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Human eosinophil major basic protein, a mediator of allergic inflammation, is expressed by alternative splicing from two promoters

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Human eosinophil major basic protein (MBP) is one of the principal mediators of injury to parasites and tissues in allergic inflammation. MBP is stored in eosinophil crystalloid granules and released with other granule constituents during eosinophil action. Previous studies have identified an MBP gene promoter that generates a 1.0 kb mRNA transcript encoding MBP pre-proprotein which undergoes processing to the mature storage form. To investigate how the MBP gene is regulated, we have examined the identity and levels of the MBP transcripts both in precursor cells and in blood eosinophils. It was found that the gene was expressed from two upstream promoters, a distal promoter P1 in addition to the previously described promoter P2. Evidence for the second promoter was initially provided by isolation from a human HL-60 leukaemic cell cDNA library of a novel 1.6 kb MBP cDNA that was distinct from the known 1.0 kb cDNA. The complete nucleotide sequence of the 1.6 kb cDNA was determined, and showed that the two cDNAs had identical coding and 3′ untranslated regions but differed in their 5′ sequences. By isolating and sequencing MBP genomic clones from an arrayed chromosome 11 library, it was demonstrated that the MBP gene is composed of nine upstream exons and five coding exons. The 1.6 and 1.0 kb cDNAs arise by differential splicing of alternate MBP transcripts from promoters P1 and P2 respectively, located 32 kb apart in the genomic DNA. Primer extension analysis identified two transcription start sites at P1, neither associated with a typical TATA box motif. Northern blotting and reverse-transcription PCR analysis showed that the 1.0 kb mRNA was present at higher levels than the 1.6 kb species in immature cells including HL-60 and bone-marrow cells. By contrast, low levels of 1.6 kb mRNA transcripts predominated in differentiated blood eosinophils. The results are compatible with differential use of P1 and P2 promoters as a mechanism for regulation of MBP expression during eosinophil maturation.

INTRODUCTION

Eosinophils are specialized blood cells that can carry out a wide range of functions in allergic, parasitic and chronic inflammatory diseases [1]. Some of their properties are thought to be protective as they can kill helminths *in vitro* [2], but others can cause tissue injury in the lungs [3] and heart [4]. Eosinophil granules contain lysosomal enzymes, eosinophil major basic protein (MBP), eosinophil peroxidase, and two ribonucleases: eosinophil cationic protein and eosinophil-derived neurotoxin. MBP forms the crystalloid core of eosinophil granules in man [5] and is a 13.8 kDa arginine-rich polypeptide [6]. It has no recognized enzymic activity but is toxic for some helminths [7] and mammalian cells [8] *in vitro*. MBP is expressed principally in bone-marrow eosinophils, but it is also synthesized in basophils and placental trophoblast x-cells [9].

A human MBP cDNA corresponding to a 1.0 kb mRNA species has been isolated from a cDNA library derived from the HL-60 promyelocytic leukaemic cell line. The cDNA sequence suggested that MBP was translated as a slightly acidic pre-proprotein containing a highly acidic proprotein segment [10]. The acidic proprotein may neutralize the highly basic and toxic properties of the mature MBP polypeptide within the cell [11].

Restriction analysis and Southern-blot hybridization of human genomic DNA established that MBP was encoded by a single-copy gene [12] on chromosome 11 [13]. The gene was reported to span 3.3 kb with six exons and five introns, preceded by a putative promoter containing typical TATA boxes [14]. MBP mRNA was abundant in immature eosinophils and decreased during eosinophil differentiation. In mature eosinophils from patients with eosinophilia, MBP mRNA was not detectable by Northern-blot hybridization [15]. The mechanisms controlling the tissue-specific and differentiation-dependent expression of MBP remain to be elucidated.

While carrying out cDNA cloning studies using an HL-60 cDNA library, we isolated a 1.6 kb MBP cDNA which specified the full MBP coding sequence but differed at its 5′ end from the previously reported 1.0 kb MBP cDNA species [10]. We show here by genomic cloning, PCR, DNA sequencing, Northern-blot and primer extension analysis that the 1.6 kb cDNA originates from a second upstream MBP gene promoter. We have fully characterized the structure of the human MBP gene and its two promoters, and have shown that the relative levels of 1.6 kb and 1.0 kb mRNA species changed dramatically during eosinophil development. These results could be important in defining the tissue and developmental expression of the MBP gene.

MATERIALS AND METHODS

Isolation of cDNA clones

A λ gt 10 HL-60 cDNA library containing 2×10^9 independent clones was kindly provided by Dr. David Bentley (ICRF, London, U.K.) [16]. The sequences of two oligonucleotide probes, which were used for screening the library, were chosen from the MBP cDNA sequence [10]: MBP1, 5′-CCT GGG TGC CAG ACC TGC CGC-3′, and MBP2, 5′-GGC TCG GGT CGC TGC AGA CGC-3′. Oligonucleotides were synthesized by Oswel DNA Service, University of Edinburgh, U.K. The screening procedure followed previously published methods [17,18]. Briefly, oligo-

Abbreviations used: MBP, major basic protein; RT, reverse transcription; AP-2, activator protein 2; APRE-1, acute-phase response element; APRRE, acute-phase reactant regulatory element; SDR, sterol-dependent repressor; SSC, 0.15 M NaCl/0.015 M sodium citrate; HES, idiopathic hypereosinophilic syndrome.

