An enhancer with cell-type dependent activity is located between the myeloid and epithelial aminopeptidase N (CD 13) promoters

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The 5' flanking region of the gene encoding the small intestinal brush-border peptidase aminopeptidase N (APN) was screened for the presence of enhancer regions. A 300 bp region with enhancer activity was identified 2.7 kb upstream of the transcriptional start site which is used in epithelial cells. The enhancer stimulated transcription from a heterologous promoter (the simian virus 40 early promoter) in a position- and orientation-independent manner. The activity of the enhancer is cell-type dependent and it is active in liver (HepG2), intestinal (Caco-2) and myeloid (K562) cells. As the epithelial APN promoter is active in the first two cell-types and the myeloid APN promoter in the last, the results may suggest that the enhancer, through

a cooperation with either of the promoters, is important for the tissue-specific expression of APN. A detailed analysis of the enhancer led to the identification of four functionally important regions that are protected against DNase I digestion by Caco-2 nuclear extract. Sequence analysis suggests that two of the regions may interact with members of the Ets transcription factor family (Ets is a transformation-specific protein first discovered in the E26 avian erythroblastosis virus), one region with a CCAAT enhancer-binding protein and one region with Sp1, a transcriptional activator first described as a factor binding to the simian virus 40 early promoter.

INTRODUCTION

Brush-border enzymes are hydrolytic membrane-bound ectoenzymes found in the apical membrane of the small intestinal epithelial cell [1–3]. Peptidases and disaccharidases are the most abundant brush-border enzymes [2]. Unlike the disaccharidases, the peptidases are also found in other tissues, mainly in specialized epithelial cells such as pancreatic exocrine cells [4], hepatocytes [5], endometrial cells [6,7] and the cells lining the kidney proximal tubules [1]. The expression of intestinal brush-border peptidases and disaccharidases varies during development, along the length of the small intestine [2] and along the crypt–villus axis [8–11]. The promoters of the genes encoding these enzymes are therefore well suited to identify *cis*-elements and corresponding transcription factors involved in the transcriptional regulation of temporal and spatial gene expression in the small intestine. Accordingly, the promoters of genes encoding brush-border enzymes have been studied and interacting transcription factors identified [9,12–16]. The gene for the brush-border peptidase aminopeptidase N (APN; also known as CD13) is controlled by two different promoters [15,17]. In cell-types of the myelomonocytic lineage and in some T-cells [18,19] transcription occurs from an upstream promoter, which for the human gene is approx. 8 kb upstream of a downstream promoter and is separated from it by an intron [17]. The downstream promoter is adjacent to the coding part of the gene. A similar arrangement is found in the porcine APN gene, but the structure of the upstream promoter and the size of the intron have not been determined [15]. The downstream epithelial promoter, which is the active promoter in intestinal epithelial cells, has three identified *cis*-elements upstream of the TATA box. These are: a binding site for members of the hepatocyte nuclear factor 1 (HNF1) homeo-

domain family of transcription factors [12,15], a binding site for the ubiquitously expressed Sp1 transcription factor [15] [Sp1 is a transcriptional activator first described as a factor binding to the simian virus 40 (SV40) early promoter], and a binding site for a member of the steroid hormone receptor family, presumably COUP-TF [7]. The HNF1 site is necessary for promoter function and the Sp1 site might also be required [15]. The COUP-TF binding site, however, does not influence the expression in liver or intestinal cells [12,15]. Tissue-specific transcription from a gene is the result of combinatorial cooperation between protein complexes bound to promoter elements, which are close to the transcriptional initiation site, and enhancer elements placed in more remote positions 5' or 3' of the transcription unit [20]. Therefore, knowledge of the structure of both promoters and enhancers operating in intestinal cells is necessary in order to understand the complete spectrum of *cis*-elements and transcription factors that regulate small-intestine-specific transcription. It is the purpose of the present work to identify enhancers in the 5' flanking region of the APN gene and to initiate the characterization of their important *cis*-elements and corresponding transcription factors. The present report represents, to the best of our knowledge, the first characterization of an enhancer for a gene encoding a brush-border enzyme.

EXPERIMENTAL

Materials

Restriction enzymes were from different suppliers: Promega, U.S.A.; Life Technologies, U.S.A.; and Amersham, U.K. Taq DNA polymerase was from Life Technologies, U.S.A., T4 polynucleotide kinase from Amersham, U.K., and T7 RNA

Abbreviations used: ANOVA, analysis of variance; APN, aminopeptidase N; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT enhancer-binding protein; Ets, a transformation-specific protein first discovered in the E26 avian erythroblastosis virus; HNF1, hepatocyte nuclear factor 1; IMDM, Iscove's modified Dulbecco's medium; PEA3, polyoma enhancer activator 3; RLU, relative light unit; Sp1, a transcriptional activator first described as a factor binding to the simian virus 40 early promoter; SRF, serum response factor; SV40, simian virus 40.

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The nucleotide sequence data for the aminopeptidase enhancer reported in this paper appears in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y10081.

polymerase from Promega, U.S.A. Commercially obtained plasmids were pBluescript SK+ (Stratagene, U.S.A.), pSP72, pGL3-Basic, pGL3-Promoter and pGEM 5Z- (Promega, U.S.A.) and PCH110 (Pharmacia, Sweden). The Dual Light kit for luminometric assays of firefly luciferase and *Escherichia coli* β -galactosidase was from Tropix, U.S.A. The ThermoSequenase cycle sequencing system was obtained from Amersham, U.K. The Chameleon double-stranded site-directed mutagenesis kit was from Stratagene, U.S.A. Bioluminescence was measured using a Lumat LB9501 luminometer (Berthold, Germany). Electroporation was performed using a Gene Pulser apparatus (Bio-Rad, U.S.A.). Oligonucleotides were purchased from Pharmacia, Sweden. [α - 32 P]UTP and [γ - 32 P]ATP were from Amersham, U.K. Ingredients for cell culture were from Life Technologies, U.S.A. The MCF-7 cell-line was a gift from Dr. Bo van Deurs (Structural Cell Biology Unit, Department of Medical Anatomy, University of Copenhagen, Copenhagen, Denmark), the Jurkat and K562 cell-lines were gifts from Dr. Astrid Kehlen (Institute of Medical Immunology Martin-Luther-University, Halle, Germany) and the C33A cell-line was obtained from the American Type Culture Collection, U.S.A.

