Differential regulation of the human nidogen gene promoter region by a novel cell-type-specific silencer element

Marlies ZEDLACHER, Marion SCHMOLL, Katrin ZIMMERMANN, Oliver HORSTKORTE and Roswitha NISCHT¹

Department of Dermatology, University of Cologne, D-50924 Cologne, Germany

Transfection analyses of the human nidogen promoter region in nidogen-producing fibroblasts from adult skin revealed multiple positive and negative *cis*-acting elements controlling nidogen gene expression. Characterization of the positive regulatory domains by gel mobility-shift assays and co-transfection studies in *Drosophila* SL2 cells unequivocally demonstrated that Sp1-like transcription factors are essential for a high expression of the human nidogen gene. Analysis of the negative regulatory domains identified a novel silencer element between nt –1333 and –1322, which is bound by a distinct nuclear factor, by using extracts from adult but not from embryonal fibroblasts. In embryonal fibroblasts, which express significantly higher amounts of nidogen mRNA as compared with adult fibroblasts, this inhibitory

nidogen promoter region did not affect nidogen and SV40 promoter activities. The silencer element seems to be active only in nidogen-producing cells. Therefore this regulatory element might function *in vivo* to limit nidogen gene expression in response to external stimuli. However, none of the identified regulatory elements, including the silencer, contribute significantly to cell-specific expression of the human nidogen gene. Instead we provide evidence that gene expression in epidermal keratinocytes that are not producing nidogen is repressed by methylation-specific and chromatin-dependent mechanisms.

Key words: fibroblasts, gene activation, gene repression, keratinocytes, Sp1.

INTRODUCTION

Nidogen (entactin) is a widespread basement membrane glycoprotein that consists of a single 150 kDa polypeptide chain [1,2]. Recombinant mouse and human nidogen have been shown to bind with high affinity to the three major basement membrane components, laminin, collagen IV and perlecan, at independent binding sites, allowing the formation of ternary complexes [3–6]. It is therefore assumed that nidogen is an essential mediator, connecting the independent networks of collagen IV and laminin, and is required for stable basement membrane formation.

Mouse nidogen was shown to be of mesenchymal origin during embryonic development in many organs and to be integrated into basement membranes in close vicinity to epithelial cells. In contrast with nidogen, the other basement membrane components are expressed in mesenchymal and/or epithelial cells, depending on the organ and developmental stage [7–9]. The importance of epithelial–mesenchymal co-operation has been shown by using antibodies that block nidogen–laminin interaction. Inhibition of this interaction resulted in perturbation of epithelial differentiation processes in kidney and lung [9] and the submandibular gland [10], thereby suggesting that nidogen might be one of the mesenchymal proteins directly involved in epithelial–mesenchymal interactions.

These observations clearly suggest that nidogen gene expression has to be regulated differentially in a cell-type-specific manner. However, analysis of the 5′-end sequences of the human and murine nidogen genes revealed a GC-rich promoter with multiple transcription start sites, features usually associated with so-called housekeeping genes [11–13]. Indeed, preliminary transfection studies showed the activity of human and murine nidogen promoter fragments in cell lines of different origins, indicating that these promoter sequences do not contribute significantly to the cell-type-specific expression of the nidogen gene [11,12]. Because nidogen is a critical component of all

basement membranes and contributes functionally to the regulation of epithelial processes by the mesenchyme, it is important to clarify the regulatory roles performed by the proximal 5′ region of the nidogen gene.

Here we demonstrate the roles performed by Sp1-like transcription factors in fibroblasts (cells expressing nidogen) and keratinocytes (cells not expressing nidogen) and have identified a novel transcriptional silencer complex active in adult fibroblasts but not in embryonal fibroblasts.

MATERIALS AND METHODS

Cell culture techniques

The human SV40-transformed fibroblast cell line Wi26 (American Type Culture Collection), human fibroblasts from skin biopsies of healthy volunteers and from human embryonic skin (12 weeks of gestation, obtained at legal abortion) and keratinocytes were routinely propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), L-glutamine (300 µg/ml) and penicillin/streptomycin. Cultures of primary human fibroblasts were established by outgrowth from skin biopsies of healthy volunteers and used at confluence in passages 3–6. Keratinocytes were isolated from healthy, full-thickness skin obtained from reduction mammoplasty and abdominoplasty and cultivated in DMEM supplemented with 5% (v/v) FCS [14]. Each experiment was performed with cells derived from a different individual. The primary cell suspension was plated on collagen-coated tissue culture dishes. For treatment with 5-azacytidine (Sigma) or trichostatin A (Wako Biochemicals), fibroblasts were seeded at a density of 2×10^6 per 100 mm dish and keratinocytes at a density of 10^6 per 60 mm dish. After 24 h, 5-azacytidine (10 µM) or trichostatin A (2.5 µM) was added to the culture medium. During the 5-azacytidine treatment the cells were re-fed daily with DMEM containing 10% (v/v) FCS and 10 µM 5-azacytidine. At

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

¹ To whom correspondence should be addressed (e-mail Roswitha.Nischt@uni-koeln.de).

