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Characterization of the Neuropilin-1 Promoter; Gene Expression is Mediated by the Transcription Factor Sp1

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Abstract Neuropilin-1 (NRP1) is a receptor for the vascular endothelial growth factor (VEGF) family of angiogenesis factors and for the semaphorin family of secreted neuronal guidance polypeptides. Very little is known, however, about how NRP1 gene expression is regulated. In this study, it was demonstrated that the tumor promoter, TPA (12-*O*-tetradecanoylphorbol-13-acetate) significantly up-regulated NRP1 mRNA levels by increasing its gene transcription rate in a manner dependent on de novo protein synthesis. To determine which elements regulate functional NRP1 expression, the promoter regions of human and mouse NRP1 genes were cloned and characterized. Promoter-reporter gene transfection experiments using deletion and point mutations demonstrated that two Sp1 elements are major contributors to both the constitutive and TPA-induced activity of the NRP1 promoter. Gel shift analysis showed a specific binding of the Sp1 transcription factor to those elements. Further mutational analysis revealed that an AP-1, and a CCAAT box also contributed to NRP1 constitutive and TPA-induced promoter activity. It was concluded that NRP1 expression is regulated by the cooperation of several regulatory elements including AP-1, Sp1, and a CCAAT box. J. Cell. Biochem. 88: 744–757, 2003.

Key words: neuropilin promoters; phorbol ester; protein kinase C; VEGF; angiogenesis; semaphorin; neuronal guidance

Angiogenesis, the growth of new blood vessels by sprouting from established blood vessels, is a pivotal process in wound healing and tumor growth. Vascular endothelial growth factor (VEGF), a mitogenic and chemotactic factor for endothelial cells, is one of the most potent angiogenic stimulators in vivo [Risau, 1997]. Two high affinity receptor tyrosine kinases, VEGFR-1 and VEGFR-2, expressed on the surface of endothelial cells, mediate the multiple biological effects of VEGF [Klagsbrun and Moses, 1999; Robinson and Stringer, 2001]. Recently, neuropilins have been described as a second class of VEGF receptor. The two members of this family, neuropilin-1 (NRP1) and neuropilin-2 (NRP2), are non-tyrosine kinase transmembrane proteins that bind VEGF in an isoform specific manner [Soker et al., 1998; Gluzman-Poltorak et al., 2000; Gluzman-Poltorak et al., 2001]. NRPs were originally identified as receptors for the semaphorin family of secreted polypeptides implicated in axonal guidance and neuronal patterning [He and Tessier Lavigne, 1997; Kolodkin et al., 1997]. Semaphorin 3A (Sema3A) binds NRP1, which signals by hetero-complexing with a transmembrane kinase, plexin [Winberg et al., 1998; Takahashi et al., 1999].

Sequence data from this article have been deposited with the GenBank library under accession numbers AF482431 (human NRP1 promoter), AF482432 (mouse NRP1 promoter) and AF281074 (human NRP2 promoter).

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Several lines of evidence have demonstrated that NRP1 regulates angiogenesis and vasculogenesis (reviewed in Miao and Klagsbrun [2000] and Soker [2001]). In the normal mouse embryo, NRP1 is expressed on endothelial cells and the surrounding mesenchymal cells [Kitsukawa et al., 1995]. NRP1 overexpression in transgenic mice results in embryonic lethality associated with excessive vascular formation, dilatation, and hemorrhaging, as well as defects in skeletal morphogenesis and neuronal guidance [Kitsukawa et al., 1995]. Targeted disruption of the NRP1 gene in mice is embryonic lethal and results in insufficient vascularization, cardiovascular anomalies, and defects in neuronal patterning [Kitsukawa et al., 1997; Kawasaki et al., 1999]. On the other hand, targeted disruption of the NRP2 gene results in viable mice that display abnormal axon guidance patterns in the peripheral and central nervous systems but do not display a vascular phenotype [Chen et al., 2000; Giger et al., 2000]. Double knockout mice, in which both NRP1 and NRP2 genes are disrupted, are embryonic lethal due to defects in the vascularization of the yolk sacs and embryos. These double knockout mice have a more severe abnormal vascular phenotype than either NRP1 or NRP2 knockout mice alone [Takashima et al., 2002]. Thus, precise control of NRP1 and NRP2 expression appears to be essential for normal neuronal and vascular development. One possible mechanism for NRP1 angiogenic activity is that NRP1 acts as a co-receptor for $VEGF_{165}$, enhancing its binding to VEGFR-2 and its bioactivity [Soker et al., 1998; Whitaker et al., 2001].

Unlike the other VEGF receptors, NRP1 is expressed in many cell types and tissues. Of particular interest, NRP1 is expressed in tumor cells and its expression [Soker et al., 1998; Banerjee et al., 2000; Ding et al., 2000] is correlated with the aggressiveness of prostate tumors [Latil et al., 2000]. Furthermore, inducible overexpression of NRP1 in prostate carcinoma cells results in increased tumor cell motility, tumor size, and tumor angiogenesis [Miao et al., 2000].

Although NRP1 plays a critical role in developmental angiogenesis, tumor angiogenesis, and neuronal guidance, very little is known about the regulation of NRP1 expression. Thus, to begin with, the effects of the tumor promoter TPA on NRP1 gene expression were evaluated. TPA is a well characterized activator of protein kinase C isozymes (PKCs) which are important regulators of signaling cascades that control cell proliferation and death and that contribute to tumor growth. To define the elements that regulate constitutive and TPA-induced NRP1 expression, we cloned and characterized the NRP1 gene promoter region. In this report, we demonstrate using deletion and site-directed mutagenesis analysis in combination with gel shift analysis that an AP-1 element, two Sp1 elements, and a CCAAT box are major contributors to constitutive and TPA-induced NRP1 promoter activity.

MATERIALS AND METHODS

Materials

Gö6850 (Bisindolylmaleimide I), SB202190, and U0126 were purchased from Calbiochem, San Diego, CA. Actinomycin, cycloheximide (Cyc), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma, St. Louis, MO NRP1 antibody (H-286) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Screening of Human and Mouse Genomic Libraries

The human NRP1 promoter region was cloned from a human PAC genomic library (IncyteGenomics, Palo Alto, CA) as previously described [Rossignol et al., 2000]. The mouse Genome Walker kit (Clontech, Palo Alto, CA) was used to isolate three overlapping fragments of the mouse NRP1 promoter region. The fragment spanning −1,404 to −885, −998 to −224, and −363 to +125 were obtained with the 3′ primers;
A(5′-GCATAAAGGCGAGGATGGGTACCTGATA-3′);
B(5′-TCCAGAGCCCTTCCTTCCTTCCTCCGCCC-3′); and
C(5′-CTGCATCCTGTCATTTAGCTCCGATT-3′), respectively. Sequencing of the three fragments by automated cycle sequencing (ABI PRISM Dye Terminator Cycle sequencing, PerkinElmer, Norwalk, CT). Identified a 1,404 bp mouse NRP1 promoter sequence. A 1,443 bp fragment was subsequently amplified by PCR from mouse genomic DNA using a 3′ primer derived from the 5′-UTR of the mouse NRP1 cDNA (5′-CAAAGCTTCCTCTTCCAGGAGCCACTGC-3′) and

a 5′ primer derived from the most 5′ sequence of the −1,404 to −885 promoter fragment obtained (5′-CCTGGGTGTCCTTGCTATTC-3′). The PCR product was cloned into a TA Cloning Vector pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced.