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nucleotides were labelled with [γ-^{32}P]ATP (5000 Ci/mmol) (Amersham International plc.) and T4 polynucleotide kinase; filters were hybridized overnight at 33 °C with prehybridization solution (50 % formamide, 6 × SSC, 2 × Denhardt's, 0.5 % SDS, 0.05 % sodium pyrophosphate, 0.1 mg/ml denatured fragmented salmon sperm DNA) containing 9.6 ng/ml of labelled probes (MBP1/MBP2, 1:1). The filters were washed at 33 °C for 1 h and exposed to X-ray film with intensifying screens at −70 °C. Positive recombinant plaques were purified from phage plate lysates according to the method of Sambrook et al. [19]. The *Eco*RI inserts were subcloned into plasmid pUC18.

DNA sequencing

Subclones were sequenced by the dideoxynucleotide chain termination method [20] using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corporation, Cleveland, OH, U.S.A.) and (α-^{35}S)-dATP (Amersham International plc., Little Chalfont, U.K.) according to the manufacturer's instructions. DNA sequences were determined for both strands of the 1.6 kb MBP cDNA and were in agreement. The sequence was analysed using PC/GENE (Intelligenetics Corp., Mountain View, CA, U.S.A.). MBP cDNA sequences have been deposited in the EMBL/GeneBank Data Libraries under accession number Z26248.

Manipulation of cells

Eosinophils were purified from the peripheral blood of patients with the idiopathic hypereosinophilic syndrome (HES) by centrifugation on discontinuous 18–24 % (w/v) metrizamide gradients [2]. The human myelomonocytic leukaemia HL-60 cell line, obtained from the American Tissue Type Collection (MD, U.S.A.), was cultured at 37 °C in RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10 % (v/v) fetal-calf serum, 2 mM L-glutamine, and antibiotics (100 i.u./ml penicillin, 100 μg/ml streptomycin). Human bone-marrow cells were used directly for RNA preparation.

RNA preparation and Northern-blot analysis

Total cellular RNA was isolated by the guanidinium isothiocyanate method [21]. Approximately 2 μg of RNA was obtained from 1 × 10^6 cells. For Northern blots, total RNA was denatured in glyoxal and dimethyl sulphoxide. Up to 10 μg of total RNA were applied to a 1.2 % agarose gel and electrophoresed at 50 V for 3 h [22]. The RNA was transferred to a nylon membrane (Hybond-N, Amersham International plc, U.K.) by capillary elution and hybridized to MBP cDNA probes that had been labelled with [γ-^{32}P]dATP by random priming. Hybridization was carried out at 42 °C in a solution containing 50 % formamide, 5 × SSC, 5 × Denhardt's solution, 0.5 % SDS and 100 μg/ml of denatured salmon sperm DNA [23].

Reverse transcription (RT) and PCR analysis

Aliquots (30 μl per reaction) of RT mix were prepared in 1 × RT buffer (50 mM Tris/HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$) containing oligo-dT (125 ng/μl), 5 mM dithiothreitol, 0.625 μM of each dNTP, BSA (52.5 ng/μl), RNAguard (61.3 units/μl) and AMV reverse transcriptase (0.5 unit/μl). RT mix was made for all samples and aliquoted (30 μl per tube) with 10 μl of 0.6 μg/μl total RNA. Tubes were vortexed, spun briefly and left for 10 min at room temperature and then at 42 °C for 40 min. Reverse transcriptase was inactivated at 72 °C for 10 min. Samples were stored at −20 °C. PCR was carried out essentially as described by Staynov and Lee [24]. The PCR primers (Figure 1a) were:

