Biochemical and Biophysical Research Communications **267,** 91–95 (2000)
doi:10.1006/bbrc.1999.1911, available online at http://www.idealibrary.com on IDEAL®

AT Motif Binding Factor 1-A (ATBF1-A) Negatively Regulates Transcription of the Aminopeptidase N Gene in the Crypt–Villus Axis of Small Intestine

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Received November 19, 1999

This is the first study to demonstrate that the AT motif binding factor 1-A (ATBF1-A) is expressed in the crypts and the bases of villi of the small intestine and negatively regulates transcription of brush-border enzyme gene, aminopeptidase-N (APN). *In situ* hybridization visualized a limited ATBF1-A mRNA expression in the crypts and the bases of villi. Transient transfection and dual luciferase-reporter assay demonstrated that ATBF1-A suppressed the activity of APN promoter, but did not that of AT motif deleted promoter. These results imply that ATBF1-A inhibits the transcription of APN gene through its direct binding to the AT motif element. Furthermore, butyrate-induced differentiation of Caco-2 cells, retaining the enterocytic phenotypes such as a villus structure and the expression of brush-border enzymes, leads to a reduced expression of ATBF1-A mRNA. We proposed that ATBF1-A regulating APN gene expression in the crypt–villus axis of the small intestine is a landmark of enterocyte differentiation and maturation. © 2000 Academic Press

Epithelial cells (enterocytes) in the small intestine are derived from stem cells in the intestinal crypts. The stem cells give rise to daughter cells which migrate out of the crypts to the villus tip for only 2 or 3 days (1, 2). They differentiate into mature epithelial cells during the migration, and eventually undergo apoptosis. Brush border enzymes for the nutrient absorption are abundant and functioning at the differentiated epithelial cells in the upper villi of the small intestine. One of the enzymes, aminopeptidase N (ANP/CD13), is uniquely expressed in the upper cells of the villi, but not in the crypts (3). The APN gene expression is up-regulated in the bases of the villi by a hepatocyte nuclear factor 1 (HNF1), which is known to activate the transcription of α-fetoprotein (AFP) in hepatocytes (4–9).

In hepatic cells, HNF1 interacts with an AT-motif binding element of the AFP promoter, where another transcription factor, the AT-motif binding factor (ATBF1), is considered to competitively bind to suppress the transcription of AFP (10, 11). In fact, HNF1 binds AT-motif to activate the expression of AFP, albumin, and various hepatic genes in human hepatoma cells (12). However, the competitive binding of ATBF1 with HNF1 at the AT-motif element is only evident in the AFP promoter, but not others. Accordingly, there should be somewhat of difference between HNF1 and ATBF1 in the binding specificity to AT-motif probably due to a small change of AT-motif alignments depending on a variation of target genes.

Our aims of this study are first to demonstrate the site-specific expression of ATBF1 in the crypt-villus axis of the small intestine, and secondly the regulatory action of ATBF1 on the APN promoter comparing to the HNF1 action.

MATERIALS AND METHODS

Immunohistochemistry. Immunohistochemical studies for analyzing the production and localization of APN protein were performed using goat polyclonal antibody to APN (Santa Cruz Biotechnology). Sections of formaldehyde-fixed, paraffin-embedded mouse small intestinal tissue were used. The sections were incubated with 0. 5% normal mouse serum for 20 min and with a 1:250 dilution of the first antibody for 4 h. After incubation with biotinylated second antibody, the streptavidin-biotin-horseradish peroxidase (SAB-PO)

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0006-291X/00 $35.00

method (HISTOFINE, Nichirei CO, Japan) was used in which 3, 3′-diaminobenzidine tetrahydrochloride (Sigma) was used as the chromogen.

Preparation of digoxigenin labeled cRNA probe. To prepare BX151 probe for mouse ATBF1-A mRNA, a 151-bp sequence of mouse ATBF1-A cDNA corresponding to nucleotides 3353-3503 of human ATBF1-A cDNA was inserted into pBluescript II KS(+). This was digested with *Bam*HI (Run off site) and transcribed by T3 RNA polymerase in the presence of DIG-UTP to produce a digoxigenin labeled 228-bp fragment consisting of 151 bp of the mouse ATBF1 sequence and 77 bp of the vector sequence (Boehringer Mannheim).

