The zinc finger transcription factor early growth response (Egr)-1 regulates the expression of numerous genes involved in differentiation, growth, and in response to environmental signals. Egr-1 activity is modulated in part through the binding of corepressors Nab1 and Nab2. Nab2 appears crucial for controlling Egr-1-mediated transactivation because it is a delayed early response gene, induced by the same stimuli that induce the immediate early gene Egr-1. To identify important elements regulating Nab2 expression, we cloned the human Nab2 gene and investigated the 5’-region. The TATA- and initiator-less Nab2 promoter, located from −679 to −74 bp, contains a total of 11 Egr binding sites, including a cluster of multiple overlapping Egr/Sp1 sites between −329 and −260 bp. This region is critical for basal promoter activity as well as for maximum induction by phorbol esters. Electromobility shifts show that Sp1 binds to this region in normal and stimulated cells, whereas stimulation induces binding of Egr-1. In addition Egr-1 activates the Nab2 promoter in a pattern similar to phorbol esters, suggesting that Egr-1 is a major inducer of protein kinase C-mediated Nab2 induction. Depletion of Egr-1 by each of two distinct Egr-1 short-interfering RNAs reduces Nab2 expression and inducibility, confirming that Egr-1 is an important regulator of Nab2 expression. Transfection experiments show that Egr-1-induced Nab2 promoter activity is itself repressed by Nab2. These results indicate that Egr-1 mediates the induction of its own repressor, thereby preventing a permanent transactivation of Egr-1 target genes and a damaging overreaction in response to environmental signals.

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The zinc finger transcription factor early growth response (Egr)-1 is an immediate early response gene that couples extracellular signals to the induction of cellular programs of differentiation, growth, and cell death through changes in the expression of Egr-1 target genes. Although Egr-1 expression is low or undetectable in resting cells, it is rapidly and transiently induced by a wide variety of environmental signals including growth factors, cytokines, and toxic substances (1). Egr-1 in turn activates the transcription of numerous target genes including growth factors that themselves induce Egr-1, as e.g. platelet-derived growth factor (PDGF), thereby establishing a positive autocrine feedback loop. Egr-1 activity is tightly regulated, in part through the function of two proteins, NGFI-A-binding protein (Nab1) and Nab2, which prevents the permanent activation of Egr-1 target genes and signaling pathways such as those for PDGF (2), which could lead to cellular transformation. Nab proteins were first identified as Egr-1-binding proteins in a two-hybrid assay (3, 4). Nab2, which is also known as mader (melanoma-associated delayed early response gene), was independently identified as a nuclear protein overexpressed in human malignant melanoma (5). Binding of Nab1 and Nab2 to Egr-1 through interaction between the NCD1 (Nab conserved domain) and the R1 domain of Egr-1 has been shown to inhibit Egr-1 activity.

Although Nab1 is constitutively expressed in most cells, Nab2 is a delayed early response gene and is induced by the same environmental signals that lead to Egr-1 expression. This suggests that Nab2 is the more important Egr-1 regulator, crucial for controlling Egr-1-mediated activation of transcription. Although this indicates the importance of understanding how the expression of Nab2 is regulated, little is known regarding the transcriptional regulation of the Nab2 gene. In the study presented here we show that Egr-1 has a significant influence on the regulation of the expression of its repressor protein Nab2. Depletion of Egr-1 by transfection of each of two distinct short-interfering (si)RNAs directed against Egr-1 reduced Nab2 expression. In addition the Nab2 promoter was shown to contain multiple putative Egr binding sites and a regulatory region, containing a cluster of multiple overlapping Egr/Sp1 sites, which is required for basal promoter activity and maximum phorbol ester-mediated induction. Although Sp1 was shown to bind to this region both in normal and phorbol ester-stimulated cells, stimulation with phorbol ester induced Egr-1 expression and Egr-1 binding to the Nab2 promoter. In addition Egr-1 activates the Nab2 promoter in a manner similar to that of phorbol ester. The Egr-1-mediated activation of Nab2 transcription was repressed by Nab2 in a dose-dependent manner, supporting the existence of an Egr-1-Nab2 negative feedback mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Materials**—Cell lines were obtained from the ATCC (Manassas, VA), established in our laboratory or obtained through exchange. The human melanoma cell lines Mel 888 and Mel E1, the human colon carcinoma cell lines Colo 320DM and LoVo and the human cervix carcinoma HeLa L cell line were cultured in RPMI 1640 Medium (Biochrom AG, Berlin, Germany) supplemented with 5% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C and 5% CO2. Cells were stimulated by the addition of phorbol myristate acetate (PMA, final concentration 10 ng/ml) obtained from Sigma-Aldrich for indicated times. All cells were routinely tested for mycoplasma contamination.
Egr-1 Activates the Nab2 Promoter