Figure 1 Functional analyses of the human nidogen gene promoter in dermal fibroblasts producing nidogen and in epidermal keratinocytes not producing nidogen

Adult epidermal keratinocytes (open bars), adult dermal fibroblasts (filled bars) and embryonic dermal fibroblasts (hatched bars) were transiently transfected with various 5'-deletion/luciferase nidogen promoter constructs as described in the Materials and methods section. The position of the 5' end of each construct is indicated. The putative Sp1-like binding sites are marked by circles. The luciferase activities, corrected for transfection efficiency, were calculated relative to the activity of the longest promoter construct NDP-9, which in each cell type was set arbitrarily at 100%. Luciferase activities obtained with the promoter construct NDP-9 expressed in relative light units (means $\pm$ S.E.M.) after normalization for β -galactosidase activity were: 18.5 ± 3.2 in adult fibroblasts, 17.1 ± 0.6 in embryonic fibroblasts, and 35.9 ± 3.6 in keratinocytes. At least three independent transfections were performed in duplicate with each construct.

indicated times, total cellular RNA was isolated. *Drosophila* Schneider SL2 cells were grown in Schneider's *Drosophila* medium (Gibco BRL) supplemented with 12% (v/v) FCS at 25 °C.

Plasmids and constructs

The Sp1 expression plasmid pPacSp1 and the empty pPac0 were kindly provided by Dr. Robert Tjian (Howard Hughes Medical Institute, Berkeley, CA, U.S.A.) and the Sp3 expression plasmid pPacUSp3 was provided by Dr. Guntram Suske (Klinikum der Philipps-Universität Marburg, Marburg, Germany). The promoterless chimaeric pGL2-basic (Promega) containing the coding region of the firefly luciferase gene was used to establish nidogen promoter/reporter gene constructs. The longest nidogen promoter construct, NDP-9, was subcloned from the previously described cosmid cos 5 [13] into pBluescript KS(+) (Stratagene). All 5'-deletion constructs were generated in this plasmid by using endogenous restriction sites. Subsequently these plasmids were digested at appropriate restriction sites in the polylinker to allow insertion into the vector pGL2-basic. All promoter constructs had the same 3' end, including the transcription start sites and 86 bp of the 5' untranslated region and the first exon. Nucleotides are numbered relative to the transcription start site at nt +2381 (GenBank Accession Number X82245). The promoter constructs were prepared by using the following restriction enzymes (the numbers given in parentheses correspond to the 5' end of the promoter fragment): NDP-9 (*Stu*I, -1915), NDP-7 (*Bsp*HI,

-1351), NDP-6 (*Pvu*II, -1102), NDP-5 (*Eco*RI, -807), NDP-4 (*Pst*I, -635), NDP-2 (*Eag*I, -360) and NDP-1 (*Bss*HII, -122). The promoter fragment -1351 to -1102 was isolated from NDP-7 by digestion with *Sma*I/*Pvu*II and ligated into the *Sma*I-digested plasmid pGL2-SV40 in both orientations upstream of the SV40 promoter and downstream of the reporter gene transcription unit. In addition, two overlapping (73 bp) fragments spanning the 5' half (-1351 to -1177) and the 3' half (-1250 to -1102) of this region were generated by PCR and inserted upstream of the SV40 promoter into the plasmid pGL2-SV40/promoter. The plasmid pAS-Sp1 was generated by introducing the coding sequence for Sp1 in an anti-sense orientation into an expression plasmid driven by the CMV promoter and enhancer.

Transfection and assessment of promoter activity

The above-described luciferase expression plasmids were used to transfect adult and embryonic fibroblasts by electroporation and adult keratinocytes by lipofectamine, in accordance with the supplier's protocol (Gibco BRL). A β -galactosidase reference plasmid (pCMV-gal) was co-transfected (molar ratio 1:10) for monitoring the transfection efficiencies and normalizing the data accordingly. As a negative control, the promoterless vector pGL2-basic was used. For transfection of fibroblasts 2×10^6 cells were mixed with 25 μ g of the luciferase promoter construct in 200 μ l of DMEM supplemented with 1% (v/v) FCS. After

30 min on ice, the mixture was rapidly warmed and then transfected by the application of an electric pulse (Gene Pulser II; Bio-Rad; 0.2 kV, 200 μ F). After 30 min at room temperature cells were plated in six-well plates and further cultivated in standard growth medium. Primary keratinocytes were seeded on six-well plates and transfected at 50–70% confluence in serum-free DMEM with 1 μ g of plasmid DNA and 8 μ g of lipofectamine. After 6 h the medium was replaced by standard growth medium. *Drosophila* SL2 Schneider cells (5×10^6 cells per 60 mm dish) were transfected with 6 μ g of the promoter/reporter gene construct together with 1.5 μ g of pPacSp1, pPacUSp3 or pPac0 by the calcium phosphate/DNA co-precipitation method. The DNA precipitates were left on the cells for 48 h. For *Drosophila* SL2 cells, the values obtained for luciferase activity were normalized to identical amounts of protein in the cell lysates, which were determined with a commercial assay (Bio-Rad). For all cell types the reporter gene assays were performed 48 h after transfection. In brief, for the enzyme assays the cells were harvested and resuspended in lysis buffer in accordance with the luciferase reporter gene system purchased from Promega (Madison, WI, U.S.A.). Samples (50 μ l) of the supernatants were used to determine luciferase activity (Lumat LB 9501; EG-G Berthold, Bad Wildbad, Germany); 7 μ l samples were used for the determination of β -galactosidase activity in accordance with the supplier's protocol (Tropix; Perkin Elmer).

