Regulation of human methylthioadenosine phosphorylase gene by the CBF (CCAAT binding factor)/NF-Y (nuclear factor-Y)

Yuwaraj KADARIYA^{*1}, Kaname NAKATANI^{*}, Junji NISHIOKA^{*}, Takahiko FUJIKAWA[†], Warren D. KRUGER[‡] and Tsutomu NOBORI^{*}

^{*}Department of Laboratory Medicine, Mie University School of Medicine, Tsu, Mie 514-8507, Japan, [†]Department of Biochemistry, Mie University School of Medicine, Tsu, Mie 514-8507, Japan, and [‡]Division of Population Science, Fox Chase Cancer Center, Cottman Avenue 333, Philadelphia, PA 19111, U.S.A.

hMTAP (human 5'-deoxy-5'-methylthioadenosine phosphorylase) is a key enzyme in the methionine salvage pathway and is frequently inactivated in human tumour cells. To understand the mechanism of the transcriptional regulation of the MTAP gene, we have cloned the 1.29 kb fragment of the hMTAP promoter and identified *cis*-acting regulatory sequences using a luciferase reporter gene assay. Maximal promoter activity was associated with sequences between –446 and –152, where two CCAAT elements were located. Electrophoretic mobility-shift assay reveals binding of specific complexes at both CCAAT motifs within the MTAP promoter, although more prominent bands were associated with the distal motif (–372 to –368). Supershift experiments and

chromatin immunoprecipitation assays indicate that both the proximal and distal complexes bind CBF (CCAAT-binding factor; also known as nuclear factor-Y), and that the distal CCAAT motif has increased levels of CBF binding. We have mapped seven different transcriptional start sites between –135 and –58. Our results show that the hMTAP expression is regulated by a CBF and that the distal one of two CCAAT motifs plays a major role in the transcriptional activation of hMTAP gene.

Key words: CCAAT motif, chromatin immunoprecipitation, 5'-deoxy-5'-methylthioadenosine phosphorylase (MTAP), luciferase, nuclear factor-Y (NF-Y), promoter.

INTRODUCTION

MTAP (5'-deoxy-5'-methylthioadenosine phosphorylase) is a key enzyme in the methionine salvage pathway, whose function is to metabolize MTA (5'-methylthioadenosine) produced as a by-product of polyamine biosynthesis. MTAP uses P_i to cleave MTA into adenine and 5'-methylthio-D-ribose-1-phosphate, which are then recycled to adenine nucleotides and methionine respectively. Cleavage by MTAP is the sole catabolic pathway for MTA to produce adenine and methionine that are needed for DNA and protein synthesis [1–3]. This pathway exists in bacterial cells [4], in rat [5,6] and in the yeast *Saccharomyces cerevisiae* [7,8]. All normal cells and tissues have abundant MTAP activity, suggesting that the entire salvage pathway is present in all of the cells in the human body [9,10].

Loss of MTAP activity is frequently observed in tumour cells. In the 1970s, Toohey first recognized that certain murine malignant haematopoietic cell lines lacked MTAP activity [11]. Since then, loss of MTAP expression (either at the protein or mRNA level) has been observed as a frequent event in a variety of tumours including lung cancer [12,13], bladder cancer [14], head and neck cancer [15], endometrial carcinoma [16], breast cancer [17], ovarian cancer [18], glioma [19] and in myxoid chondrosarcoma and soft-tissue sarcoma [20,21]. More recently, it has been shown that MTAP can act as a tumour-suppressor gene and that this tumour-suppressor activity may be mediated by the activation of the polyamine biosynthetic pathway [17,22].

The MTAP gene has been cloned and mapped to chromosome 9p21 in a region of the genome that is often deleted in tumour cells [3,23–25]. In most cases, it appears that the loss of MTAP activity or mRNA is the result of homozygous deletions of MTAP gene [26–30]; however, this is not always the case. A recent

study on MTAP expression in malignant melanoma shows that in most malignant melanoma-derived cell lines MTAP expression is reduced by mechanisms other than deletion, as these cells have an intact MTAP gene [31]. In addition, there is at least one MTAP-deficient lymphoma cell line with an intact MTAP gene that fails to express MTAP mRNA [32]. These observations suggest that there may be defects in the production of MTAP mRNA in some cell lines.