The human Egr-1 cDNA, kindly provided by Erhard Hofer (Department of Vascular Biology and Thrombosis Research, Vienna International Research Cooperation Centre, University of Vienna (6)), was subcloned into HindIII/EcoRI-digested pCDNA3 (Invitrogen). The IMAGE Clone 5721972, containing full-length human Nab1 cDNA, was purchased from MRC geneservice (Cambridge, UK) and was subcloned into EcoRI/NotI-digested pCDNA3. Human Nab2 cDNA, kindly provided by John Saven (Department of Pathobiological Sciences, University of Wisconsin, Madison (3)), was subcloned into HindIII-digested pCDNA3. The expression construct for human Phlda-1 was described previously (7). Inserts of all constructs were validated by sequencing. Jetstar 2.0 plasmid kit (Genomed, Germany) was used for DNA preparation.

Isolation and Characterization of the Nab2 Genomic Sequence—2.5 × 10^5 independent clones of a lambda-Gem-11 human leukocyte genomic library (Promega) were screened with a 463-bp Nab2 cDNA fragment corresponding to base pairs 223–685 of the GenBank genomic library (Promega) were screened with a 463-bp Nab2 cDNA fragment corresponding to base pairs 223–685 of the GenBank sequence NM_005967. The probe was labeled with [α-32P]dATP (Amersham Biosciences) by random priming and hybridization was performed as described (8) using Hybrid N filters (Amersham Pharma- cia Biotech). Purified DNA from two positive clones (m1, m4) was digested with restriction endonucleases and analyzed by Southern blotting for hybridization with the screening probe. Positive fragments were subcloned into pBlueScriptII vectors for further analysis. The Nab2 genomic sequence was deposited with GenBank (AF268380). Identification of the potential promoter region and transcription factor binding sites was conducted using the Genomatix suite of programs (9).

Production of Nab2 5′-Region Deletion Reporter Constructs—All constructs were generated by PCR amplification using Pfu Turbo (Stratagene) with antisense primer Nab2-21 containing HindIII restriction site 5′-GAGAACGTCCTGCCCTTTCTCGGTGCC-3′ plus the following sense primers containing KpnI restriction site: Nab2-872, 5′-TCAGGGCAGACGGGCAGGCAGC-3′ (forward) and 5′-TGCGCAGCTCAGGGGTGGGC-3′ (reverse), human Nab2, 5′-GACCTGCAAGCAGACTC-3′ (forward) and 5′-CCAGGCAGTGTGATAGCTTTC-3′ (reverse) and human GAPDH, 5′-AATTTCCAAGACGGCTCAAG-3′ (forward) and 5′-GCCGTGTTCCACCCCTTT-3′ (reverse). To check for linear amplification each PCR was performed with 1 μl of undiluted cDNA and serial dilutions of 1:10, 1:100, and 1:1000. Linear amplification for each PCR was achieved with 1 μl of 1:10 diluted cDNA. Therefore, each PCR was performed using 1 μl of 1:10 diluted cDNA in a 20-μl PCR with the use of Taq polymerase and buffer Y from Peqlab (Erlangen, Germany). Products were separated by electrophoresis through a 1.2% agarose gel. To control for the integrity and uniformity of the sample preparations, GAPDH mRNA was amplified. Densitometric analysis was performed using SigmaGel software (SPSS science).

Nuclear Extract Preparation—Mel 888 cells were either left unstimulated or stimulated for 3 h with PMA. Nuclear extracts were prepared from 5 × 10^4 cells using the high salt extraction as described (10).

EMSAs—Double-stranded oligonucleotides were end-labeled with [γ-32P]ATP (Hartmann Analytic, Braunschweig, Germany) using T4 polynucleotide kinase (New England Biolabs). Nuclear extracts (12 μg of protein) were incubated for 40 min on ice with 32P-labeled oligonucleotide in a final volume of 15 μl of binding buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 50 mM NaCl, 5% glycerol, and 2 μg of poly(dI-dC). For oligonucleotide competitions and antibody supershift/blocking experiments, the gel shift mixture was preincubated on ice for 30 min with 40 ng of oligonucleotide competitor or 2 μg of antibody. Preincubation with Egr-1 antibody was performed at room temperature. DNA-protein complexes were separated on a 4% nondenaturing polyacrylamide gel containing 0.5X Tris borate EDTA at 125 V for 3 h at room temperature. Gels were dried and exposed to x-ray film with intensifying screens at −80 °C. Antibodies against Egr-1 (sc-110 X) and Sp1 (sc-59 X) as well as oligonucleotides for consensus and mutant DNA-binding domains of Egr-1 (sc-2529/sc-2530) and Sp1 (sc-2502/sc-2503) were purchased from Santa Cruz (Santa Cruz, CA). Rabbit control serum (X903) was obtained from DAKO (Hamburg, Germany).