RNA isolation and RNase protection assays

Total cellular RNA from cultured cells and tissue was isolated with the RNazol reagent by following the supplier's protocol (Wak-Chemie, Bad Soden, Germany). A 317 bp nidogen cDNA fragment spanning the region from nt +1222 to +1539 [15] was generated by PCR and inserted into the TA-cloning vector pCR2.1 (Invitrogen). The anti-sense transcript was synthesized after linearization of the plasmid by using T7 polymerase and [32 P]UTP (800 Ci/mmol; Amersham). The full-length transcript (389 nt) was purified on a 4% (w/v) polyacrylamide gel. Samples of total RNA (20 μ g) were hybridized overnight in 80% (v/v) formamide/40 mM Pipes (pH 6.4)/400 mM NaCl/1 mM EDTA at 46 °C with the labelled anti-sense transcript. Hybrids were digested for 45 min at 37 °C with 10 μ g of RNase A (Sigma) and 200 units of RNase T1 (Boehringer Mannheim). The reaction was stopped by the addition of 40 μ g of Proteinase K and 0.25% SDS, followed by incubation at 37 °C for 15 min. Protected fragments were separated on 4% (w/v) polyacrylamide/8 M urea gels and analysed by autoradiography. tRNA (50 μ g) was used as a negative control. As an internal control for the quality and amount of RNA used in the assays a riboprobe for the S26 ribosomal protein (205 nt) was included [16].

Electrophoretic mobility-shift assays

Nuclear protein extracts were prepared as described by Schreiber et al. [17]. The protein concentration of nuclear extracts was determined with a commercial assay (Bio-Rad). Oligonucleotides annealed in equimolar amounts and PCR fragments were end-labelled with [γ - 32 P]ATP. For binding assays, 1 μ g of nuclear extract was preincubated for 5 min at room temperature with 2.5 μ g of poly(dA-dT) in binding buffer [25 mM Hepes (pH 7.9)/60 mM KCl/2 mM MgCl₂/0.1 mM EDTA/2 mM dithiothreitol/4 mM spermidine/10% (v/v) glycerol; final volume 20 μ l]. When indicated, unlabelled competitor oligonucleotides or PCR fragments were preincubated with nuclear proteins 10 min before the addition of the labelled probe. Labelled double-stranded probes (15 fmol) were added to the binding reaction and incubation continued for 15 min. Samples were analysed on

0.5 $\times$ TBE (1 $\times$ TBE is 50 mM Tris/50 mM boric acid/1 mM EDTA)/5% (w/v) polyacrylamide gels. Oligonucleotides used were as follows: Sp1 (5'-GTTAGGGGCGGGATGGGCG-GAGTT-3'), PEA3 (5'-ATCAAGAGGATGTTATAAAGCA-3'); 5'-CCCCGGACGCGGCCG-3' and 5'-GCGGGCGCGC-CGCGG-3' for the PCR fragments F1 (–360 to –122), and 5'-CCCGCCGTCCCACCTGC-3' and 5'-GTGTACAACACA-AGCC-3' for F2 (–635 to –561).

RESULTS

Functional analyses of the human nidogen gene promoter in dermal fibroblasts producing nidogen and epidermal keratinocytes not producing nidogen

To identify regulatory elements controlling human nidogen gene transcription and to test whether these elements contribute to cell-type-specific transcription of this gene, comparative transfection analyses were performed with a series of 5'-deletion/luciferase reporter gene constructs (Figure 1). Adult and embryonal skin fibroblasts, expressing high levels of nidogen mRNA, and epidermal keratinocytes, which do not express nidogen mRNA (results not shown), were transiently transfected with the indicated nidogen promoter constructs. Transfection of adult and embryonal fibroblasts with progressively longer nidogen promoter constructs, NDP-1, NDP-2 and NDP-4, did not reveal significant differences in luciferase activities between these two cell types. Lengthening these constructs resulted in a gradual increase in luciferase activity, achieving 80% of the activity of

Table 1 Sp1 and Sp3 transactivate human nidogen promoter constructs

Induction of promoter activity by Sp1 and Sp3 was determined in *Drosophila* SL2 cells after transient co-transfection of nidogen promoter constructs along with constant amounts (1500 ng) of an Sp1 or Sp3 expression vector. Luciferase activity was calculated relative to the normalized luciferase activities obtained by transfecting the nidogen promoter constructs along with the expression vector pPac0, which does not express Sp1-like proteins. Results are means $\pm$ S.E.M. for duplicates in at least three independent experiments.

Construct	Relative luciferase activity	
	With pPacSp1	With pPacUSp3
NDP-1	10.3 $\pm$ 1.2	7.5 $\pm$ 1.4
NDP-2	36.7 $\pm$ 2.3	31.8 $\pm$ 1.9
NDP-5	36.9 $\pm$ 4.6	30.8 $\pm$ 5.1
NDP-9	48.3 $\pm$ 3.7	56.8 $\pm$ 3.5

Table 2 Sp1 and Sp3 simultaneously bind and transactivate the human nidogen promoter construct NDP-9

The longest nidogen promoter construct NDP-9 was co-transfected with constant amounts of pPacSp1 and increasing amounts of pPacUSp3 as indicated. Luciferase activities were calculated relative to the normalized luciferase activity obtained by transfecting the nidogen promoter construct along with the expression vector pPac0, which does not express Sp1-like proteins. Results are means $\pm$ S.E.M. for duplicates in three independent experiments.

