A Novel Activation Function for NAB Proteins in EGR-dependent Transcription of the Luteinizing Hormone β Gene*

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The EGR1/NGFI-A transcription factor directly activates the luteinizing hormone β (LHβ) subunit promoter, and female mice lacking EGR1 are infertile due to LHβ deficiency. The NGFI-A-binding proteins NAB1 and NAB2 are corepressors of EGR1/NGFI-A and of the related proteins EGR2/Krox20 and EGR3. Here we report that at certain promoters, including LHβ, NAB proteins display a novel ability to stimulate EGR-directed transcription. NAB coactivation requires the conserved NCD2 protein domain, previously implicated in NAB corepression, is strictly dependent upon EGR binding to the LHβ proximal promoter and is independent of EGR activation domains. Furthermore, we report that NAB-activated promoters such as LHβ contain EGR consensus sites that are fewer in number and lower in binding affinity than those found at NAB-repressed promoters such as basic fibroblast growth factor. Analysis of mutant and synthetic promoters confirms that both the strength and multiplicity of EGR-binding sites influence the transcriptional outcome of NAB recruitment. These results suggest a novel means by which EGR target genes could be differentially regulated in cells where EGR and NAB proteins are coexpressed.

Transcriptional control plays a key role in fundamental cellular processes throughout the life of an organism and is regulated by the interactions of DNA-bound transcription factors with other nuclear proteins. Although direct contacts between transactivators and components of the core RNA polymerase II complex provide one method of controlling transcriptional activation, interactions between DNA-bound factors and coregulatory proteins also regulate gene transcription. One example of a corepressor is the retinoblastoma protein, which, by interacting with the E2F transcription factor, converts E2F into a transactivator or with N-CoR-Rpd3 corepressor complexes to achieve both positive and negative effects on transcription.

These complexes may influence transcription through effects on chromatin structure and/or through interactions with the transcriptional initiation machinery. EGR proteins are zinc finger transcription factors that have been implicated in the control of cell growth, differentiation, and apoptosis in the nervous system, the immune system, and elsewhere (4–6). As immediate-early genes that are rapidly synthesized following a wide range of extracellular stimuli, EGR proteins transduce extracellular signals into a rapid transcriptional response. Although members of the EGR family are frequently coexpressed and may be functionally redundant, targeted gene disruption of EGR family members has revealed specific roles for individual EGR proteins. Female mice lacking EGR1 display infertility due to reduced transcription of LHβ (7, 8), whereas mice without EGR3 fail to develop muscle spindles (9). Loss of EGR4 results in male infertility due to increased germ cell apoptosis and defective spermiogenesis (10). EGR2 knockout mice exhibit defects in hindbrain patterning, peripheral nerve myelination, and bone formation (11–14).

Transcriptional activation by EGR family members is modulated by interactions with the NAB family of corepressors. These proteins represent a broadly conserved family with homologues in mammals, Caenorhabditis elegans, and Drosophila melanogaster.1 The mammalian NAB1 and NAB2 proteins possess a conserved N-terminal domain necessary for the interaction with EGR proteins and for NAB self-association (NAB Conserved Domain 1, NCD1) and a conserved C-terminal domain implicated in transcriptional corepression (NCD2). NAB proteins down-regulate the activity of EGR1, EGR2, and EGR3, which contain a conserved domain (R1) that lies N-terminal of the DNA-binding zinc fingers, but do not interact with EGR4, which lacks this domain (15). NAB repression normally requires the recruitment of NAB-EGR complexes to promoters via EGR-binding sites, but direct tethering of NAB proteins by fusion to the Gal4 DNA-binding domain can repress various Gal4-responsive promoters in the absence of EGR proteins (16). NAB proteins could potentially coregulate a wide variety of EGR target genes, including bFGF2 (17, 18), TGF-β1 (19, 20), tissue factor (21), several Hox genes (22–24), platelet-derived growth factor (A and B chains) (25, 26), Fas ligand (27, 28), and LHβ (7, 8). In PC12 cells, NAB2 overexpression blocked NGF-dependent differentiation and prevented NGF induction of TGF-β1, MMP-3, and p21WAF1 (29). Interestingly, a recessive mutation in the NAB-binding domain of EGR2 has recently been linked to human myelopathy, as have dominant mutations in the EGR2 DNA-binding domain (30).

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1 M. Clements and J. Milbrandt, unpublished data.

