Human Nidogen Gene: Structural and Functional Characterization of the 5′-Flanking Region

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Nidogen is a sulfated multifunctional glycoprotein present in basement membranes. In this study, we have cloned the 5′-flanking region of the human nidogen gene. Initially, an ~35-kb DNA clone (NCos4) was isolated from a human cosmid genomic library. Southern hybridization of EcoRI-digested NCos4 allowed isolation of a 3.7-kb fragment, which was shown to contain a portion of intron 1, the entire exon 1, and ~0.9 kb of 5′-flanking sequences of the nidogen gene. Nucleotide sequencing of the 5′-flanking DNA revealed the presence of two canonic CCAAT consensus sequences in the antisense strand and a potential variant of the TATA motif, TATTT, in the sense strand. One putative AP-2 and six putative SP1 binding sites were also present. To test the functional promoter activity of the 5′-flanking genomic DNA, two nidogen promoter/CAT reporter gene constructs, with the promoter segment spanning from −864 to −1 and from −534 to −1, respectively, were developed and analyzed in transient transfections of human and mouse cell cultures. Both constructs showed clearly detectable promoter activity, and the activity of the larger construct could be up-regulated by 12-O-tetradecanoyl phorbol 13-acetate up to 2.5 times. The results indicate that the nidogen promoter/CAT gene constructs developed in this study provide a means to examine the transcriptional regulation of nidogen gene expression in human diseases of the basement membrane zone. *J Invest Dermatol 97:281–285, 1991*

Nidogen/entactin is a sulfated multifunctional glycoprotein present in basement membranes [1,2]. High-resolution immunoelectron microscopy has co-localized nidogen with laminin to a narrow area of the epithelial basement membrane [3]. Because of the intimate association of nidogen and laminin, it is conceivable that nidogen shares biologic activities previously assigned to laminin, including stimulation of neurite outgrowth, as well as attachment, migration, growth, and differentiation of cells.

Nidogen was initially isolated from mouse EHS (Englebreth-Holm-Swarm) tumors and cell cultures, and was shown to consist of a polypeptide of M_r ~150 kD [4–6]. Recent analyses of human nidogen cDNA have delineated the entire primary nucleotide sequence that encodes 1,247 amino acids, including a characteristically hydrophobic signal sequence [7,8]. The deduced amino acid sequence contained seven epidermal growth factor-like cysteine-rich repeats, one putative tyrosine O-sulfation site, and a possible N-glycosylation site. The tripeptide sequence, arginine-glycine-aspartic acid (RGD), a potential cell attachment site, was also identified [8].

The human nidogen cDNA have been utilized in Northern hybridizations of RNA isolated from a variety of human cell cultures, including skin fibroblasts, HT-1080 fibrosarcoma, and JEG-3 choriocarcinoma cells [7], as well as from human HeLa and mouse NIH-3T3 fibroblast cultures (O'Leary et al, unpublished results). Transcripts of ~6 kb were noted in RNA extracted from human skin fibroblast and HT-1080 fibrosarcoma cell cultures [7], but no detectable message was observed with HeLa or 3T3 cell cultures. An interesting finding was that human JEG-3 choriocarcinoma cells, which actively express other genes for basement membrane components, including the B1 chain of laminin, did not contain detectable levels of nidogen mRNA [7,9]. These observations suggested that the expression of genes encoding different basement membrane zone macromolecules can be uncoordinate and under differential regulatory control.

The recently developed human nidogen cDNA have also been used for preliminary characterization of the nidogen gene [7,8]. In situ chromosomal hybridizations suggested that nidogen is a single-copy gene at locus 1q43 of the human genome [7]. Southern analyses of human genomic DNA blots with nidogen cDNA revealed evidence for multiple restriction fragment-length polymorphisms (RFLP), and suggested that the entire nidogen gene encompasses ~50 kb [8,10]. However, the structural organization and transcriptional regulation of the nidogen gene are largely unknown.

To elucidate regulation of nidogen gene expression at the transcriptional level, we have isolated and characterized ~0.9 kb of 5′-flanking DNA of the human nidogen gene. Functional activity of the nidogen promoter was assessed by constructing chimeric human nidogen promoter/chloramphenicol acetyltransferase (CAT) reporter gene plasmids and utilizing them in transient tranfection experiments of cultured human and rodent cells. Our results provide novel information on the regulatory *cis*-elements and functional promoter activity of the 5′-flanking region of the human nidogen gene.