pPacSp1 (ng)	pPacUSp3 (ng)	Relative luciferase activity
0	100	22.7 $\pm$ 5.3
100	0	21.5 $\pm$ 5.4
100	10	36.3 $\pm$ 4.8
100	100	46.9 $\pm$ 11.4
100	500	57.8 $\pm$ 8.4
100	1000	57.4 $\pm$ 10.4

Figure 2 Sp1-like transcription factors bind to nidogen promoter fragments

Gel mobility assays were performed with nuclear extracts from SV40/Wi26 cells incubated directly (lanes 1) with the PCR fragment F1 spanning nt -122 to -360 (**A**) or with F2 spanning nt -561 to -635 (**B**). Competition with 10-fold, 50-fold and 100-fold molar excesses of unlabelled F1 [lanes 2, 3 and 4 in (**A**); lanes 8, 9 and 10 in (**B**)], F2 [lanes 2, 3 and 4 in (**B**); lanes 8, 9 and 10 in (**A**)] and a double-stranded Sp1 consensus oligonucleotide [lanes 5, 6 and 7 in (**A**) and (**B**)] is shown. Complexes C1', C2' and C3' indicate the DNA–protein complexes formed without competitor. The unlabelled arrows on the left mark the nucleoprotein complexes formed after incomplete competition with the Sp1 oligonucleotide or the fragment F2.

Figure 3 Induction of nidogen gene expression in keratinocytes

Total RNA ($20 \mu\text{g}$) isolated from keratinocytes after treatment for different durations with either $10 \mu\text{M}$ 5-azacytidine (upper panel) or $2.5 \mu\text{M}$ trichostatin A (lower panel) was analysed by RNase protection assays. Total RNA was isolated after 24 h (lane 4), 48 h (lane 5), 72 h (lane 6), 96 h (lane 7) and 120 h (lane 8) after treatment with 5-azacytidine, and after 2 h (lane 4), 4 h (lane 5), 6 h (lane 6) and 12 h (lane 7) after treatment with trichostatin A. Total RNA isolated from untreated keratinocytes at the earliest time point is shown in lane 3 in both panels, and at the latest time point in lane 9 (upper panel) and lane 8 (lower panel). The hybridization probe (lanes 1) and the control hybridization with $50 \mu\text{g}$ of tRNA (lanes 2) are shown in both panels. The sizes of the nidogen-specific hybridization probe and the protected probe are indicated at the left.

the longest construct, NDP-9. Therefore the first 635 bp of the 5'-flanking region is essential in providing high basal activity to the nidogen promoter. Interestingly, extending the constructs

Table 3 In keratinocytes the nidogen promoter activity is repressed by co-transfection of an Sp1 anti-sense expression plasmid

Epidermal keratinocytes were transfected with constant amounts of the longest nidogen promoter construct NDP-9 ($0.4 \mu\text{g}$) along with increasing amounts of the Sp1 anti-sense expression plasmid (pAS-Sp1) as described in the Materials and methods section. The degree of induction was calculated relative to the normalized luciferase activity obtained by transfecting the promoterless plasmid pGL2-basic, which was set at unity. Results are means $\pm$ S.E.M. for duplicates in at least three independent experiments.

pAS-Sp1 (μg)	Induction relative to pGL2-basic (fold)
0	21.1 ± 5.4
0.1	17.6 ± 5.9
0.2	15.0 ± 4.2
0.3	10.2 ± 2.3
0.4	8.7 ± 1.4
0.5	6.6 ± 1.3
0.6	5.5 ± 1.4

farther in the 5' direction, up to nt -1351 (NDP-5, NDP-6 and NDP-7), led to a marked decrease in promoter activity in adult fibroblasts, whereas in embryonal fibroblasts no significant changes were observed. Maximum promoter activity was obtained in both types of fibroblast with the longest promoter construct NDP-9, indicating the presence of additional up-regulatory *cis*-elements between nt -1351 and -1915 . Surprisingly, in keratinocytes, high luciferase activities were detected with all nidogen promoter constructs, even though keratinocytes do not express nidogen-specific mRNA. Therefore, although this promoter region contains elements differentially utilized by adult fibroblasts compared with embryonal fibroblasts, it does not contribute significantly to cell-type-specific repression of nidogen gene transcription by keratinocytes. Because transfection analysis in SV40-transformed Wi26 fibroblasts showed promoter activities

Figure 4 Repression of SV40 promoter activity by the nidogen promoter fragment spanning nt -1351 to -1102

(A) Schematic diagram of the SV40/nidogen promoter constructs. (B) The SV40 promoter constructs containing the indicated nidogen promoter fragment in opposite orientations upstream of the SV40 promoter (pN1, pN2), downstream of the reporter gene transcription unit (pN3), the 5' part (pN4) and the 3' part (pN5) of this region were transiently transfected in SV40/Wi26 cells. (C) Luciferase activities obtained in fibroblasts (filled bars), embryonal fibroblasts (hatched bars) and keratinocytes (open bars) after transfection with pN1 or pN4. The luciferase activities, corrected for transfection efficiency, were calculated relative to the activity of pGL2-SV40 obtained in each cell type, which was set arbitrarily as 100%. Values shown are averages for at least three independent experiments in each cell type with duplicate determinations. (D) The 32 P-labelled 5' part of the indicated nidogen promoter fragment was incubated directly with nuclear protein extracts isolated from SV40/Wi26 cells (lanes 1 and 5), adult keratinocytes (lane 2), adult fibroblasts (lane 3) and embryonal fibroblasts (lane 4) as described in the Materials and methods section. Competitions with 10-fold, 50-fold and 100-fold molar excesses of the unlabelled 3' fragment (lanes 6, 7 and 8), the 5' fragment (lanes 9, 10 and 11), Sp1 (lanes 12, 13 and 14) and 50-fold and 100-fold molar excesses of PEA3 (lanes 15 and 16) consensus oligonucleotides are shown. The positions of the complexes are indicated at the left.

comparable to those obtained in adult fibroblasts (results not shown), this cell line was used in some of the following experiments.