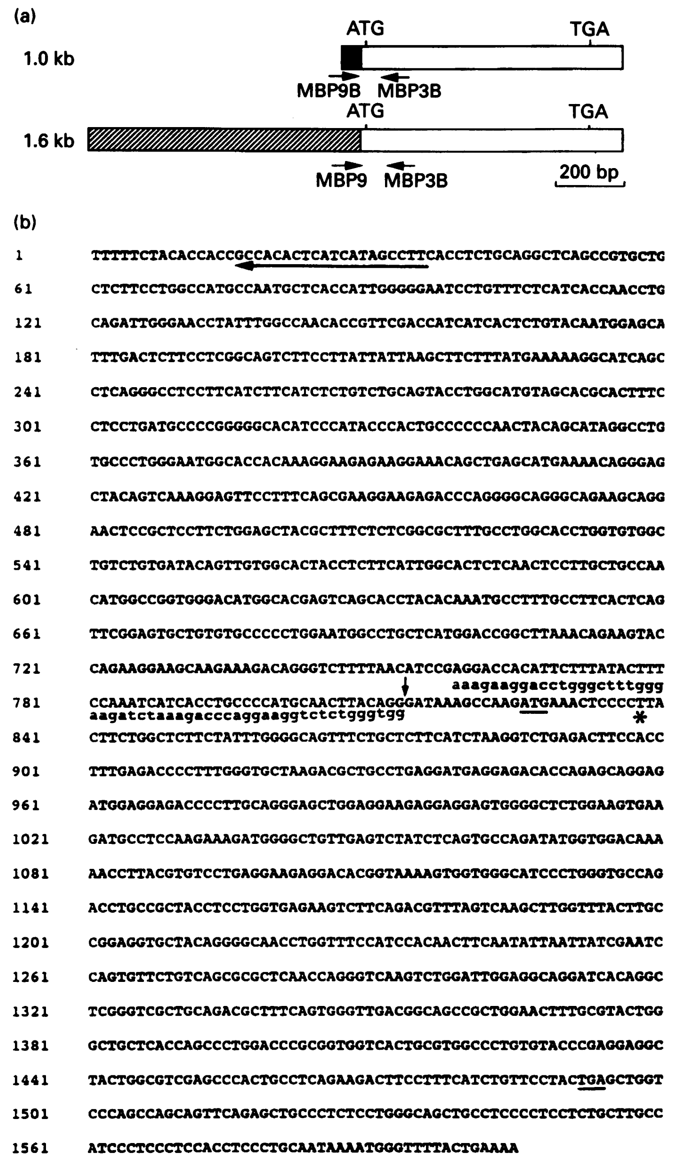


Figure 1 Structure and DNA sequences of human MBP cDNAs

(**a**) Schematic comparison of the 1.6 kb MBP cDNA sequence with a previously reported 1.0 kb cDNA species. Initiation (ATG) and termination codons (TGA) are shown. Coding and 3′ sequences are identical (open boxes) but diverge 12 nucleotides upstream of the initiation codon (black and hatched boxes). The positions of primers MBP9, MBP9B and MBP3B used for PCR amplification (see text) are indicated under the corresponding cDNAs. Bar denotes 200 bp. (**b**) DNA sequence of the 1.6 kb MBP cDNA. Nucleotides are numbered on the left of the figure. The MBP start and stop codons are underlined. Vertical arrow indicates position of a splice site: DNA sequence 3′ of this position was identical to that determined for a 1.0 kb cDNA by Barker et al. [10] except for a silent substitution denoted by an asterisk. DNA sequence for the 1.0 kb cDNA lying 5′ of the splice site is shown by lower-case letters. A horizontal arrow indicates the position of a reverse oligonucleotide primer used in primer extension analysis.

MBP9 (Forward 1) 5′-TGCCCCATGCAACTTACAGG-3′; MBP9B (Forward 2) 5′-AAGATCTAAAGACCAGGAAGG-3′; and MBP3B (Reverse) 5′-AGATGAAGAGCAGAAACTGCC-3′.

The reaction was started with a 94 °C step for 4 min, followed by up to 34 PCR cycles (1 min at 94 °C, 2 min at 55 °C, and 2 min at 72 °C). Samples (10 μl) of each PCR product were electrophoresed on a 3.0 % agarose gel in glycine buffer and

stained with ethidium bromide. Bands on photographic negatives were quantified with a laser densitometer (LKB 2202 Ultrascan).

Isolation and characterization of genomic MBP clones from an arrayed chromosome 11 genomic library

Chromosome 11-specific cosmid library filters were obtained from the Reference Library-Database, ICRF, London (courtesy of Dr. Günther Zehetner). Duplicate filters were hybridized separately with each of three different radiolabelled MBP probes obtained by PCR: MBP genomic upstream sequence from position 24 to 764 from the reported genomic sequence [14], MBP coding region cDNA and the upstream MBP cDNA sequence determined for the 1.6 kb cDNA, from position 871 to 1540 and 20 to 793 in Figure 1(b), respectively. The hybridization solution contained 50% formamide, 4×SSC, 50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 10% dextran sulphate, 1% SDS, 50 μg/ml denatured salmon sperm DNA and 10× Denhardt's solution (0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% BSA). Hybridization was performed at 42 °C for 16 h. After a high stringency wash at 42 °C for 1–2 h, the filters were exposed to X-ray film with intensifying screens for a few hours.

Southern-blot analysis

DNA was prepared by the alkaline lysis method [19] and digested to completion with appropriate restriction enzymes according to manufacturers' instructions. After electrophoresis in 0.8% agarose, the DNA fragments were blotted on to Hybond-N nylon membranes (Amersham International) by capillary transfer [19]. The membranes were hybridized to radiolabelled MBP probes (see previous section and Figure 2c) in 6×SSC, 5×Denhardt's solution, 50% formamide containing 100 ng/ml denatured salmon sperm DNA at 42 °C for 16 h. After a high-stringency wash at 68 °C for 1 h, the filters were exposed to X-ray film with intensifying screens for a few hours at −70 °C.