In situ hybridization. *In situ* hybridization with paraffin tissue sections was performed essentially as described by Panoskaltsis-Mortari and Bucy (13). Sections were hybridized with 3 ng of heat-denatured cRNA probe for 16 h at 50°C in 100 μl of hybridization solution (50% formamide, 10 mM Tris-HCl, pH 7.6, 200 μg/ml yeast tRNA, 1× Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS and 1 mM EDTA pH 8.0). After hybridization, slides were successively washed in 2× SSC for 30 min at room temperature, treated at 37°C for 30 min with RNase A (10 μg/ml in 500 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA), washed at 50°C for 15 min in 2× SSC containing 50% formamide, and at 50°C for 15 min in 1× and 0.2× SSC. DIG-labeled RNA probes used for hybridization were immunodetected with anti-digoxigenin-alkaline phosphatase Fab antibody (1:2000; Boehringer Mannheim), and nitroblue tetrazolium 6-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Boehringer Mannheim) as a substrate for the alkaline phosphatase. Staining was allowed to proceed overnight in the dark at room temperature and the reaction was stopped in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA.

Cell culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 4 mM L-glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml) and gentamicin sulfate (50 μg/ml) in 5% CO_2 in a humidified incubator at 37°C.

RNase protection assays. To prepare MD14 probes for RNase protection assays of human ATBF1-A and ATBF1-B transcripts, a 256-bp sequence from nucleotides 3353-3608 of human ATBF1-A cDNA was inserted into pBluescript II KS(+) (Stratagene). This was digested with *Bam*HI and transcribed by T7 RNA polymerase (Boehringer Mannheim) in the presence of [α-^{32}P]CTP to produce a radioactive 287-bp fragment consisting of 256 bp of the ATBF1-A sequence and 31 bp of the vector sequence. This probe yielded a 256-bp fragment with ATBF1-A mRNA and 216-bp fragment with ATBF1-B mRNA (Fig. 2). To prepare the HNF1 probe, a 162-bp sequence from nucleotides 641-802 of human HNF1 cDNA was inserted into pBluescript II KS(+) (Stratagene). This was digested with *Hin*dIII and transcribed by T7 RNA polymerase (Boehringer Mannheim) in the presence of [α-^{32}P]CTP to produce a radioactive 288-bp fragment consisting of 218 bp of the HNF1 sequence and 70 bp of the vector sequence.

Total RNA (5 μg) was hybridized at 45°C overnight with 5×10^5 cpm of probe in 40 mM PIPES (pH 6.4), 80% formamide, 0. 4 M NaCl, and 1 mM EDTA. The reaction mixture was digested with 40 μg/ml RNase A and 2 μg/ml RNaseT1 at 30°C for 30 min. The sample was then treated with 130 μg/ml proteinase K, electrophoresed on 5% polyacrylamide/urea gel, and autoradiographed.

Construction of plasmids. Plasmid containing firefly luciferase (promoterless) (pGV-B vector) was purchased from Toyo-inki. Co., Tokyo. The APN promoter genomic sequence (from −350 to +43) was amplified by anchored polymerase chain reaction (PCR). The following primers that included restriction sites were used for amplification: APN-*Sma*I−350 (5′-TCCCCCGGGACCTCAGTTCTGATGCTGTT-3′, in which the *Sma*I site is underlined), and APN-*Hin*+43 (5′-CCCAAGCTTGATGGTGGGGAGGCGGCTCA-3′, in which the *Hin*dIII site is underlined). After TA cloning (pGEM-T Easy vector, Promega) and sequencing, a −350 to +43 promoter APN gene fragment was subcloned into the *Sma*I and *Hin*dIII sites of pGV-B (reporter plasmid; pGV-B-APN). For the deletional analysis of APN promoter, the AT motif deleted region (from −68 to +43) was amplified by anchored PCR. The following primer that included restriction sites were used for amplification: APN-*Sma*I−68 (5′-TCCCCCGGGTGCCCAGTCTGCCTG-TTGTG-3′, in which the *Sma*I site is underlined) and APN-*Hin*+43. After TA cloning and sequencing, a −68 to +43 AT motif deletion APN promoter fragment was subcloned into the *Sma*I and *Hin*dIII sites of pGV-B (reporter plasmid; ΔpGV-B-APN) (Fig. 3a). Full-length ATBF1-A expression vector (pHbSMEdXba) was used as the effector plasmids and antisense ATBF1-A expression vector (pHbSMER) were used as a control (mock).

