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Onset of transcription of the aminopeptidase N (leukemia antigen CD 13) gene at the crypt/villus transition zone during rabbit enterocyte differentiation

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The sequence of a cDNA clone (2.82 kbp) of rabbit intestinal aminopeptidase N (CD 13) is reported. Using the corresponding anti-sense RNA probe, the distribution of aminopeptidase N mRNA along the crypt/villus axis of the rabbit small intestine was studied by in situ hybridization. The aminopeptidase N gene is expressed along the whole length of the villus with a maximum at its base. Expression was not detected in the crypt cells. The distribution of aminopeptidase N mRNA correlates with the presence of active enzyme as monitored by histochemical staining. The results are compatible with onset of transcription of the aminopeptidase N gene at the crypt/villus transition zone during the enterocyte differentiation.

Rabbit aminopeptidase N; CD 13; cDNA sequence; In situ hybridization; Histochemical staining

1. INTRODUCTION

The intestinal epithelium is renewed every third/fourth day. This is accomplished by the extrusion of the epithelial cells from the tips of villi and compensated by cell division, differentiation and migration from the crypt stem cells (for a review see [1]). The enterocyte is the dominating cell type in the epithelium and it carries out hydrolysis and absorption in the terminal phase of digestion. To accomplish this the luminal plasma membrane is organized as a brush border carrying several hydrolases [2,3] and transporters [4].

Enzymatic analyses of horizontal sections of fresh frozen small intestine [5-7] or on sequentially isolated enterocytes [8,9] as well as histochemical staining of vertical sections of the small intestine [10] show that the activity of the brush-border hydrolases is maximal in the mid-villi enterocytes and that the enzymatic activity is proportional to the amount of enzyme protein [6]. This might indicate that the genes of the microvillar hydrolases are inactive in the enterocytes of the crypts of Liberkühn and then are turned on late during their differentiation, i.e. when they during their migration reach the base of the villi. Alternatively the enzyme expression could be regulated at the post-transcriptional, translational or at the post-translational level.

Studies on the biosynthesis of aminopeptidase N, one of the dominating hydrolases in the intestinal brush-border, along the crypt/villus axis [7] points at the exclusion of a post-translational regulation of its expression but did not discriminate between a transcriptional, post-transcriptional or translational regulation.

The human intestinal aminopeptidase N cDNA has recently been cloned using a partial rabbit cDNA clone for the screening [11]. Cloning of the leukemia marker antigen CD13 cDNA [12] has demonstrated that CD13 is identical to aminopeptidase N. Recently also the rat kidney aminopeptidase N cDNA has been cloned [13]. The aminopeptidase N/CD13 is encoded by one single gene [14] which is located to the human chromosome 15ter [15,16]. Thus specific probes are now available for aminopeptidase N making it feasible to study the expression of its mRNA along the crypt villus axis in order to investigate the site of regulation of its expression.

2. MATERIALS AND METHODS

2.1. *DNA-sequencing*

The rabbit cDNA clone was isolated by immunological screening of a rabbit intestinal λ-gt11 library [17], the insert subcloned in Bluescript$^+$, nestled deletions created by the *Exo*III/mung bean method [18] and single-stranded DNA prepared (Stratagene, San Diego, CA) all described in detail for the cloning of the human aminopeptidase N cDNA [11]. DNA sequencing was carried out using the dideoxy sequencing method [19] using either the Klenow fragment or the T_7 DNA polymerase (Sequenase, USB, Cleveland, OH). Se-

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Abbreviations: 1 × SSPE, 0.18 M NaCl/0.01 M Na-phosphate, pH 7.7/0.001 M EDTA; 1 × SSC, 0.15 M NaCl/0.015 M Na-citrate, pH 7.0; PBS, 0.140 M NaCl/0.0027 M KCl/0.0015 M KH_2PO_4/0.0081 M Na_2HPO_4; Denhardt's solution, 1% Ficoll/1% polyvinylpyrrolidone/1% bovine serum albumin

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quencing (in both directions when appropriate) was carried out on several overlapping clones so each stretch was sequenced 3–9 times. [α-^{35}S]dATP was used as radiolabel and the analyses were carried out using salt gradient polyacrylamide gels [20]. The PC-GENE software (Genofit, Grand-Lancy, Switzerland) was used to organize and analyse the DNA-sequence.