Primer extension

High-quality total RNA was purified by CsCl ultracentrifugation [19] and digested with RNAase-free DNAase I for 30 min at 37 °C. The primer extension essentially followed the method of Kong et al. [25] with modifications. Briefly, DNA-free RNA samples were annealed with the primer 5′-AAGGCTATGAT-GAGTGTGGC-3′ by heating to 70 °C and cooling slowly to 50 °C. Primer extension was performed at 50 °C for 15 min in a final volume of 40 μl of 50 mM Tris/HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, BSA (52.5 ng/μl), RNAguard (61.3 units/μl), 0.03 mM each of dATP/dGTP/dTTP, 0.03 mM [α-^{32}P]dCTP and 10 units of AMV reverse transcriptase. A further 10 units of reverse transcriptase were added and the reaction was continued for another 15 min. After phenol/chloroform extraction and ethanol precipitation the primer extension product was redissolved in water plus sequencing stop solution and electrophoresed on a 6% polyacrylamide/8 M urea gel. The gel was fixed, dried and exposed to X-ray film.

RESULTS

Isolation and DNA sequence analysis of a 1.6 kb human MBP cDNA

To obtain MBP cDNA clones, an HL-60 λ gt 10 library was screened. Two radiolabelled 21-mer oligonucleotides, MBP1 and MBP2, were used as probes for the known human MBP coding sequence. A single positive cDNA clone was found after screening 22000 independent phage recombinants, which contained a 1.6 kb *Eco*RI insert. A second independent screening of the library yielded eight positive clones all containing approx. 1 kb cDNA inserts (results not shown) in agreement with the 1.0 kb MBP cDNA previously reported [10]. The new 1.6 kb cDNA was cloned into pUC18 to give plasmid pUC18M1. DNA sequence analysis of the insert confirmed the presence of the MBP coding sequence and extended into the 3′ untranslated region (Figure 1a). The coding region of the 1.6 kb mRNA was identical to that published by McGrogan et al. [12], but differed from that reported by Barker et al. [10] by a single nucleotide change (T in place of C) at position 838 (Figure 1b). This change did not affect the MBP protein sequence. The sequences of the 5′ untranslated regions of the 1.6 kb and 1.0 kb cDNAs differed from a point 12 bp upstream of the initiation codon and just upstream of the eukaryotic initiation signal [26] at position 817 (Figures 1a and 1b). No significant homology was found between the 1.6 kb MBP cDNA sequence and other sequences deposited in the GeneBank and EMBL databases. These results suggested that human MBP was expressed via two distinct mRNA species.

Relationship of the 1.6 kb MBP cDNA sequence to the MBP gene isolated from a chromosome 11-specific library

A chromosome 11-specific human genomic library was screened with transcript-specific probes to study the possibility that the 1.6 and 1.0 kb cDNAs were alternative transcripts of the same MBP gene. First, a 1.6 kb cDNA-specific probe was used to identify MBP genomic clones. This identified six spots on duplicate filters, consistent with the known location of the MBP gene on chromosome 11 (Figure 2a). The six spots also probed positive with sequences from the MBP coding region (Figure 2a) and MBP genomic upstream sequence (results not shown) (for details of the probes, see the Materials and methods section). This indicated that both the 1.6 kb and the 1.0 kb transcripts were derived from a single-copy MBP gene within a 40 kb region defined by the inserts of the cosmid library of chromosome 11.

The six positive spots represent three independent clones which gave similar patterns of DNA fragments by restriction analysis and hybridization of Southern blots with the MBP transcript-specific probes used in genomic library screening. One of these genomic clones (ICRFc107D124) containing a 40 kb insert spanning the entire MBP gene was analysed further. It produced 4 kb *Eco*RI and 2.5 kb *Hin*dIII bands, each of which hybridized to both transcript-specific probes (Figure 2b). Inspection of Figure 2(b), panel A, revealed that these bands were doublets, a result confirmed by subcloning and sequence analysis. Coincidentally, each partner of the two doublets carried sequence corresponding to one of the two different cDNA transcripts. A 3 kb *Eco*RI fragment also hybridized to the 1.6 kb cDNA-specific probe (Figure 2b, panel B). In order to clarify the genomic structure of the human MBP gene and its relation to the upstream sequences of the two transcripts, a 'mini chromosome walking' strategy [27] was employed. The initial probes are shown in Figures 2(b) and 2(c): a 2.5 kb *Hin*dIII fragment which carried upstream sequences of the 1.0 kb transcript and two *Eco*RI fragments, 3 kb and 4 kb, which contained the upstream sequences for the 1.6 kb transcript. By using overlapping DNA fragments in a series of successive Southern hybridizations, in combination with subcloning and partial sequence analysis, it was established that the whole MBP gene extended over 35 kb on chromosome 11. Genomic sequence specifying the 5′ untranslated part of the 1.6 kb transcript was located in a 15 kb region upstream of sequence specifying the 5′ region of the 1.0 kb