Transfection experiments. Transfection of Caco-2 was performed in a 12-well plate with the cells at 80% confluency. The reporter and effector plasmids were co-transfected with the control vector pRL-TK Vector (Promega) containing renilla luciferase as a transfection efficiency control. We used the Chen and Okayama transfection method (14). Luciferase activity was quantified in a luminometer using the dual-luciferase-reporter system (Promega).

For this, the culture medium was removed and 120 μl of 1× PLB (Passive Lysis Buffer, Promega) was added to each well. Firefly luciferase activity was measured after the addition of a mix containing beetle luciferase substrate (LAR I: Luciferase Assay Reagent I, Promega) to the cell lysate. Then renilla luciferase activity was measured by adding a mix containing coelenterazine (Stop & Glo: Renilla Assay Reagent, Promega). All experiments were performed in triplicates.

Butyrate-induced differentiation of Caco-2 cells. Subconfluent Caco-2 cells were plated in MEM with 10% fetal bovine serum for 24 h; after 24 h, the medium was replaced, at which time Caco-2 cells were treated with either sodium butyrate (final concentration of 5 mM) or vehicle (control).

RESULTS

Immunohistological Detection of APN in the Small Intestine

APN was immunohistochemically detected in the brush-border of absorptive epithelial cells residing from the middle to the top of the villi (Fig. 1A). APN was not detected in the cells of the crypt and the base of villi. The results showed that well-differentiated and mature epithelial cells expressed APN.

In Situ Hybridization of ATBF1 in the Small Intestine

In situ hybridization revealed that ATBF1 mRNA was expressed in the crypts and the bases of villi. ATBF1 mRNA was not detected in well-differentiated and mature epithelial cells that expressed APN (Fig. 1B).

Expression of ATBF1 and HNF1 mRNA in Caco-2 Cells

Caco-2 cells, which have a villus structures and express the genes of intestinal brush-border enzymes such as sucrase-isomaltase (SI) and APN, are widely