2.2. *Northern blotting*

Crude RNA was isolated from N_2-frozen rabbit small intestine [21]. The RNA was after denaturation in formamide separated in formaldehyde containing agarose gel (1%) [22], transferred to nitrocellulose membranes and hybridized with the nick translated 5′-*Eco*R1 fragment (in 5 × SSPE, 50% formamide, 5 × Denhardt's solution, 10% Dextran sulphate, 42°C; final wash 0.1 × SSPE, 0.1% SDS, 20°C, 30 min).

2.3. *Preparation of probes*

The 5′-*Eco*R1-fragment (1.26 kbp) was cloned in both orientations in a Bluescript$^+$ plasmid. CsCl-banded plasmid DNA was linearized by an appropriate restriction enzyme not cutting within the cDNA and the in vitro transcription was carried out (according to recipe delivered by Stratagene, San Diego, CA) by T_3 RNA polymerase using rATP, rGTP, rCTP (400 μM, final concentration) in the presence of 50 μCi [α-^{35}S]UTP (48 μM, final concentration) in order to obtain sense and anti-sense probes. The DNA was removed by RNase-free DNase 1, extracted with phenol/chloroform and precipitated with ethanol in the presence of 2 M ammonium acetate [23]. Reduction of probe fragment length to average 200 bp was carried out by incubation in 0.2 M sodium carbonate buffer, pH 10.2 (60°C, 40 min).

The size of the in vitro transcription products was analysed before and after the alkaline treatment by electrophoresis in formaldehyde containing agarose (1%). The specific activity of the probes was 2.5×10^8 dpm/μg.

2.4. *Tissue and section preparation*

Adult rabbits were killed by neck dislocation. Immediately after this, pieces of jejunum were taken about 10 cm from the ligament of Treitz.

For in situ hybridization 5 mm pieces were fixed in 4% paraformaldehyde in PBS, pH 7.4 overnight. After fixation the tissues were paraffin-embedded according to the routine histological methods. 5 μm thick sections were cut and mounted on aminoalkyl silane-treated slides [24] and kept at 4°C.

For histochemical staining 1 cm cylinders of jejunum were cut and immediately frozen (2 min) in precooled hexane (−70°C). The frozen tissue was mounted on a chuck and 8 μm sections were cut by a Bright's cryostat (−25°C). The sections were transferred to clean glass slides and then used directly for histochemical staining.

2.5. *In situ hybridization*

Generally the procedures recommended by Angerer et al. [23] were used. Sections were deparaffinated through a graded series of xylol in ethanol and then hydrated through a graded series of ethanol in water. The slides were incubated (30 min, 37°C) in a solution containing proteinase K (1 μg/ml) in 0.1 M Tris-HCl, pH 8.0, 50 mM EDTA. The digestion was stopped by a brief wash in distilled water and transferred directly into 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, and incubated (10 min, 20°C). The acetylation was stopped by brief wash in 2 × SSC and the slides were dehydrated through a graded ethanol series up to 99% ethanol and left to air-dry. The hybridization took place in 50% formaldehyde, 0.3 M sodium chloride, 20 mM sodium acetate, pH 5.0, 1 mM EDTA, 1 × Denhardt's solution, 500 μg *E. coli* tRNA per ml, 10% dextran sulphate and 100 mM dithiothreitol (60°C, 20 h) under mineral oil. The probe concentration was varied between 0.2 and 0.8 μg/ml. After removal of the mineral oil the excess probe was washed off in 4 × SSC, containing 10 mM dithiothreitol for 15 min. The washing step was repeated 3 times. The slides were dehydrated through a graded ethanol series and air-dried. They were then incubated in 50% formamide, 0.3 M sodium chloride, 20 mM sodium acetate, pH 5.0, 10 mM EDTA, 10 mM dithiothreitol (60°C, 10 min) followed by a brief wash in 2 × SSC. Treatment with RNAase A (20 μg/ml in 0.5 M sodium chloride, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 37°C, 30 min) was carried out and the stringent washing performed in 0.1 × SSC (50°C, 5 min) followed by a repeated washing (20°C, 30 min). The slides were then dehydrated and initially analysed by autoradiography on a film (Fuji RX). They were then covered by film emulsion (Kodak NTB-2) and exposed for an appropriate period (2–8 weeks). After development the slides were counterstained by hematoxylin.