To understand the molecular mechanism behind decreased MTAP mRNA production in these cells, it is first necessary to characterize the MTAP promoter. In the present study, we have performed a series of experiments defining important *cis*- and *trans*-acting elements required for MTAP expression.

MATERIALS AND METHODS

Cloning of human MTAP promoter

A phage library constructed from human placenta DNA (Stratagene, La Jolla, CA, U.S.A.) was screened with hMTAP (human MTAP) cDNA probe as described previously [33]. A 1.29 kb fragment of the hMTAP promoter was obtained and cloned into pBlueScript vector and then sequenced by conventional dideoxy-nucleotide chain termination sequencing procedures [34].

MTAP-luciferase reporter plasmid construction

The 1.29 kb fragment of hMTAP promoter subcloned in pBlueScript was digested with *KpnI* and *PstI* restriction endonucleases, followed by 0.8% agarose gel electrophoresis. The resulting fragment was purified with the gel extraction kit (Qiagen, Valencia, CA, U.S.A.) and subcloned into pGEM-3z cloning vector (Promega, Madison, WI, U.S.A.) linearized

Abbreviations used: AP2, activated protein 2; CBF, CCAAT-binding factor; ChIP, chromatin immunoprecipitation; HRP, horseradish peroxidase; MTA, 5'-methylthioadenosine; (h)MTAP, human 5'-deoxy-5'-methylthioadenosine phosphorylase; NF-Y, nuclear factor-Y; 5'-RACE, 5'-rapid amplification of cDNA ends.

¹ To whom correspondence should be addressed, at Division of Population Science, Fox Chase Cancer Center, Cottman Avenue 333, Philadelphia, PA 19111, U.S.A. (email yuwaraj.kadariya@fccc.edu).

with *KpnI* and *PstI*. Since the *KpnI*–*PstI* fragment inserted into pGEM-3z was flanked by *HindIII* sites, single *HindIII* digestion yielded the 1.29 kb *HindIII* fragment containing MTAP promoter. Purified *HindIII* fragment of MTAP promoter was subcloned into *HindIII*-cut pGL2-basic reporter vector (Promega). JM 109 *Escherichia coli* was transformed with the final ligation mixture and colonies were analysed. The resultant plasmid construct containing the 1.29 kb fragment of MTAP promoter was purified with Midi preparation kit (Qiagen), followed by sequencing. The orientation of the insert was determined by restriction enzyme mapping. We designated this MTAP promoter construct as MTAP – 1291/– 20 plasmid. The structure of pGL2-basic vector carrying the luciferase gene has been described in detail elsewhere [35].

Generation of deletion constructs

Nested deletions were created from the 5'-end of the promoter using the Erase-a-Base[®] system (Promega). This system uses exonuclease III (ExoIII) and S1 nuclease by the method of Henikoff [36] with modification by Hoheisel and Pohl [37]. Using this methodology, we obtained ten unidirectionally deleted constructs of the 5'-flanking region of MTAP promoter (see Figure 3). One more construct comprising a small part of exon 1, designated as MTAP – 20/+ 60 was prepared by PCR amplification using the sense primer 5'-GCCCACTGCAGATTCCTTTCCCGTG-3' and the antisense primer 5'-AACCGCTGCGGCTGGGAGGG-CTCATCTCAC-3'.

Cell lines and culture conditions

293T (A.T.C.C. CRL 11268; human kidney cell lines), HeLa S3 (A.T.C.C. CCL 2.2; human cervical carcinoma), Hs 766T (A.T.C.C. HTB 134; human pancreatic carcinoma metastatic to lymph node), LS 180 (A.T.C.C. CL 187; human colon adenocarcinoma) and SW 837 (A.T.C.C. CCL 235; human rectal adenocarcinoma) cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, U.S.A.), supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO, U.S.A.). Cultures were maintained at 37°C in a humidified 5% CO₂-containing atmosphere.