Deletion constructs with CAT as reporter gene

The construction of -1.1 kb chloramphenicol acetyltransferase (CAT) has been described previously (referred to as pAMPCAT in [15]). In essence this construct consists of the porcine APN gene from the *Hind*III site at position -1122 to the *Sac*I site at position $+28$, in front of a *Hind*III/*Bam*HI CAT cassette fragment. This CAT cassette fragment is derived from the pSV2CAT construct [21] and is cloned into pBluescript SK+. For the generation of the -1.1 kb CAT construct the 1150 bp APN fragment was inserted as a *Hind*III fragment. This was made possible by taking advantage of a subclone used for the sequencing of the pig APN gene [22]. From this construct, the pig APN promoter fragment from position -1122 to $+28$ can be excised as a *Hind*III fragment, because the APN *Sac*I site at position $+28$ is followed by a pBluescript SK+ polylinker fragment from *Sac*I to *Hind*III. An important feature of the -1.1 kb CAT construct is that the CAT cassette is flanked on either site by a *Sac*I restriction site and the CAT cassette can therefore be excised as a *Sac*I fragment. When this *Sac*I CAT fragment is cloned in the proper orientation into the *Sac*I site at position $+28$ of the various APN deletion constructs they get identical polylinker regions between the transcriptional initiation site and the CAT cassette.

The -4.8 kb deletion was constructed by cloning the 4.8 kb *Sal*I-*Sac*I fragment, which corresponds to the 5'-most part of the pig genomic G19-1 APN clone [22], into pBluescript SK+ digested with *Sal*I and *Sac*I. The *Sal*I site is derived from the EMBL3 polylinker and the *Sac*I site corresponds to position $+28$ in the pig APN gene. A construct ($-1122/+28$ SK+) was constructed by cloning the 1150 bp *Hind*III-*Sac*I fragment, reaching from positions -1122 to $+28$ of the APN gene, into pBluescript SK+ digested with *Hind*III and *Sac*I. The *Hind*III fragment corresponding to the region from positions -3800 to -1122 was cloned into the *Hind*III site of ($-1122/+28$ SK+) in the correct orientation to generate the -3.8 kb deletion. A construct ($-1103/+28$ SK+) was constructed by cloning the 1131 bp *Apal*/*Sac*I fragment corresponding to the region from positions -1103 to $+28$ of the APN gene into pBluescript SK+ digested with *Apal* and *Sac*I. The *Apal* fragment corresponding to the region from position -2600 to -1103 was cloned into the *Apal* site of $-1103/+28$ SK+ in the correct orientation to generate the -2.6 kb deletion. The CAT cassette was inserted in

the *Sac*I site at position $+28$ of the -4.8 kb, -3.8 kb and -2.6 kb deletion constructs as the *Sac*I CAT cassette fragment described above.

Cloning of the enhancer

The enhancer was localized to a region around position -2.8 kb (see the Results section), and the region from position -3.0 to -2.7 was therefore amplified by PCR using the primers -3.0 kb 5' GATCTCGAGCCAACTTGGAGGACCAGAGG 3' and -2.7 kb 5' CCAGTCGACCTCCATCAGAGCTAGACTTTG 3', and the resulting 300 bp fragment was cloned into the *Sal*I site in the pBluescript SK+ polylinker in front of the -1.1 kb CAT deletion construct to give the construct (3.0-2.7 kb) + 1.1 kb CAT.

The enhancer region was sequenced on both strands using the ThermoSequenase cycle sequencing system. 32 P-labelled primers were used and as template a pBluescript SK+ based construct, which contains the genomic *Hind*III fragment corresponding to positions -3800 to -1122 , was used. The 300 bp PCR-amplified enhancer fragment was sequenced on both strands and the sequence was identical with the genomic sequence.

Deletion constructs with rabbit β -globin as reporter gene

The rabbit β -globin gene was excised from OVEC [23] as a *Pst*I-*Hind*III fragment and cloned into pSP72 to generate the construct glob72. To generate the -3.8 kb, -2.6 kb and -1.1 kb globin constructs the corresponding fragments were excised from the CAT deletion constructs with *Clal* (which is in the pBluescript SK+ polylinker) and *Sac*I. The *Clal*-*Sac*I fragments were subsequently cloned into glob72 digested with *Clal* and *Sac*I. The -4.8 kb β -globin construct is generated from the -4.8 kb CAT construct by replacing the CAT cassette (removed by digestion with *Sac*I) with the β -globin gene as a *Sac*I fragment. The β -globin gene *Sac*I fragment was produced by excising the β -globin gene from glob72 with *Clal* and *Hind*III and cloning it into pBluescript SK+ digested with *Clal* and *Hind*III. From this construct (globSK+) the β -globin gene can be excised as a *Sac*I fragment. The polylinker region between the APN transcription-initiation site and the β -globin gene is identical in all four constructs.