2.6. *Enzyme cytochemistry*

Cryostat sections were incubated at 37°C for 15, 30 and 45 min in freshly prepared medium containing 1.5 mM L-leucyl-4-methoxy-2-naphthylamide dissolved in 0.5% *N,N*-dimethylformamide, 0.05 M sodium-phosphate buffer, pH 7.4, containing 60 mM sodium chloride, 1.0 mM potassium cyanide and 2.5 mM Fast blue B. The medium was replaced every 15 min. The incubations were stopped by rinsing in 0.15 M sodium chloride (2 × 5 min), transferred to a 0.1 M copper sulphate solution (2 × 5 min) and distilled water (2 × 5 min) prior to mounting in glycerin-jelly containing 0.1 M copper sulphate.

3. RESULTS AND DISCUSSION

3.1. Sequence of rabbit aminopeptidase N

Fig.1 shows the DNA sequence of the cloned rabbit aminopeptidase N cDNA together with the most important restriction enzyme sites and the deduced amino acid sequence. The 5′-*Eco*R1 fragment of the cDNA clone hybridises to a mRNA of 3.4 kB (fig.2) and no other hybridization signal is seen. The rabbit clone (2819 bp) corresponds to 84% of the 3′-part of the human clone and does not cover the cytoplasmic and transmembrane domains, i.e. the N-terminal segment. Consequently the sequence earlier published [25] for a part of this segment is not found in the amino acid sequence in fig.1. The rabbit clone has a long open reading frame ending at a stop codon (nt 2375) corresponding to that in the human cDNA. The DNA sequence in the coding region is strongly conserved (84% identity). The 3′-noncoding sequence, which is polyadenylated, is similar in length (0.44 kbp) to that of the human enzyme and is much less conserved. The protein sequence which is strongly conserved (80% identity) encompasses the part showing similarity to the *E. coli* peptidase N [26,27]. Thus it contains the Zn^{2+}-binding signature proposed to form a part of the active site [11,28]. There are 8 potential glycosylation sites and one potential tyrosine sulfation site (aa 737) corresponding to one of two found in the human aminopeptidase N sequence.

3.2. *Distribution of aminopeptidase N mRNA in the small intestine*

This report is the first in which the mRNA expression of a gene for an intestinal brush-border hydrolase has been studied along the crypt/villus axis. The distribution of aminopeptidase N mRNA in the different layers of the small intestine is analyzed by the in situ