Generation of promoter mutants

On the basis of transfection results of deletion constructs, we amplified the fragments of MTAP promoter over the range – 216 to – 152, – 446 to – 321 and – 446 to – 332 to characterize further the dominant element by transfection. We named these amplified fragments as MTAP – 216/– 152, MTAP – 446/– 321 and MTAP – 446/– 332 respectively. We used oligos 5'-GGTACCC-GGGAACCTTCGATTTG-3' (sense) and 5'-GAGCTCGACAGCT-GGATTGGCTG-3' (antisense) for MTAP – 216/– 152; 5'-GG-TACCTCCGACGCCACTAATATTG-3' (sense) and 5'-GAGC-TCCATTATAGAGCGCTG-3' (antisense) for MTAP – 446/– 321; and 5'-GGTACCTCCGACGCCACTAATATTG-3' (sense) and 5'-GAGCTCCGCTGTATTGTTCCAGAC-3' (antisense) primers for MTAP – 446/– 332. We added an *XhoI* site at one end and a *KpnI* site at the other to facilitate the subcloning of the PCR amplification products into pGL2-basic reporter vector. The PCR-amplified fragments of MTAP promoter were first cloned into a pCR 2.1 vector (Invitrogen, San Diego, CA, U.S.A.) and then subcloned into luciferase expression reporter vector pGL2-basic linearized with *XhoI* and *KpnI*. All constructs were sequenced to confirm that no mutations occurred during PCR amplification.

Site-directed mutagenesis

Mutagenesis of distal CCAAT was performed using QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions using MTAP – 446/– 332 as a template. PCR amplification was performed using the following primers 5'-GCGAAAGTTAAGTAAGTAAAGTGCAAAACAC-ACAGTCTGG-3' and 5'-CCAGACTGTGTGTTTTGCACTTT-ACTTACTTAACTTTTCGC-3'. After thermal cycling, the product was treated with *DpnI* restriction enzyme and incubated at 37°C for 1 h to digest the parental (non-mutated) supercoiled double-stranded DNA. The nicked plasmid DNA containing the desired mutation was then transformed into Epicurian Coli[™] XL1-Blue supercompetent cells (Stratagene). The clone was isolated and sequenced for the confirmation of deletion and screened for PCR-induced errors. The other construct with a substitution mutation at the CCAAT motif (TG mutant, shown underlined below) was prepared using the same template and a technique described previously [38]. The mutagenic primers designed were sense primer, 5'-GCGAAAGTTAAGTAAGCTGATTAAAGTGC-3' and anti-sense primer, 5'-GCACTTTAATCAGCTTACTTAACTTTTCGC-3'. Similarly, the proximal CCAAT motif was also mutated using specific primers.

Transfection and luciferase reporter assay

Cells (3×10^5 /well) were seeded in 12-well tissue culture plates for 24 h before the transfection procedure. Cells were transfected at 90–95% confluence using LIPOFECTAMINE[™] 2000 reagent (Invitrogen) according to the manufacturer's instructions. To obtain the optimum transfection efficiency, conditions were pre-optimized for different cell lines. The luciferase gene driven by an enhancerless simian virus 40 promoter, pGL2-promoter (positive control) and an empty pGL2-basic (negative control) vector was introduced as an internal control. To normalize the transfection efficiency, cells were co-transfected with pCMV β vector, which is designed to express β -galactosidase from the cytomegalovirus promoter (Clontech, Palo Alto, CA, U.S.A.) in each set of experiments. Each well was exposed to a total of 2 µg of plasmid construct for 24 h. The cells were washed twice with PBS and harvested. Cell lysate were prepared using 1× reporter lysis buffer (Promega). Luciferase activity was measured by the luciferase assay system using a 'Pica Gene' luminescence kit (Toyo Ink, Tokyo, Japan) on the same day that cell lysates were prepared. The β -galactosidase activity was determined using the β -galactosidase enzyme system (Promega). Total protein levels of cell lysates were estimated with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.). Results were normalized to β -galactosidase activity. At least three independent experiments were performed and mean values were used for the graphic presentation.

Preparation of nuclear extract and gel-shift assay

Nuclear extracts were prepared from exponentially growing 293T and HeLa S3 cells in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, essentially as described by Dignam et al. [39]. Protein concentration was measured with bicinchoninic acid protein assay kit (Pierce) with BSA as the standard.