2 The abbreviations used are: bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; LHβ, luteinizing hormone β; CMV, cytomegalovirus; HA, hemagglutinin; kb, kilobase pair; PCR, polymerase chain reaction; NGF, nerve growth factor; GnRH, gonadotropin releasing hormone.
NAB1 is widely expressed in the adult mouse, whereas NAB2 mRNA expression is highest in brain and thymus (31). NAB2 is induced as a delayed-early gene by several stimuli that also up-regulate EGR expression, such as serum stimulation of fibroblasts or NGF treatment of PC12 cells. NAB1 induction has also been observed in several systems, including glucocorticoid treatment of a leiomyosarcoma cell line (32).

The infertility phenotype of female EGR1 knockout mice has demonstrated the physiological relevance of EGR1 transactivation at the LHβ promoter. The GnRH-responsive element of the LHβ promoter encompasses two broadly conserved EGR1-binding sites as well as two binding sites for steroidogenic factor-1 (SF-1), which synergizes with EGR1 to dramatically up-regulate LHβ transcription (33, 34). LHβ is up-regulated in putative gonadotropes following stimulation by the hypothalamic peptide GnRH, and several studies have reported up-regulation of EGR1 in gonadotrope cell lines following GnRH administration (34, 35). In addition, the homeobox transcription factor PTX1 has recently been reported to enhance LHβ transcription through synergistic interactions with SF-1 and EGR1 (36).

In this study we report a novel ability of NAB proteins to enhance EGR-mediated activation of the LHβ gene. NAB activation was found to require the protein domain (NCD2) previously implicated in NAB repression and was not dependent upon the presence of EGR activation domains or of SF-1-binding sites at the LHβ promoter. Additionally, analysis using chimeric, synthetic, and mutant promoters demonstrated that both the number and relative affinity of EGR-binding sites combine to determine the effect of NAB proteins on transcription.

MATERIALS AND METHODS

Plasmid and DNA Manipulation—All protein-coding sequences were placed under the control of the cytomegalovirus (CMV) immediate-early promoter in the pCB6 mammalian expression vector (37). Expression constructs for wild type and mutant EGR and NAB proteins have been previously described, as have the construction of R1-ZnF, R1(mut)-ZnF, and HA-EGR2 (16, 38, 39). N-terminal HA-tagged versions of EGR1, EGR2, and EGR3 were generated using forward PCR primers bearing the Kozak consensus sequence (40) and HA epitope used in constructing and HA-EGR2 (GAATTTCACTGCTGTTTCGCTAGCTGGCAGTCTACGAGTTTCC) fused to the 5′ end of each open reading frame. The proximal LHβ promoter construct with a native or mutated downstream EGR site has been described previously (6), as has the luciferase gene. NAB2 is induced as a delayed-early gene by several stimuli that also up-regulate EGR expression, such as serum stimulation of fibroblasts or NGF treatment of PC12 cells. Lysates were boiled for 10 min, electrophoresed on a sodium dodecyl sulfate-10% polyacrylamide gel, and transferred to a nitrocellulose membrane (Midwest Scientific). Membranes were blocked in Tris-buffered saline/Triton (TBST: 25 mM Tris-HCl, pH 7.4, 145 mM NaCl, 5 mM KCl, 1% Triton X-100) containing 5% milk prior to incubation with TBST containing 3% milk and a 1:2000 dilution of the 12CA5 anti-μA monoclonal antibody. Protein blots were washed five times in TBST, incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), washed five times in TBST, and visualized by chemiluminescence detection (Amersham Pharmacia Biotech). To confirm the migration size of the relatively faint HA-EGR1 band, an additional lane was loaded with an HA-EGR1 lysate and probed with the anti-EGR1 antibody A311 (data not shown).

RESULTS

NAB2 Enhances EGR Activation of the LHβ Promoter—To determine the effect of NAB2 expression on the proximal rat LHβ promoter (−156 to +6), NAB2 was cotransfected into CV-1 cells along with the four EGR proteins (EGR1–4) and an LHβ promoter-luciferase reporter employed in our previous studies. Surprisingly, NAB2 did not repress but instead potentiated LHβ transactivation by all EGR family members except EGR4/NFPM-I, which lacks the NAB-binding R1 domain (Fig. 1A). NAB2 coactivation of EGR3 was particularly potent. In contrast, EGR-dependent activation of a synthetic promoter-luciferase reporter containing four canonical EGR-binding sites (GGCGGGGCG) was repressed by NAB2 except in the case of EGR4 (Fig. 1B), as previously observed (15).