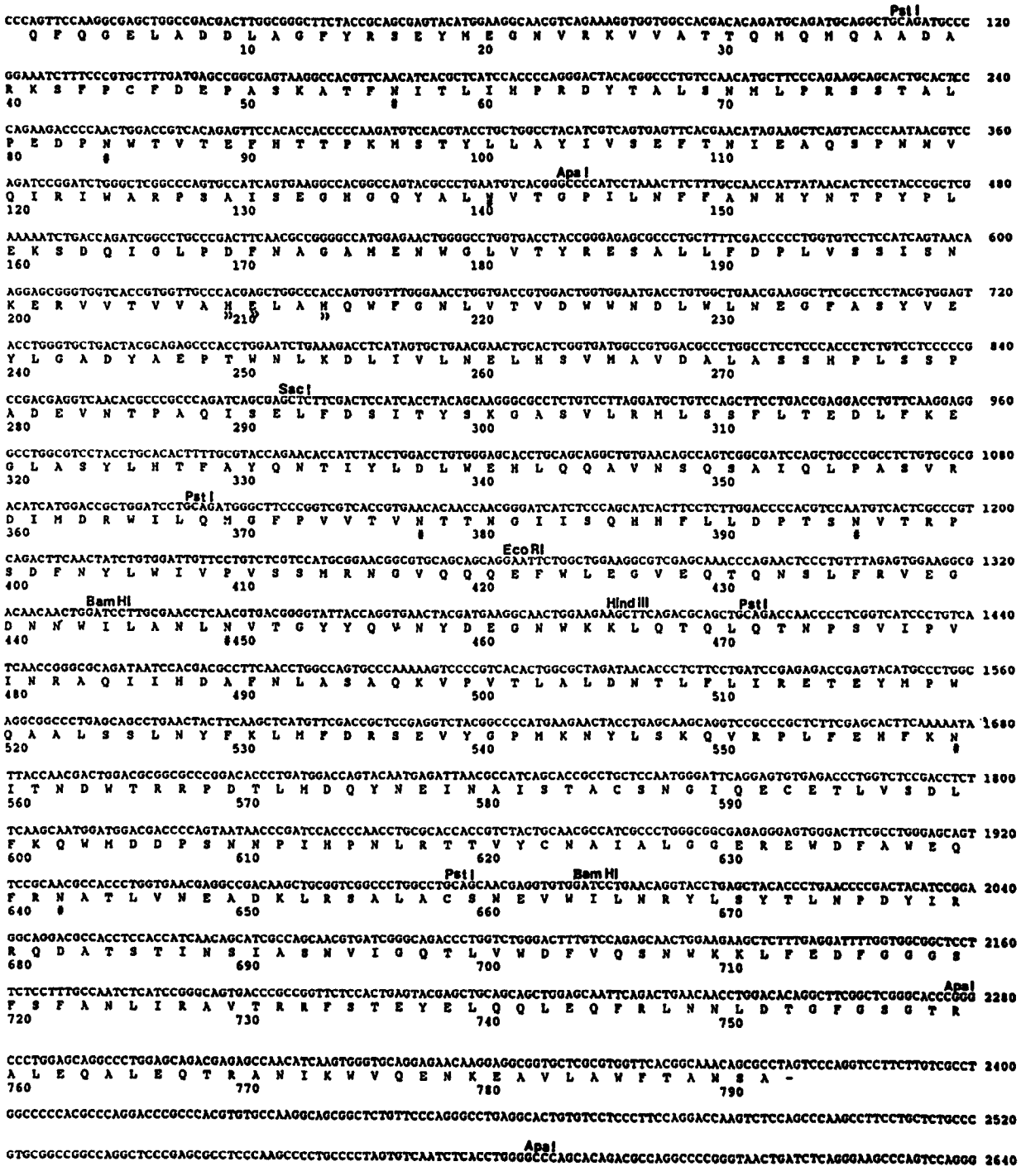

Fig.1. DNA sequence of rabbit intestinal aminopeptidase N and the deduced amino acid sequence. >>, predicted ligands of Zn^{2+}; #, potential glycosylation sites.

hybridization technique. Using the anti-sense RNA probe (fig.3A) a very strong signal is seen in the intestinal epithelium of the villi. The sense RNA (fig.3B) used on a consecutive section under the same experimental conditions resulted in diffuse labelling of the tissue as well as outside the tissue. The signals seen in lamina propria and the submucosa using the anti-sense RNA probe does not exceed those obtained by the sense RNA probe or the labelling obtained outside the tissue demonstrating that these tissues contain low or no

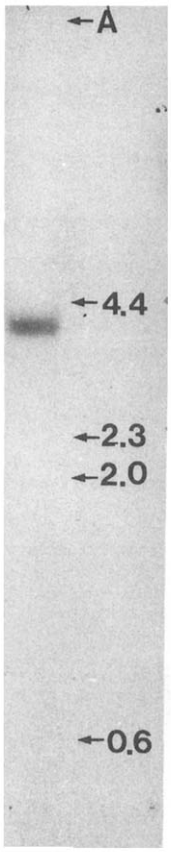

Fig.2. Northern blotting of rabbit intestinal mRNA. The probe used was the nick translated 1.26 kbp 5′-*Eco*R1 fragment of rabbit aminopeptidase N cDNA.

aminopeptidase N mRNA. Smooth muscle cells and the cells in the connective tissue are known to contain very low levels of aminopeptidase N [6,29]. The in situ hybridization results clearly show that this is due to low levels of aminopeptidase N mRNA most probably due to lack of effective transcription of the gene in the corresponding cells although a less likely rapid post-transcriptional degradation of the mRNA cannot be excluded.

The aminopeptidase N mRNA in the enterocytes is limited to the villi and is maximal at their bases. In contrast, no significant labelling is seen in the crypt enterocytes. The fully differentiated villi enterocytes have been shown to have the highest potential for synthesis of RNA in the small intestinal epithelium [30]. Our data show that aminopeptidase N mRNA are among the mRNAs synthesized by the fully differentiated enterocytes.

The distribution of the aminopeptidase N mRNA is in good accordance with that of the active enzyme as demonstrated by histochemical staining (fig.4). The enzymatic activity is also in this case maximal at the base of the villi (data not shown). In most crypts no activity is detected even after prolonged incubation. Only in crypts close to the base of the villi is a weak enzymatic activity detected which, within a narrow zone, increases to full activity. This result is in agreement with those reported for rat, guinea pig and human [10] using the same technique.