Northern Blot Analysis

HeLa cells were grown to 80% confluence on 100-mm tissue culture dishes, washed three times in serum-free DMEM, and then treated 40 h later with TPA in serum-free DMEM. Total RNA was isolated with the RNeasy mini kit (Qiagen, Valexcia, CA) and 15 μg of total RNA were resolved by electrophoresis in a 1.2% formaldehyde-agarose gel and then blotted onto nylon membranes (PerkinElmer Life Sciences, Boston, MA, NEN). The blots were prehybridized in 1 M NaCl, 10% dextran sulfate (Appligen Oncor, Illkirch, France), 1% SDS, and 100 μg/ml salmon sperm DNA for 4 h at 65°C. Hybridization was carried out overnight at 65°C in the same buffer at a final concentration of 10^6 cpm labeled probe per milliliter hybridization solution. After hybridization, blots were washed twice with 2 × SSC and 0.1% SDS and once with 0.5 × SSC and 0.1% SDS for 10 min at 65°C. Autoradiography was performed using intensifying screens at −70°C. Quantitation of mRNA levels was performed using a PhosphorImager (Amersham Biosciences, Piscataway, NJ).

Plasmid Constructs

The pGL2 basic plasmid contained the firefly luciferase gene without a promoter (Promega). The plasmid pCMVβ-gal contained the β-galactosidase gene driven by the cytomegalovirus (CMV) promoter and enhancer. Reporter constructs containing fragments of the human NRP1 5′-flanking region were inserted into pGL2 basic and numbered according to the length of the fragment starting at the transcription start site in the 5′ and 3′ directions. For example, plasmid pGL2−823/+79 contained a human NRP1 promoter fragment extending from position −823, 5′ of the transcription start site, to position +79, 3′ of the transcription start site cloned into pGL2 basic. Plasmids pGL2−823/+79, pGL2−401/+79, pGL2−192/+79, pGL2−173/+79, pGL2−97/+79, and pGL2−22/+79 were created from promoter fragments generated by PCR of human NRP1 PAC DNA. All constructs were sequenced from the 5′ and 3′ ends to confirm orientation and sequence.

Transient Transfections and Reporter Gene Assays

At 24 h prior to transfection, HeLa cells were plated in 12-well plates in DMEM, 5% FBS at 1.1×10^5 cells/well. Cells were transfected for 16 h at 37°C by the calcium phosphate method using for each transfection, 1.5 μg of the appropriate reporter construct along with 0.3 μg of pCMVβ-gal. Cells were washed, incubated in DMEM, 5% FBS for 8 h, starved for 20 h in DMEM, and then treated for 24 h with or without 10^{-7} M TPA. Cell were lysed in 100 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM 1, 2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 2 mM DTT, 10% glycerol, and 1% Triton X-100) for 10 min at room temperature, and cell debris was removed by centrifugation. The extract (20 μl) was assayed for luciferase activity in a MicroBeta TRILUX luminescence counter (PerkinElmer, Norwalk, CT) in 50 μl of luciferase buffer (20 mM Tricine, 1.07 mM $(MgCO_3)Mg(OH)_2$, $5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferine, and 530 μM ATP). The extract (5 μl) was assayed for β-galactosidase activity using the Galacto-Light Chemiluminescent Reporter Assay kit (Tropix, Bedford, MA). The ratio of luciferase activity to β-galactosidase activity in each sample was a measure of normalized luciferase activity. The normalized luciferase activity was divided by the luciferase activity of pGL2−817/+79, and expressed as relative luciferase activity. Each construct was transfected at least four times in triplicate, and data for each construct are presented as the mean ± SD.

Gel Shift Assays

HeLa cells were grown to 90% confluence on 100-mm dishes, serum-starved for 24 h, and treated with or without 10^{-7} M TPA for 6 h. Nuclear extracts were prepared as described [Paya et al., 1992]. The nuclear extracts were then concentrated five times on an Ultrafree-4 Centrifugal Filter Unit, Biomax-10 (Millipore, Bedford, MA), washed with dialysis buffer (20 mM Tris, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM protease inhibitor AEBSF, 0.5 mM NaF, and 0.5 mM orthovanadate) and stored at −70°C. Each synthetic double stranded oligonucleotide

(5 pmol) was labeled with T4 polynucleotide kinase (NEB) and 50 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 45 min at 37°C, and labeled primers were purified over a G-25 Sephadex column (Roche, Basel, Switzerland). Binding reactions consisted of 5 μg of HeLa nuclear extracts, binding buffer (7.5 mM HEPES, pH 8, 35 mM NaCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 7.5% glycerol, 0.5 mM DTT), 2 μg of poly(dI-dC), 3 μg bovine serum albumin (BSA), 100 ng salmon sperm DNA, and 250 fmol of labeled probe. Unlabeled competitors were added 10 min prior to addition of labeled probe. Reactions were carried out for 35 min on ice and analyzed by 6% polyacrylamide gel electrophoresis in 0.5 × TBE buffer. The Sp1 consensus sequence was 5′-ATTCGATCGGGGCGGGGCGAGC-3′ (Promega). Supershift antibodies (200 ng) Sp1 (R&D Systems, Minneapolis, MN) and Sp3 (Santa Cruz Biotechnology) were added to the reaction for 15 min on ice before adding radiolabeled probe.

RESULTS

TPA Induces NRP1 Gene Expression

The effect of the tumor promoter, TPA, on NRP1 gene expression in HeLa and human umbilical vein endothelial (HUVE) cells was analyzed (Fig. 1 and data not shown). TPA had a similar inductive effect on NRP1 mRNA levels in both cell types. Figure 1 shows by phosphorimaging analysis that 10^{-7} M TPA increased the steady-state levels of HeLa NRP1 mRNA at 4 h and that the induction was maximal at 8 h, about eightfold. Western blot analysis demonstrated that TPA also increased HeLa NRP1 protein levels at 24 h (Fig. 1B). Because of the relative ease of working with HeLa cells compared to HUVEC, for example, in promoter transfection experiments, further regulation studies were carried out in HeLa cells.