Deletion constructs with luciferase as reporter gene

The luciferase cassette was excised from pGL3-Basic as a *Kpn*I-*Bam*HI fragment and cloned into pBluescript SK+ digested with *Kpn*I and *Bam*HI. From this construct the luciferase cassette can be excised as a *Sac*I fragment, which can be inserted into the *Sac*I site at position $+28$ in the APN gene. The -1.1 kb and the (3.0-2.7 kb) + 1.0 kb luciferase constructs were generated by replacing the CAT cassette in the corresponding CAT constructs with the *Sac*I luciferase fragment. The 5' and 3' deletions of the enhancer (Figure 5) were prepared by PCR using the primers:

position [-2967 (+)]
 5' CTAGTCGACGAGTAAAGACTCCAGCCAACC 3'
 position [-2897 (+)]
 5' GTTGTCGACAGGCCGGCCTCTTCCTTTG 3'
 position [-2837 (-)]
 5' CTAGTCGACGAAAGACACACAAGCCTGCCT 3'
 position [-2796 (-)]
 5' GTGGTCGACGTTTCACTCTGCAGGCCTTCAG 3'

The numbers represent the position in the APN gene and the + and - signs refer to the upper and lower strand respectively.

The -2967 bp to -2700 bp and the -2897 bp to -2700 bp constructs were prepared using the -2.7 kb primer as 3' primer. For the -3.0 kb to -2837 bp and the -3.0 kb to -2796 bp constructs the -3.0 kb primer was used as 5' primer. The respective fragments were cloned in front of the -1.1 kb luciferase construct by use of the *SaI* site in the pBluescript polylinker. The sequences of the fragments generated by PCR were verified by sequencing of both strands using ³²P-labelled primers and the ThermoSequenase cycle sequencing system.

APN enhancer and SV40 promoter hybrids

The construct pGL3-promoter, which consists of the SV40 early promoter placed in front of the luciferase gene, was used. The enhancer fragment was excised from the (3.0–2.7 kb) + 1.1 kb construct by digestion with *XhoI* and *SaI* and cloned in either orientation into the *XhoI* site immediately upstream of the SV40 early promoter in pGL3-promoter to give the constructs →SV40 and ←SV40 respectively. The →SV40 construct contains the enhancer in the orientation found in the APN 5' flanking region. The cloning of the enhancer fragment in either orientation into the *SaI* site, which is immediately downstream of the luciferase transcription unit in the pGL3-promoter construct, resulted in the constructs SV40 → and SV40 ← respectively.

Site-directed mutagenesis

Mutations were introduced into the enhancer sequence of the (3.0–2.7 kb) + 1.1 kb luciferase construct by the unique-site elimination procedure [24] using the Chameleon site-directed mutagenesis kit. The selection was performed against the *XmnI* site present in the ampicillin-resistance gene of pBluescript SK + using the selection primer 5' CATCATTTGGAAAACGCTCTTCGGGGCG 3'

The primers used for the generation of the enhancer mutations were:

Mut. 1 5' CCAACCAGGCCCGGCTAGCGTGTCCAAAGC 3'

Mut. 2 5' GGTCTGTGTCCAGCTAGCACAGGAAGAGG 3'

Mut. 3 5' GGCCGGGCCGCTAGCTTTGTTTGGAG 3'

Mut. 4 5' GCCTCTTCCTTGCTAGCGAGTAGGGATTAG 3'

Mut. 5 5' CTTTGTGTTGGAGGCTAGCTTAGGCCAGGC 3'

Mut. 6 5' GATTAGGCCAGGCCGCTAGCGTGTGTCTTTC 3'

Mut. 7 5' CAGGCTTGTGGCTAGCTCTGCAGCAAAGG 3'

Mut. 8 5' GCAGCAAAGGCTAGCGGCTGAAGGCC 3'

The primers were synthesized with a 5' phosphate group and purified by PAGE. The 6 bp mutation is underlined in each oligonucleotide and introduces an *NheI* restriction site. The sequences of the mutations were verified using the ThermoSequenase cycle sequencing system.

The mutations of the HNF1 site, the Sp1 site and the TATA box were produced by PCR using the primers:

HNF1(–)

5' GTGCATGCTCGATTCACTCAGACAGACGAAGGT-CAC 3'

HNF1(+)

5' GAGCATGCACTGTAGTCTGCAGGCTGTGGGGCTC 3'

Sp1(–)

5' GAGCATGCCTACTGAGTCTATGCAGACTGGGCAA-AAATTAACCAG 3'

Sp1(+)

5' TAGACTCAGTAGGCATGCTCTCAGGGATATAAGCC-TGGTCCG 3'

TATA(–)

5' GTTGCATGCAGTGCCCTGAAGGGGAGGAGCCCC-AC 3'

TATA(+)

5' ACTGCATGCGGTCCGAAGCTGCCCTGTCCCCTG 3'

The oligonucleotides introduce an *SphI* site (underlined). PCR reactions were carried out with the (+) or (–) primers, together with an appropriate pBluescript specific primer [M13 primer for the (+) primer and the pBluescript SK + reverse primer for the (–) primer] and the plasmid G14EM as template. G14EM consists of the 1760 bp fragment of the APN gene reaching from the *EcoRI* site at position -1182 to the *EcoRI* site in the first intron of the APN gene [22]. The M13 primer which was used contains a 5' extension with an *SmaI* restriction site. The M13- and (+) primer [Sp1(+) or TATA(+)]-generated PCR fragments were digested with *SmaI* and *SphI* and cloned into pSP72 digested with the same restriction enzymes. The corresponding constructs are referred to as pSP1M13 and pTATAM13 respectively. The pBluescript SK + reverse- and (–) primer [Sp1(–) or TATA(–)]-generated PCR fragments were digested with *HindIII* and *SphI* and the resulting PCR fragments were cloned into pSP1M13 or pTATAM13, which had been digested with *HindIII* and *SphI*. In this manner the PCR fragments were fused at the *SphI* site. Fragments containing the mutated sequence were excised using *NheI* and *SacI* and used to replace the homologous fragment from position -738 to +28 in the -4.8 kb APN-globin construct. The sequence of the region generated by PCR was verified by sequencing of both strands.