Gel-shift assays were performed using the Gel Shift Assay System (Promega). Probes were created from double-stranded oligonucleotides spanning the region between – 381 and – 352 of the hMTAP promoter (including distal CCAAT motif): wild-type (WT1) 5'-TTAAGTAAGCCAATTAAAGTGCAAAACA-CA-3' (CCAAT motif underlined) and mutated (mWT1) 5'-TTAAGT-AAGCt_gATT-AAAGTGCAAAACACA-3' (lowercase boldfaced letter represents substituted bases). Similarly, another set of

probes was prepared from double-stranded oligonucleotides spanning the region from -177 to -149 of the hMTAP promoter (consisting of proximal CCAAT): wild-type (WT2) 5'-CCA-CACAAGCAGCCAATCCAGCTGTCCCG-3' and mutated (mWT2) 5'-CCACACAAGCAGctgATCCAGCTGTCCCG-3'. All reaction mixtures were incubated at room temperature (25°C) for 30 min and were then electrophoresed on a 4% (w/v) non-denaturing polyacrylamide (37.5:1 acrylamide/bisacrylamide) gel in $0.5 \times \text{TBE}$ buffer (5.4 g of Tris, 2.75 g of boric acid and 2 ml of 0.5 M EDTA in a 1 litre solution) for 30 min. The gel was dried and analysed by autoradiography. For antibody supershift assay, 2 or 4 μg of anti-CBF-A/anti-NF-Y-B (C-20 $\times$) antibody (sc-7711X; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.; CBF stands for CCAAT-binding factor and NF-Y stands for nuclear factor-Y) was added to the reaction mixture before the addition of the probes and incubated at room temperature for 30 min. Anti-mouse IgG-HRP (sc-2031; Santa Cruz Biotechnology; HRP stands for horseradish peroxidase) was used as a non-specific antibody for the control reaction. Protein-DNA complexes were fractionated on a 4% (w/v) non-denaturing polyacrylamide gel in $0.5 \times \text{TBE}$ buffer at 4°C for 30 min and visualized by autoradiography.

ChIP (chromatin immunoprecipitation) assay

Cells were grown in a 10 cm dish (90–95% confluence) and histones were cross-linked to DNA by adding formaldehyde to a final concentration of 1% for 10 min at 37°C . Cells were washed three times with ice-cold PBS and scraped into a conical tube. Subsequent steps were performed at 4°C using NE-PERTM reagent kit (Pierce) to extract nuclear proteins. Cells were pelleted by centrifugation at 326 g for 4 min and suspended in 500 μl of ceramide-I with vortex-mixing. After 10 min of incubation on ice, 27.5 μl of ceramide-II was mixed and again incubated for 1 min on ice. Cells were pelleted and resuspended in 250 μl of nuclear extraction reagent. The mixture was kept on ice for 40 min with brief vortex-mixing at 10 min intervals. The supernatant was collected as a nuclear fraction and was subjected to sonication (20 strokes, each stroke lasting for 6 s) at maximum power. The sonicated nuclear fraction containing chromatin was precleared with normal rabbit serum and Protein G-Sepharose beads for 4 h followed by centrifugation at 11752 g for 3 min. The supernatant was then mixed with antibodies (specific or non-specific), salmon sperm DNA and Protein G-Sepharose beads, and incubated at 4°C overnight with continuous agitation. Next morning the pellet was collected and washed twice with Triton X-100-containing TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 7.6). Antibody-bound chromatin fragments were eluted from the beads with 200 μl of freshly prepared 1% SDS in 0.1 M NaHCO_3 . Cross-links were reversed by heating at 65°C overnight with agitation followed by 4 h incubation at 65°C with addition of 20 μl of 4 M NaCl. Then, the mixture was added to 10 μl of 0.5 M EDTA and 20 μl of Tris/HCl, pH 7.8 and then incubated at 45°C for 1 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation and resuspended in 20 μl of TE buffer. PCRs were performed to amplify -446 to -332 and -216 to -152 fragments of MTAP promoter using 1 μl of the extracted DNA (with or without antibody) as a template.