MATERIALS AND METHODS

Materials The following reagents were obtained from commercial sources, as indicated: restriction endonucleases and T4 DNA

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Abbreviations:
CAT: chloramphenicol acetyltransferase
TPA: 12-O-tetradecanoyl phorbol 13-acetate

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Table I. Oligonucleotide Primers Used for Amplification of Nidogen Promoter Sequences by PCR[a]

5′ primer for pNP864CAT:
5′-GGCTCGAGCTAAATTTGTGATTCAAGGCTGTA-3′
5′ primer for pNP534CAT:
5′-GGCTCGAGCGCGCAATGAATGGGTGGATGAGC-3′
3′ primer for both constructs:
5′-GGAAGCTTGTTCCCGAACTGCGGTCCCGCAAA-3′

[a] Conditions for PCR were: 94°C, 10 min; [94°C, 2 min; 54°C, 1.5 min; 73°C, 1 min] × 30; 73°C, 10 min. Note the presence of XhoI restriction sites in the 5′ primers and of a Hind III restriction site in the 3′ primer (underlined).

ligase, Boehringer Mannheim Biochemicals; polynucleotide kinase, New England Biolabs; Sequenase Version 2.0, U.S. Biochemicals; Bluescript, Stratagene; pSV2CAT, Pharmacia; nick translation kit, Promega; α[^{32}P]dCTP, γ[^{32}P]ATP and [^{35}S]dATP, New England Nuclear; Taq DNA polymerase, Perkin-Elmer; protein assay kit, BioRad. Oligonucleotides were synthesized with an automated DNA synthesizer (Coder 300, Dupont). The pHV4 cosmid vector was graciously provided by Dr. Henrik Vissing.

Construction and Screening of a Cosmid Genomic DNA Library DNA was prepared from human peripheral blood lymphocytes, partially digested with Sau3A and fractionated on a 1.25–5.0 M NaCl gradient in 10 mM Tris-HCl (pH 7.4), containing 1 mM EDTA [11,12]. The 35–50-kb fraction was isolated and ligated to pHV4 cosmid arms. The pHV4 vector is a variant of the cosmid vector pTCF [12], in which the HpaI site is replaced by an XhoI site and a polylinker cloning region containing a BamHI site is inserted. The cosmid vector pHV4 polylinker region is also flanked by the T3 and T7 RNA polymerase promoters. The Rec A$^-$ strain ED8767 cells were infected with packaged cosmids, and approximately 4×10^5 colonies, representing five human genomes, were plated and screened [13].

The genomic library was screened with a 0.7-kb 5′ subclone of a previously characterized human nidogen cDNA (cHFN-7; [8]). The cDNA probe was radiolabeled by nick translation and hybridized to the genomic DNA library filters [14]. Prehybridization and hybridization of the cosmid filters were performed in 5 × SSC, 2 × Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA, at 65°C for 15 h. The filters were washed twice, 15 min each, at room temperature in a solution consisting of 2 × SSC and 0.1% SDS, followed by one wash in 0.2 × SSC and 0.1% SDS at 65°C for 45 min. The primary screen yielded three positive clones, which were purified and amplified, as previously described [15].

Construction of Nidogen Promoter/Chloramphenicol Acetyltransferase Reporter Gene Plasmids A promoterless chimeric plasmid, pBS0CAT, was previously generated by subcloning a 1.6-kb HindIII-BamHI fragment of pSV2CAT, containing the entire CAT gene, small t intron, and the polyadenylation signal, into the polylinker region of the plasmid Bluescript (pBSKS) [15]. The plasmid pBS0CAT was used to develop two different nidogen promoter/CAT reporter gene constructs. First, a double-stranded DNA fragment extending from −1 to −534 was generated by polymerase chain reaction (PCR) amplification. The primers used for PCR were 32 nucleotides in length and contained an XhoI site in the 5′ oligomer and a HindIII site in the 3′ oligomer, to enable direct cloning of the PCR product into pBS0CAT (Table I). The resultant chimeric plasmid, pNP534CAT, contained nidogen promoter sequences directly upstream of the entire CAT gene, small t intron, and polyadenylation signal, all within the polylinker region of the plasmid, Bluescript. Secondly, a construct, pNP864CAT, containing nidogen promoter DNA sequences from −1 to −864, was generated in a similar manner (Table I).