Template for *in vitro* transcription of the globin antisense RNA probe

A construct (globmap) for the generation of a globin antisense riboprobe was constructed by cloning the 950 bp *NheI*–*NcoI* fragment spanning the APN-globin junction in the APN-globin constructs into pGEM-5Z. The *NheI* site is at position -738 in the pig APN gene [22], the *NcoI* site is at position +166 in the rabbit β -globin gene [25] and 46 nucleotides are derived from the pSP72 polylinker.

Cell culture

The origins and culture conditions for Caco-2, HepG2 and HeLa cells have been described previously [12,15]. MCF-7 cells [26] were cultured in Dulbecco's modified Eagle's medium supplemented with fetal calf serum [10% (v/v)], L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 units/ml). C-33A cells were cultured in minimum essential medium supplemented with potassium acetate (1 mM) and non-essential amino acids (1 mM) in addition to the supplements used for MCF-7 cells. Jurkat and K562 cells were cultured in GlutaMax containing RPMI 1640 medium supplemented with the antibiotics described above.

Transfection with constructs containing CAT as reporter gene

HepG2 cells (6×10^6) or 8×10^6 Caco-2 cells were transfected in 100-mm dishes using either the standard calcium phosphate coprecipitation procedure (HepG2) or the modified procedure described for epithelial cells [27]. A known amount (2.7 pmol) of each APN expression construct (corresponding to 10 μ g of the -1.1 kb APN-CAT construct) was used, and in the case of HepG2 cells 10 μ g of the β -galactosidase expression construct PCH110 was co-transfected. Extracts were prepared and CAT and β -galactosidase activities were measured as described earlier [12,15]. The results from the experiments with Caco-2 cells were

Figure 1 Deletion analysis in Caco-2 and HepG2 cells of the 5' flanking region of the APN gene

The indicated 5' deletions (the numbers represent 5' position relative to the transcriptional start site) were placed in front of the CAT gene and transfected into either Caco-2 or HepG2 cells. The (3.0-2.7) + 1.1 kb is a construct with a PCR fragment covering the indicated part of the 5' flanking region cloned in front of the -1.1 kb deletion. The S.D.s of the samples are shown. The resulting average CAT activities are normalized to the activity observed with the -4.8 kb construct in each series.

obtained by assaying CAT activity from an extract corresponding to 400 µg of protein. For the experiments with HepG2 cells, one half of the extract volume was used to measure β-galactosidase activity. Volumes of extracts containing identical amounts of β-galactosidase activity were subsequently assayed for CAT activity.

Transfection with constructs containing firefly luciferase as reporter gene

Cells (1×10^6) propagated as monolayers (Caco-2, HeLa, MCF-7 and C33A) were transfected in 35-mm dishes using either the modified calcium phosphate co-precipitation procedure (Caco-2) or the standard procedure. For the SV40-based constructs, 150 fmol (corresponding to 0.5 µg of the pGL3-promoter construct) of the indicated constructs were co-transfected with 0.2 µg of PCH110. For the enhancer deletion constructs, 360 fmol (corresponding to 1.5 µg of the -1.1 kb luciferase construct) of the deletion constructs were co-transfected with 0.2 µg of PCH110. DNA was introduced into the Jurkat and K562 cell-lines by electroporation. Exponentially growing cells were washed twice in Iscove's modified Dulbecco's medium (IMDM) and resuspended in IMDM at a concentration of 1×10^8 cells/ml. A 0.2 ml volume of the cell suspension was transferred to a 0.4 cm electroporation cuvette and incubated with DNA for 10 min at room temperature before a pulse was delivered with the Gene Pulser set to 960 µF and 170 V. Following the pulse the cells were transferred to tissue-culture flasks and incubated for 24 h in IMDM supplemented with 5% (v/v) fetal calf serum, L-glutamine and antibiotics as described above. The SV40-based construct (2.4 pmol), 8 µg of PCH110 and 20 µg of sonicated salmon sperm DNA were included in each electroporation. Preparation of cell extracts and analysis of firefly luciferase and β-galactosidase activities were carried out using the Dual-Light kit according to the recommendations of the manufacturer.

Transfection with constructs containing the rabbit β-globin gene as reporter gene

APN-β-globin expression constructs (2.2 pmol), 0.5 µg of pRSVNEO [21] and 0.25 µg of the OVEC-ref [23] plasmids were

co-transfected. Selection of stably transfected clones was carried out in the presence of G418 (1 mg/ml). RNA was extracted [28] from pools of resistant clones 1 week after a confluent monolayer of cells was obtained. Specific transcripts were detected by RNase mapping as described earlier [15] with an [α - 32 P]UTP-labelled antisense riboprobe transcribed with the T7 RNA polymerase, using the globmap construct linearized with *NotI* as template. The protected bands corresponding to transcriptional initiation from position +1 in the APN gene or from the SV40 promoter were quantified using a PhosphorImager. The cells were fully differentiated at the time of harvesting, as judged by the expression of APN (measured by Northern blotting; results not shown) and dome formation [29], which indicates that the cells form a tight monolayer and are able to perform a directional transport of ions.

Preparation of nuclear extract and DNase I footprinting

The preparation of the Caco-2 nuclear extract has been described previously [30]. The enhancer fragment was labelled on either strand with [γ - 32 P]ATP, and the gel-purified fragments were used for DNase I footprinting, which was performed in the presence of 40 µg of nuclear extract, as described previously [15].