To determine whether NAB1 could also enhance EGR-dependent transcription of the LHβ promoter, increasing amounts of NAB1 were cotransfected with EGR3. As shown in Fig. 1C, the maximum amount of cotransfected NAB1 resulted in approximately 10-fold stimulation of EGR3-mediated transcription. To test whether NAB coactivation could be observed in the context of a more extensive LHβ promoter fragment, we cotransfected NAB2 along with wild type or mutant EGR1 and a reporter construct containing a 1.7-kb fragment (−1700 to +5) of the rat LHβ promoter (Fig. 1D). NAB2 stimulated EGR1 transcription of this larger LHβ promoter by approximately 4-fold.

These experiments demonstrated that both NAB1 and NAB2 possessed an unexpected ability to coactivate EGR-mediated transcription of the LHβ promoter and that this coactivation depended upon interactions with EGR proteins. In the absence of cotransfected EGR1, NAB2 expression had no effect on the LHβ promoter (Fig. 1D). Conversely, EGR1 with a point mu-
tation (I293F) in its NAB-binding domain was not responsive to NAB coactivation. Notably, EGR1(I293F) was no more active than wild type EGR1 in the absence of NAB. This observation contrasts sharply with results previously reported using NAB-repressed promoters, where EGR1(I293F) is typically 10–15-fold more active than wild type EGR1 (38), presumably due to its inability to interact with endogenous NAB proteins, and provides further evidence that the LHβ promoter is not subject to NAB repression. Additionally, a deletion of the EGR-binding NCD1 domain of NAB1 was found to abrogate coactivation of EGR-dependent LHβ transcription (see Fig. 4, mutant Δ2–210), further confirming that a specific NAB-EGR interaction is required for NAB activation.

Differential Responses of EGR Proteins to NAB2 Coactivation—In performing the experiments depicted in Fig. 1A, we noticed differences in the ability of NAB2 to coactivate EGR family members. For instance, NAB2 coactivation was observed in transfections employing 10 ng of an EGR3 expression vector, but greater than 100 ng of an EGR1 expression vector was required to see a similar effect (data not shown). To test whether protein expression levels influence these differences in NAB responsiveness, the hemagglutinin (HA) epitope was fused to the N terminus of all four EGR proteins. In transfection experiments using the promoters employed in Fig. 1A and B, HA-tagged EGR proteins behaved indistinguishably from the untagged versions (data not shown). Equal amounts of HA-EGR proteins were transfected into CV-1 cells, and 3-fold dilutions of cell lysates were probed on a Western blot using an anti-HA monoclonal antibody. This experiment revealed striking differences in expression levels among HA-tagged EGR proteins (Fig. 2). HA-EGR1 was expressed roughly 10-fold less robustly than EGR2 or EGR3, which were detected at essentially equal levels. EGR4 appeared at least 10-fold more highly expressed than EGR2.

This result provided a possible explanation for the relative difficulty of observing NAB2 coactivation of EGR1 compared with EGR2 or EGR3 at comparable levels of expression. The apparent instability of EGR1 may reflect its short half-life within the cell, consistent with the observation that many EGR1 inductions involve a massive burst followed by a rapid (2–4 h) disappearance of the EGR1 protein (31), whereas EGR3 is reported to be significantly more stable (43). Due to the relatively poor expression level of transfected EGR1, we included EGR2 and EGR3 throughout our analysis, as these more highly expressed proteins facilitated the evaluation of NAB-EGR interactions at various mutant promoters.

Similarities between NAB1 Corepression and Coactivation—NAB proteins are active transcriptional repressors that function as part of a DNA-bound EGR-NAB complex (Ref. 16 and data not shown). NAB1 represses activation domains other than those of EGR1, and tethered NAB proteins can repress
several types of constitutively active promoters. To determine whether NAB2 coactivation of the LHβ promoter could occur independently of EGR transcriptional activation domains, NAB2 was recruited to the LHβ promoter using an EGR1 expression construct consisting solely of the NAB-binding R1 domain and DNA-binding domain of EGR1 (R1-ZnF). R1-ZnF does not activate transcription by itself, but cotransfection of wild type NAB2 with R1-ZnF resulted in a 4-fold activation of the LHβ promoter (Fig. 3). Cotransfection of R1-ZnF with a non-EGR-binding NAB2 mutant, or of a mutant R1-ZnF with NAB2, failed to enhance transcription above background levels. Therefore, NAB2 appears to contain an independent coactivation domain that can be specifically recruited to the LHβ promoter independently of EGR transcriptional activation domains.