RESULTS

Isolation of the Nab2 Genomic Sequence—The full-length Nab2 gene was isolated from a human leucocyte genomic library screened with a 463-bp probe from the 5′-region of the cDNA. Two phage clones, m1 and m4, were obtained. A 7.4-kb BamH1 fragment (4B7) from m1 and a 2.1-kb EcoRI fragment (1E10) from m4 were subcloned and sequenced. Clone 4B7 was found to contain the entire Nab2 gene as well as an 1845-bp region 5′ to the first ATG (Fig. 1). As previously reported the
Nab2 gene consists of 7 exons (3). All 6 introns fulfill the GT-AG rule. Comparison of the exon sequence with the sequence of the drop8 variant cDNA (GenBank AJ011081), which was isolated from a human melanoma cell line, showed that this is a splice variant lacking exon 6 (Fig. 1).

Identification of Nab2 Promoter Region—The Nab2 expression is driven by a promoter with high GC content, which contains no typical core promoter elements such as TATA box, initiator sequence, or CAAT box. In silico analysis indicated a CpG island at bp /H11002/876 to /H11002/82, a putative promoter region at bp /H11002/705 to /H11002/82 relative to translation start site and several putative Egr, Sp1, NFκB, and AP2 transcription factor binding sites as well as a cAMP-responsive element (Fig. 2). To identify promoter elements that control Nab2 transcription, the 5'-region of Nab2 was used to drive a promoterless firefly luciferase reporter plasmid. This construct as well as serially 5'-truncated fragments were assessed for activity by transient transfection in Nab2 expressing Colo 320DM and Mel 888 cells (Fig. 2). The activity of the luciferase reporter maluc-679 was comparable with that of maluc-872 and a construct containing the 5'-full-length genomic DNA of Nab2 (data not shown), whereas the activity of maluc-558, lacking two Egr sites, one Sp1, and one AP2 binding site, was reduced by 20–30%. These findings indicate that maluc-679 includes the complete Nab2 promoter. The removal of an additional 90 bp, including one Egr and one Egr/Sp1 binding site, up to nucleotide —468 reduced the activity by another 20%, whereas further deletion up to bp —379 had no influence on the luciferase activity. This indicates that the Egr/Sp1 binding site at bp —422 has no important function in basal expression. Further 5'- truncation of the Nab2 promoter to nucleotide —263 resulted in a loss of luciferase activity of 72% in Mel 888 and 60% in Colo 320DM, compared with maluc-379, suggesting the presence of a major regulatory element in the promoter region between bp —379 to —263. This region contains multiple overlapping Egr/Sp1 sites and one AP2 binding site (see Fig. 6A). The activity of maluc-195, lacking one Egr/Sp1 binding site and a cAMP-responsive element compared with maluc-263, declined by a further 60%. Further truncation of an additional 121 nucleotides, comprising one Egr binding site, up to bp —74 essentially abolished transcriptional activity.

These results indicate that the Nab2 promoter is located from bp —679 to —74 relative to translation start site, corresponding precisely to the putative promoter region predicted by in silico analysis software. This region includes four Egr, three overlapping Egr/Sp1 binding sites, one Sp1, one AP2 site, a cAMP-responsive element, and one cluster of multiple Egr/Sp1 binding sites. Analysis of the deletion constructs indi-
**Egr-1 Activates the Nab2 Promoter**

**FIGURE 3.** Egr-1 induces endogenous Nab2 expression and activates the Nab2 promoter. A, Egr-1 induces endogenous Nab2 expression. Colo 320DM cells were transfected in 6-well plates with 1 μg of human Egr-1 or human Phlda-1 as control. Levels of Egr-1 and Nab2 mRNA were determined by RT-PCR after 48 h as described under “Experimental Procedures.” – DNA, control for PCR. B, Egr-1 activates the Nab2 promoter. Mel 888, Mel Ei, Colo 320DM, and LoVo cells were transfected with 60 ng of maluc-872 and 40 ng of human Egr-1 or Phlda-1 control plasmid DNA. Luciferase activity was determined as described under “Experimental Procedures.” Results are presented as fold activation over luciferase activity of maluc-872 cotransfected with Phlda-1 control plasmid DNA. The results are presented as the mean and S.D. of triplicates from a representative experiment. C, Egr-1 activates the Nab2 promoter in a dose-dependent manner. Mel 888 cells were transfected with 60 ng of maluc-872 and increasing amounts, as indicated, of human Egr-1 or Phlda-1 control DNA. DNA was filled up to 120 ng with empty vector. Luciferase activity was determined as described under “Experimental Procedures.” Average and S.D. of triplicates from a representative experiment is shown. RLU, relative light units/μg of protein.