5'-RACE (5'-rapid amplification of cDNA ends) reaction

To analyse the transcription-initiation sites of MTAP promoter, 5'-RACE was performed using Marathon-ReadyTM cDNA (Clontech) according to the manufacturer's instructions. In brief, 2.5 μl of Marathon-Ready cDNAs were subjected to the first

Figure 1 Promoter activity of hMTAP promoter in cell lines

The pGL2-basic vector containing full-length hMTAP promoter (MTAP $-1291/-20$) was transfected into different cell lines as indicated. A promoterless vector (pGL2-basic) was used as a negative control in 293T cell line and pCMV β was co-transfected to measure the transfection efficiency. The results are means $\pm$ S.D. for quadruplicate determinations and expressed as the normalized luciferase activity.

round of touchdown PCR amplification using Marathon adaptor primer 1 and MTAP GSP1 (gene-specific primer 1; 5'-GATGG-TGTGCTGCCTTCCATGCCTT-3'). Second round PCR was performed using 0.5 μl of first round PCR product with Marathon adaptor primer 2 and MTAP GSP2 (gene-specific primer 2; 5'-CCTTCAAAGCCCAGATGTTTCGCCTG-3'). After thermal cycling, 5 μl aliquots of PCR products were analysed by agarose gel electrophoresis followed by cloning in the pCR 2.1 vector and DNA sequencing.

RESULTS

To search for functional elements in the hMTAP promoter, we first examined the activity of a 1271 bp fragment of the hMTAP promoter using a luciferase reporter gene in a transient transfection experiment in five different human cell lines. Although all cell lines showed significant promoter activity, the highest activities were observed in the kidney-derived cell line 293T and cervical carcinoma cell line HeLa S3 (Figure 1). These results indicate that this 1271 bp promoter region can direct MTAP expression and that there may be some cell-type preference regarding its usage. On the basis of these initial experiments, we chose to use 293T and HeLa S3 cells for further study.

To determine the critical elements required for promoter activity, eleven 5'-deletion mutants were constructed and analysed for promoter activity using the luciferase reporter in 293T and HeLa S3 cells (Figure 2). Removal of the sequences from -1291 to -446 actually increased promoter activity by approx. 30%, suggesting that there might be a weak repressor-binding site within this region (Figure 3). We found that deletion of the -446

Figure 2 The sequence of hMTAP promoter region

Indicated in the sequence are relevant restriction sites and consensus sequences, which may act as binding sites for factors that regulate promoter activity. Potential consensus sequences for regulatory elements, transcription-binding sites, relevant restriction enzyme sites and the ATG start codon are in boldface. All nucleotide positions are numbered in relation to the start of translation, which is indicated as +1. The nucleotide sequences coding MTAP protein are in upper case letters. Sequences in italics are not included in construct preparation for luciferase reporter assay. Arrows indicate the 5'-end of each deleted construct and PstI restriction site indicates the 3'-end of all constructs. Arrowheads indicate the major transcription start sites at -58 and -135 locations.

to -321 sequence decreased the promoter activity to half its previous value, indicating that there are sequences in this region that are critically important for basal promoter activity (Figure 3). Analysis of additional deletion constructs also suggests that there are additional important promoter activation elements between -216 and -152 and between -152 and +60.

Sequence analysis in the region between -446 and +60 revealed three potentially important regulatory motifs; two CCAAT motifs (-372 to -368 and -165 to -161), a GC element (-226 to -221) and a TATA box (-328 to -322). The importance of GC box [presumably the Sp1 (specific protein 1)-binding element] in promoter activation seems minor as deletion of this sequence did not result in a noticeable decline in luciferase activity (compare MTAP -321/-20 with MTAP -216/-20; Figure 3). In contrast, when the regions with both the distal CCAAT element and the TATA box were lost (compare MTAP -446/-20 with MTAP -321/-20), promoter strength was significantly impaired, suggesting that either or both of these sequences may be important in promoter activation. We did not

identify these obvious regulatory sequences in the region of -152 to +60 despite a decrease in promoter activity.

To characterize further these elements, three new constructs were created. MTAP -446/-321 contains the distal CCAAT element and the TATA box. MTAP -446/-332 contains the distal CCAAT element and lacks the TATA box, and MTAP -216/-152 contains only the proximal CCAAT element. As shown in Figure 4, MTAP -446/-321 has about two-thirds of the full-length MTAP promoter activity. There was no significant decrease in promoter strength with deletion of the TATA element at position -328 to -322, suggesting that this element is not functional. MTAP -216/-152, containing the proximal CCAAT element, had approx. 25% of the activity of the total hMTAP promoter strength, suggesting that this element may be important in promoter activation.

