated mutations was transformed into yeast strain Y190 bearing a fusion of the Gal4 DBD with the R1 domain of Egr1 (Russo *et al*., 1995). To test for NAB multimerization, the same mutants in pRS425LN2G were transformed into Y190 bearing a fusion of the Gal4 DBD with residues 7–133 of mouse NAB1 NCD1 (Svaren *et al*., 1996). Output of the yeast two-hybrid assay was determined by measuring β-galactosidase activity (Rose *et al*., 1990) produced by the *GAL1*-lacZ reporter gene in Y190.

Transfections

African green monkey CV-1 cells and JEG-3 cells were cultured as described previously (Paulsen *et al*., 1992). Transfections were performed in 6-well plates (Corning) using 7×10^4 cells per well. Luciferase reporter plasmids include five Gal4 binding sites upstream of a minimal E1B promoter (Figures 3 and 7), five Gal4 binding sites upstream of the Adenovirus major late promoter (Figure 6), two Egr1 binding sites upstream of the prolactin minimal promoter (Figure 8), and –500 to +160 of the bFGF promoter (Figure 9) (Russo *et al*., 1993; Wang *et al*., 1997; Swirnoff *et al*., 1998). All transfections were performed essentially as described (Russo *et al*., 1993), using the indicated expression plasmids, 500 ng of the luciferase reporter, 100 ng of a CMV-driven lacZ reporter, and the indicated amounts of the expression plasmids. Bluescript plasmid (Stratagene) was added as required to make a total of 2 μg DNA per

transfection. The average luciferase activity of duplicate samples was normalized to the β-galactosidase activity from the transfected lacZ reporter. Means and standard deviations of two separate transfections are shown. Transfections to examine the protein levels of mutant NAB proteins were performed as above using 2 μg of the CMV expression construct per well.

Coimmunoprecipitations and Western blot analysis

Coimmunoprecipitations were performed using quail fibroblast (QT6) cells (2×10^5 cells per 3.5 cm dish) transfected with 5 μg each of expression constructs for both NAB1 and various NAB2 derivatives as described in Figure 5. After 48 h, cells were washed twice with phosphate-buffered saline and then lysed for 10 min in 250 μl buffer A (20 mM Tris pH 7.6, 5 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol (DTT), 0.1% NP-40, 10 mM NaF, 140 mM NaCl) containing 0.5% aprotinin and 0.2 mM phenylmethysulfonyl fluoride (PMSF). For each sample in Figure 5A, 3 μg of NAB1 polyclonal antiserum (Swirnoff *et al*., 1998) was incubated with 50 μl protein A–Sepharose beads (Gibco-BRL) for 45 min at 25°C. Fifty microliters of each lysate was diluted into 500 μl buffer A containing 0.1% bovine serum albumin (BSA) and combined with the protein A–Sepharose beads prebound with the NAB1 antibody, then incubated with agitation for 60 min at 25°C. Immunoprecipitates were washed twice with buffer A supplemented with BSA, then once with buffer A alone. Pellets were boiled in Laemmli buffer, fractionated on 10% SDS–PAGE, and transferred to nitrocellulose (Midwest Scientific). After overnight blocking with Tris-buffered saline containing 5% milk, blots were incubated first with a 1:50 dilution of the 1C4 NAB2 monoclonal antiserum (Kirsch *et al*., 1996), and then with horseradish peroxidase-conjugated anti-mouse secondary antibodies (Jackson Laboratories) at a dilution of 1:10 000, and subjected to autoradiography following incubation with ECL reagents (Amersham). For Figure 5B, QT-6 cells were transfected with NAB2-Flag and NAB1 constructs. Lysates were incubated with recombinant Egr1 for 2 h prior to the addition of 25 μl of agarose-coupled M2 anti-Flag antibody (Sigma) for another hour. The beads were washed 5 times with 500 μl buffer A as described above, and protein complexes were eluted in Laemmli buffer. The presence of NAB1 in the samples was detected by Western blot analysis using an anti-NAB1 polyclonal antibody diluted 1:125 (Swirnoff *et al*., 1998). The same blot was then reprobed with an anti-Egr1 monoclonal antibody 6H10 (Day *et al*., 1990) diluted 1:50. Recombinant Egr1 was made by fusing the rat Egr1 sequence with the 6× His tag in pET30a (Novagen), and purifying the protein from bacteria using Ni-NTA agarose (Qiagen) according to the manufacturer's protocol.

Acknowledgements

We would like to thank Judith Johnson for providing the NAB2/Mader monoclonal antibody, Mark Johnston for providing yeast strains and plasmids, and Elizabeth Apel for providing a rat brain two-hybrid library. We also thank Wolfram Hörz for helpful comments on the manuscript. J.S. was supported by National Institutes of Health National Research Award 1F32GM18058-01 from the National Institute of General Medical Sciences. T.G. was supported by a grant from the Deutsche Akademische Austausch Dienst (DAAD). This work was supported by NIH grant 5 P01 CA49712-08 and a grant from The Association for the Cure of Cancer of the Prostate (CaP CURE).

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Received March 26, 1998; revised July 29, 1998;
accepted August 27, 1998