Statistical tests and computer analyses

The effects of addition of the enhancer to the SV40 promoter in different cell-types (Table 1) were evaluated using a *t*-test. Comparison of the activity of the ← SV40 construct in different cell-types, the results of the deletion analysis of the enhancer (Figure 4) as well as the mutational analysis (Table 2) were evaluated by one-way analysis of variance (ANOVA) as described in [31].

The nucleotide sequence of the enhancer was searched for potential *cis*-elements using the signal scan program [32] and the transcription factor (TFD) database. Only elements potentially corresponding to the protected regions (Figure 4) are discussed.

Figure 2 APN enhancer is active in differentiated Caco-2 cells

The indicated 5' deletions with (–4.8 kb and –3.8 kb) or without (–2.6 kb and –1.1 kb) the enhancer were placed in front of the rabbit β -globin gene and co-transfected into Caco-2 cells together with a reference construct (OVEC-ref) and an expression plasmid conferring resistance to G418. RNA was harvested from pools of stably transfected cells 1 week after the cultures had reached confluence. At this stage the Caco-2 cells were differentiated and dome formation, which indicates the presence of tight junctions and directional ion transport, was evident. Specific transcripts from the APN promoter (APN) or the reference construct (Ref.) were detected by RNase mapping. The samples labelled –1.1 kb ref. and OVEC-ref represent individual clones only transfected with the –1.1 kb globin or the OVEC-ref constructs respectively. The bands indicated by APN and Ref. were quantified using a PhosphorImager. The mean APN/Ref values ($\pm$ S.D.) and the number of experiments (*n*) are shown below each construct. End-labelled DNA fragments derived from pBluescript KS+ digested with *Hae*III were used as markers and the positions of these molecular-mass markers are shown on the left.

RESULTS

A putative enhancer is located within a 0.3 kb region, 2.7 kb upstream in the 5' flanking region of the porcine APN gene

A 4.8 kb sequence of the 5' region flanking the porcine APN gene [22] was used for the construction of a series of deletions which were placed in front of the bacterial CAT gene. The constructs were transfected into either exponentially growing human colon

Table 1 Activation of the SV40 early promoter by the enhancer in different cell-types

The region from position –3.0 kb to –2.7 kb in the APN 5' flanking region was cloned either 5' or 3' to a firefly luciferase transcription unit driven by the SV40 early promoter. The region is represented by an arrow and the rightward arrow ($\rightarrow$) represents the natural 5' (–3.0 kb) to (–2.7 kb) 3' orientation. The constructs were transfected into the cell-lines listed and the resulting luciferase activity is normalized to the activity of the SV40 promoter without the enhancer region in each cell-line (results given $\pm$ S.D.). Except for the Jurkat cell-line, the addition of the enhancer region resulted in a significant (Student's *t*-test, $P < 0.05$) increase in luciferase activity. Using ANOVA the various cell-lines could be divided into different groups (MCF-7 > Caco-2 and K526 > HeLa and C33A > Jurkat) with significantly different ($P < 0.05$) luciferase activity of the $\rightarrow$ SV40 construct.

Construct	Normalized luciferase activity					
	Caco-2 (<i>n</i> = 4)	HeLa (<i>n</i> = 6)	C33A (<i>n</i> = 6)	MCF-7 (<i>n</i> = 4)	K562 (<i>n</i> = 4)	Jurkat (<i>n</i> = 4)
SV40	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.75	1.0 $\pm$ 0.0
$\rightarrow$ SV40	2.1 $\pm$ 0.4	1.7 $\pm$ 0.5	1.5 $\pm$ 0.2	3.4 $\pm$ 0.8	2.7 $\pm$ 0.6	1.1 $\pm$ 0.5
$\leftarrow$ SV40	3.3 $\pm$ 0.4					
SV40 $\rightarrow$	2.0 $\pm$ 0.5					
SV40 $\leftarrow$	2.4 $\pm$ 0.5

Figure 3 Sequence of the APN enhancer

The 300 bp sequence of the enhancer between positions –3.0 kb and –2.7 kb is shown. The regions protected in DNase I footprinting (Figure 4) are indicated and labelled with Roman numerals. Hypersensitive sites are indicated with arrows.

carcinoma (Caco-2) or human hepatoma (HepG2) cells (Figure 1). Deletion of the region from position –4.8 kb to –3.8 kb did not have any significant effect. Deletion of the region from position –3.8 to –2.6 kb, however, resulted in a significant ($P < 0.05$) decrease in reporter gene activity in both Caco-2 and HepG2 cells. Deletion of the region from position –2.6 kb to –1.1 kb did not produce any additional significant effects in either of the cell-lines. These results indicate the presence of a putative enhancer in the region between –3.8 kb and –2.6 kb, and preliminary results obtained with constructs with 5' ends in this region (results not shown) suggested that the enhancer was located around position –2.8 kb. This was confirmed by cloning the region from position –3.0 kb to –2.7 kb in front of the –1.1 kb construct. This construct (3.0–2.7 kb) + 1.1 kb has the same activity as the –4.8 kb construct. Thus the putative enhancer increases the transcription from the –1.1 kb construct 3- to 4-fold (Figure 1).