Sp1 and Sp3 transactivate the human nidogen gene promoter

In the human nidogen gene, the 5'-flanking region from nt -400 to +600 is embedded in a CpG island [13]. Because Sp1-like DNA binding sites occur very frequently in CpG islands and because the activating nidogen promoter constructs contain putative Sp1-like binding sites (Figure 1) the requirement of Sp1-like transcription factors for nidogen gene transcription was examined. *Drosophila* Schneider SL2 cells, which have been reported to lack Sp1-like activity [18,19], were transfected with nidogen promoter constructs and expression plasmids for either Sp1 (pPacSp1) or Sp3 (pPacUSp3). As shown in Table 1, transcription factors Sp1 and Sp3 each transactivated the nidogen promoter regions to comparable levels. In addition, the increase in luciferase activity observed with the constructs NDP-1, NDP-2 and NDP-9 clearly indicate that multiple Sp1-like binding sites, located in different promoter regions, are required for full-level transcription of the nidogen gene. Furthermore, transfection of the longest nidogen promoter construct NDP-9 along with constant amounts of pPacSp1 and increasing amounts of pPacUSp3 revealed a dose-dependent transactivation of the nidogen promoter, demonstrating that both factors can simultaneously bind and activate the human nidogen promoter (Table 2).

The crucial role of Sp1-like transcription factors was corroborated by gel mobility-shift analyses with radiolabelled PCR fragments spanning the regions between nt -360 to -122 (F1) and -635 to -561 (F2). With nuclear extracts from SV40/Wi26 cells, only one nucleoprotein complex (C1') was formed with F1 (Figure 2A, lane 1), whereas with F2 two complexes (C2' and C3') could be identified (Figure 2B, lane 1). All of these complexes were specifically competed for by a 100-fold molar excess of the unlabelled cognate in the binding reactions (Figures 2A and 2B, lanes 2, 3 and 4). The addition of a 100-fold molar excess of an Sp1 consensus oligonucleotide completely abolished the complexes C1' and C2' but did not affect complex C3' (Figures 2A and 2B, lanes 5, 6 and 7). In contrast with unlabelled F1, which completely inhibited the formation of the nucleoprotein complex C2' (Figure 2B, lanes 8, 9 and 10), the addition of a 100-fold molar excess of unlabelled F2 resulted in only partial competition of complex C1' (Figure 2A, lanes 8, 9 and 10), indicating a lower Sp1-binding capacity of the promoter fragment F2. The same complexes were obtained with nuclear extracts from keratinocytes (results not shown).

Nidogen gene expression in keratinocytes is repressed by methylation-specific and chromatin-dependent mechanisms

Because high nidogen promoter activity was obtained after transient transfection of keratinocytes not expressing nidogen (Figure 1), other mechanisms that might be involved in cell-specific nidogen gene repression were analysed by treating keratinocytes with either 5-azacytidine, a specific inhibitor of DNA methyltransferase [20], or trichostatin A, a specific inhibitor of histone deacetylase [21]. As shown by RNase protection analyses (Figure 3), each of these inhibitors was able to induce nidogen gene expression in keratinocytes, whereas the expression in fibroblasts was not affected by these inhibitors (results not shown). This result suggests that cell-specific expression of the human nidogen gene is methylation- and chromatin-dependent. On the basis of these results, we examined whether the nidogen promoter activity demonstrated in keratinocytes could be due to

Figure 5 Mapping of the silencer binding site within the region -1351 to -1250

Complexes are differentially inhibited by double-stranded oligonucleotides derived from different DNA sequences within the regulatory region as indicated (A). For simplicity, only one strand of the double-stranded oligonucleotide sequences is shown. The putative AP-1-binding site is underlined. The boxed region represents the sequences determined by the inhibition studies to be critical for complex formation. Gel mobility-shift assays were performed with the 174 bp 5'-probe (B) or with the oligonucleotide S1 (C) with the use of nuclear extracts from adult fibroblasts. Double-stranded oligonucleotides were added as unlabelled competitors at 10-fold, 50-fold and 100-fold molar excesses in (B) or at a 100-fold molar excess in (C).

the binding of the ubiquitously expressed transcription factor Sp1 to the transiently transfected promoter constructs, which might lack a repressed or highly organized chromatin structure. To test this possibility, co-transfection experiments with an Sp1 anti-sense expression plasmid were performed. As shown in Table 3, transfection of constant amounts of the longest promoter construct NDP-9 along with increasing amounts of an Sp1 anti-sense expression plasmid induced strong inhibition of promoter activity, clearly demonstrating that Sp1 binds and activates the transiently transfected nidogen promoter in keratinocytes not expressing nidogen.