Previous work in our laboratory (16) mapped the NAB1 corepression activity to the C-terminal NCD2 domain using deletion, insertion, and replacement mutations in the NAB1 protein. To learn whether NAB1 coactivation might involve a region of the protein distinct from that required for NAB1 corepression, we assayed the effect of various NAB1 mutants on EGR3-mediated activation of either or both SF-1-binding sites had no effect upon LHβ transcription in CV-1 cells (7), and this synergistic effect upon LHβ transcription in CV-1 cells (7), and this synergy has been attributed to protein-protein interactions between EGR1 and SF-1 (36). The rat LHβ promoter contains SF-1-binding sites at −127 and −59 (33). To learn whether these SF-1-binding sites might influence NAB2 coactivation, mutant proximal LHβ promoters were tested in which one or both SF-1-binding sites were deleted. As shown in Fig. 5, mutation of either or both SF-1-binding sites had no effect upon NAB2 coactivation of EGR-mediated transcription, suggesting that SF-1-binding sites are not required for NAB activity.

To determine whether the two EGR-binding sites in the LHβ promoter are both required for NAB activation, we tested an LHβ promoter-reporter with a mutated downstream EGR1-binding site (TTGGGGGTG to CTAAGAATA). This mutant promoter still demonstrated NAB2 coactivation, although the overall level of transcription was attenuated compared with that seen with wild type LHβ (Fig. 5). When the upstream EGR-binding site (TTGAGGCG) was mutated to TTGGAAAGCG in the context of the native downstream EGR site, the resulting promoter was still NAB-activated but showed lower activity (Fig. 5). If both potential EGR-binding sites were mutated, the promoter lost all EGR and NAB responsiveness. Therefore, NAB2 coactivation is absolutely dependent on the presence of a functional EGR-binding site in the LHβ promoter but does not require that both sites be intact.

**Comparison of NAB Function at Potential EGR Target Genes**—To determine the effect of NAB2 on several potential Egr target genes, we cotransfected the NAB2 and EGR3 expression plasmids with reporter constructs for the bFGF/FGF-2, tissue factor, TGF-β1, and Fas ligand promoters (Fig. 6) in CV-1 cells. NAB2 coexpression repressed transcription of the bFGF, tissue factor, and TGF-β1 promoters. However, the promoter for Fas ligand, which has been implicated as a target gene of Egr2 and Egr3 (27, 28), demonstrated robust NAB coactivation, as did LHβ. Therefore, NAB2 coactivation is not
FIG. 5. NAB2 coactivation of the LHβ promoter requires EGR1-binding sites. CV-1 cells were transfected with the indicated promoter-luciferase constructs and expression constructs for NAB2 (20 ng), and EGR1 (60 ng), EGR2 (20 ng), or EGR3 (10 ng). An X denotes a missense mutation in the designated consensus site (see “Materials and Methods”). Normalized luciferase activity was divided by basal promoter activity to determine fold activation.

FIG. 6. NAB-repressed EGR target promoters possess more GC-rich domains than NAB-activated promoters. Black rectangles depict stretches of at least six nucleotides consisting exclusively of guanosine or cytosine and are drawn to scale for the −100 to +100 region of each promoter. CV-1 cells were transfected with promoter-luciferase constructs (250 ng) for bFGF (−500 to +160), tissue factor (−264 to +15), TGF-β1 (−190 to +20), LHβ (−156 to +6), and FasL (−511 to −2) along with expression constructs for EGR3 (10 ng) and NAB2 (20 ng). Fold activation was calculated by dividing normalized luciferase activity in the presence of NAB2 by normalized luciferase activity in the absence of NAB2 for NAB-activated promoters; the calculation was performed in the opposite manner for NAB-repressed promoters. A negative value indicates NAB2 repression.

Comparison of these EGR target promoters revealed striking differences in the abundance of sequences rich in the nucleotides guanine (G) and cytosine (C) according to whether the genes were repressed or activated by NAB coexpression (Fig. 6; Table I).