In order to identify the possible signaling pathways involved in TPA induction of NRP1, specific pharmacological inhibitors of signal transduction pathways were tested. The primary intracellular target of TPA are the conventional protein kinases C (cPKCs isoforms α, β, and γ) and the novel PKCs (nPKCs isoforms δ, ϵ, η, and θ) [Nishizuka, 1995]. Activation of PKC has been demonstrated to involve the Ras pathway, which couples membrane-associated protein-tyrosine kinases to the MAP kinase

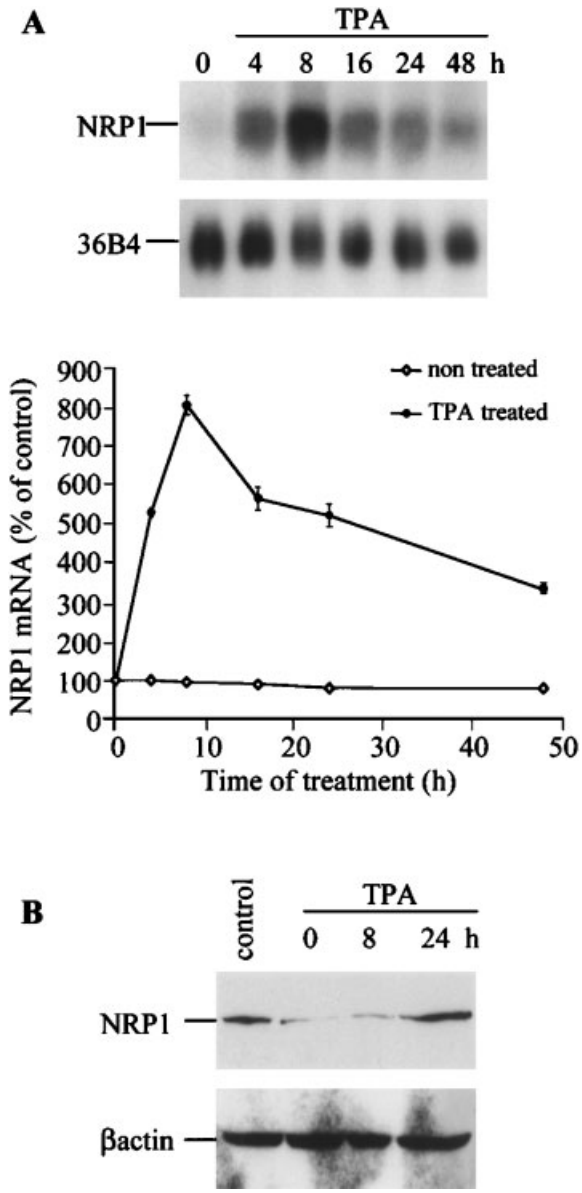

Fig. 1. Time course of TPA-induced NRP1 expression in HeLa cells. **A**: Cells were treated without (○) or with (●) 10^{-7} M TPA for 0–48 h, total RNA was prepared at the indicated times, and 15 μg were analyzed by Northern blotting. Bands corresponding to NRP1 mRNA (**top**) and the control gene 36B4 mRNA (**bottom**) are indicated. The **upper panel** is a representative Northern blot and the **lower panel** shows the quantitation of the Northern blots whereby signal intensities were quantified by phosphorimaging and normalized to the control gene, 36B4. Values represent the mean ± SD of four separate experiments each performed in duplicate. **B**: Cells were treated with 10^{-7} M TPA for 0–24 h, cell lysates were prepared at the indicated times, and 40 μg were analyzed by Western blotting. Bands corresponding to NRP1 (top) and the control, β-actin (bottom) are indicated. A positive control of non-starved and non-TPA treated HeLa cells is shown (control).

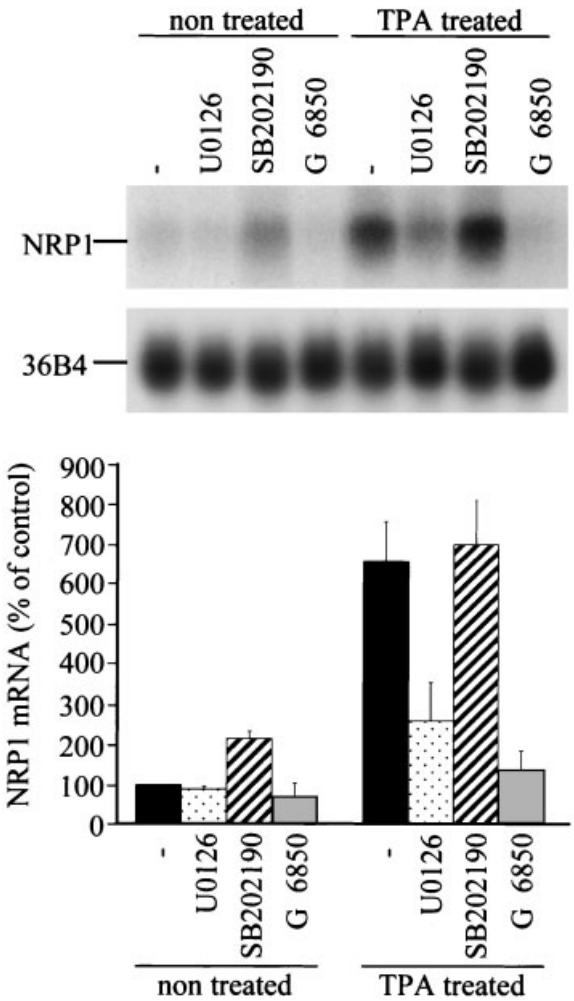

Fig. 2. Effects of signal transduction inhibitors on TPA induction of NRP1 mRNA levels. Cells were pretreated for 45 min without or with 10 μM U0126 (MEK1/2 inhibitor), 5 μM SB202190 (p38 MAP kinase inhibitor), or for 3 h with 5 μM Gö6850 (PKCα, β, γ, δ, and ϵ inhibitor) and then incubated for 6 h without or with 10^{-7} M TPA. The **upper panel** is a representative Northern blot and the **lower panel** shows results from quantitation of Northern blots by phosphorimaging as described in Figure 1.

kinase 1/2 (MEK1/2)–MAP kinase (ERK1/2) pathway via the Raf protein kinase [Ueda et al., 1996; Marais et al., 1998]. Pretreatment of the cells with Gö6850, a specific PKCα, β, γ, δ, and ϵ inhibitor totally blocked the TPA effect (Fig. 2). Pretreatment with U0126, a pharmacological inhibitor of MEK1/2, reduced the TPA induction of NRP1 mRNA by 60% (Fig. 2). Since stimulation of the JNK-p38 MAP kinase pathway has also been shown to be activated in response to mitogens and stress, the contribution of p38 MAP kinase to NRP1 induction was tested. Pretreatment with SB202190, the p38 MAP kinase-specific inhibitor, did not inhibit, and even slightly increased, NRP1 induction by TPA (Fig. 2). These results show that activation of PKC and MEK1/2–ERK1/2 but not the p38 MAP kinase pathways are necessary steps in TPA regulation of NRP1 expression.