The Caco-2 cell-line is a widely used model for small-intestinal epithelial cells. Post-confluent monolayers of Caco-2 cells spontaneously differentiate and express increased amounts of brush-

Figure 4 DNase I footprinting of the enhancer

The enhancer fragment shown in Figure 3 was labelled on either the upper (+) or lower (–) strand and used for DNase I footprinting. A Maxam/Gilbert G/A sequencing lane (MG) is used as marker. 0 indicates DNase I digestion of naked DNA, and Caco-2 indicates DNase I digestion in the presence of 40 µg of nuclear proteins extracted from differentiated Caco-2 cells. The protected regions are indicated by rectangles and labelled with Roman numbers. Hypersensitive sites are indicated with arrows. The 5' and 3' deletions of the enhancer (1, full enhancer; 2, nucleotides –2967 to –2700; 3, nucleotides –2897 to –2700; 4, nucleotides –3000 to –2837; 5, nucleotides –3000 to –2796) which are schematically shown below the DNase I footprint were placed in front of the –1.1 kb–luciferase construct (6) and transfected into Caco-2 cells. An expression vector for β-galactosidase was co-transfected to correct for variations in transfection efficiency. Reporter-gene activities were measured by luminometry and the luciferase relative light unit/β-galactosidase relative light unit ratios (± S.D.) are shown. The positions of the protected regions are indicated. On the (+) strand, numbers refer to the boundaries of the footprints. On the (–) strand, numbers refer to DNase I hypersensitive sites.

border enzymes (for a review see [33]). In order to analyse the activity of the enhancer in differentiated Caco-2 cells, –4.8 kb, –3.8 kb, –2.6 kb and –1.1 kb deletion constructs, carrying the rabbit β-globin gene as reporter gene, were generated. The constructs were co-transfected with the internal control plasmid OVEC-ref [23], which consists of the SV40 early promoter in front of a deleted version of the β-globin gene, into Caco-2 cells. Pools of post-confluent (1 week) differentiated cells, having stably integrated these constructs into the genome, were analysed by RNase mapping using a riboprobe (Figure 2). Transcription from the APN promoter was detected as a 239 nt protected fragment. The less-intense protected band, which is

seen between the 254 and 267 marker bands, was invariably detected using the APN–globin expression constructs. However, the intensity of this band co-varied with the same RNA sample in different experiments and it is therefore most likely that it represents a partially RNase-resistant secondary structure in the probe–target mRNA hybrid. Transcription from the SV40 promoter is expected to give the 139 nt protected fragment observed (Figure 2). The presence of additional protected bands using this construct has been reported previously [23]. The average steady-state β-globin mRNA level originating from the APN promoter was 2.4-fold higher from cells transfected with constructs containing the enhancer (the –4.8 kb and –3.8 kb constructs)

compared with cells transfected with the constructs lacking the enhancer (the -2.6 kb and -1.1 kb constructs). This shows that the putative enhancer is active also in differentiated Caco-2 cells.

The enhancer is a *bona fide* enhancer with cell-type-dependent activity

To further characterize the putative enhancer, it was cloned in front of the SV40 early promoter either immediately upstream of the promoter or immediately downstream of the luciferase transcription unit, which is driven by the SV40 early promoter. In each case the fragment was cloned in either orientation. In all constructs the addition of the -3.0 kb to -2.7 kb APN fragment resulted in significant ($P < 0.05$) stimulation (2- to 3-fold) of the transcription from the SV40 promoter, when the constructs were transfected into Caco-2 cells (Table 1). Thus this region from position -3.0 kb to -2.7 kb in the APN 5' flanking part is a *bona fide* enhancer.

The SV40 enhancer is considered to be active in most cell-types and the effect of adding the APN enhancer to the SV40 promoter was analysed in various cell-types (Table 1). The enhancer resulted in a significant ($P < 0.05$) stimulation of transcription from the SV40 promoter in the cervical carcinoma cell-lines HeLa and C33A, in the mammary carcinoma cell-line MCF-7 as well as in the chronic myelogenous leukaemia cell-line K562. In the acute T-cell leukaemia cell-line Jurkat, no significant stimulation was observed. By using one-way ANOVA the various cell-lines could be divided into four groups with significantly different ($P < 0.05$) activities from the APN enhancer-SV40 promoter hybrid. In decreasing order the groups are: MCF-7 > Caco-2 and K562 > HeLa and C33A > Jurkat. These results demonstrate that the enhancer has a cell-type-dependent activity.

Fine structure of the enhancer

The sequence of the fragment containing the enhancer was determined (Figure 3). DNase I footprinting of both strands using a nuclear extract from differentiated Caco-2 cells revealed the presence of five footprints (Figures 3 and 4). Footprint I covers nucleotides -2937 to -2913 on the upper (+) strand. It is divided by hypersensitive sites at positions -2931 and -2919 . On the lower (−) strand only one hypersensitive site at position -2923 is seen. Footprint II covers nucleotides -2887 to -2864 on the (+) and nucleotides -2887 to -2868 on the (−) strand. Hypersensitive sites are seen at position -2875 on the (+) strand and positions -2878 and -2868 on the (−). Footprint III covers nucleotides -2852 to -2833 on the (+) strand. A hypersensitive site is seen at position -2844 . On the (−) strand hypersensitive sites are seen at positions -2857 and -2837 . Footprint IV covers nucleotides -2831 to -2822 on the (+) strand. On the (−) strand the protection starts at position -2834 . The end of the footprint cannot be determined precisely but it ends before position -2811 . Footprint V starts with a hypersensitive site at position -2752 and extends to position -2728 on the (+) strand. In addition a hypersensitive site is found at position -2740 . On the (−) strand nucleotides -2753 to -2737 are protected with a hypersensitive site at position -2746 .

A series of 5' and 3' deletions of the enhancer region were produced based on the DNase I footprinting results. The resulting constructs were placed in front of the -1.1 kb construct and the transcriptional activity measured in Caco-2 cells (Figure 4). One-way ANOVA showed that the constructs could be divided into three groups with significantly different ($P < 0.01$) luciferase activity. Constructs 1, 2 and 5 have full enhancer activity.