To confirm that the CCAAT elements themselves were directly involved in promoter activation, we created mutations in the CCAAT element by site-directed mutagenesis. The CCAAT motifs in both the distal element construct (MTAP -446/-332) and

Figure 3 A deletion analysis of the hMTAP promoter

On the left side of the Figure, the deletion mutations are presented schematically as black bars, representing the portion of sequence that is not deleted. Dotted lines, the deleted sequences during unilaterally nested deletion (5'–3') by ExoIII exonuclease. The portions of the CCAAT elements are shown as open rectangles, GC elements as open circles and the TATA boxes as grey rectangles. The position of the last remaining bases of promoter sequence for each mutant is indicated in the middle row of the Figure in decreasing order of deletion and without deletion at the lowermost construct (MTAP – 1291/– 20). Arrows near the luciferase gene indicate the transcription-initiation sites. Right side shows relative reporter activity of deleted mutants in transiently transfected 293T (black bars) and HeLa S3 (grey bars) cells. The pGL2-basic plasmid was a promoterless negative control. The bars show luciferase activity relative to β -galactosidase activity expressed in arbitrary units. The results are means $\pm$ S.D. for at least three independent experiments with quadruplicate assays in each.

Figure 4 Promoter activity of wild-type and mutant MTAP promoter fragments

Promoterless vector (pGL2-basic) was used as a negative control in 293T cell line and pCMV β was co-transfected to measure the transfection efficiency. The results are means $\pm$ S.D. for quadruplicate determinations and expressed as the normalized luciferase activity. +, Mut or Δ indicates that the CCAAT motif of the indicated MTAP fragment is present, mutated or deleted respectively.

the proximal element construct (MTAP – 216/– 152) were either entirely deleted or altered such that the sequence was changed from CCAAT to CTGAT. In both the distal and proximal element, deletion of the CCAAT motif caused a 90 % decrease in luciferase

activity (Figure 4). The 2 bp substitution resulted in a decrease of approx. 70 % activity in both elements. These results show that the CCAAT motifs are critical for transcriptional activation of the human MTAP gene promoter.

Protein binding to the CCAAT element

To identify and characterize protein factors that could bind to the CCAAT elements in the MTAP promoter, gel-shift assays were performed using double-stranded oligonucleotides spanning each CCAAT motif. Nuclear extracts from HeLa S3 or 293T cells contained the factor that bound to a labelled WT1 oligonucleotide (probe for the distal CCAAT motif) showing a strong retarded band (Figure 5, lane 2) in gel-shift assays. Formation of the retarded band was almost completely inhibited while competing with 100 times the molar excess of unlabelled oligonucleotide (Figure 5A, lane 4 and Figure 5B, lane 5). Addition of 200 times the molar excess of a non-specific probe containing the AP2 (activated protein 2)-binding site or containing a mutant CCAAT-binding site failed to compete. We found essentially identical results using a labelled WT2 oligo containing the proximal CCAAT and flanking (Figure 6). These results show that the protein(s) binding to these CCAAT elements is specific and found in both HeLa S3 and 293T cells.

The transcription factor CBF (NF-Y) is a ubiquitous factor known to bind to the CCAAT motif [40]. We examined if our protein–DNA complex contained CBF by verifying that incubation with CBF antibodies caused a supershift in our complex. To identify the specific nature of protein complexes, we performed antibody supershift assays using CBF-A antibody as a specific antibody and anti-mouse IgG–HRP (sc-2031, Santa Cruz Biotechnology) as a non-specific antibody. When the CBF-A antibody was added to the gel-shift experiment, it resulted in the appearance of a slowly migrating species (Figure 7, lane 3). In contrast, addition of non-specific antibody had no effect (Figure 7,

Figure 5 Formation of a DNA–protein complex on the distal CCAAT motif of MTAP promoter by electrophoretic mobility-shift assay

A labelled fragment representing nt –381 to –352 of MTAP promoter (WT1) was incubated with HeLa (**A**) or 293T (**B**) cell nuclear extracts in the absence (–) or presence (+) of 25, 50 or 100 times the molar excess of unlabelled fragment. Competition with non-specific or the mutant oligonucleotides (mWT1) was performed simultaneously. Lane 1, control without added nuclear extract; lane 2, with nuclear extract; lanes 3 and 4 in (**A**), and 3–5 in (**B**) with nuclear extract, competition with unlabelled fragments (–381 to –352); lane 5 in (**A**) and lane 6 in (**B**) with nuclear extract, competition with unlabelled non-specific oligonucleotide (AP2); lane 6 in (**A**) with nuclear extract, competition with unlabelled mutant fragment of –381 to –352 (mWT1). WT1 and mWT1 sequences are described in the Materials and methods section.

lane 5), confirming the specificity of immunoreaction with CBF. Together these results indicate that the protein interacting with the MTAP promoter DNA is indeed CBF.