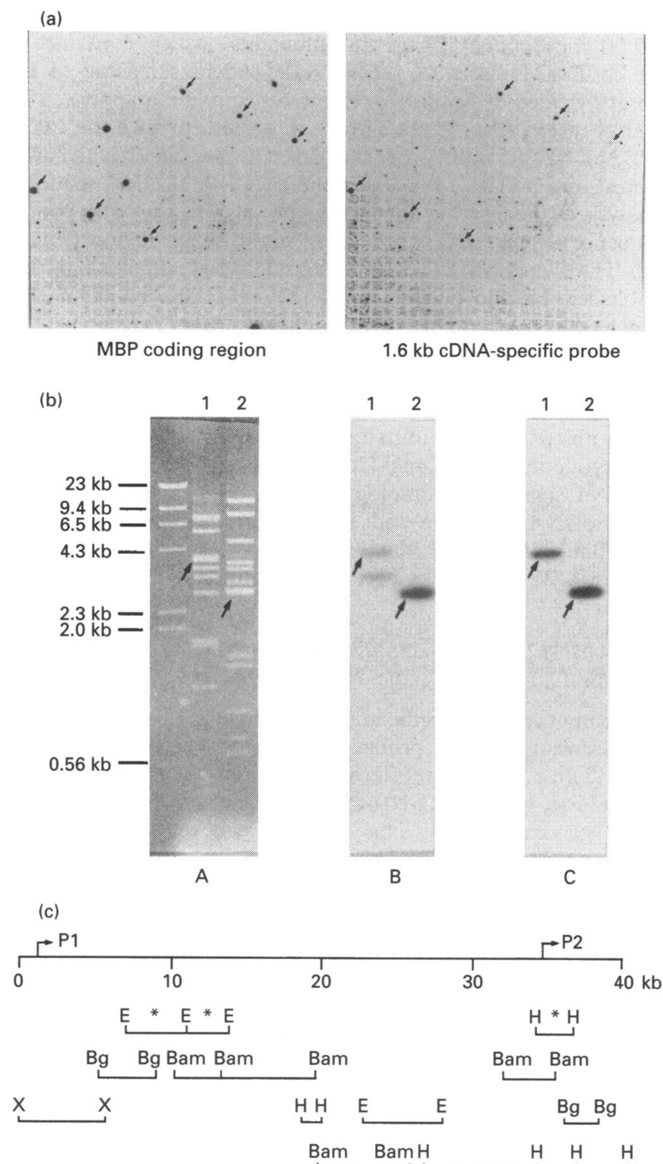


Figure 2 Isolation and characterization of human MBP genomic clones

(**a**) Autoradiograms of chromosome 11-specific library filters. A cosmid library was hybridized to MBP probes as indicated (MBP coding region probe and 1.6 kb cDNA-specific probe spanned cDNA positions 871–1540 and 20–793 in Figure 1b, respectively.). Six cosmid clones positive to both MBP probes in separate hybridizations are indicated by arrows. Several other clones contained MBP coding region only. (**b**) Southern-blot analysis of the MBP genomic clone, ICRFc107D124. DNA was digested with *Eco*RI (lanes 1) or *Hin*dIII (lanes 2), and the fragments were separated by agarose gel electrophoresis (A). DNA size markers were run alongside. Southern blots of A were hybridized to probes specific to the upstream sequences of the 1.6 kb cDNA [see legend for (**a**)] (B) or MBP genomic upstream sequence (position 24–764 of [14]) specific to the 1.0 kb cDNA (C). Arrows denote bands positive to the two probes. (**c**) Schematic presentation of the human MBP gene. Two arrows indicate the distal promoter P1 and proximal promoter P2. The positions of various DNA restriction fragments are shown. E, H, Bam, Bg and X denote sites for *Eco*RI, *Hin*dIII, *Bam*HI, *Bgl* and *Xba*I. Three genomic DNA fragments corresponding to positive bands in (**b**), used as initial probes in Southern blotting, are indicated by asterisks.

transcript and the MBP coding region. Thus, the 1.6 and 1.0 kb transcripts were derived from the same MBP gene but originate from distinct promoters P1 and P2. The relationship between the two promoters and various subclones or DNA fragments used as probes in Southern blotting is shown in Figure 2(c).

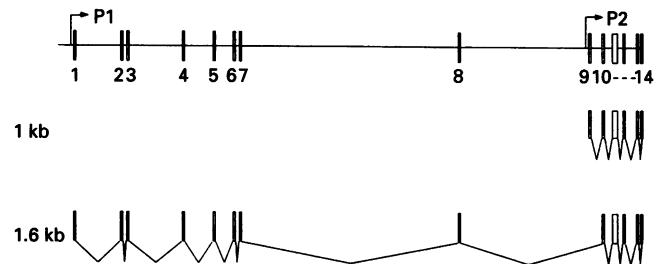


Figure 3 Structure of the human MBP gene

Exons are denoted by open boxes and numbered according to convention from 5′ to 3′ in the MBP gene. Lines indicate introns. P1 and P2 are promoters generating 1.6 kb and 1.0 kb mRNAs respectively. Splicing patterns for the two mRNA species are shown underneath.