Our results based on in situ hybridization and

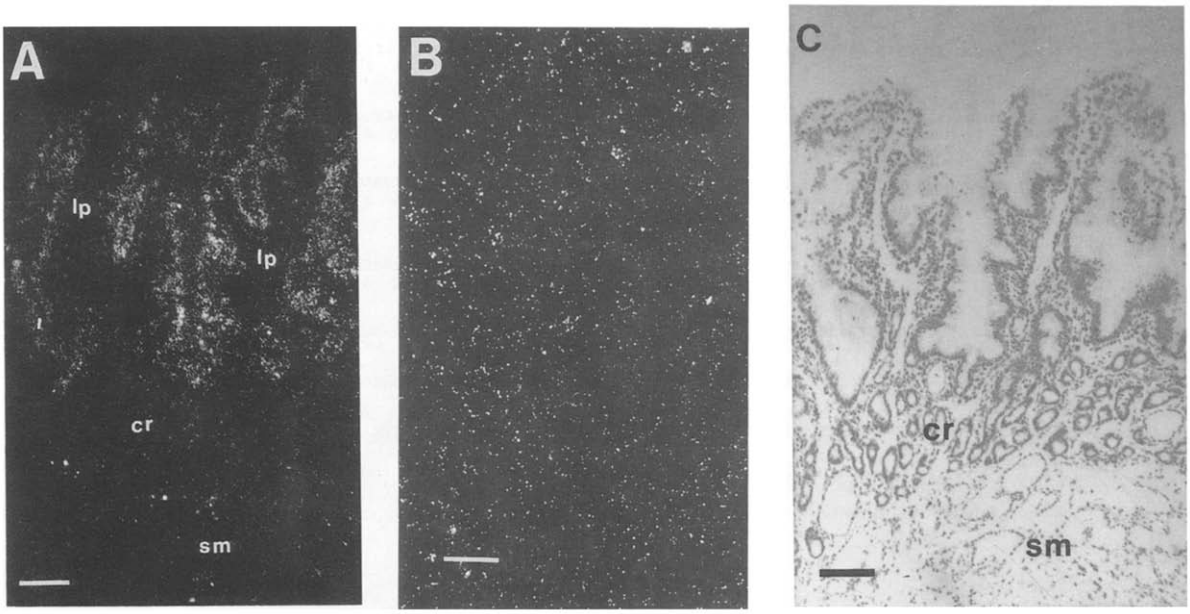

Fig.3. Distribution of aminopeptidase N mRNA in the rabbit jejunum as studied by in situ hybridization. The hybridization was carried out as described in section 2. The sections were covered by film emulsion and developed after 6 weeks of exposure. They were then stained with hematoxylin. (A) and (B) are consecutive sections shown in dark-field illumination. Strong hybridization is seen all along the villi using the anti-sense RNA probe (A) with a maximum at the base. The labelling in the lamina propria (lp), crypts (cr) and submucosa (sm) do not differ from the labelling outside the tissue and the diffuse labelling obtained by using the sense RNA probe (B). (C) shows the bright-field picture of (A). Bar 100 μm.

Fig.4. Distribution of aminopeptidase N activity in rabbit jejunum. Frozen sections of jejunum were stained for aminopeptidase N activity by use of a simultaneous coupling technique. Incubation time 15 min. No enzymatic activity is seen in the crypts (open arrow) even after prolonged incubations. An increasing activity in the brush border is visible in the transition zone between the crypt and villus (small solid arrows) reaching fulminant activity in the villus (large solid arrow). Bar 100 μm.

histochemical staining studies thus exclude the existence of large amounts of inactive precursor forms of aminopeptidase N in the rabbit crypt cells.

Our data are compatible with an onset of production of aminopeptidase N mRNA in a narrow zone of developing enterocytes at the transition zone between the crypts and villi. The distribution of the aminopeptidase N mRNA thus is very similar to that reported for the villin mRNA [31]. This is most probably due to an abrupt start of transcription at this stage of enterocyte differentiation due to expression of a complement of regulatory factors and/or changes in chromatin structure. It could, however, also theoretically depend on a diminished breakdown of aminopeptidase N transcripts either in the nuclei or in the cytoplasm. The result excludes a regulation along the crypt/villus axis at the translational or post-translational level of aminopeptidase N as the corresponding mRNA cannot be detected in the crypts. However, direct studies on the rate of biosynthesis of aminopeptidase N mRNA has to be carried out to settle the exact level of regulation. The regulation mechanism along the crypt/villus axis thus differs from that of the foetal to adult development which have been shown to be controlled at the translational level [32].

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