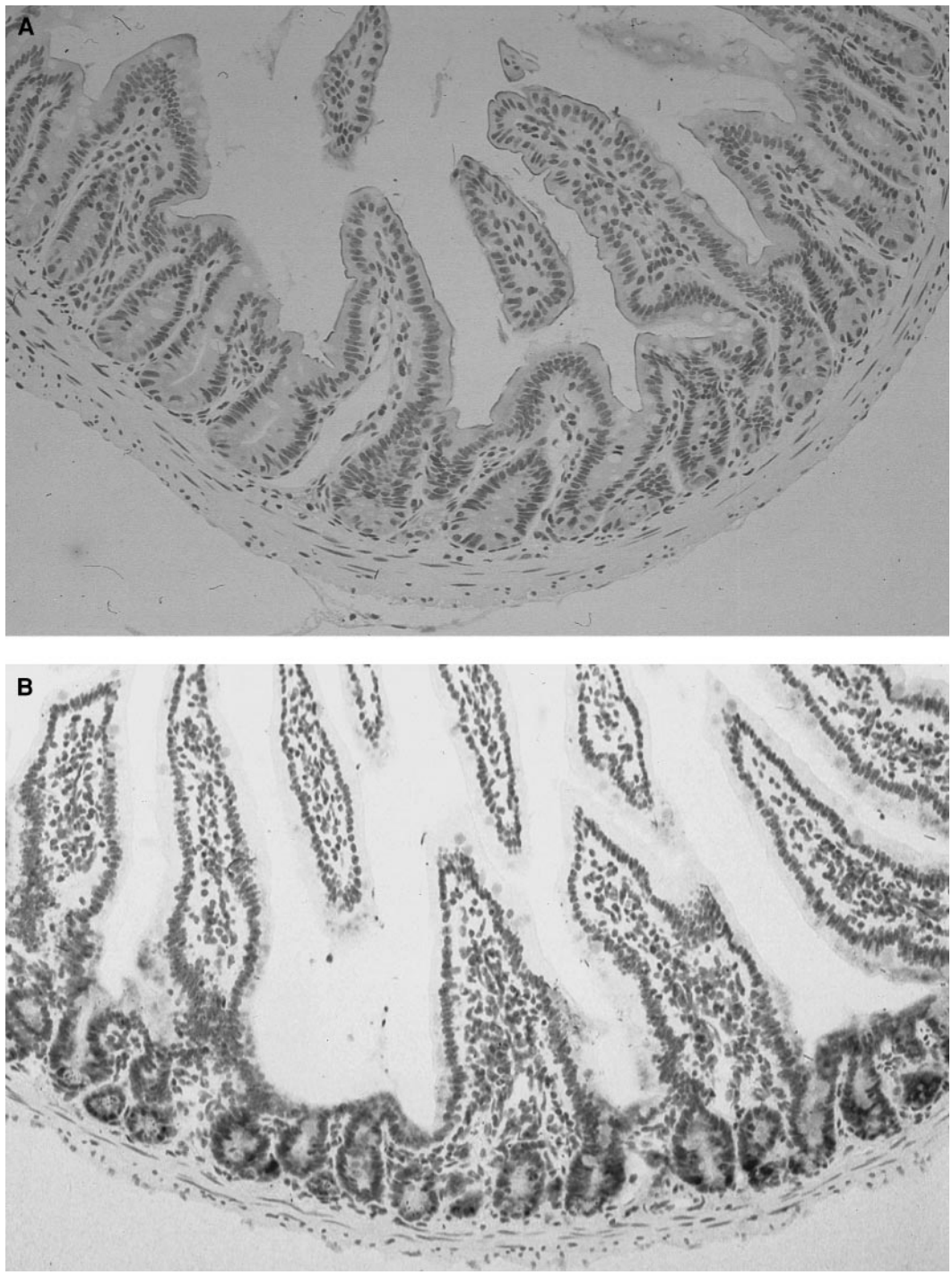

FIG. 1. (A) Immunohistochemical staining of APN in the mouse small intestinal mucosa. APN expression is detected in the brush-border of the villous enterocytes, but not at the base of the villi and crypts. (B) *In situ* hybridization for ATBF1 mRNA of the mouse small intestine. ATBF1 mRNA is expressed at the base of the villi and crypts, but not from the middle to the top of the villi.

used as a model system for the study of enterocytic function (9, 15). RNase protection assays revealed that Caco-2 cells express mRNAs for ATBF1-A, -B and HNF1 (Fig. 2). The expression level of ATBF1-A mRNA was higher than that of ATBF1-B.

APN Promoter Activity by ATBF1-A

To investigate whether ATBF1 negatively regulates APN promoter, we performed a transient transfection assay with Caco-2 cells. The constructs of reporter

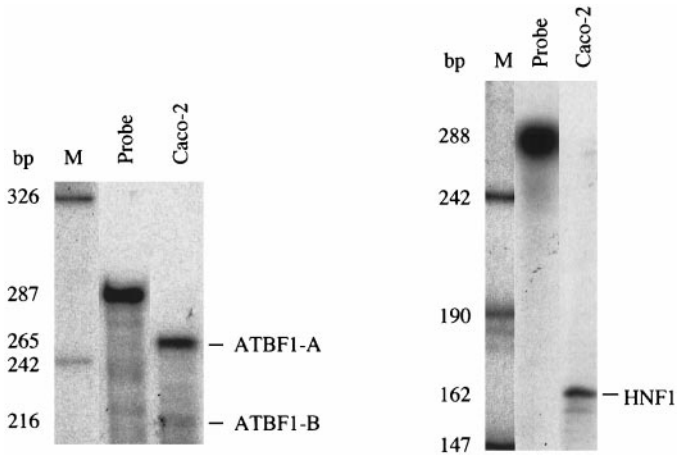

FIG. 2. RNase protection assay of ATBF1 and HNF1 mRNAs in Caco-2 cells. ATBF1-A and ATBF1-B are produced by alternative splicing and alternative promoter usage. In order to examine the expression levels of ATBF1-A and -B mRNAs, a α-^{32}P-labeled RNA probe (287 bp) was produced covering the alternative splicing site. ATBF1-A, and -B mRNA were detected as 265- and 216-bp bands.