**FIGURE 4.** Depletion of Egr-1 reduces Nab2 expression. HeLa L cells were transfected with negative control siRNA and two distinct siRNAs directed against Egr-1 (Egr-1-1 and Egr-1-2, as indicated) as described under “Experimental Procedures.” 48 h later the cells were stimulated with PMA, and RNA was isolated after 20 min and 1 h. A, levels of Egr-1, Nab2, and GAPDH mRNA were determined by RT-PCR (as described under “Experimental Procedures”). – DNA, control for PCR. B, densitometric analysis of Egr-1 expression (as described under “Experimental Procedures”). Results are presented as the ratio of Egr-1 to GAPDH. C, densitometric analysis of Nab2 expression. Results are presented as the ratio of Nab2 to GAPDH.

Egr-1 has been shown to induce Nab2 mRNA expression when transfected into murine neuroblastoma cells (11) and was identified as a Egr-1 target gene by Affymetrix microarray (12). As can be seen in Fig. 3A transfection of Egr-1 leads to Nab2 gene expression in human colon carcinoma cells. Colo 320DM cells were transfected with human Egr-1 or control vector and RT-PCR was performed 48 h later. Nab2 mRNA was increased after Egr-1 transfection, but remained unaffected after transfection of the control protein Phlda-1. To determine whether Egr-1 influences the Nab2 promoter activity, human Egr-1 was transfected together with the human Nab2 promoter reporter construct. Sixty ng of the reporter construct maluc-872, which contains the complete Nab2 promoter, were transfected into Colo 320DM, LoVo, Mel 888, and Mel Ei cells together with 40 ng of Egr-1 or Phlda-1 as control. In all cell lines, Egr-1 increased Nab2 promoter activity compared with Phlda-1 (Fig. 3B). Induction was higher in melanoma cell lines (4–5-fold) than in colon carcinoma cell lines (2–3-fold). Additional experiments indicated that activation of the Nab2 promoter by Egr-1 is dose-dependent, reaching a plateau at 20 ng of Egr-1 (Fig. 3C). Transfection of Phlda-1 did not influence the Nab2 promoter activity. These results indicate that Egr-1 induces the expression of its own corepressor by stimulating Nab2 promoter activity.

**Depletion of Egr-1 Reduces the Inducibility of Nab2 in Response to Phorbol Ester**—To examine the significance of Egr-1 in the regulation of Nab2 expression two distinct siRNAs specific for Egr-1 (Egr-1-1 and Egr-1-2) and a negative control siRNA, with no similarity to human gene sequences, were transfected into HeLa L cells. After 48 h the cells were stimulated with PMA, and RT-PCR analysis of Egr-1 and Nab2 expression was performed at 20 min and 1 h. As can be seen from Fig. 4 this cell line expresses both Egr-1 and Nab2 under normal growth conditions. Transfection of control siRNA did not influence the typical mRNA expression patterns of either Egr-1 or Nab2. A 3-fold Egr-1 induction in response to PMA was observed after 20 min, which increased to 4-fold after 1 h, whereas a 2-fold induction of Nab2 expres-
tion was first seen 1 h after PMA treatment. Transfection of two siRNAs directed against Egr-1 (Egr-1-1 and Egr-1-2) resulted in a decrease of basal Egr-1 mRNA expression of 40% (Egr-1-2) and 70% (Egr-1-1), respectively (Fig. 4B). Although PMA stimulation induced Egr-1 expression after 20 min in Egr-1 siRNA-treated cells, the Egr-1 mRNA levels were reduced by 60% in Egr-1-1 and 70% in Egr-1-2-transfected cells compared with control cells. In contrast to control siRNA-treated cells, no further increase in Egr-1 expression was detectable after 1 h. Depletion of Egr-1 influenced Nab2 expression as well. Transfection of Egr-1 siRNAs reduced basal Nab2 mRNA levels by 65% (Egr-1-2) and 80% (Egr-1-1) (Fig. 4C). Stimulation with PMA increased Nab2 mRNA levels at 1 h, but this was reduced by 70% compared with cells transfected with control siRNA.

Transfection of each of the two siRNAs directed against Egr-1 inhibited not only Egr-1 expression but also resulted in a decrease of Nab2 expression, confirming that endogenous Egr-1 has a significant influence on the regulation of Nab2 expression.