ChIP assay

Previous experiments clearly show that CBF binds to the CCAAT elements *in vitro*, but what about *in vivo*? To examine this question, we have used a ChIP assay. We immunoprecipitated chromatin from asynchronously growing 293T cells using a specific or non-specific antibody against CBF. We then performed PCR to assay for various fragments of the MTAP promoter. As expected in

Figure 6 Electrophoretic mobility-shift assay with HeLa and 293T cell nuclear extracts on proximal CCAAT element

A labelled fragment representing nt –177 to –149 of MTAP promoter (WT2) was incubated with HeLa or 293T cell nuclear extracts in the absence (–) or presence (+) of 50, 100 or 200 times the molar excess of unlabelled fragment. Competition with non-specific or the mutant oligonucleotides (mWT2) was performed simultaneously. Lane 1, control without added nuclear extract; lane 2, with nuclear extract; lanes 3–5, competition with unlabelled fragments (–177 to –149); lane 6, competition with unlabelled non-specific oligonucleotide (AP2); lane 7, competition with unlabelled mutant fragment of –177 to –149 (mWT2). WT2 and mWT2 sequences are described in the Materials and methods section.

Figure 7 CBF-A antibody supershift of the DNA–protein complex formed with 293T cell nuclear extract

In supershift assays, specific antibody (CBF-A antibody) was added before incubation with the labelled –381 to –352 (WT1) fragment of MTAP promoter. Lane 1, incubation without nuclear extracts; lane 2, incubation with nuclear extracts; lanes 3 and 4, incubation with 2 or 4 µg anti-CBF-A antibody; lane 5, incubation with non-specific antibody (anti-mouse IgG–HRP). *, DNA–protein complexes; arrowhead indicates the super-shifted band.

the absence of antibody (Figure 8A, lanes 2 and 5), no MTAP promoter DNA was detected by PCR. However, in the presence of specific antibody, we successfully amplified a band using primers

Figure 8 ChIP assay was performed using CBF-A antibody (specific) in cultured 293T cells

Samples extracted after ChIP assay (with or without antibody) or plasmid DNA was subjected to PCR amplification using specific primers as described in the Materials and methods section. PCR products were electrophoresed on 0.8% agarose gel stained with ethidium bromide and visualized under UV light. Lane M, 100 bp ladder. **(A)** Lanes 1–3, amplified by primers specific for –446 to –332 and template: ChIP sample with specific antibody in lane 1, ChIP sample without antibody in lane 2 and 20 ng of –446 to –332 plasmid DNA in lane 3. Lanes 4–6, amplified by primers specific for –216 to –152 and template: ChIP sample with specific antibody in lane 4, ChIP sample without antibody in lane 5 and 20 ng of –216 to –152 plasmid DNA in lane 6. **(B)** Lanes 1–4, amplified by primers specific for –446 to –332 and template. Lane 1, ChIP sample with specific antibody; lane 2, ChIP sample without antibody; lane 3, ChIP sample with non-specific antibody (anti-mouse IgG–HRP); and lane 4, –446 to –332 plasmid DNA.

specific for the MTAP –446/–332 region from the immunoprecipitated sample (Figure 8A, lane 1), but we were not able to amplify DNA using primers from the MTAP –216/–152 region. In contrast, we could not amplify a band using primers specific for the MTAP –446/–332 in the presence of non-specific antibody (Figure 8B, lane 3). The identity of the –446/–332 fragment was confirmed by cloning into a T/A vector and DNA sequencing. These experiments show that the distal CCAAT motif binds CBF *in vivo*, and suggests that the distal CCAAT motif is stronger and more important when compared with the proximal CCAAT in the transcriptional activation of MTAP gene promoter.