Table I: EGR consensus sites in target gene promoters

| Gene | Speciesa | Site | Sequenceb |
|------|----------|------|-----------|
| FGF-2/bFGF | H | −260 | GCGGGGGGAGG |
| | | −165 | GCGGGGGGTTG |
| | | −63 | ATGGGGGGAG |
| Tissue factor | M | −74 | GCGGAGGCG |
| TGF-β1 | H | −165 | GAGTTGGGG |
| | | −178 | GCGGGGGC |
| LHβ | R | −50 | GTCGGGGGTTG |
| Fas ligand | H | −214 | GAGTTGGGTG |

a H, human; M, mouse; R, rat. b Promoters scanned using EGR consensus site GHGKRGGHG.

Filled boxes depict GC stretches of 6 nucleotides or more. NAB-repressed genes displayed abundant GC-rich sequence from −100 to +100. Conversely, no significant GC-rich domains were observed in NAB-activated promoters. To assess the number of consensus EGR-binding sites located within the promoter-reporter constructs tested in Fig. 6, we analyzed these proximal promoter sequences using an algorithm derived from previous studies defining the high affinity EGR consensus-binding site as TGCCG(T/g)/(A/G)(C/a/t)G/(G/T) (44). As expected, the GC-rich EGR consensus sequence was found more frequently in the GC-rich NAB-repressed promoters than in the GC-poor NAB-activated promoters (Table I). Three potential EGR-binding sites were found in the bFGF promoter, for example, compared with one potential site (GAGTGTTG) in the FasL promoter. It should be noted, however, that this algorithm will not identify EGR-binding sites that deviate from the consensus, such as the upstream LHβ site (TTGGGGGCG) described earlier. Although computer-based search methods do not provide a completely reliable means of identifying EGR-binding sites, it nonetheless appears that GC-rich, NAB-repressed promoters generally possess more consensus EGR-binding sites than do GC-poor, NAB-activated promoters.

NAB Repression at bFGF/LHβ Promoter Chimeras—To gain insight into the determinant(s) of NAB function at different promoters, we constructed reciprocal chimeras of the NAB-repressed bFGF proximal promoter and the NAB-activated LHβ proximal promoter, and we tested their function in CV-1 cells. The GC-rich bFGF promoter (−500 to +160) contains three EGR-binding sites as defined by the algorithm described above, and at least two of these sites are involved in promoter activation (18). In addition, the promoter contains five Sp1 consensus sites, and Sp1 binding at some of these sites has been observed in gel mobility shift assays (18). Whereas the LHβ promoter contains a functional TATA element near the transcriptional start site when tested in CV-1 cells, both the LHβ/bFGF and the bFGF/LHβ chimeric promoters were repressed by NAB2 (Fig. 7A). The bFGF/LHβ chimera, which features multiple EGR and Sp1 consensus sites derived from the bFGF promoter, was strongly activated by EGR proteins and strongly repressed by NAB2. This result indicated that NAB repression of bFGF could be observed in a promoter with heterologous elements controlling transcriptional initiation and that sequences surrounding the TATA element of the LHβ promoter do not dictate NAB function.

More surprisingly, fusion of the upstream LHβ promoter with the downstream bFGF sequences (LHβ/bFGF) resulted in
FIG. 7. NAB proteins repress EGR-dependent transcription in chimeric reporters containing bFGF and LHβ promoter sequence. A, EGR-binding sites, Sp1-binding sites, and GC-rich stretches in the LHβ and bFGF promoters are depicted by the indicated symbols. Only consensus EGR-binding sites identified by the algorithm used in Table I are shown; a nonconsensus site has been identified in the LHβ promoter (Fig. 4), however, and additional EGR-binding sites may be present in the bFGF promoter (18). The −35 position in each promoter was used as a crossover point in constructing the chimeras and is indicated by a *vertical line*. Wild type or chimeric promoters fused to luciferase were transfected along with expression constructs for EGR1 (60 ng), EGR2 (20 ng), EGR3 (10 ng), and NAB2 as indicated. Normalized luciferase activity was divided by basal promoter activity to determine fold activation. NAB2 coactivation of EGR1 at the wild type LHβ promoter is diminished relative to that shown in Fig. 1A due to the use of nearly 10-fold less expression construct. Solid bar, 0 ng of NAB2; shaded bar, 20 ng of NAB2; solid triangle, EGR consensus site; solid oval, Sp1 consensus site; solid square, GC stretch ≥6 nucleotides. B, replacement of 18 base pairs of the native LHβ promoter (~24 to −6) with GC-rich sequence has no effect upon NAB2 coactivation of EGR-dependent LHβ transcription. CV-1 cells were transfected with the a promoter that no longer supported NAB coactivation and showed approximately 2-fold NAB corepression. This chimeric promoter included both LHβ EGR-binding sites in addition to downstream bFGF sequence (~35 to +160); this GC-rich bFGF sequence includes a consensus Sp1 site and might contain noncanonical EGR-binding sites as well. Taken together, these data indicate that both the LHβ/bFGF and the bFGF/LHβ chimeric promoters resembled the intact bFGF promoter in their response to NAB proteins and suggest a dominant influence of GC-rich bFGF sequence on NAB function.