Identification of Elements Mediating the Nab2 Induction—Nab2 is a delayed early response gene and can be induced by exposure to growth factors and mitogens as well as by stimulation with phorbol esters such as PMA. To identify elements in the Nab2 promoter mediating induction by phorbol esters the 5′-truncated fragments were transfected into Colo 320DM and Mel 888 cells and cultured in the presence or absence of PMA for 24 h (Fig. 5A). In Colo 320DM cells truncation of the Nab2 complete promoter to bp −379 relative to translation start site had no influence on luciferase activity in response to PMA. Further deletion up to bp −263 reduced luciferase activity by 80%. A 4–5-fold stimulation in response to PMA is still observed with maluc-263, and this is reduced to 2–3-fold in maluc-195. These results indicate an important element between bp −379 and −263, which is necessary for maximum activation of the Nab2 promoter in response to PMA in Colo 320DM cells, as well as responsive elements between bp −263 and −74. The region between bp −379 and −263 has already been shown to be important for basal promoter activity and contains a complex cluster of binding sites, including several Egr/Sp1 motifs (Fig. 6A). An additional Egr/Sp1 binding site, a cAMP-responsive element, and a single Egr binding site are located between bp −263 and −74. The importance of the region between bp −379 and −263 in mediating PMA induction is also observed in Mel 888 cells. However, in these cells additional elements located further upstream contribute significantly to PMA induction. In Mel 888 cells luciferase activity of maluc-872, containing the full Nab2 promoter, was 2.5-fold higher than that of maluc-379 and maluc-468 after PMA treatment. This region contains additional four Egr binding sites, one Egr/Sp1 site, one AP2, and a single NFκB binding motif. This implies that a cluster of multiple Egr/Sp1 binding sites and one AP2 binding site located at bp −329 to −260, present in maluc-379 but not in maluc-263, is the major regulatory element in the Nab2 promoter for induction of transcription after PMA stimulation.

Identification of Egr-1-responsive Elements in the Nab2 Promoter—The expression of transcription factors of the Egr family but not Sp1 (2,
Egr-1 Activates the Nab2 Promoter

FIGURE 6. Members of the Egr family bind to the Nab2 promoter. A, a detailed representation of the Nab2 oligonucleotides 1, 2, and 3, spanning the region between bp −289 and −260 of the Nab2 promoter, used for EMSAs. Positions relative to translation start site (+1) in the Nab2 promoter are indicated. Dotted lines represent putative Sp1 binding sites, solid lines represent putative Egr binding sites, and an AP2 binding site is noted. B, binding of Egr family members to the Nab2 oligonucleotide 3 is induced by stimulation with PMA, whereas binding of Sp1 is not influenced. Nuclear extracts (12 μg of protein) of PMA stimulated or unstimulated Mel 888 cells were incubated with the 32P-labeled Nab2 oligonucleotides as described under “Experimental Procedures.” Nab2 oligo, no competitor added; Cons, consensus; Mut, mutated. The observed complexes are indicated with roman numerals. C, members of the Egr family bind to the region between bp −289 and −260 of the Nab2 promoter after PMA treatment. Nuclear extracts (12 μg of protein) of Mel 888 cells stimulated for 3 h with PMA were incubated with the indicated 32P-labeled Nab2 oligonucleotides in the presence or absence of an Egr consensus sequence oligonucleotide (Egr cons) as described under “Experimental Procedures.” The observed complexes are indicated with roman numerals.

13, 14) is induced after stimulation with PMA. To investigate whether Egr-1 activates the Nab2 promoter deletion reporter constructs in the same manner as PMA, the 5′-truncated fragments were assessed for activity in Colo 320DM and Mel 888 cells after transfection with 40 ng of human Egr-1 or Phlda-1 DNA as control (Fig. 5B). In Colo 320DM cells the pattern of induction of the Nab2 promoter by Egr-1 was nearly identical to that after PMA stimulation, although the activation after PMA treatment was higher than after Egr-1 transfection. In addition the construct maluc-195 responds to PMA, whereas it does not respond to Egr-1 transfection in Colo 320DM cells. These results indicate the involvement of additional transcription factors in response to PMA in this cell line. Corresponding to the results obtained after PMA stimulation, the cluster of multiple Egr/Sp1 sites and one AP2 binding motif, located between bp −379 and −263, was necessary for maximum Egr-1-mediated activation of the Nab2 promoter in Colo 320DM cells. These results indicated that in this cell line the same promoter regulatory elements that respond to PMA are also responsive to Egr-1, suggesting that Egr-1 binding sites mediate the PMA stimulation. In Mel 888 cells both the pattern and strength of induction after Egr-1 transfection coincides with that observed after PMA stimulation. The region between bp −379 and −260 is also of major importance in response to Egr-1, but an overlapping Egr/Sp1 site and an Egr binding site, located between bp −558 and −468, are necessary for highest Egr-1-dependent induction of Nab2 expression in Mel 888 cells.

These results indicated that Egr-1 is a major activator of Nab2 transcription in response to PMA but also that other transcription factors might be involved. Furthermore the major regulatory element at bp −329 to −260, comprising a cluster of multiple Egr/Sp1 binding sites and one AP2 site, is important for basal promoter activity, induction of transcription after stimulation with PMA, and for Egr-1-induced activity.

Egr-1 Binds to the Nab2 Promoter after PMA Stimulation—To investigate whether Egr-1 and/or Sp1 are present in nuclei of PMA-stimulated and control cells and can bind to the cluster of overlapping putative Sp1/Egr-1 binding sites, located at bp −329 to −260, EMSAs were performed.