plasmids (pGV-B-APN and ΔpGV-B-APN) are shown in Fig. 3A. Transient transfection assays revealed that the overexpressed ATBF1-A lowered APN promoter activity (61.3 ± 11.3%). The half dose of its expression vector slightly suppressed the promoter activity (96.1 ± 11.3%). By mutant reporter plasmid without AT motif (ΔpGV-B-APN), the overexpressed ATBF1-A lost the suppressive activity (94.7 ± 4.2%) (Fig. 3B). These results suggest that a binding of ATBF1-A to AT motif is essential to inhibit the APN promoter activity.

Butyrate-induced Differentiation of Caco-2 Cells and ATBF1-A mRNA Expression

It is well known that butyrate induce the differentiation of Caco-2 cells in well-differentiated state, i.e., a slow proliferation rate and inductions of brush border enzymes (16, 17). RNase protection assay demonstrated a time-dependent reduction of ATBF1-A mRNA expression within 24 h in differentiated Caco-2 cells by butyrate (Fig. 4). These results including the ATBF1-A

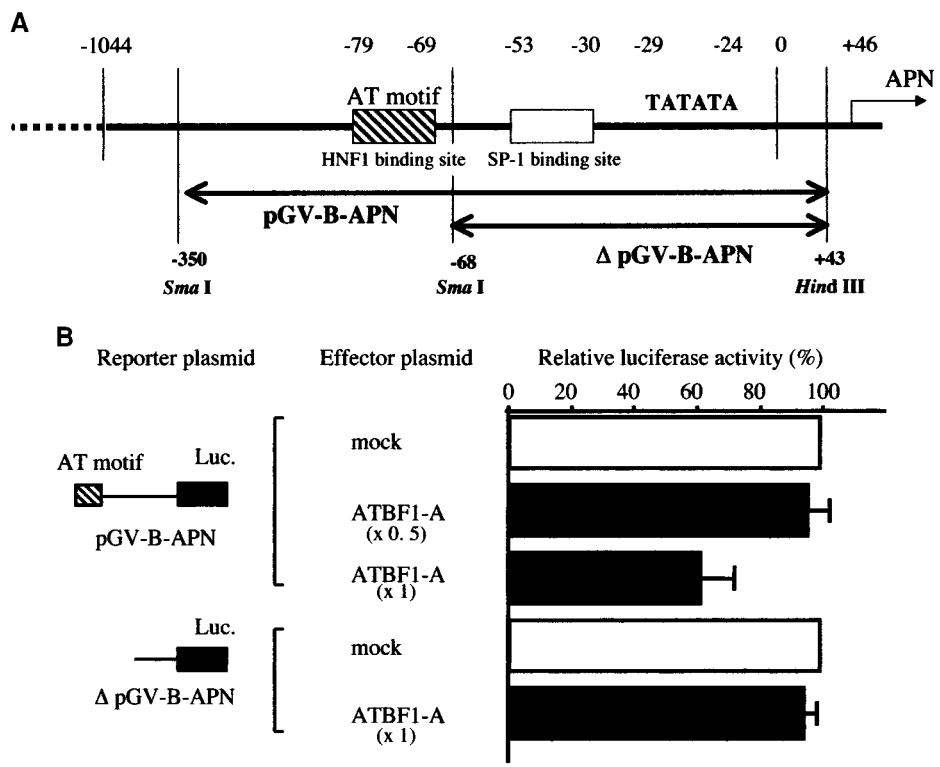

FIG. 3. (A) The constructions of APN reporter plasmids for luciferase assay. Two distinct promoters, arranged in tandem, myeloid promoter and epithelial promoter, control the APN gene. The epithelial promoter is closest to the coding part of the APN gene and is active in the enterocyte and other epithelial cells such as hepatocytes and endometrial cells. The other myeloid promoter is placed 8 kb upstream of the epithelial promoter and is active mainly in myeloid cell (18). The promoter DNA fragment from −350 to +43 of APN epithelial promoter region was amplified by anchor PCR and inserted into the *Sma*I, *Hin*dIII site of firefly luciferase pGV-B vector (pGV-B-APN). The promoter DNA fragment from −68 to +43 was amplified by anchor PCR and inserted into the *Sma*I, *Hin*dIII site of firefly luciferase pGV-B vector to construct AT motif deletion mutant vector, ΔpGV-B-APN. (B) Transient transfection assay using a dual luciferase-reporter assay. The ratios between firefly luciferase activity and renilla luciferase activity (pRL-TK vector) were compared between the mock (full length antisense ATBF1-A expression vector, pHbSMER) group and the full length sense ATBF1-A expression vector (pHbSMEdXba) group. Data represent the ratio between both activity of firefly luciferase and renilla luciferase for triplicates (the mean relative activities ± SE).

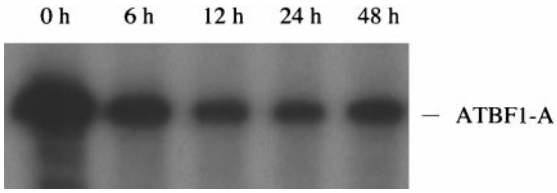

FIG. 4. Expression of ATBF1-A mRNA in butyrate-induced Caco-2 cell differentiation. Total RNA (5 μg) from butyrate treated Caco-2 cells was analyzed by RNase protection assay.

mRNA expression in the small intestine suggest that ATBF1-A expression might be regulated by the cell differentiation.