Transcriptional Regulation of the NRP1 Gene by TPA

Gene expression can be regulated through both transcriptional and post-transcriptional mechanisms. To assess whether TPA treatment affected NRP1 gene transcription or mRNA stability, inhibitors of transcription (actinomycin D, ActD), and translation (Cyclohexamide Cyc) were tested. ActD (5 μg/ml) or Cyc (10 μg/ml) was added with or without TPA (10^{-7} M), and NRP1 mRNA levels were measured 8 h after treatment. Simultaneous addition of TPA and ActD, or TPA and Cyc, completely blocked the induction of NRP1 gene expression (Fig. 3A). These results suggested that TPA increased the rate of NRP1 gene transcription in a manner that was dependent on de novo protein synthesis. In order to test whether the stability of the NRP1 transcript was modified by TPA, HeLa cells were pretreated for 4 h without or with TPA, and ActD was added to block transcription. Pretreatment of cells with TPA did not significantly increase the half-life of the NRP1 mRNA indicating that TPA did not affect the stability of the NRP1 transcript (Fig. 3B).

Isolation and Characterization of the Human and Mouse NRP1 Promoters

In order to investigate further, the transcriptional regulation of the human NRP1 gene, a 4.7 kb of genomic sequence upstream to the known 5′-UTR sequence of human NRP1 cDNA was isolated from a human PAC library and sequenced [Rossignol et al., 2000]. The transcription start site of the human gene was determined by 5′RACE PCR analysis (data not shown) and is located 330 bp upstream of the start codon (Fig. 4B). The start site identified is highly homologous to the initiator (Inr) consensus sequence Py–Py–A_{+1}–N–$^{T}/_{A}$–Py–Py (where A_{+1} is the transcription start site). Inr elements have been characterized in various TATA-less and TATA-containing promoters and are implicated in the positioning of the

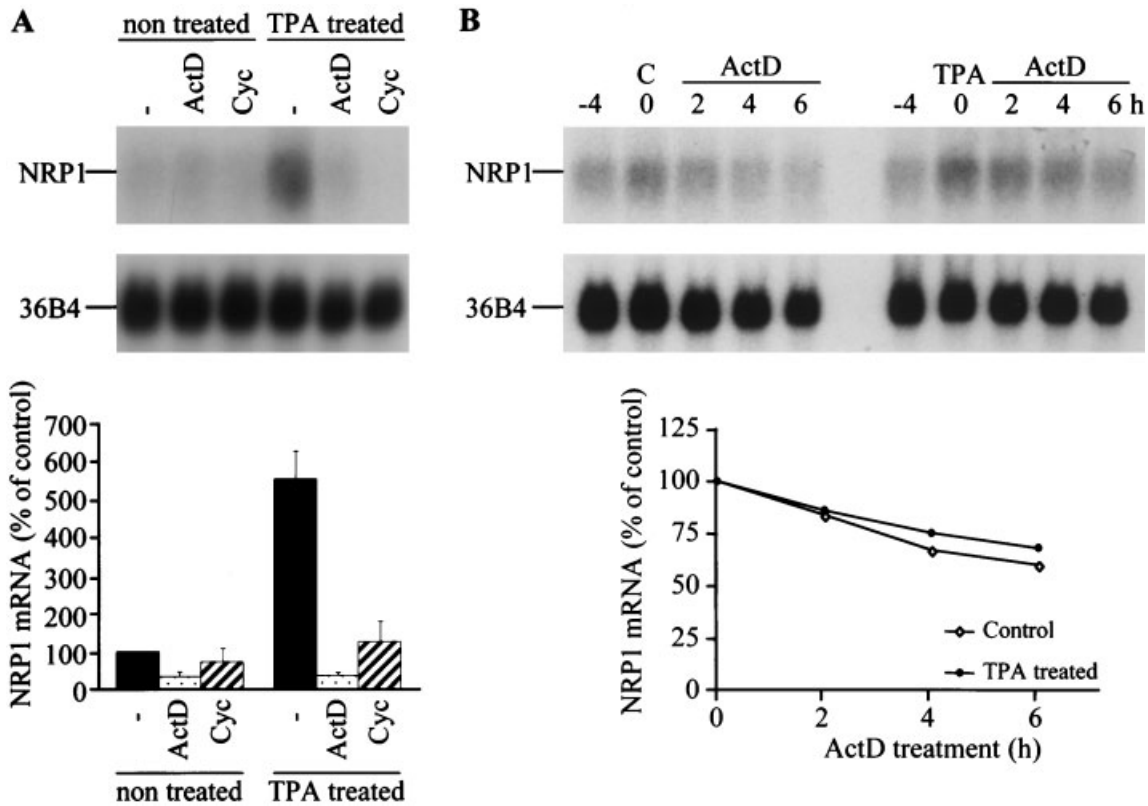

Fig. 3. TPA effects on NRP1 gene transcription and mRNA stability. The respective **upper panel** of **A** and **B** are representative Northern blot analyses performed as described in Figure 1. The respective **lower panels** of A and B represent quantification of the Northern blots by phosphorimaging as described in Figure 1. A: HeLa cells were treated for 8 h in the absence or in the presence of 10^{-7} M TPA in combination with 5 μg/ml actinomycin D (ActD) or 10 μg/ml cycloheximide (Cyc). B: Cells were treated for 4 h without or with 10^{-7} M TPA, and 5 μg/ml ActD was added to control and to TPA-treated cells. NRP1 mRNA and control gene 36B4 mRNA levels were determined after 0, 2, 4, and 6 h.

basal transcription machinery through interaction with the transcription factor, TFIID [Smale and Baltimore, 1989; Goodrich et al., 1996]. The 5′-flanking region of the mouse NRP1 gene was also cloned. Three partially overlapping fragments of the mouse NRP1 promoter ranging in size from 488 to 775 bp were successively amplified from a mouse genomic DNA adapter-ligated library by nested PCR. All four fragments were sequenced and assembled to generate a sequence of 1.4 kb. This 1.4 kb was amplified subsequently by PCR from mouse genomic DNA.

Alignment of the human and mouse NRP1 promoters revealed a region of high homology with 80% nucleotide identity within the first 330 nucleotides upstream from the transcription start (Fig. 4B). Nucleotide homology dropped significantly in the more upstream sequences, suggesting that the proximal conserved 330 nucleotides of the promoter is important for transcriptional regulation of NRP1 in both species. Sequence analysis of the promoters through the MatInspector program [Quandt et al., 1995] demonstrated the presence of several potential regulatory elements that are conserved between mouse and human NRP1 promoters and that may have functional importance in NRP1 regulation (Fig. 4A,B). There is a conserved CCAAT box (−42 to −50) in the reverse orientation (ATTGG). The position of the CCAAT box, ~50 bp upstream of transcriptional initiation, and its orientation in the NRP1 promoter, is consistent with the typical location of this element in TATA-less promoters [Mantovani, 1998]. Although we have not mapped the mouse transcriptional start site, sequence alignment between human and mouse promoter regions reveals that there is also a CCAAT box at about the same distance upstream of the ATG in the mouse promoter. CCAAT boxes have been demonstrated to bind