Nuclear extracts of either unstimulated or PMA-stimulated Mel 888 cells were incubated with 32P-labeled oligonucleotides corresponding to the Nab2 promoter region from bp −329 to −294 (oligo 1), bp −315 to −285 (oligo 2), and bp −289 to −260 (oligo 3) (Fig. 6A). In extracts from nonstimulated cells formation of four specific complexes can be detected binding to oligo 3 (Fig. 6B). Formation of complexes I and II is blocked by unlabeled Sp1 consensus oligonucleotide, but not by the mutated Sp1 consensus sequence, suggesting that complex I or II consists of Sp1 and/or Sp3 (which share a similar consensus sequence) bound to oligo 3. Complex III is blocked by Egr consensus oligonucleotide but not by the mutated Egr consensus sequence indicating that complex III may contain a member of the Egr family. Formation of complex IV is not influenced by either Egr or Sp1 consensus competition.

After PMA stimulation two intense bands, complexes IVa and V, appear. These complexes are both inhibited by the Egr consensus oligo-
Egr-1 Activates the Nab2 Promoter

FIGURE 7. Egr-1 binds to the region between bp −329 and −260 of the Nab2 promoter after PMA treatment, whereas the binding behavior of Sp1 remains unaffected by PMA. A, Sp1 binds to the region between bp −329 and −260 of the Nab2 promoter. Nuclear extracts (12 μg of protein) of Mel 888 cells either left unstimulated or stimulated for 3 h with PMA were incubated with the indicated 32P-labeled Nab2 oligonucleotide after preincubation at 4 °C with a specific Sp1 antibody or control serum as described under “Experimental Procedures.” The observed complexes are indicated with roman numerals. B, Egr-1 binds to the region between bp −329 and −260 of the Nab2 promoter after PMA treatment. Nuclear extracts (12 μg of protein) of Mel 888 cells either left unstimulated or stimulated for 3 h with PMA were incubated with the indicated 32P-labeled Nab2 oligonucleotide after preincubation at room temperature with a specific Egr-1 antibody or control serum as described under “Experimental Procedures.” The observed complexes are indicated with roman numerals.

nucleotide, but not by the mutated Egr consensus sequence or the Sp1 oligonucleotides. This indicates that members of the Egr family not detectable in unstimulated cells can bind to the specific Nab2 oligo 3 after PMA induction. Although complexes IV and IVa demonstrate a similar electrophoretic mobility, complex IV migrates slightly faster and is not influenced by the presence of competitive Egr consensus sequence. Complex III is also detectable after PMA stimulation but does not appear to be significantly stronger than in unstimulated cells. The intensity of complexes I and II is not influenced by stimulation with phorbol ester, which is consistent with the presence in these complexes of the noninducible Sp1 or Sp3.

Similar results were obtained using the labeled Nab2 oligos 1 and 2. Formation of the inducible complexes IVa and V is prevented by preincubation with the Egr consensus oligonucleotide (Fig. 6C) but not by the mutated Egr consensus sequence or the Sp1 oligonucleotides (data not shown). An additional band complex # can be observed after incubation of labeled oligo 1 or oligo 2 with nuclear extracts of unstimulated or PMA-treated cells (Figs. 6C and 7A), which is neither inducible (Fig. 7A) nor influenced by Egr-1 (Fig. 6C) or Sp1 consensus oligonucleotides (data not shown).

Previous results (Fig. 5A) indicate that the region between −329 and −260 bp is critical for the induction of the Nab2 promoter by phorbol esters. The EMSA analyses of this region suggest that the Egr family members forming complexes IVa and V with the Nab2 oligonucleotides are relevant for this induction, because these complexes are not detectable in nonstimulated cells. Proteins bound in complexes I, II, III, and # would seem to be important for basal activity of this region in the Nab2 promoter because they are found in stimulated and nonstimulated cells. To identify the proteins bound to the three Nab2 oligonucleotides the nuclear extracts were preincubated with Egr-1-, Sp1-, and Sp3-specific antibodies. Similar results were obtained for all Nab2 oligonucleotides. Preincubation with a Sp1-specific antibody eliminated formation of complex I, reduced complex II, and led to the appearance of supershifted complexes in extracts of unstimulated and stimulated cells (Fig. 7A). This indicates that complex I observed with each of the three Nab2 oligos is composed of Sp1. Similar experiments using an antibody to Sp3 indicated that this transcription factor is present in complex II (data not shown). Under the conditions required for the anti-Egr-1 antibody incubation, the Sp1 and Sp3 complexes are not detectable, but the Egr complexes IV and IVa are clearly seen in PMA-stimulated cells. The Egr-1 antibody blocked formation of complex V (Fig. 7B), thereby identifying Egr-1 as the protein forming complex V with the three Nab2 oligonucleotides spanning the region between bp −329 and −260 of the Nab2 promoter. The fact that complex IVa was not influenced by anti-Egr-1 antibody but was inhibited by a nonlabeled egr consensus oligonucleotide suggests that a second member of the egr family is also induced by PMA stimulation and can bind to this region of the Nab2 promoter. These studies demonstrate that stimulation with PMA induces binding of Egr-1 to multiple sites within the cluster of overlapping putative Sp1/Egr-1 binding sites, located at bp −329 to −260, whereas the binding behavior of Sp1 and Sp3 remained unchanged.