Determination of the transcription start site

Marathon-Ready™ hMTAP cDNA was used to analyse the transcription start site using a 5'-RACE reaction. Multiple locations of the transcription start points were found. The most extended transcription start site for hMTAP is a 'G' nucleotide 135 bp upstream from the translation start site. One of the major transcription start sites that is located closest to the first codon is a 'C' nucleotide, which is at –58 position. In between –135 and –58, there were five more major transcription-initiation sites that are located at –68 (C), –70 (C), –81 (G), –84 (G) and –111 (C) positions. All these sites are located downstream to the major transcription regulatory elements.

DISCUSSION

In the present study, we report the first characterization of the human MTAP reporter. We found that a 1271 bp fragment of the MTAP promoter was sufficient to drive a luciferase expression construct in a variety of human cell types. Although MTAP is supposed to be ubiquitously expressed in all tissues, we did find that different cell lines showed significantly different levels of MTAP promoter activity. The highest level of promoter activity was

observed in 293T cells (embryonic kidney) and HeLa S3 (cervical carcinoma), whereas significantly lower levels of expression were observed in LS180 and SW 837, two colon cell lines. We assume that these differences in expression levels reflect differences in promoter strength; however, it is possible that these differences actually reflect differences in luciferase mRNA stability in the different cell lines.

Our studies show that the distal CCAAT motif (–372 to –368) plays an important role in the activation of the MTAP promoter. Deletion or mutation of this element resulted in a significant reduction of promoter activity in our reporter assay. This element binds a protein complex and we have identified that CBF is part of this complex. Our results also suggest that the proximal CCAAT motif (–165 to –161) may have some role, but both our *in vivo* and *in vitro* experiments suggest that it is less important than the distal site. We did not see any significant functional role for the putative Sp1-binding site located at position –226 to –221 or the TATA element at position –328 to –322.

A number of mammalian promoters have been shown to contain transcription elements with the sequence motif CCAAT [41–43]. The CCAAT motif is one of the most common promoter elements present in the proximal promoter of numerous mammalian genes transcribed by RNA polymerase II, which is often found in between 80 and 100 bp upstream of the transcription start site [44]. In the MTAP promoter, the proximal CCAAT site was 98 bases upstream from its first major transcription start site, whereas the distal CCAAT element is >100 bases upstream. Our results indicate that CBF (NF-Y) binds to the CCAAT motif in the MTAP promoter. Both CCAAT motifs were bound by specific protein complexes in HeLa S3 and 293T cell nuclear extracts in gel-shift assays. These complexes were specific to CCAAT DNA as oligonucleotides containing a 2 bp mutation failed to compete. Furthermore, mutations in the CCAAT motif severely compromised the promoter activity, which is in accordance with the previous findings [45–47]. The transcription factors CBF (NF-Y), CCAAT enhancer-binding protein, nuclear factor-1 and CCAAT displacement protein can all potentially bind to the CCAAT motif [43]. Addition of CBF-A antibody caused a distinct supershift, clearly showing that it is part of the binding complex. CBF is an abundant transcription factor that binds to DNA with a strong preference for specific CCAAT-box sequences [48]. CBF consists of three subunits named CBF-A, CBF-B and CBF-C. Initially, CBF-A and CBF-C interact with each other to form a heterodimeric CBF complex, which later forms a complex with CBF-B to form a heterotrimeric CBF molecule. CBF has been shown to interact with the TATA-binding protein [49], with different cofactors and to promote the binding of other transcription factors to nearby sequences.

A CBF complex activates the CCAAT box containing promoters *in vivo* through a physical association with related histone acetyltransferases. Over the past decade, a strong link has emerged between chromatin structure, gene expression and histone acetylation [50–52]. Acetylation of histones leads to an open chromatin structure that facilitates the binding of additional activator proteins and allows easier access of RNA polymerase II to begin transcription. Thus transcriptional activation of MTAP may be mediated by the recruitment of histone acetyltransferases to the promoter by CBF bound at the CCAAT box.

Our characterization of the MTAP promoter may be useful in understanding the molecular mechanism of MTAP down-regulation found in some tumours with an undeleted MTAP locus. Such tumours may have mutations in key regions of the MTAP promoter (i.e. the CCAAT-box) or they may have reduced levels of *trans*-acting factors such as CBF. The work described here provides a framework to analyse this question.

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