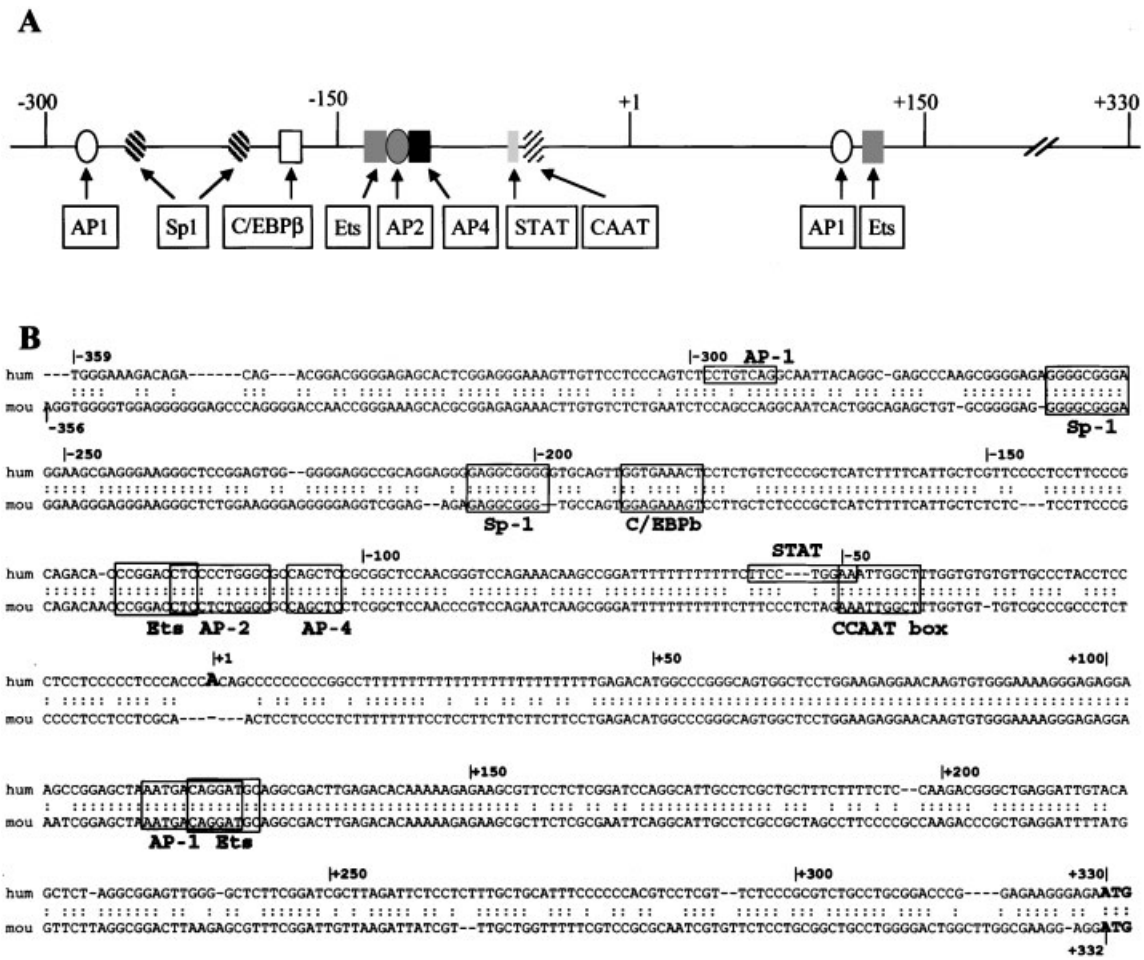

Fig. 4. Structure of the human NRP1 promoter and homology to the mouse NRP1 promoter. **A**: Schematic structure of the human NRP1 promoter with potential regulatory elements shown. **B**: Nucleotide homology between human and mouse NRP1 promoters. The numbers correspond to the human NRP1 promoter. The transcription start site (+1) is indicated in bold. Dots indicate homologous nucleotides between human and mouse. Dashed lines represent gaps in the homology. Consensus transcription binding sites are boxed.

a number of transcription factors including NF-Y (also called CBF or CP1), CDP, NF-1, and C/EBP [Dorn et al., 1987; Chodosh et al., 1988]. An AP-4 consensus binding site (−108 to −103), an AP-2 consensus binding site (−111 to −121), an Ets factor binding site (−127 to −119), a C/EBPβ consensus binding site (−190 to −182), and two consensus Sp1 factor binding sites (−199 to −207 and −253 to −261) are located in the conserved region of the promoter and may play a role in transcriptional regulation of NRP1. An AP-1 consensus binding site (−297 to −291) is present in the human but not the mouse NRP1 promoter. A conserved sequence located upstream of the transcription start site contains an AP-1 binding site (+112 to +122) that overlaps with a sequence homologous to an Ets factor binding motif (+117 to +124). The NRP1 binding sites are summarized in Figure 4A.

Functional Analysis of the Human NRP1 Promoter

To identify the functional promoter elements involved in NRP1 promoter constitutive activity and in gene induction by TPA, progressive 5′ deletion mutants were constructed based on the location of consensus factor binding sites on the promoter (Fig. 5, center panel). Deletion constructs were transiently transfected into HeLa cells, and their relative luciferase activity was assayed in the absence or presence of TPA. The constitutive basal activity of each vector is shown in the left panel of Figure 5. The empty

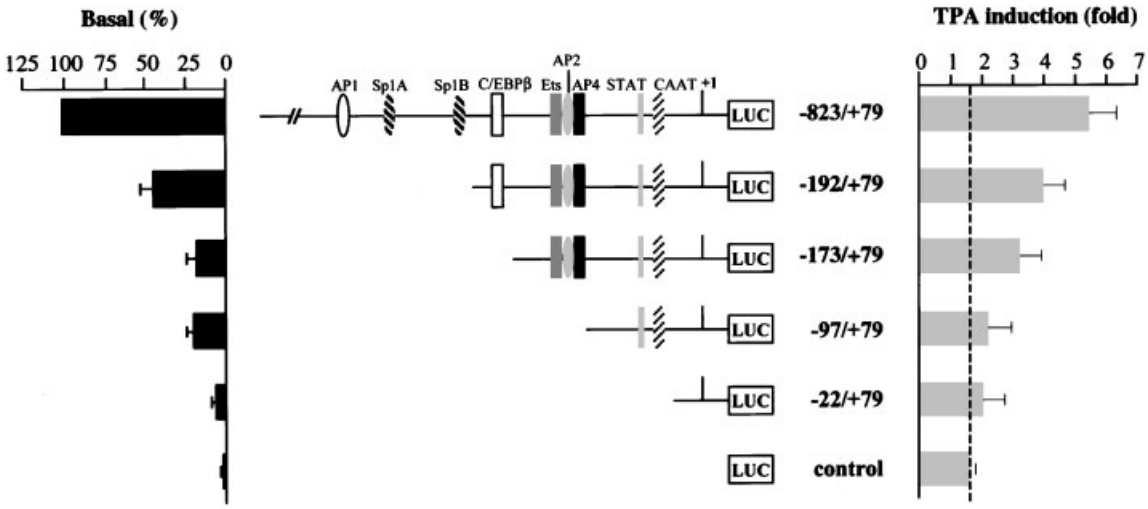

Fig. 5. Effect of progressive 5′ deletions on constitutive and TPA induction of NRP1 promoter activity. Each promoter construct (**center**) was transiently transfected into HeLa cells, and luciferase activity was expressed as fold induction of TPA-treated over untreated cells for each construct. Values represent the mean ± SD from at least four separate experiments, each done in triplicate wells. The histogram on the left indicates the impact of NRP1 promoter deletions on the constitutive activity of each construct. The constitutive activity of the −823/+79 construct was set at 100%. The histogram on the right shows the transcriptional activity in the presence of 10^{-7} M TPA for each NRP1 promoter deletion construct.

pGL2 basic vector had no detectable luciferase activity. A deletion from −823 to −192, which removed 632 bp of promoter sequence including a potential AP-1 site and two potential Sp1 binding sites caused a 55% decrease in constitutive activity of the promoter. The removal of the promoter region between −192 and −173 that contained one potential C/EBPβ binding site reduced the constitutive activity of the promoter by an additional 40%. Finally, the removal of the −97 to −22 region, including a potential STAT-binding site and the CCAAT box abrogated the remaining constitutive activity of the promoter.