Egr-1-mediated Induction of the Nab2 Promoter Is Inhibited by Nab Corepressors—Because Nab1 and Nab2 proteins can act not only as corepressors but also as coactivators of Egr-dependent transcription (15), the ability of these proteins to repress or to stimulate the Egr-1-induced Nab2 promoter activity was examined.

To investigate the influence of Nab2, the Nab2 promoter construct, maluc-872, was cotransfected with Egr-1 or Phlda-1 as control and increasing amounts of Nab2 expression plasmid. The expression of Nab2 led to a dose-dependent inhibition of Egr-1-mediated Nab2 pro-
Egr-1 Activates the Nab2 Promoter

**FIGURE 8.** Egr-1-mediated induction of the Nab2 promoter is inhibited by Nab corepressors. A, Mel 888 cells were transfected with 60 ng of maluc-872 and Egr-1 or Phlda-1, as control for basal promoter activity, and increasing amounts of human Nab-2 as indicated in ng of DNA. DNA was filled up to 100 ng with empty vector. Luciferase activity was determined as described under "Experimental Procedures." Average and S.D. of triplicates from a representative experiment is shown. The control activity of the Nab2 promoter construct in the absence of Egr-1 or Nab2 expression constructs (first column) is indicated by the line. RLU, relative light units/μg of protein. B, Mel 888 and Colo 320DM cells were transfected with 60 ng of maluc-872 and 60 ng of human Egr-1, Nab1, Nab2, or Phlda-1, as control for basal promoter activity, as indicated. DNA was filled up to 240 ng with empty vector. Luciferase activity was determined as described under "Experimental Procedures." Results are presented as fold activation over luciferase activity of maluc-872 cotransfected with control plasmid Phlda-1 and empty vector (first two columns, set to 1). The results are presented as the mean and S.D. of triplicates from a representative experiment.

The transcription factor Egr-1 plays a key role in the regulation of proliferation, differentiation, and other cellular responses to extracellular stimuli (1). Egr-1 is an immediate early gene and is rapidly and transiently induced in response to growth factors, cytokines, stress, and phorbol esters such as PMA. Egr-1 itself activates the transcription of numerous target genes, including growth factors such as platelet-derived growth factor and fibroblast growth factor (2, 16), establishing a positive feedback loop that serves to amplify and sustain transcription through Egr-1-mediated mechanisms. This positive feedback loop is controlled in part through the action of two proteins, Nab1 and Nab2, which bind to Egr-1 and inhibit its activity. Of the two proteins Nab2 appears to be the more important Egr-1 regulator because its expression is induced by the same stimuli that induce Egr-1. As a delayed early response gene, Nab2 expression follows that of Egr-1 by several hours. Although the importance of Nab2 in controlling Egr-1 activity indicates the relevance of understanding how the expression of Nab2 is regulated, the transcriptional regulation of Nab2 expression remains unclear.

In the study presented here the 5′-region of the Nab2 gene and the regulation of Nab2 expression have been analyzed. The Nab2 promoter was shown to comprise the region from bp −679 to −74 relative to the translation start site, containing three overlapping Egr/Sp1 sites, four Egr sites, two AP2 sites, one Sp1 site, a cAMP-responsive element, and a cluster of multiple overlapping Egr/Sp1 sites extending over a 70-bp region. Transient transfection experiments in colon carcinoma and melanoma cell lines indicated that the region between bp −329 and −260 is critical for basal promoter activity as well as for induction of transcription after stimulation with PMA. This region contains the cluster of overlapping transcription factor binding motifs, consisting of six Egr sites, four Sp1 sites, and a single AP2 site (Fig. 6). Overlapping Egr-1 and Sp1 sites have been observed in a number of promoters (2, 14, 16–19). Generally these are found as a single unit (e.g. 1 Egr-1/1 Sp1 site), and a promoter may contain several as in the Nab2 promoter (2, 20). A cluster of multiple overlapping Egr/Sp1 sites, comparable to that in the Nab2 promoter, has been found in the 5-lipoxygenase promoter (21). This element in the 5-lipoxygenase promoter is also essential for basal promoter activity and for induction by PMA stimulation.