Transient DNA Transfections and CAT Assays Mouse NIH-3T3 cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, glutamine, penicillin, and streptomycin. Human skin fibroblasts and human HT-1080 fibrosarcoma cells were grown in the same medium, but supplemented with 10% fetal calf serum instead of calf serum. The cells were transfected with plasmid DNA (10 or 20 μg) using the calcium phosphate co-precipitation method [16]. For transfections, $2-3 \times 10^5$ cells were plated on 28-cm^2 plastic dishes. The following day the cells were incubated with calcium phosphate/DNA precipitate for 4 h and then exposed to 15% glycerol in 0.15 M NaCl, 1.4 mM Na_2HPO_4, 25 mM Hepes, pH 7.12, for 2 min ("glycerol shock"). Subsequently, the cells were washed twice with phosphate-buffered saline and incubated for 48 h in the growth medium supplemented either with 1% or 10% fetal calf serum. In some experiments, 12-O-tetradecanoyl phorbol 13-acetate (TPA) (100 ng/ml, dissolved in DMSO) or all-*trans*-retinoic acid (10^{-6} M, dissolved in ethanol) was added at approximately 30 min following the exposure of the cells to glycerol. The cells were then harvested, lysed by freeze-thawing in 0.25 M Tris-HCl, pH 7.8, and appropriate amounts of protein from each cell extract were used to determine the CAT activity, as described previously [17]. The acetylated and non-acetylated forms of [^{14}C]chloramphenicol were separated using thin-layer chromatography (TLC) and visualized by autoradiography. The CAT activity was quantified by cutting all forms of [^{14}C]chloramphenicol out of the TLC plate and measuring the radioactivity using liquid scintillation counting. The transfection efficiencies were determined by assaying the relative plasmid copy number in cell cultures transfected with

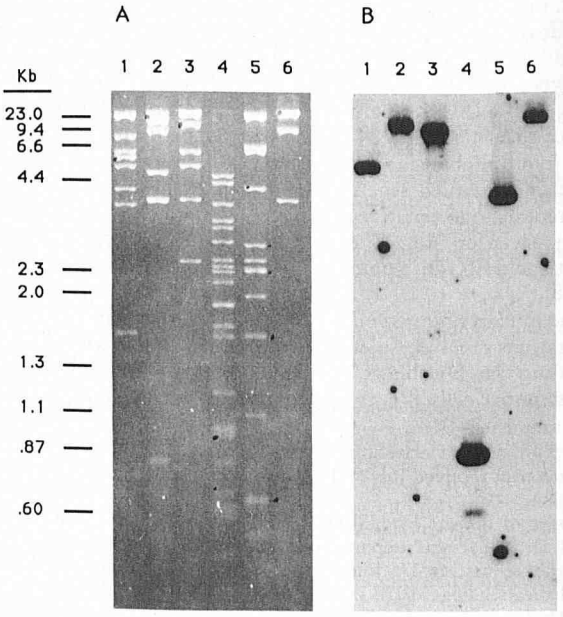

Figure 1. Restriction endonuclease digestions and Southern analyses of human nidogen genomic DNA. *A*: One microgram of NCos4 was digested with XhoI, *lane 1;* XbaI, *lane 2;* ClaI, *lane 3;* PstI, *lane 4;* EcoRI, *lane 5;* SalI, *Lane 6.* The fragments were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed under UV light. The sizes of molecular weight DNA standards are indicated on the *left side of panel A. B:* Autoradiograph of the Southern blot of fragments shown in *panel A,* after hybridization to a radiolabeled antisense oligonucleotide corresponding to the signal sequence of the human nidogen mRNA (see Fig 2B; [8]).

pNP534, pNP864, pSV2CAT, and pBS0CAT, as described previously [18].