The level of TPA induction of luciferase activity by various constructs was defined to be that observed above the background control vector induction, 1.6-fold (Fig. 5, control). Background induction by TPA of a variety of vectors has been described previously and is presumably mediated through cryptic sites in the various plasmids [Kushner et al., 1994]. Deletion of the region comprising −823 to −192, which removed a potential AP-1 site and two potential Sp1 binding sites, caused a 30% decrease in TPA-induced activity. Deletion of the region from −192 to −173 that contained one potential C/EBPβ binding site, and the region from −173 to −97 that contained potential binding sites for Ets, AP-2, and AP-4, reduced further the TPA-induced activity by 20 and 45%, respectively. Further deletion of the promoter (−97 to −22) abolished the remaining TPA-induced activity.

Sp1, AP-1, and CCAAT Elements are Implicated in Constitutive and TPA-Induced Activity of the Human NRP1 Promoter in HeLa Cells

In order to understand the contribution of each individual consensus binding site to constitutive and TPA-induced activity, point mutations were introduced, and tested in HeLa cells (Fig. 6). The −823/+79-deletion construct was chosen as a template for mutagenesis because this construct had the highest constitutive and TPA-induced luciferase activity compared to the promoterless plasmid. However, in the case of the Sp1A and Sp1B double mutants a shorter promoter fragment (−401/+79) was used because the two potential Sp1 binding sites located at −688 and −554 were capable of interfering with the activity of the double mutant. Anyhow, the deletion from −823 to −401 had no significant effects on constitutive and TPA-induced activity of the NRP1 promoter (data not shown).

Mutation of either C/EBPβ, Ets, AP-2, or AP-4 binding sites did not affect the constitutive or the TPA-induced activity of the promoter. Mutation of either the AP-1 site (−297) or the

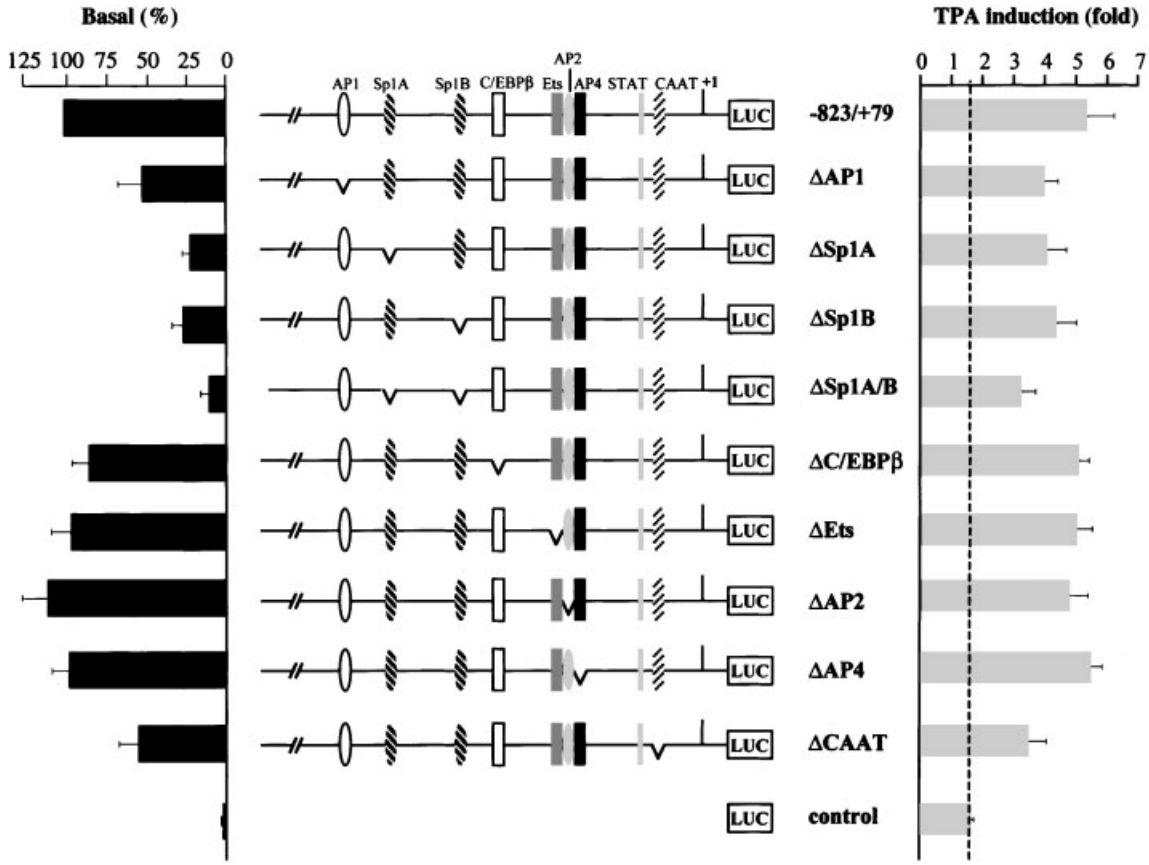

Fig. 6. Effect of NRP1 promoter mutations on constitutive and TPA induction of NRP1 promoter activity. Each promoter construct (**center**) was transiently transfected into HeLa cells, and luciferase activity was expressed as fold induction of TPA-treated over untreated cells. Values represent the mean ± SD from at least four separate experiments, each done in triplicate wells. The histogram on the left shows the impact of each mutation on constitutive activity of the construct. The histogram on the right shows the transcriptional activity in the presence of TPA 10^{-7} M for each mutation.

CCAAT box reduced promoter constitutive activity by 50% and TPA-induced activity by 30%. Mutation of either Sp1A or Sp1B or both Sp1 binding sites resulted in a dramatic decrease in the constitutive activity of the NRP1 promoter. Mutation of the first or the second Sp1 sites resulted in a 20% reduction of the TPA-induced activity. Consistent with these results, the mutation of both Sp1 sites reduced the TPA-induced activity of the promoter by 40%. Together these results demonstrate that the two Sp1 elements, the AP-1 element, and the CCAAT box are critical for constitutive and TPA-induced NRP1 gene expression.