EMSAs with the region of the Nab2 promoter containing this element (bp −329 to −260) showed that Sp1 binds in both unstimulated and stimulated cells, whereas expression and binding of Egr-1 to multiple sites is induced by stimulation with phorbol ester. Transfection of each of two distinct siRNAs directed against Egr-1 resulted not only in a reduction of Egr-1 but also in a decrease of Nab2 expression, confirming the importance of Egr-1 in the regulation of Nab2 expression. In addition transfection of Egr-1 together with Nab2 promoter constructs showed that Egr-1 induces Nab2 promoter activity. Furthermore, a nearly identical pattern of promoter activity induced by transfection of Egr-1 and by phorbol ester stimulation was observed. This suggests that the ubiquitous, constitutively expressed Sp1 may be the major transcription factor driving basal promoter activity, whereas Egr-1 is a major inducer of protein kinase C-mediated Nab2 induction.

The presence of a PMA-induced complex specifically inhibited by the Egr consensus oligonucleotide but not influenced by antibody against Egr-1 suggests that a second not yet identified member of the Egr family is also expressed and binds to the cluster of overlapping Sp1/Egr binding sites in the Nab2 promoter. This suggests that Nab2 expression in response to extracellular stimuli is regulated by Egr-1 and a second member of the Egr family. Nab2 expression was shown in several systems to be associated with Egr-2 (Krox-20) expression (22–24), and transfection of breast cancer cells with Egr-3 led to up-regulation of Nab2 mRNA (25), suggesting that protein kinase C-mediated Nab2 induction may also be influenced by Egr-2 and Egr-3. The studies presented here show directly that Egr-1, the most widely expressed member of the egr family, not only up-regulates Nab2 expression, but also...
binds to and activates the Nab2 promoter. Taken together, these data suggest a complex regulation of the induction of Nab2 expression by several members of the Egr family.

In EMSA competition experiments formation of two complexes was blocked by the Sp1 consensus sequence oligonucleotide in unstimulated and stimulated cells. Although one of these complexes was shifted by an antibody to Sp1, the second was shown to be shifted by an antibody to Sp3 (data not shown), which shares a similar DNA-binding consensus sequence with Sp1. Regulation of basal transcription by interaction of Sp1 and Sp3 has been observed in several genes. Although Sp1 and Sp3 usually act in cooperation (26, 27), Sp1-mediated expression of activation has been described to be inhibited by Sp3 as well (28).

Results from PMA and Egr-1 experiments suggest a difference in the regulation of Nab2 expression in melanoma and colon carcinoma cell lines. Egr-1-mediated activation of the Nab2 promoter was higher in the tested melanoma cell lines, as compared with the colon carcinoma cells. In addition, although ectopic expression of Egr-1 was sufficient in Mel 888 cells for maximum activation of the Nab2 promoter, in Colo 320DM cells Egr-1-induced promoter activity was only half of that observed after PMA stimulation, suggesting the involvement of additional transcription factors. Furthermore in Mel 888 cells additional elements located further upstream of the major regulatory element at bp −329 to −260 contribute significantly to PMA and Egr-1 mediated induction, whereas in Colo 320DM cells the major regulatory element is sufficient for highest activation of the Nab2 promoter.

Nab1 and Nab2 proteins can act not only as corepressors but also as coactivators of Egr-dependent transcription depending on special promoter properties, such as the number of putative Egr binding sites (15). The experiments described here demonstrate that human Nab1 as well as Nab2 repress Egr-1-mediated Nab2 promoter activation, an expected result given the large number of Egr binding sites. Nab1 and Nab2 inhibition is synergistic, although Nab2 seems to be a more potent repressor of Egr-1-mediated Nab2 promoter activation.

The negative feedback loop established between Nab2 and Egr-1 to control Egr-1 activity is comparable to that proposed for the regulation of Egr-2 activity by the Nab proteins in hindbrain development (22). In this case transcriptional activity of Egr-2 (Krox-20) was shown to be under the control of a negative feedback mechanism exerted by Nab1 and Nab2. Furthermore expression of the Nab proteins in the hindbrain was found to be dependent on Egr-2 expression. However it is not known whether the involvement of Egr-2 in Nab expression is direct or indirect, and this negative feedback appears to be tissue-specific because the lack of Egr-2 does not influence Nab protein expression in the heart. However given the results presented here confirming a direct role of Egr-1 in the induction of Nab2 expression, it might be expected that a similar interaction occurs between Egr-2 and Nab2.

In conclusion, the studies described here demonstrate that Egr-1 is a major activator of Nab2 transcription in response to extracellular stimuli. This Egr-1-induced Nab2 expression can be repressed by Nab proteins, supporting a negative feedback mechanism (22, 29), which is crucial to prevent a damaging overreaction in response to environmental signals. Alterations in expression of Egr-1 and Nab proteins have been associated with deficiencies in neuronal development (22, 30) and with malignancy (31, 32), indicating the importance of understanding the precise mechanism by which Nab2 expression is regulated. The experiments presented here give further insight concerning this issue by identifying Egr-1 as a potent regulator of Nab2 transcription and by identifying a major regulatory element in the Nab2 promoter, comprising multiple overlapping Egr/Sp1 sites and an AP2 site.

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