RESULTS

Isolation of Genomic DNA Containing the 5′-Flanking Region of the Nidogen Gene A 0.7-kb 5′ fragment of a recently characterized human nidogen cDNA (cHFN-7; [8]) was used to screen a human cosmid genomic DNA library. A genomic clone (NCos4), ~35 kb in size, was isolated and characterized by multiple restriction endonuclease digestions, followed by Southern hybridizations (Fig 1). Hybridization with a synthetic 21-bp oligomer, corresponding to the signal sequence of human nidogen, allowed isolation of a 3.7-kb EcoRI fragment (Fig 1*B, lane 5*). Characterization of this 3.7-kb fragment revealed that it contained sequences corresponding to intron 1, the entire exon 1, and ~0.9 kb of 5′-flanking sequences (Fig 2*A*). Sequencing of this DNA fragment, using exon 1-specific oligomers as primers, allowed delineation of the exon 1/intron 1 border, 5′-GAGTCTACGT/CACCACAAAT-3′. These genomic sequences, when compared with the nucleotide sequence of nidogen cDNA [8], indicated that the segment of exon 1 extending from the ATG translation initiation site downstream to the intron 1 border consists of 226 nucleotides.

Sequence Analyses of the 5′-Flanking DNA Nucleotide sequencing of the ~0.9 kb upstream from the ATG site revealed several interesting features (Fig 2*B*). Specifically, two canonic CCAAT motifs were present in the antisense strand at positions −190 to −194 and −431 to −435. No canonical TATA sequence was found, although a variant of this sequence (TATTT) [19] was located at −120 to −124. The absence of a canonical TATA sequence probably accounts for the presence of multiple transcription initiation sites in the nidogen gene ([8] and unpublished observations), in analogy to similar findings in other genes lacking a TATA box [20–22]. In addition to these motifs, six putative SP1 binding sites and one putative AP-2 binding site (−235 to −242) were identified (Fig 2). A potential TPA-responsive element (TGATTCA) [23] was found at position −848 to −855. Furthermore, two sequences resembling a recently characterized retinoic acid-responsive element (GTTCAC) [24] were recognized at positions −282 to −287 and −617 to −622 (Fig 2). In general, the 5′-flanking region was relatively rich in guanine and cytosine (64.3%). Collectively, the lack of a canonical TATA sequence and the abundance of C and G are features associated with promoter regions of so-called "housekeeping genes," genes encoding proteins involved in general metabolic activity of many cells [25]. The above data suggest that this ~0.9-kb fragment of 5′-flanking DNA is representative of the promoter region of the nidogen gene.

Expression of Nidogen Promoter/Chloramphenicol Acetyltransferase Reporter Gene Constructs in Cultured Cells To test the functional promoter activity of the 5′-flanking region of the nidogen gene, we constructed two chimeric nidogen promoter/CAT reporter gene plasmids: pNP534CAT (−534 to −1) and pNP864CAT (−864 to −1) (Fig 2*C*). These two constructs were then utilized in transient transfections of several different mammalian cell lines. All transfections were performed in parallel with