Transcription Factor Sp1 Binds to NRP1 Sp1A and Sp1B Elements

To determine whether DNA–protein complexes are formed on the Sp1A and Sp1B elements of the NRP1 promoter, nuclear extracts from unstimulated HeLa cells were analyzed by electrophoretic mobility shift assay (EMSA) using labeled oligonucleotides spanning either the Sp1A or the Sp1B elements (Fig. 7A). DNA–protein complexes were detected using the labeled Sp1A or Sp1B oligonucleotides (Fig. 7B, lanes 2 and 7). An excess of unlabeled Sp1A (lane 3), Sp1B (lane 8) oligonucleotides, or of consensus Sp1 oligonucleotide (lanes 5 and 10) competed the formation of the complexes, showing that these complexes were specific. On the other hand, unlabeled mutant Sp1A (lane 4) or Sp1B (lane 9) oligonucleotides did not inhibit formation of the complex. To investigate further the identity of the complex, supershift experiments using antibodies against the two ubiquitously expressed Sp family members (Sp1 and Sp3) were carried out. The complex was supershifted in the presence of Sp1 antiserum (Fig. 7C, lanes 2 and 5), but not in the presence of Sp3 antiserum (lanes 3 and 6), thus demonstrating that the observed complex is

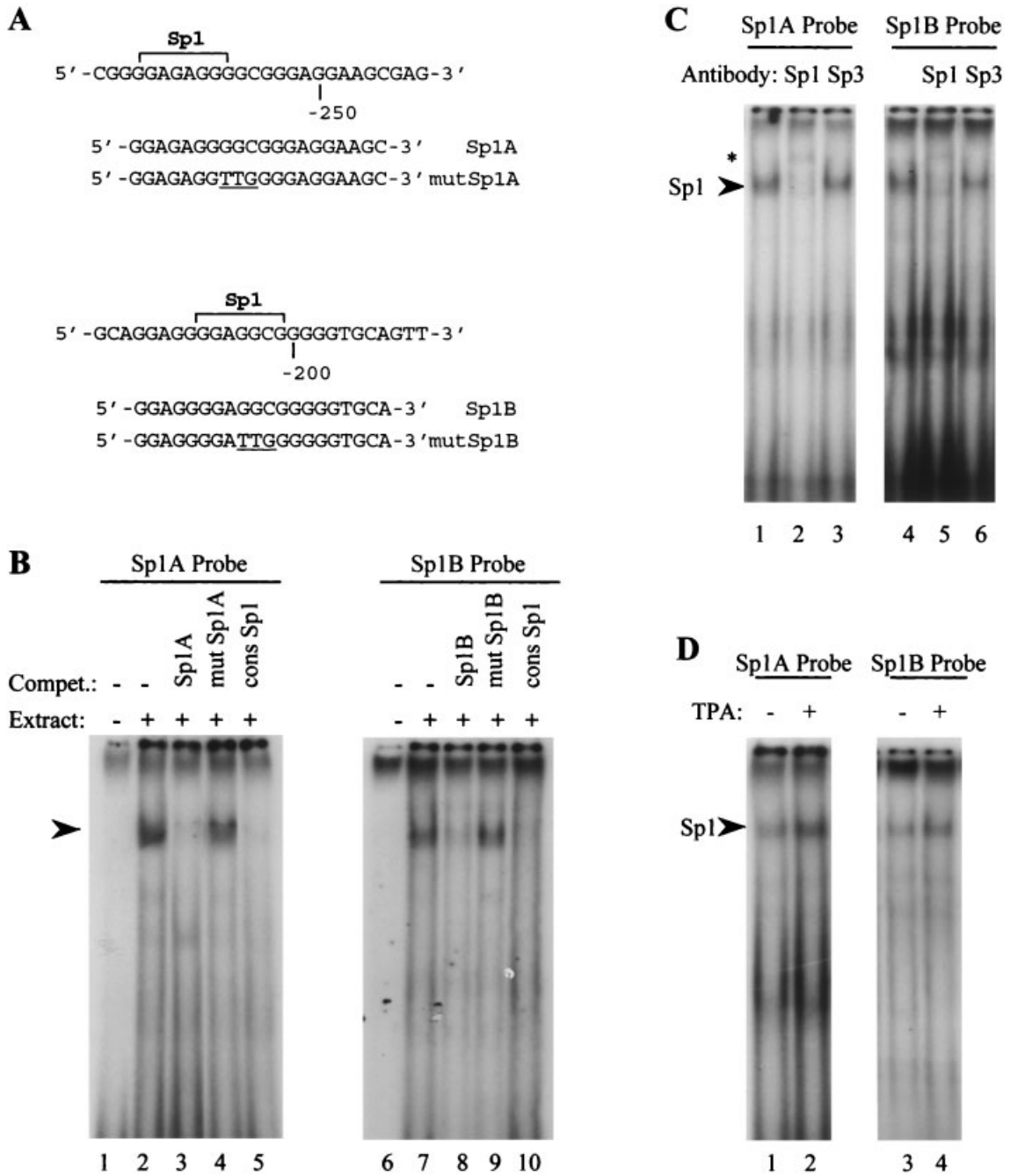

Fig. 7. Sp1 binding to NRP1 promoter elements. **A**: Double-stranded oligonucleotide sequences of NRP1 promoters elements used for gel shift analysis. Point mutations are underlined. **B**: Gel shift assays with ^{32}P-labeled NRP1 promoter sequence −264 to −245 (Sp1A probe) and −212 to −193 (Sp1B probe) either alone (**lanes 1** and **6**) or in the presence of HeLa nuclear extract (**lanes 2–5** and **7–10**). Competition for binding was carried out with 50-fold (lanes 3–5, and 8–10) molar excess of the unlabeled oligonucleotides as indicated. **C**: Supershift analysis in the presence of 200 ng of anti-Sp1 (lanes 2 and 5) or anti-Sp3 (lanes 3 and 6).The arrow indicates the Sp1 complex and the asterisks the supershifted complex. **D**: Gel shift assay with ^{32}P-labeled NRP1 promoter fragments, Sp1A and Sp1B, in the presence of untreated (lanes 1 and 3) or TPA-treated (lanes 2 and 4) HeLa nuclear extracts.

composed of Sp1. Sp1 binding to Sp1A and Sp1B elements was reproducibly induced upon TPA treatment (Fig. 7D, compare lanes 1 and 2, and lane 3 and 4). Taken together, our data show that the transcription factor Sp1 binds to the functional NRP1 Sp1A and Sp1B elements and that this binding is enhanced upon TPA treatment.