Characterization of the inhibitory nidogen promoter regions

For characterization of the inhibitory promoter regions, fragments comprising nt -807 to -635 , -1102 to -807 and

-1351 to -1102 were tested for silencer activity by introducing these segments into a vector upstream of the reporter gene driven by the SV40 promoter (Figure 4). Transfections of Wi26 fibroblasts with the constructs containing the nidogen promoter region -1102 to -635 did not inhibit SV40 promoter activity, indicating that this region exerts its inhibitory effects only in close association with other *cis*-elements in the nidogen promoter (results not shown). However, the fragment -1351 to -1102 (pN1) decreased SV40 promoter activity to approx. 40% of the activity of the basic construct pGL-SV40, which was set arbitrarily at 100%. The inhibitory activity was largely independent of the orientation relative to the transcription start site, as demonstrated with the promoter constructs pN1 and pN2, containing this fragment in opposite orientations upstream of the SV40 promoter. Furthermore, a similar inhibition was observed with construct pN3, which contains this inhibitory domain

downstream of the reporter gene transcription unit, indicating distance-independence of the silencing effect. These characteristics resemble the basic features of silencers [22,23]. Further shortening of the inhibitory region in the promoter constructs pN4 and pN5 showed that the functionally essential sequences must be located between nt -1351 and -1250. Transfection of adult fibroblasts with the SV40 promoter construct pN1 and pN4 revealed no significant differences when compared with SV40/Wi26 cells (Figure 4C). However, transfection of embryonal fibroblasts and keratinocytes with the same promoter constructs did not result in any significant decrease in SV40 promoter activity.

To characterize this region further, nuclear factor binding within the region -1351 to -1102 was investigated (Figure 4D). PCR fragments comprising the 5' and 3' parts of the inhibitory region (see Figure 4A) were generated. By using nuclear extracts from SV40/Wi26 cells or adult fibroblasts, three prominent nucleoprotein complexes (C1, C2 and C3) were formed with the segment -1351 to -1171 (Figure 4D, lanes 1 and 3). All of these complexes were specifically competed for by a 100-fold molar excess of the unlabelled cognate in the binding reactions (Figure 4D, lanes 9, 10 and 11). However, with nuclear extracts from keratinocytes and embryonal fibroblasts, which did not show a decrease in nidogen or SV40 promoter activities, only two complexes, C1 and C2, were formed (Figure 4D, lanes 2 and 4), indicating that complex C3 is involved in the silencing activity of this promoter region. Because the 5' part of the silencing region contains putative transcription-factor-binding sites for Sp1, PEA3 and AP-1, competition experiments were performed with consensus oligonucleotides. Formation of complex C1 was completely abolished by the addition of an excess of either an Sp1 oligonucleotide (Figure 4D, lanes 12, 13 and 14) or the unlabelled 3' fragment (lanes 6, 7 and 8). The latter observation is due to the presence of an Sp1 consensus binding site in the overlapping region of the PCR fragments. Complex C2 was specifically competed for by 50-fold and 100-fold molar excesses of the PEA3 consensus oligonucleotide (Figure 4D, lanes 15 and 16), whereas none of the complexes could be disrupted by the AP-1 consensus oligonucleotide (results not shown).

Mapping of the silencer binding site in the promoter region -1351 to -1250

The position of the silencer element was further narrowed down by using double-stranded, overlapping oligonucleotides (S1, S2 and S3) spanning the entire 101 bp DNA segment from -1351 to -1250, as unlabelled competitors in gel-retardation assays (Figure 5). As shown in Figure 5(B), with nuclear extracts from adult fibroblasts, only oligonucleotide S1 was able to inhibit the formation of complex C3 completely, whereas S2 and S3 did not affect complex formation. However, the oligonucleotides S2 and S3, which both contained a PEA3 consensus binding site, did compete with the formation of complex C2 (see also Figure 4D). These results indicate that the silencer element is most probably located within the region -1351 to -1301. For further analysis of this 50 bp region, the indicated double-stranded oligonucleotides (Figure 5A) were used as unlabelled competitors for complex C3 formed with the labelled oligonucleotides S1. By using nuclear extracts from adult fibroblasts, complex formation was completely abolished only by an excess of the oligonucleotides S1A, S1E or S1G, whereas none of the other oligonucleotides was able to do so (Figure 5C). This defines the sequences involved in interaction with the silencer binding protein/s between positions -1333 and -1322.

DISCUSSION

The aim of this study was to identify regulatory elements controlling human nidogen gene transcription and to elucidate the mechanisms underlying cell-type-specific expression of this gene. Therefore transient transfection studies in adult dermal fibroblasts and epidermal keratinocytes were performed with various promoter constructs spanning approx. 2 kb of the nidogen promoter region. In adult fibroblasts, which express nidogen-specific mRNA, multiple positive and negative *cis*-acting elements regulating nidogen gene transcription were identified. Surprisingly, the transfection of keratinocytes, which do not express nidogen-specific mRNA, also revealed high nidogen promoter activity.