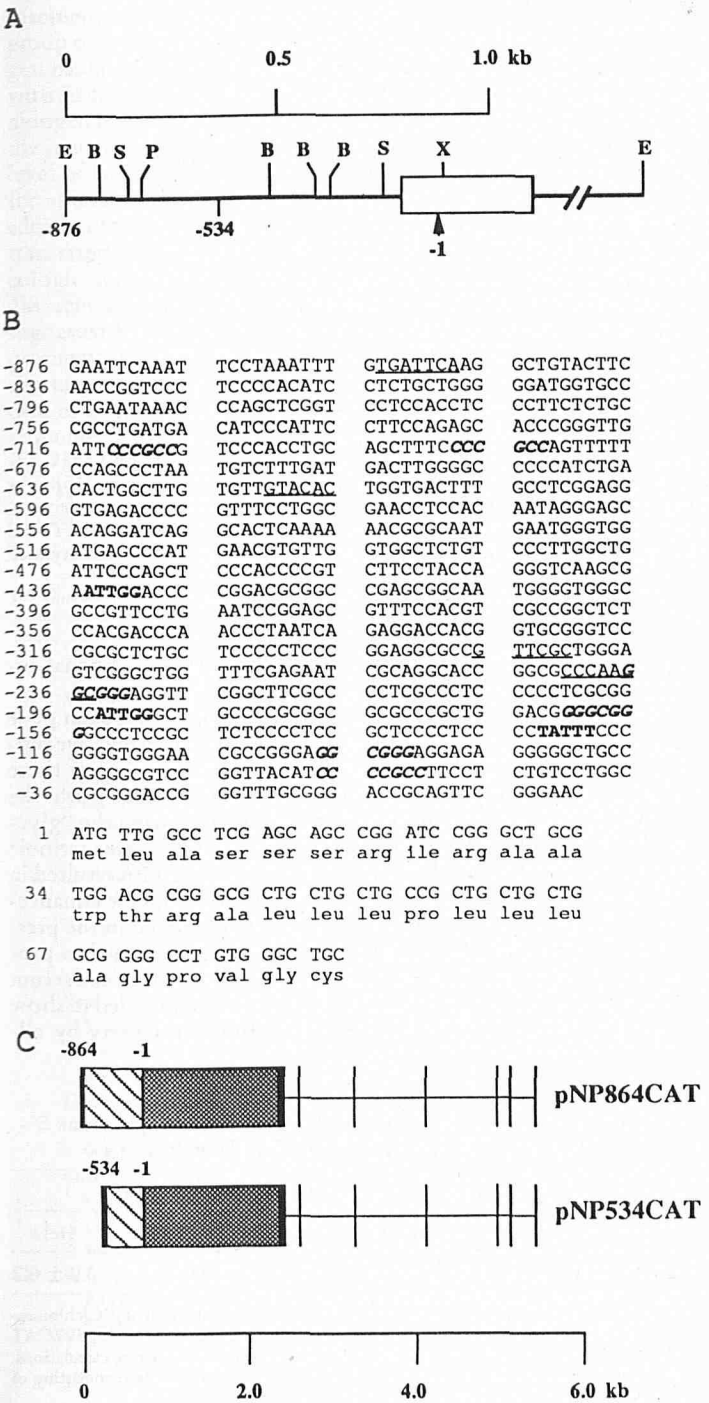

Figure 2. Schematic illustration of the 5′-flanking region of the human nidogen gene *(A)*, the nucleotide sequences of the promoter region and the coding region corresponding to the signal peptide *(B)*, and the structures of chimeric nidogen promoter/CAT reporter gene plasmids *(C)*. *A:* The segment extending from −1 to −876 of the 3.7-kb EcoRI DNA fragment (see *Fig 1*) was characterized by restriction endonuclease digestions. E, EcoRI; B, BanI; S, SmaI; P, PstI; X, XhoI. The *open box,* containing the translation initiation site *(arrowhead),* corresponds to exon 1 of the human nidogen gene. *B:* Nucleotide sequence of the 5′ end of the human nidogen gene. The sequences comprising the 5′-flanking region from −1 to −876 are numbered from the ATG translation initiation site. The nucleotide sequences from 1 to 84 correspond to the signal peptide of the human nidogen [8], and the deduced amino acid sequence is indicated in the *line* below the nucleotide sequence. The following putative *cis*-acting elements are indicated: six putative SP1 binding sites *(boldface italics);* a TATTT motif *(boldface)* at −120 to −124; two CCAAT motifs *(boldface)* within the antisense strand at −190 to −194, and −431 to −435; a putative AP-2 binding site CCCAAGGC *(underlined)* at −235 to −242; two putative retinoic acid responsive elements *(underlined)* at −282 to −287 and −617 to −622; a TPA-responsive element *(underlined)* at −848 to −855. *C:* Schematic presentation of linearized human nidogen promoter/CAT reporter gene plasmids. The pNP864CAT contains human nidogen promoter region sequences extending from −864 to −1 and pNP534CAT promoter segment extends from −534 to −1 (▨). The promoter DNA was cloned upstream of the entire CAT structural gene (▩) in the plasmid Bluescript.