Table 1 Exon–intron organization of the human MBP gene corresponding to the 1.6 kb cDNA transcript

Exon and intron sequences are in upper- and lower-case letters respectively. *n* is the exon number according to Figure 3.

n	Exon size (bp)	Exon (*n*)	Intron	Exon (*n*+1)
1	73–78	CTGCAG	gtgagtgc—3 kb—tggtccag	GCTCAG
2	77	CTGCAG	gtgggagc—232 bp—atccccag	ATTGGG
3	93	ATTAAG	gtaaatat—3.2 kb—ttctgcag	CTTCTT
4	140	CATAGG	gtaaggac—1.8 kb—ttccatag	CCTGTG
5	98	AGGAAG	gtgaagcc—1.5 kb—cccattag	AGACCC
6	171	CACGAG	gtggtagg—196 bp—ttctgcag	TCAGCA
7	117	AGACAG	gtgagatg—13 kb—ttaaccag	GGTCTT
8	71	TACAGG	gtaagtta—9 kb—cgttgcag	GATAAA
10	70	ATCTAA	gtaagtgt—569 bp—cctgccag	GGTCTG
11	308	GCTTGG	gtgagtgg—301 bp—ccctgtag	TTTACT
12	132	GGCTCG	gtaagaga—710 bp—ttctccag	GGTCGC
13	112	CCCGAG	gtgaggtg—220 bp—cttcacag	GAGGCT

Structure of the human MBP gene

To determine the detailed organization of the MBP gene, fragments of the ICRFc107D124 genomic clone were subcloned on the basis of the Southern blotting results, and sequenced. In total, 14 kb of genomic DNA sequence was obtained. Each exon was located and sequenced in full, as were the intron–exon boundaries. The structure of the human MBP gene is summarized in Figure 3. The gene consisted of 14 exons, nine upstream exons (1–9) and five coding exons (10–14). Our results confirmed the sequence and genomic organization of these coding exons. The 1.0 kb cDNA transcript was derived from exon 9 and the coding exons, which was consistent with the previous report [14]. The 1.6 kb cDNA transcript was derived from exons 1–8 and 10–14. Exon 8 and exon 10 were separated by a 9 kb, non-coding region containing exon 9, the only upstream exon of the 1.0 kb transcript. In contrast, exon 9 and the first coding exon, 10, are separated only by a 639 bp intron. The longest intron had a size around 13 kb located between exon 7 and 8. The distal P1 and proximal P2 promoter regions were separated by a distance of 32 kb. All of the exon–intron–exon junctions were conserved in agreement with the GT-AG model [28]. The sizes of the exons and introns, and their junction sequences, are summarized in Table 1.

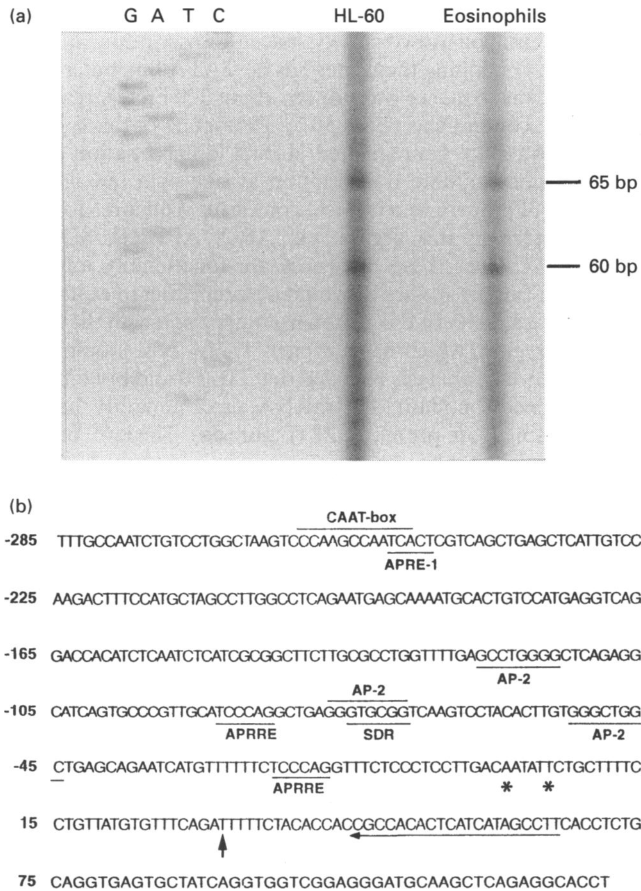


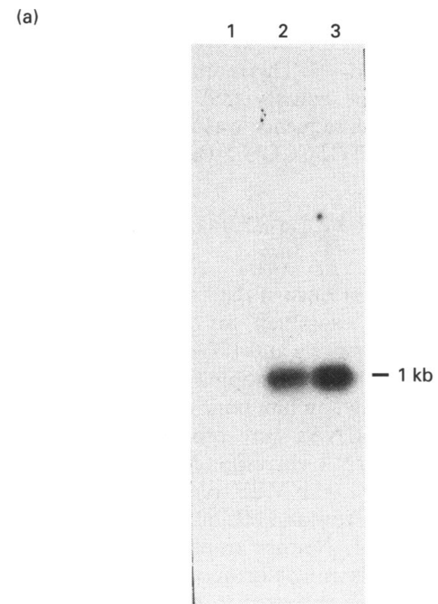
Figure 4 Transcription start sites for the 1.6 kb cDNA transcript and analysis of the P1 promoter