DISCUSSION

Immmunoreactive APN protein was detected in brush-border of differentiated enterocytes (Fig. 1a) in support of the reported observation on a limited localization of APN mRNA to the villi, but very little in crypts (3). It is well documented by the previous reports that there is a single HNF1 binding site (AT motif) and Sp1 binding site in the epithelial promoter of APN, and that HNF1 is capable of inducing a 10-fold activation of APN epithelial promoter (8, 9, 18, 19). There is a declining gradient of HNF1 mRNA expression levels from the crypt to the villus tips with the highest expression in the crypt (5). It has long remained unclear as to why HNF1 mRNA is strongly expressed in the crypt cells, and why APN mRNA is not expressed there. As shown in Figure 1b, ATBF1-A mRNA expression is uniquely detected in the crypts and the bases of villi. Considering the opposing action of ATBF1-A to HNF1 on the AFP expression observed in hepatocytes, these results explain that abundant ATBF1-A overcomes the action of HNF1 in the crypts resulting in the suppression of APN mRNA expression.

Pluripotent stem cells are located in the intestinal crypt. During a ascending migration in intestinal villus, they acquire the structural features of mature enterocytes and express specific gene products such as the brush-border enzymes, SI and APN (6, 7). The characteristic pattern of intestinal gene transcription appears to be regulated by the complimentary combination of multiple transcription factors including ATBF1-A and HNF1. In the early stages of enterocytic differentiation and maturation, ATBF1-A and HNF1 might competitively bind to the AT-motif element to regulate APN transcription in the crypts and the bases of villi.

In this study, we have demonstrated that ATBF1 is strongly expressed in the enterocytes retaining a potential of differentiation in the crypts and the bases of villi but little in the mature cells in the upper villi, and that the expression is markedly suppressed by the induction of differentiation of Caco-2 by butyrate. Taken together with these observations, ATBF1-A may be uniquely expressed in the proliferating stem cells of intestinal tissue, while little expressed in the embryonal carcinoma cells such as P19 (11). Although the actual mechanism to explain the tissue-specific difference in ATBF1-A remains unclear, both ATBF1-A and HNF1 transcription factors are most likely involved in the initiation of enterocytic differentiation in the crypt-villus axis of the small intestine.

ACKNOWLEDGMENTS

We thank Manami Yamamoto and Yukimi Itoh, Nagoya City University Medical School, for technical assistance.

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