Table 2 Point mutations in the enhancer

Point mutations were introduced into the regions covered by footprints I–IV (Figure 4) in the (3.0–2.7 kb) + 1.1 kb luciferase construct (Figure 1). The footprint targeted by each mutation and the exact position of the changed nucleotide sequence (Mut.) relative to the wild-type sequence (Wt.) are indicated. The constructs were transfected into Caco-2 cells and the luciferase activity is normalized to the activity obtained with the -1.1 kb construct without the enhancer (results given $\pm$ S.D.). ANOVA showed that mutation 1 is without significant ($P < 0.05$) effect on the enhancer activity. All other mutations reduce the enhancer activity significantly.

Construct	Footprint with mutation	Sequence	Normalized activity
1.1 kb (3.0–2.7 kb) + 1.1 kb		No enhancer Entire enhancer	1.0 $\pm$ 0.3 6.4 $\pm$ 1.2
Mut. 1	I	Wt. 5' CCGTGGTCTGTG 3' Mut. 5' GCTAGC 3'	7.4 $\pm$ 1.9
Mut. 2	I	Wt. 5' CCAAAGCAAACA 3' Mut. 5' GCTAGC 3'	2.4 $\pm$ 0.6
Mut. 3	II	Wt. 5' GCCTCTTCCTTT 3' Mut. 5' GCTAGC 3'	2.8 $\pm$ 1.6
Mut. 4	II	Wt. 5' CTTTGTTTGGAG 3' Mut. 5' GCTAGC 3'	3.1 $\pm$ 1.15
Mut. 5	II	Wt. 5' GAGTAGGGATTA 3' Mut. 5' GCTAGC 3'	1.9 $\pm$ 0.5
Mut. 6	III	Wt. 5' GGCAGGCTTGTG 3' Mut. 5' GCTAGC 3'	5.2 $\pm$ 1.5
Mut. 7	III	Wt. 5' GTGTGTCTTCT 3' Mut. 5' GCTAGC 3'	3.3 $\pm$ 1.2
Mut. 8	III	Wt. 5' AAGGGAGGGGGC 3' Mut. 5' GCTAGC 3'	3.3 $\pm$ 1.1

Construct 4 has intermediate enhancer activity, whereas constructs 3 and 6 (construct 6 is the -1.1 kb–luciferase construct) have no enhancer activity. These results imply that the region containing footprints I–IV is sufficient for the full activity of the enhancer, whereas the region containing footprint V is not of importance under the experimental conditions used.

Individual mutations were introduced into each of the regions corresponding to footprints I–IV and the effect on the transcriptional activity in Caco-2 cells was analysed (Table 2). All mutations except mutation 1, which is located in the 5'-most part of footprint I, result in a significant ($P < 0.05$) reduction of enhancer activity. The Mut. 2 construct, which is mutated in the centre of footprint I, and the Mut. 5 construct, which is mutated in the 3' end of footprint II, have the lowest transcriptional activity among all the Mut. constructs ($P < 0.05$).

Table 3 Point mutations in the APN promoter

The wild-type sequences (Wt.) of the HNF1 region, the Sp1 region and the TATA box from the APN epithelial promoter are shown. The actual binding site is underlined for each factor respectively. Point mutations (Mut.), which introduce a *Sp1* site (shown in bold), of the elements were generated and the effects evaluated in differentiated Caco-2 cells (Figure 5).

Cis-element	Sequence of wild-type and mutation
HNF1	Wt. 5' CCCTGGTTAATTTTGGCCAG 3' Mut. 5' TGAATCGAGCATGCACTGTAG 3'
Sp1	Wt. 5' GGCTGTGGGGCTCCTCCCTT 3' Mut. 5' TAGACTCAGTAGGCATGCTCT 3'
TATA box	Wt. 5' ATATAAGCCT 3' Mut. 5' CACTGCATGC 3'

Figure 5 Both the HNF1 and Sp1 sites in the promoter are required for full activity of the enhancer–promoter combination

The -4.8 kb globin construct, or versions with mutations in the HNF1 site, the Sp1 site or TATA box respectively, were transfected into Caco-cells. The OVEC-ref reference construct and an expression vector conferring resistance to G418 were co-transfected. RNA was harvested from pools of stably transfected cells 1 week after the cultures had reached confluence. Specific transcripts from the APN promoter (APN) or the reference construct (Ref.) were detected by RNase mapping. The bands indicated by APN and Ref. were quantified using a PhosphorImager. The mean APN/Ref. values are shown below each construct. Two independent experiments were carried out with each construct. The protected bands labelled Rt. represent read-through transcripts initiated at spurious initiation sites in the vector. The different sizes of these bands produced by the different constructs are due to the difference in nucleotide sequences between the riboprobe and the constructs containing the mutations.

Cooperation between the enhancer and proximal promoter elements

The construct containing the full 4.8 kb APN $5'$ flanking region placed in front of the rabbit β -globin gene was used for the construction of three additional constructs with individual point mutations (Table 3) in the HNF1 site, the Sp1 site and the TATA box respectively. RNA was extracted from pools of stably transfected Caco-2 cells and analysed by RNase mapping (Figure 5). Mutations in either the HNF1 site or the Sp1 site resulted in a 10-fold reduction of correctly initiated transcription. Mutation of the TATA box virtually abolished correctly initiated transcription. Thus the results show that the APN TATA box is a

bona fide TATA box and that the HNF1 and Sp1 sites are both required for full activity of the enhancer–promoter combination. In addition to the correctly initiated transcripts (marked APN on Figure 5) additional protected bands corresponding to transcriptional initiation from other sites are observed. Incorrectly initiated transcription originating at poorly defined sites often occurs in plasmid vectors. Transcripts which are initiated from such vector sites and extended through a promoter being investigated can potentially give rise to a translation product which will generate a background of reporter gene activity. The OVEC system has the advantage that correctly initiated transcription can be identified directly [23]. The β -globin antisense probe used in this work ends at position -738 of the APN gene, and in Figure 2 the clones containing the -2.6 kb construct contain some read-through transcripts, which are seen as protected fragments with the same size as the probe. In the Caco-2 clones containing the DNA constructs with mutations in the promoter (Figure 5), the read-through transcripts are present in relatively high amounts. In this case they are cleaved by the RNases into two bands of different sizes (marked Rt. on Figure 5) because the probe corresponds to the wild-type sequence whereas the read-through transcripts contain a mutated sequence.