(**a**) Primer extension analysis. Total RNA from HL-60 cells and human eosinophils was subjected to primer extension and the resulting radiolabelled DNA products were separated by denaturing PAGE. Two primer extension products, 65 and 60 bp in length, were sized using M13mp18 DNA-sequence size markers (lanes marked G, A, T and C). (**b**) Nucleotide sequence of the putative MBP promoter P1 region. Transcription start sites are indicated by asterisks. Potential binding sites for different transcription factors are indicated. A vertical arrow denotes the first nucleotide of the 1.6 kb cDNA and a horizontal arrow indicates the position of a reverse primer used in primer extension (also see Figure 1b and text).

Transcription initiation sites and structure of the putative P1 promoter region

Primer extension was performed on total RNA from HL-60 and bone-marrow cells to specify the transcription start site of the 1.6 kb cDNA transcript. The primer was a 20 bp oligonucleotide complementary to the 1.6 kb cDNA sequence from position 16 to 34 (Figure 1b and the Materials and methods section). Due to the low level of transcription (see below), a sensitive method was used in which the primer extension product was labelled by incorporation of radioactive nucleotides throughout reverse transcription. Two bands sized at 60 bp and 65 bp were obtained (Figure 4a), showing that transcription initiated with similar efficiency at two sites, 27 and 32 bp upstream of the cDNA sequence shown in Figure 2(b). Scrutiny of this region in genomic DNA (Figure 4b) showed that the sequences surrounding the two transcription start sites were homologous to cap signal consensus sequences [29].

The P1 promoter region upstream of exon 1 lacked a TATA box, but had a CAAT box located at position −220 to −209 with respect to the upstream transcription start site at position 1 (Figure 4b). This region contains three activator protein 2 (AP-2) consensus sequences [30] at −52, −77 and −121. Few GC-rich regions were present and the overall GC content was about 53%. Two acute-phase reactant regulatory elements (APRREs) [31] and one acute-phase response element (APRE-1) [32] consensus sequences were present at −88, −23 and −211 respect-



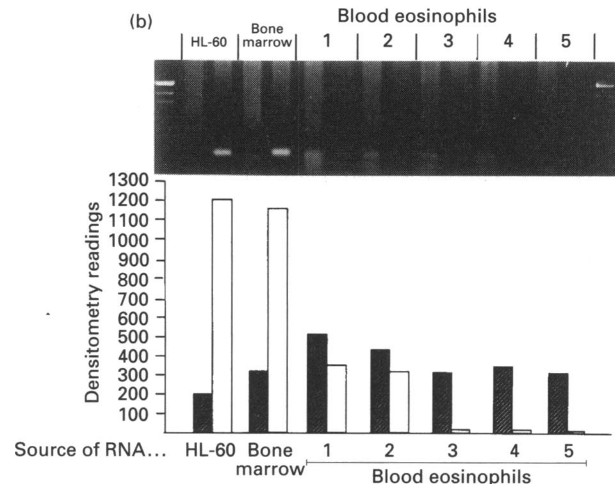


Figure 5 Analysis of MBP expression in human cells

(**a**) Northern blotting analysis. Total RNA prepared from human blood eosinophils (lane 1), human bone-marrow cells (lane 2) and HL-60 cells (lane 3) was separated by agarose gel electrophoresis and hybridized to an MBP coding region probe. RNA sizes were determined from the mobility of RNA markers run alongside. (**b**) Semi-quantitative PCR analysis of MBP expression in eosinophils and other tissues. PCR detection of specific MBP transcripts in HL-60, bone-marrow cells and eosinophils from five patients with HES. Size markers were run alongside. First-strand cDNA was prepared from total cellular RNA and used for PCR in adjacent lanes with primers specific for the 1.6 kb (shaded) and 1.0 kb cDNA (open). Specific 104 and 110 bp PCR products were detected. Bands on the photograph were scanned using a laser densitometer and the relative amounts of each PCR product are indicated below the relevant lanes in the bar chart. Densitometry readings are shown on the ordinate, and give relative estimates of RNA levels except for two reactions: the PCR products amplified from RNA samples from human bone-marrow and HL-60 cells with the primers specific to the 1.0 kb cDNA transcript had reached saturation and gave underestimated RNA levels.

ively. A short repeated motif, consisting of a short series of imperfect duplications of the sequence 5′-TCCC-3′, was located between −22 and −6. This motif has been shown to be necessary for optimal gene activity [33]. A sterol-dependent repressor (SDR) consensus sequence was also apparent at −77 [34]. The sequence, 5′-TTTCTCCC-3′, was repeated at −27 and −16.