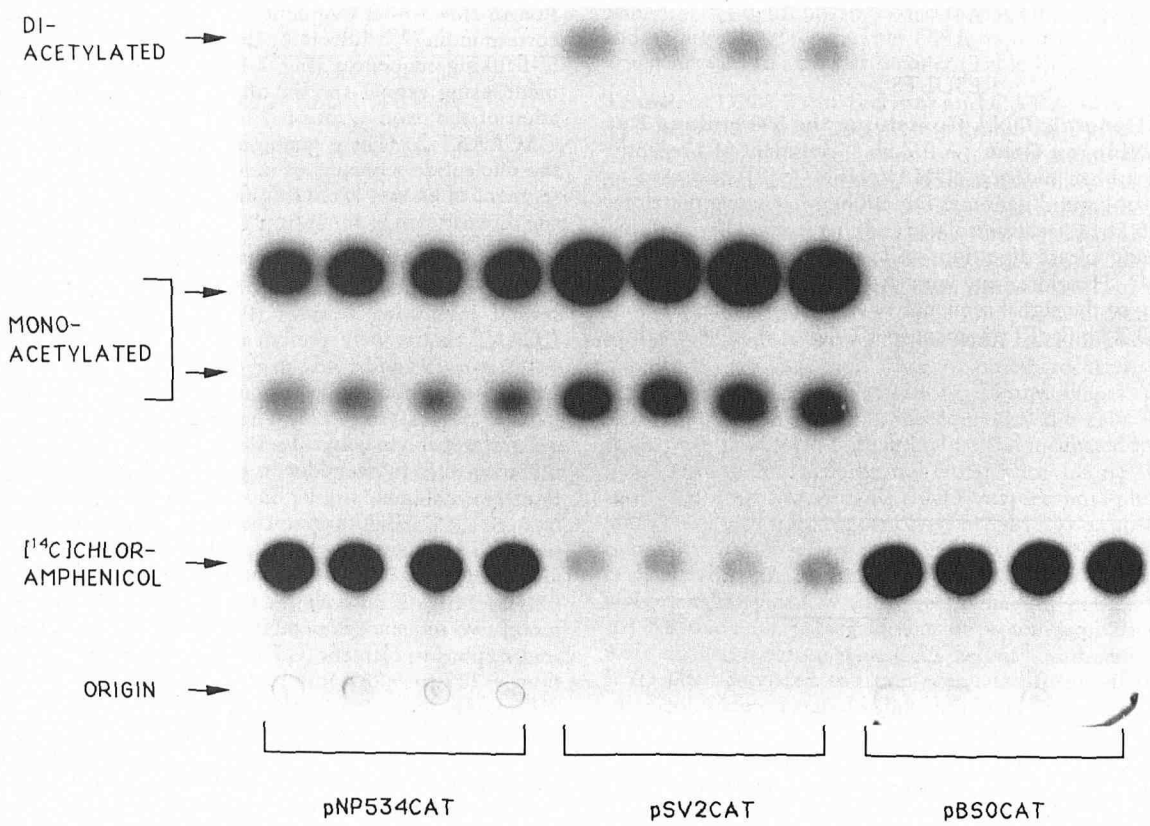

Figure 3. Autoradiogram of a CAT assay of cultured human HT-1080 fibrosarcoma cells transfected with the following chimeric plasmids: pNP534CAT, containing the nidogen promoter DNA fragment; pSV2CAT, containing the early region promoter of SV40; pBS0CAT, a promoterless plasmid [15]. The cells were transfected with 20 μg of plasmid DNA using the calcium phosphate/DNA co-precipitation method, and harvested at 48 h after transfection. Protein content of the cell extracts was quantified and aliquots containing 170 μg of protein from cultures transfected with pNP534CAT and pBS0CAT, and 20 μg from cultures transfected with pSV2CAT, were used in the CAT assay. The positions of [^{14}C]chloramphenicol and its monoacetylated and diacetylated derivatives, as well as the origin of the chromatograph, are indicated.

pSV2CAT, containing the early region promoter of SV40 (positive control) [17], and with pBS0CAT (negative control) [15].

Transfection of human fibrosarcoma HT-1080 cells with pNP534CAT resulted in significant CAT activity, well above that noted with pBS0CAT (Fig 3, Table II). Expression of the CAT gene was also noted in mouse NIH-3T3 fibroblasts, and HeLa cells (Table II), as well as in human skin fibroblasts (not shown) in culture. In three separate experiments, each carried out as 3 or 4 parallel determinations, the relative level of promoter activity observed in HT-1080 cells transfected with pNP864CAT was 50.8 ± 2.9% (mean ± SD) of that noted with pNP534CAT. The transfection efficiency of these two constructs was similar, as determined by the relative plasmid copy number [18]. Thus, these results suggest that the upstream sequences between −534 and −864 contain down-regulatory *cis*-elements.