Characterization of the positive regulatory domains by gel mobility-shift analyses with an Sp1 consensus oligonucleotide as unlabelled competitor showed that Sp1-like transcription factors contribute to all major DNA-protein complexes formed with activating promoter regions. Furthermore, transfection studies in *Drosophila* Schneider cells demonstrate that the human nidogen promoter constructs are transactivated in a comparable manner by Sp1 and one other Sp family member, Sp3. Sp3, like Sp1 and also Sp4, are transcription factors binding with similar affinities to GC/GT boxes. However, only Sp1 and Sp3 are expressed ubiquitously [24,25]. Interestingly, co-transfection experiments with both transcription factors indicated that Sp1 and Sp3 can simultaneously bind and transactivate the human nidogen promoter. This result is in good agreement with a number of recently published studies, showing that Sp3 can function as a positive regulatory factor [26–30]. However, it has also been reported that Sp3 functions as a repressor of Sp1-mediated transcription [26,31–34]. Because it is now apparent that Sp3 encodes at least three distinct proteins *in vivo* and that internally initiated Sp3 isoforms function as transcriptional inhibitors [26], these conflicting results might be due to the use of different Sp3 expression vectors, leading preferentially to the biogenesis of full-length or shorter Sp3 isoforms. The Sp3 expression vector used in this study leads exclusively to the biosynthesis of full-length Sp3 [35], which allows Sp3 to serve here as a transcriptional activator.

Three lines of evidence indicate that the luciferase activities obtained with nidogen promoter constructs in keratinocytes could be due mainly to the binding of the ubiquitous transcription factors Sp1 and/or Sp3 to the transiently transfected DNA: (1) co-transfection with an Sp1 anti-sense expression plasmid strongly inhibited nidogen promoter activity in keratinocytes; (2) no major differences were detected, between extracts from keratinocytes and fibroblasts, in nuclear factor binding within the positive regulatory regions; (3) nidogen gene repression in keratinocytes could be relieved by treatment with either 5-azacytidine, a demethylating agent, or trichostatin A, a substance decreasing histone deacetylation thereby opening chromatin structure [21]. Collectively, these experiments demonstrate that in keratinocytes the transactivation of the transiently transfected nidogen promoter is most probably due to the binding of Sp1 to the plasmid DNA, which lacks the repressed and/or highly organized chromatin structure of the endogenous nidogen gene in keratinocytes. *In vivo*, the structure of the human nidogen gene might exclude the binding of Sp1-like transcription factors, preventing transcription. These results agree with a growing number of reports showing similar differences between the activity of transiently transfected promoters and their endogenous counterparts [36–40]. Our results provide strong evidence that methylation-specific and chromatin-dependent repression mechanisms, which might operate in concert as has been sug-

gested recently [41], have important roles in controlling the cell-type-specific expression of the human nidogen gene.

Characterization of the inhibitory nidogen promoter regions by transfection analyses revealed that one of these regions contains a silencer element located between nt -1351 and -1250 that is able to repress SV40 promoter activity largely independently of its relative position and orientation. However, this silencing effect was observed in adult fibroblasts but not in embryonal fibroblasts and keratinocytes. Comparative analyses of nuclear factor binding *in vitro* within the silencing region identified the nucleoprotein complex C3 to be exclusively formed with nuclear extracts from adult fibroblasts, indicating that this complex is involved in the silencing activity. Preliminary experiments identified a single protein in this complex. Further narrowing down of the position of the inhibitory element by competition experiments localized the binding site for the protein forming complex C3 to a 12 bp element between nt -1333 and -1322 that does not display DNA sequence similarity to other known silencers.

The function of this silencer element might be to modulate or limit the level of transcription in nidogen-producing fibroblasts, as has been already suggested for the COL4 silencer [42]. However, cell-type-specific expression of this gene is instead achieved by a distinct methylation- and chromatin-dependent repression mechanism. Because the expression of basement membrane proteins is spatially and temporally regulated during development [7–9] as well as during growth or regeneration processes such as wound healing [43], the silencer element might have an important role in regulating the overall nidogen expression in response to different external stimuli. In this context it is interesting to consider that the silencer element is located in very close vicinity to, or even overlapping with, a putative AP-1-binding site. Binding of the silencer factor might preclude binding of the AP-1 enhancer complex. It is therefore reasonable to propose that this close association of a silencing and enhancing element might provide an excellent system for controlling overall nidogen gene expression.

We thank Dr. R. Tjian (Howard Hughes Medical Institute, Berkeley, CA, U.S.A.) and Dr. G. Suske (Klinikum der Philipps-Universität, Marburg, Germany) for generously sharing the Sp1 and Sp3 expression plasmids; Dr. D. Petersohn (Dermatology Department, University of Cologne, Cologne, Germany) for advice on the RNase protection assay; and Dr. B. Eckes, Dr. T. Krieg and Dr. M. Hafner for helpful discussion and critical reading of the manuscript. The work was supported by a grant from the German Research Foundation DFG 403/4-3.