To determine whether the dominant influence of bFGF promoter sequence in the chimeric promoters could be due solely to its high GC content, which might influence promoter melting during transcriptional initiation (46), a mutant LHβ promoter was constructed in which sequence near the transcriptional initiation site (~24 to −6) was changed to GGGCCCGGGCCGGCCC. This GC-rich sequence, which does not recruit any known transcription factor (47), permitted strong NAB coactivation (Fig. 7B), suggesting that GC content per se is not a determinant of NAB function.

Alternatively, GC-rich bFGF promoter sequence might suppress NAB coactivation by recruiting Sp1 to the bFGF/LHβ promoter. Sp1 is a widely expressed transcriptional activator that binds to a GC-rich consensus site (48). To test the possible role of Sp1 without interference from endogenous Sp1 protein or complications from cryptic EGR-binding sites, we recruited a Gal4-Sp1 fusion protein to an LHβ promoter bearing a Gal4-binding site at its 5′ end (5′Gal4-LHβ, Fig. 7C). Coexpression of EGR3 and NAB2 resulted in strong NAB2 coactivation of this promoter, whereas expression of Gal4-Sp1 by itself resulted in dose-dependent activation. Coexpression of Gal4-Sp1 with EGR3 and NAB2 did not diminish NAB coactivation and in fact resulted in increased transcriptional activity. Therefore, recruitment of Sp1 to a NAB-activated promoter does not alter NAB function.

Effects of Increased Binding Affinity and Number of EGR-binding sites on NAB Function—The ability of bFGF promoter sequences to prevent NAB2 coactivation in the LHβ/bFGF promoter chimera could involve recruitment of additional EGR molecules to the promoter. To explore the influence of additional EGR-binding sites upon NAB2 function, EGR-binding sites of varying number and binding strength were fused to the 5′ end of the LHβ proximal promoter fragment (Fig. 8A). These additional sites included one or two copies of the downstream LHβ EGR site (GTGGGGGTG), which is a relatively weak EGR-binding site (35), or one or two copies of the optimal EGR consensus site (CCGGGCC) determined by *in vitro* studies. In all four of these promoters, addition of upstream EGR-binding sites attenuated NAB2 coactivation. This effect was particularly striking when two copies of the optimal consensus sequence were employed (5′2×CCGG) and less dramatic when one optimal consensus site was included (5′1×CCGG) or when one or two LHβ downstream sites were included (5′1×GTG and 5′2×GTG). Therefore, both the number and relative strength of EGR-binding sites appear to influence NAB function.

To extend this observation, we altered the LHβ promoter’s native downstream EGR-binding site (GTGGGGGTG). When this element was changed to a double downstream EGR site (2×GTG), NAB2 coactivation was weakened but not abolished.

**NAB Proteins Coactivate LHβ Transcription**

- NAB2 coactivation with bFGF/LHβ promoter included both LHβ EGR-binding sites in addition to downstream bFGF sequence (~35 to +160); this GC-rich bFGF sequence includes a consensus Sp1 site and might contain noncanonical EGR-binding sites as well. Taken together, these data indicate that both the LHβ/bFGF and the bFGF/LHβ chimeric promoters resembled the intact bFGF promoter in their response to NAB proteins and suggest a dominant influence of GC-rich bFGF sequence on NAB function.

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**DISCUSSION**

The initial characterization of NAB proteins demonstrated that they repress transactivation by EGR1 of a synthetic promoter bearing four canonical EGR-binding sites. As widely expressed corepressors of EGR-dependent transcription that can be induced by many of the stimuli that up-regulate EGR genes, NAB proteins were predicted to negatively regulate Egr target genes. Here we have reported that NAB proteins can also function as potent coactivators of certain EGR target promoters, including LHβ, perhaps the best established physiological target of EGR1. NAB coactivation was also observed using both various synthetic minimal promoters and the promoter of the Fas ligand gene, a putative Egr2/Egr3 target gene (27, 28).