DISCUSSION

Current information on the transcriptional regulation of the NRP1 gene is limited to the demonstration that TNFα, dHAND, and Ets-1 are regulators of NRP1 expression [Giraudo et al., 1998; Yamagishi et al., 2000; Teruyama et al., 2001]. We decided to analyze the regulation of NRP1 expression in HeLa cells in response to TPA, a tumor promoter that activates a number of genes involved in cell proliferation and tumor progression such as growth factors and their receptors. The use of pharmacological inhibitors of signaling pathways activated by TPA demonstrated that TPA induction of NRP1 expression is mediated through both PKC and MEK/ERK signaling pathways. TPA enhanced NRP1 gene transcription but did not affect NRP1 mRNA stability. To identify the molecular components involved in both constitutive and TPA induced expression of the NRP1 gene in HeLa cells, the promoter regions of the human and mouse NRP1 genes were cloned and characterized. To our knowledge, this is the first report characterizing the NRP1 gene promoter. Deletion and site-directed mutagenesis analysis in combination with nuclear protein binding, competition, and supershift studies demonstrated that an AP-1 element, two Sp1 elements, and a CCAAT box contribute to constitutive and TPA-induced NRP1 promoter activity.

We demonstrated that mutation of both Sp1 binding sites almost totally abolished the constitutive activity of NRP1 promoter and reduced significantly TPA-induced activity. The commonly acknowledged role for Sp1 has been that it regulates constitutive expression levels. However, there are an increasing number of studies demonstrating that Sp1 mediates the activation of gene expression in response to extracellular stimuli as well. For example, a regulatory role for Sp1 has been reported for glucose activation of the carboxylase [Daniel and Kim, 1996] and plasminogen activator inhibitor-1 [Chen et al., 1998] promoters, and for TNF-α-induced VEGF gene expression in human glioma cells, respectively [Ryuto et al., 1996]. These results are consistent with our finding that Sp1 is not only involved in constitutive expression but also mediates inducible expression of the NRP1 gene in response to TPA.

Sp1-driven transcription can be due to an increased binding of Sp1 to its recognition sites [D'Angelo et al., 1996; Ryuto et al., 1996; Tanaka et al., 2000], which could reflect increased Sp1 abundance. Our study indicates that TPA slightly increases Sp1 binding to its recognition sites present in the NRP1 gene promoter. However, this increase can not be attributed to induced expression of Sp1, since Northern blotting as well as Western blotting analysis showed no difference in Sp1 mRNA and protein levels in quiescent or TPA-treated cells (data not shown). Sp1-driven increased transcription can also be due to recruitment of transcription factors, including GATA-1 [Merika and Orkin, 1995], NF-κB [Perkins et al., 1994], and $TAF_{II}110$ [Gill et al., 1994] or of co-activators, such as p300/CBP [Billon et al., 1999] interacting with Sp1. TPA-induced NRP1 gene expression is dependent on de novo protein synthesis, therefore, it is possible that TPA itself induces the production of a co-factor that will activate transcription of NRP1 gene through interaction with Sp1. A similar mechanism of induction of $p21^{WAF1/CIP1}$ has been described during NGF-mediated neuronal differentiation in which NGF-activated $p21^{WAF1/CIP1}$ gene transcription is due to the interaction between constitutively bound Sp1 and p300/CBP, whose production is induced by NGF [Biggs et al., 1996]. Consistent with this, we have found that co-transfection of p300 with the −823/+79 luciferase reporter construct induced the promoter activity by sixfold in HeLa cells (data not shown). Therefore, it is possible that p300/CBP is implicated in the induction of NRP1 gene transcription upon TPA treatment.

The AP-1 element present at −297 of the promoter is an imperfect consensus sequence for the AP-1 transcription factor-binding site NTGAGTCA [Angel et al., 1987]. The AP-1 transcription factor complex comprises the *c-fos* and *c-jun* proto-oncogenes that are known to be activated as a result of TPA stimulation of PKC-dependent pathways [Angel and Karin, 1991]. TPA induction of gene expression implicating AP-1 transcription factor is usually maximal as early as 2 h after stimulation [Angel and Karin, 1991; Tanaka et al., 2000]. In the case of NRP1, the induction by TPA was maximal after 8 h but was detectable by 4 h. Deletion of the AP-1 site in the human NRP1 promoter caused only a 25% reduction in the TPA effects on the NRP1 promoter. This AP-1 site is not conserved in the mouse promoter. Together these results suggest that AP-1 is not a major

contributor to the induction of NRP1 expression by TPA. However, AP-1 might be implicated in the early stage of TPA induction.

Mutagenesis analysis of the CCAAT box present at −50 of the NRP1 promoter has demonstrated that this box is implicated to some extent to the constitutive and TPA-induced activity of the promoter. CCAAT boxes are binding sites for numerous transcription factors, such as NF-Y, C/EBP, NF-1, and CDP [Mantovani, 1998]. However, according to a recent survey [Mantovani, 1998] of NF-Y binding CCAAT boxes and according to the MatInspector program [Quandt et al., 1995], the CCAAT box found in the NRP1 promoter is highly homologous to the binding site of NF-Y. NF-Y is a ubiquitous transcription factor formed by subunits A, B, and C [Kim et al., 1996] that specifically recognizes a CCAAT-box motif bound in the promoter and enhancer regions of many eukaryotic genes. Among these genes are some induced by cAMP [Mantovani, 1998]. Interestingly, NF-Y, like Sp1, binds to the coactivator p300/CBP, to activate the transcription of the human ferritin promoter upon cAMP treatment [Faniello et al., 1999].

NRP1 is part of a receptor family that includes NRP2, a receptor that has a 44% amino acid identity with NRP1 and that has a similar domain structure. The cloning and the characterization of the NRP1 and NRP2 genes have demonstrated that the two genes arose by gene duplication [Rossignol et al., 2000]. In order to determine whether NRP1 and NRP2 gene promoter regions shared sequence homology, we also cloned the human NRP2 gene promoter (accession number: AF281074). According to sequence analysis of the 5′ flanking region of the human NRP2 gene by the MatInspector program [Quandt et al., 1995] no typical mammalian TATA and CCAAT boxes were present. Although NRP1 and NRP2 promoters did not share any significant sequence homology, some potential transcription binding sites, such as Sp1 binding sites, two juxtaposed AP-2 and AP-4 binding sites, Ets-1 binding sites, and numerous myeloid zing finger-1 (MZF1) binding sites, are found in both promoters. MZF1 is a transcription factor implicated in the control of hematopoiesis. Interestingly, NRP1 was recently implicated in hematopoiesis [Tordjman et al., 1999]. The fact that the NRP1 and NRP2 gene promoters are related but not identical may explain why the two proteins have similar but not completely overlapping expression patterns. For example, during mouse brain development, NRP1 and NRP2 are expressed in neuronal cells but not necessarily the same ones [Chen et al., 1997; Kolodkin et al., 1997; Giger et al., 1998]. During chick embryonic development NRP1 and NRP2 are expressed in arterial and venous endothelial cells, respectively [Herzog et al., 2001; Moyon et al., 2001]. In neuroblastoma, both NRP1 and NRP2 are upregulated but at different stages of the disease [Fakhari et al., 2002].

In summary, we have cloned the NRP1 promoter and demonstrated that the transcription factor Sp1 and to a lesser extent the transcription factor AP-1 and a CCAAT box are modulators of constitutive and TPA-induced NRP1 activity. Future studies will further define those elements that specifically regulate NRP1 and NRP2 expression.

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