REFERENCES

- Durkin, M. E., Chakravati, S., Bartos, B. B., Liu, S.-H., Friedman, R. L. and Chung, A. E. (1988) *J. Cell Biol.* **107**, 2749–2756.
- Mann, K., Deutzmann, R., Aumailley, M., Timpl, R., Raimondi, L., Yamada, Y., Pan, T.-C., Conway, D. and Chu, M.-L. (1989) *EMBO J.* **8**, 65–72.
- Fox, J. W., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Mann, K., Timpl, R., Krieg, T., Engel, J. and Chu, M.-L. (1991) *EMBO J.* **10**, 3137–3146.
- Battaglia, C., Mayer, U., Aumailley, M. and Timpl, R. (1992) *Eur. J. Biochem.* **208**, 359–366.
- Reinhardt, D., Mann, K., Nischt, R., Fox, J. W., Chu, M.-L., Krieg, T. and Timpl, R. (1993) *J. Biol. Chem.* **268**, 10881–10887.
- Mayer, U., Zimmermann, K., Mann, K., Reinhardt, D., Timpl, R. and Nischt, R. (1995) *Eur. J. Biochem.* **227**, 681–686.
- Thomas, T. and Dziadek, M. (1993) *Exp. Cell Res.* **208**, 54–67.
- Thomas, T. and Dziadek, M. (1994) *Mech. Dev.* **45**, 193–201.
- Eklom, P., Eklom, M., Fecker, L., Klein, G., Zhang, H.-Y., Kadoya, Y., Chu, M.-L., Mayer, U. and Timpl, R. (1994) *Development* **120**, 2003–2014.
- Kadoya, Y., Salmivirta, K., Tait, J. F., Kadoya, K., Mayer, U., Timpl, R. and Eklom, P. (1995) *Development* **124**, 683–691.
- Fazio, M. J., O'Leary, J., Kähäri, V.-M., Chen, Y. Q., Saitta, B. and Uitto, J. (1991) *J. Invest. Dermatol.* **97**, 281–285.
- Durkin, M. E., Shu-Huang, L., Reing, J. and Chung, A. E. (1993) *Gene* **132**, 261–266.
- Zimmermann, K., Hoischen, S., Hafner, M. and Nischt, R. (1995) *Genomics* **27**, 245–250.
- Sudbeck, B. D., Parks, W. C., Welgus, H. G. and Pentland, A. P. (1994) *J. Biol. Chem.* **269**, 30022–30029.
- Nagayoshi, T., Sanborn, D., Hickok, N. J., Olsen, D. R., Fazio, M. J., Chu, M.-L., Knowlton, R., Mann, K., Deutzmann, R. and Timpl, R. (1989) *DNA* **8**, 581–594.
- Vincent, S., Marty, L. and Fort, P. (1993) *Nucleic Acids Res.* **21**, 1498.
- Schreiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419.
- Courey, A. J. and Tjian, R. (1988) *Cell* **55**, 887–898.
- Courey, A. J., Holtzman, D. A., Jackson, S. P. and Tjian, R. (1989) *Cell* **59**, 827–836.
- Bird, A. P. (1986) *Nature (London)* **321**, 209–213.
- Yoshida, M., Kijima, M., Akita, M. and Beppu, T. (1990) *J. Biol. Chem.* **265**, 1714–1719.
- Khoury, G. and Gruss, P. (1983) *Cell* **33**, 313–314.
- Brand, A. H., Breiden, L., Abraham, J., Sternglanz, R. and Nasmyth, K. (1985) *Cell* **41**, 41–48.
- Hagen, G., Müller, S., Beato, M. and Suske, G. (1992) *Nucleic Acids Res.* **20**, 5519–5525.
- Kingsley, C. and Winoto, A. (1992) *Mol. Cell. Biol.* **12**, 4251–4261.
- Liang, Y., Robinson, D. F., Dennig, J., Suske, G. and Fahl, W. E. (1996) *J. Biol. Chem.* **271**, 11792–11797.
- Noti, J. (1997) *J. Biol. Chem.* **272**, 24038–24045.
- Bigger, C. B., Melnikova, I. N. and Gardner, P. D. (1997) *J. Biol. Chem.* **272**, 25976–25982.
- Majello, B., De Luca, P. and Lania, L. (1997) *J. Biol. Chem.* **272**, 4021–4026.
- Kennett, S. B., Udvadia, A. J. and Horowitz, J. M. (1997) *Nucleic Acids Res.* **25**, 3110–3117.
- Hagen, G., Müller, S., Beato, M. and Suske, G. (1994) *EMBO J.* **13**, 3843–3851.
- Majello, B., DeLuca, P., Hagen, G., Suske, G. and Lania, L. (1994) *Nucleic Acids Res.* **22**, 4914–4921.
- Denning, J., Hagen, G., Beato, M. and Suske, G. (1995) *J. Biol. Chem.* **270**, 12737–12744.
- DeLuca, P., Majello, B. and Lania, L. (1996) *J. Biol. Chem.* **271**, 8533–8536.
- Denning, J., Beato, M. and Suske, G. (1996) *EMBO J.* **15**, 5659–5667.
- Archer, T. K., Lefebvre, P., Wolford, R. G. and Hager, G. L. (1992) *Science* **255**, 1573–1576.
- Bulla, G. A., DeSimone, V., Cortese, R. and Fournier, R. E. (1992) *Genes Dev.* **6**, 316–327.
- Kitabayashi, I., Kawakami, Z., Chiu, R., Ozawa, K., Matsuo, T., Toyoshima, S., Umehara, K., Evans, R. M., Gachelin, G. and Yokoyama, K. (1992) *EMBO J.* **11**, 167–175.
- Ellis, J., Talbot, D., Dillon, N. and Grosfeld, F. (1993) *EMBO J.* **12**, 127–134.
- Ellis, J., Tan-Un, K. C., Harper, A., Michalovich, D., Yannoutsos, N., Philipsen, S. and Grosfeld, F. (1996) *EMBO J.* **15**, 562–568.
- Kass, S. U., Pruss, D. and Wolffe, A. P. (1997) *Trends Genet.* **13**, 444–449.
- Haniel, A., Welge-Lüssen, U., Kühn, K. and Pöschl, E. (1995) *J. Biol. Chem.* **270**, 11209–11215.
- Fässler, R., Sasaki, T., Timpl, R., Chu, M.-L. and Werner, S. (1996) *Exp. Cell Res.* **222**, 111–116.

Received 22 September 1998/11 November 1998; accepted 10 December 1998