A comparative analysis of CAT activity in HT-1080, NIH-3T3, and HeLa cells was performed utilizing pNP534CAT (Table II). As expected from previous nidogen mRNA expression studies [7], human HT-1080 fibrosarcoma cells demonstrated significantly greater CAT activity than the other two cell lines studied. These results suggest that the 534-bp 5′-flanking segment contains some *cis*-acting elements contributing to cell-specific transcription of the nidogen gene. However, an interesting observation was the presence of CAT activity in HeLa cells, which do not express detectable levels of nidogen mRNA. These seemingly contradictory findings probably result from the presence of *cis*-acting elements, such as SP1 and AP-2 binding sites, which have been shown to enhance general transcriptional activity in a variety of genes [26]. Further analyses of the nidogen genomic DNA upstream sequences, as well as sequences within the first intron [27–29], may reveal additional *cis*-acting regulatory elements.

As indicated above, the 5′-flanking region of the nidogen gene contained a putative TPA-responsive element and two putative retinoic acid-responsive elements. To test the functionality of these *cis*-elements, transient transfections of HT-1080 cells with the pNP864CAT construct were performed, and following the "glycerol shock," either 100 ng/ml of TPA or 10^{-6} M all-*trans*-retinoic acid was added to the culture medium. Addition of TPA resulted in the up-regulation of the promoter activity, the highest enhancement being ~2.5 times over the controls when tested in the presence of 1% fetal calf serum. A variable, and somewhat less pronounced, effect was observed in the presence of 10% fetal calf serum (results not shown). In contrast, several experiments failed to show significant modulation of the nidogen promoter activity by all-

Table II. Promoter Activity of the Human Nidogen Gene 5′-Flanking DNA in Transient Cell Transfections

Chimeric Plasmid	Relative CAT Activity (%)[a]		
	HT-1080	NIH-3T3	Hela
pNP534CAT	8.2 ± 0.7	3.1 ± 1.0	3.0 ± 0.2

[a] The relative CAT activity was calculated as percent of conversion of [^{14}C]chloramphenicol to its acetylated forms in relation to the activity obtained with pSV2CAT (100%). The baseline value noted with pBS0CAT was subtracted before calculations. The values represent the mean ± SD of 2–4 separate experiments each consisting of 2–4 parallel transfections with each cell line.

trans-retinoic acid at 10^{-6} M concentration. These observations suggest that the TPA-responsive element (the AP-1 binding site) is indeed functional in the nidogen promoter. The lack of response to all-*trans*-retinoic acid does not vigorously exclude the functionality of retinoic acid-responsive *cis*-elements in the nidogen promoter, because the presence of *trans*-acting retinoic acid receptors (RAR) in HT-1080 cells is not known.

DISCUSSION

Identification of functional promoter activity within the 5′-flanking region of the nidogen gene provides a model to analyze *trans*-acting regulatory elements in a variety of cells expressing the nidogen gene. Also, detection of nidogen gene promoter activity in human skin fibroblast cultures provides a system to study transcriptional control of nidogen gene expression in genetic diseases affecting the basement membrane zone [30], such as epidermolysis bullosa, a group of primary blistering disorders. It should be noted that nidogen has been recently excluded as a candidate gene in large kindred with EBS-2 showing linkage to chromosome 1 [10]. Nevertheless, nidogen may well be a candidate gene in other forms of EB, such as the junctional variants, which affect the basement membrane at the level of lamina lucida. It should be noted that there is no evidence for altered nidogen gene expression in any form of EB as yet. In addition to disease states, our results provide a system to study the transcriptional modulation of nidogen gene expression by pharmacologic agents or by immunologic affector molecules, including different cytokines. The importance of the latter experiments is suggested by the presence of a putative AP-2 binding site in the promoter region of the nidogen gene (see Fig 2). The AP-2 binding site has been shown to mediate cAMP- and TPA-mediated induction of gene transcription [23]. Furthermore, identification of two sequence motifs resembling those recently demonstrated to be retinoic acid-responsive elements in the retinoic acid receptor β gene [24] suggests that retinoids may also play a role in the regulation of nidogen gene expression.

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