DISCUSSION

An enhancer with cell-type-dependent activity cooperates with the APN epithelial promoter

We have previously shown that removal of the HNF1 site in the APN promoter virtually eliminates promoter activity. As HNF1 proteins are only expressed in specialized epithelial cells, this finding shows that the HNF1 site plays a key role for the epithelial APN promoter by restricting its activity to cells expressing HNF1 proteins [7,12,15]. The Sp1 site interacts with the ubiquitously expressed Sp1 transcription factor, and we have speculated that the Sp1 site functions as an essential proximal promoter element working in synergy with the HNF1 site, as constructs containing only the Sp1 site and TATA box have very low promoter activity [12,15]. The importance of both promoter elements is now directly demonstrated, as both the HNF1 and Sp1 sites are required for full activity of the enhancer–promoter combination. The enhancer identified in the present work has the characteristics of a *bona fide* enhancer, i.e. its activity is independent of its orientation and position in relation to the promoter. The APN enhancer is cell-type dependent and the results presented here suggest that this may be important for the generation of high transcriptional rates from the epithelial promoter in liver and intestinal cells. In the mammary carcinoma cell line MCF-7 the enhancer has a high activity, but the epithelial promoter is inactive as these cells do not contain HNF1 proteins [34]. In the cervical carcinoma cell-lines the enhancer has a low activity and these cells do not express HNF1 proteins either [34,35]. The present work therefore suggests that for epithelial cell-types a coincidence of high epithelial promoter activity with high enhancer activity may only occur in liver and intestinal cells. With respect to the cells of the immune system, it is well established that APN (which is identical with the CD13 marker) is expressed in the highest amounts in cells of the myeloid lineage and that the myeloid promoter is preferentially active in these cell-types [17,18,36]. The finding that the enhancer has a relatively high activity in the chronic myelogenous leukaemia cell-line K562, whereas it is inactive in the acute T-cell leukaemia Jurkat cell-line, may therefore suggest that a coincidence of high myeloid promoter activity with high enhancer activity occurs in cells belonging to the myeloid lineage.

Transcription factors of the Ets and C/EBP families are potential functionally important APN enhancer-binding proteins

A computer search revealed a potential polyoma enhancer activator 3 (PEA3) binding site in both footprints I (nucleotides –2918 to –2913 on the + strand) and in footprint II (nucleotides –2881 to –2886 on the – strand), a potential NF-IL6-binding site in footprint III (nucleotides –2753 to –2744 on the – strand) and a potential serum response factor (SRF)-binding site in footprint V (nucleotides 247 to 256 on the + strand). PEA3 is a transcription factor that binds to the polyoma virus early promoter [37] and it belongs to the Ets transcription factor family characterized by a common DNA-binding motif recognizing *cis*-elements with a 5' GGA core (for review see [38]). Other Ets proteins in addition to PEA3 may therefore bind to the motifs in footprints I and II. An important feature of Ets proteins is that they often need to interact with other transcription factors for efficient DNA binding [38]. In the *c-fos* promoter an Ets protein, the ternary complex factor, combines with the SRF and generates a ternary DNA–protein complex, which mediates the immediate transcriptional response from the *c-fos* promoter following serum stimulation [39]. Thus, it might be significant that footprint V covers a CC(A/T)₆GG element, which is the consensus-binding sequence for the SRF. The response to serum stimulation, e.g. from the *c-fos* promoter, is maximal a few minutes after serum stimulation and declines to a prestimulatory level after 30 min [40]. The fact that no functional importance could be attributed to footprint V may therefore be due to the procedure used for the transient transfections. Cells were harvested at least 24 h after the last change of the culture medium, and after this time-span any serum response would have declined.

Mutational analysis of the enhancer points to the 3' parts of both footprints I and II as the most important sequences in the enhancer. No known transcription-factor-binding sites were identified in these regions by the computer search, although the Ets factors mentioned above could potentially have important DNA contact points in these regions. The identity of the factors binding to these parts of the enhancer is, however, presently unsettled.

Footprint III includes a sequence that shows similarity to the consensus-binding sequence for NF-IL6

5' T(T/G)NNGNAA(T/G) 3'

[41], which is a transcription factor induced by the cytokine interleukin-6. NF-IL6 is a member of the C/EBP family of leucine zipper proteins. Some of the C/EBP proteins are well established as important transcription factors in liver and intestinal cells [42]. Footprint III might therefore, through interaction with a C/EBP-related protein, be important for the activity of the enhancer in liver and intestinal cell-types. It is clear however that both the deletion of a part of the C/EBP site as well as the mutation of its most 3' nucleotide only have a relatively small (but significant) effect. Footprint IV is also required for full activity of the enhancer, and the sequence shows great similarity to the Sp1 site in the APN epithelial promoter; Sp1 is thus a good candidate for a protein binding to this region.

The potential Ets elements in the enhancer may cooperate with Myb bound at the myeloid promoter

The human myeloid promoter has recently been shown to contain an Myb- and an Ets-binding site and binding of Myb and Ets to their elements results in a synergistic activation of the myeloid promoter [36]. As the APN enhancer characterized here contains potential Ets-binding sites, it is possible that the enhancer can cooperate with the myeloid promoter through an interaction

between Myb bound at this promoter and Ets bound at the enhancer. The present work points towards future experiments aimed at delineating the interplay between the enhancer and the two APN promoters in different cell-types.

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