Comparison of NAB-activated and NAB-repressed EGR-responsive promoters suggested a positive correlation between the observation of NAB repression and the presence of GC-rich sequences in a given promoter. In principle, GC-rich sequences might influence NAB function through an effect on promoter melting requirements for transcriptional initiation or by recruiting the ubiquitously expressed Sp1 transactivator, which binds a GC-rich consensus site (GCGGGG). However, analysis of chimeric, mutant, and synthetic promoters demonstrated that both the nature and the number of a promoter’s EGR-binding sites ultimately determine NAB function.

An ability to both activate and repress transcriptional activity has previously been reported for a number of proteins, including WT1, YY1, p53, retinoblastoma protein, Dorsal,
RORα, various prokaryotic transactivators (49–51), and others. WT1, the Wilms tumor suppressor protein, is related to EGR proteins in its DNA-binding domain and binds a similar consensus site (52). Its differential effects on transcription have been mapped to independent activation and repression domains that may interact with various cellular proteins (53). In several other cases of activator/repressor proteins, such as RORα (54) and the HIV Tat protein (55), separate activation and repression domains have been identified. Concentration-dependent activity switches have been reported for other repressor/activators such as p53, which can activate at low levels and repress at higher levels (56), and BSAP, which at low levels reportedly activates transcription from high affinity binding sites but at higher concentrations represses transcription from low affinity sites (57). The activity of Dorsal depends on binding of factors to adjacent DNA sites (58), whereas the corepressor Rb can activate transcription through interactions with factors such as MyoD (59). YY1 activates or represses in a complex pattern that may depend both on its intrinsic DNA-bending ability and on interactions with promoter-bound factors (60, 61).

A bacteriophage activator/repressor protein, p4 from phage 429, is reminiscent of NAB proteins in the use of a single protein domain to carry out both repression and activation. The interaction between p4 and bacterial RNA polymerase (RNAP) leads to activation or repression depending on the strength of the sigma A-binding site (62). At a weak binding site, p4-directed transcription is activated through recruitment and stabilization of RNAP; at a strong binding site, bound RNAP is overstabilized and trapped at the promoter, leading to transcriptional repression. Fig. 10 depicts a similar model as applied to NAB activity. According to this hypothetical scheme, the NAB NCD2 domain might interact with the mammalian RNA polymerase complex either directly or through an unidentified bridging factor. At promoters with few or low affinity EGR-binding sites, such an interaction might result in productive recruitment of the transcriptional machinery and NAB coactivation. At promoters with higher affinity and/or greater numbers of EGR-binding sites, cooperative interactions between bound NAB-EGR complexes and the NCD2-interacting factor could result in trapping of the overstabilized polymerase complex, reduced promoter clearance, and NAB repression. Testing of this model would require the identification of NCD2 target(s) and the development of promoter clearance assays in NAB-regulated transcriptional systems.

Initial analysis of NAB proteins suggested that their interaction with EGR proteins would transform an EGR transactivator into an EGR-NAB repressor complex. Results described in this study suggest that, at certain promoters such as LHβ, NAB proteins can amplify an EGR-directed transcriptional response. This novel activation function of NAB proteins may help to explain the association of a mutation in the NAB-binding domain of EGR2 (I268N) with a recessive case of human myelinopathy (30). Myelinopathies result from Schwann cell defects leading to abnormal myelination, and these particular cases are presumably caused by misregulation of one or more EGR2 target genes. If NAB proteins function as repres-
sors of a critical target gene, one might expect a single, derepressed allele of EGR2(1268N) to up-regulate markedly target gene transcription, leading to a dominant inheritance pattern. But if NAB proteins function instead as coactivators of a critical EGR2 target gene, mutation of both EGR2 alleles might be required to decrease significantly EGR-directed transcription, and recessive disease transmission would be predicted. Further elucidation of the role of NAB proteins in human myelopathies awaits the identification of critical EGR2 target genes within Schwann cells, but the association of a mutation in the NAB-domain of EGR2 with human disease highlights the importance of coregulatory proteins such as NAB1 and NAB2 in transcriptional control.

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