Relative levels of P1 and P2 transcripts alter during eosinophil development

The relative importance of the 1.6 and 1.0 kb transcripts in MBP expression was examined for a variety of cell types using Northern-blot analysis and RT-PCR. Total RNA was prepared from purified blood eosinophils obtained from a patient with HES, from normal human bone marrow and from cultured HL-60 cells. These RNAs were probed on Northern blots with a radiolabelled cDNA corresponding to the MBP coding region (Figure 5a). The 1.0 kb MBP transcript was present at high levels in both bone marrow and HL-60 cells. No signal was seen for the 1.6 kb transcript. Neither transcript was detected in purified blood eosinophils using Northern-blot analysis (Figure 5a). Thus, the 1.0 kb species appeared to be the major MBP transcript in HL-60 and bone-marrow cells.

The relative amounts of MBP transcripts in blood eosinophils were examined using a more sensitive RT-PCR technique. Total RNA was isolated from the blood eosinophils of five patients with HES. First-strand cDNA synthesis was carried out using reverse transcriptase and an oligo-dT primer. This cDNA was used as the template for separate PCR reactions using transcript-specific forward primers MBP9 (for the 1.6 kb transcript) and MBP9B (for the 1.0 kb transcript) and the same reverse primer MBP3B made to coding region sequence (see Figure 1a and the Materials and methods section). PCR products were displayed and sized by agarose gel electrophoresis (Figure 5b). The primers were expected to generate 104 bp and 110 bp PCR products from the 1.6 and 1.0 kb transcripts respectively. Figure 5(b) shows that in each of the RNA samples from eosinophils of HES patients, the 1.6 kb transcript was present at higher levels than the 1.0 kb transcript. The opposite was seen for RT-PCR analysis of HL-60 and bone-marrow cells (Figure 5b). In these cells, the 1.0 kb transcript predominated, in agreement with the results of the Northern-blot analysis (Figure 5a).

DISCUSSION

A human HL-60 cDNA library has been screened and a variant 1.6 kb cDNA for MBP was obtained. This cDNA carried the same protein coding sequence as a previously reported 1.0 kb cDNA clone isolated from the same cell line [10]. However, the two cDNAs differed in the sequence corresponding to the 5′ untranslated region of the mRNA transcripts. Isolation and analysis of genomic MBP clones from a chromosome 11 library revealed that the 1.6 and 1.0 kb cDNA transcripts initiated at two different MBP gene promoters which we termed P1 and P2 respectively. By isolating, sequencing and characterizing MBP genomic clones from an arrayed chromosome 11 library, it was revealed that the MBP gene was composed of nine upstream exons and five coding exons. The 1.6 and 1.0 kb cDNAs were shown to arise from differential usage of the non-coding exons. The putative P1 promoter region was located about 32 kb upstream of the previously described P2 promoter [14]. These results established that the two forms of MBP messenger RNA were generated by differential splicing using two alternative tandem promoters and that the structure of the MBP gene is more complex than previously reported [14].

Primer extension analysis revealed that the 1.6 kb mRNA transcript initiated at two closely spaced transcription start sites. The region containing these sites has no TATA box but a CAAT box consensus sequence was present about 220 bp upstream. The overall GC content was about 50 %. Promoters of this type have been reported to be regulated during differentiation or development and initiate transcription at one or a few clustered start sites [35]. There were several consensus sequences for *trans*-acting factors in this region, i.e. AP-2, APRRE, SDR and APRE-1. Whether these sequences are functionally important must await further studies. It would be interesting to examine the *trans*-acting activity of this region in comparison with the activity of the P2 region, which has a typical TATA box promoter.

Northern-blot analysis revealed that HL-60 and bone-marrow cells contained abundant MBP mRNA, predominantly the 1.0 kb species arising from promoter P2 (Figure 5a). The level of 1.6 kb transcript was low. Neither transcript was present at high levels in blood eosinophils purified from patients with HES (Figure 5a). However, by using RT-PCR, MBP transcripts could be detected (Figure 5b). RNA samples for these studies were obtained from patients whose eosinophils showed different stages of maturity and activity. In all cases the 1.6 kb MBP transcript was more abundant in patient eosinophils than the 1.0 kb transcript. These results have provided information on the control of expression of MBP during differentiation of precursor cells to mature eosinophils.

Differential splicing involving dual or multiple promoters has been described in many systems and is usually associated with either tissue-specific or developmental gene regulation (for review see reference [36]). In the case of human MBP, the protein is expressed in three tissue types, namely placental trophoblasts, basophils and eosinophils and their precursors [9]. MBP expression in basophils is difficult to investigate due to the rarity of this cell type. We have used PCR to look for MBP expression in placental trophoblasts (data not shown) and found similar levels to HL-60 and bone-marrow cells with dominance of the P2 promoter transcript. This suggested that differential splicing of the MBP gene does not occur in a tissue-specific manner.

In agreement with previous work [15], we have shown that the 1.0 kb mRNA arising from promoter P2 was less abundant in mature eosinophils than in HL-60 and bone-marrow cells which contain eosinophil progenitor cells (Figures 5a and 5b). Concomitantly, the 1.6 kb transcript generated from promoter P1 became predominant (Figure 5b). It is possible that the activation of P1 during eosinophil development may inhibit transcription from the downstream P2 promoter. Transcriptional interference of this kind has been reported for the *Drosophila melanogaster* Adh gene [37], a ribosomal RNA gene from *Acanthamoeba* [38] and a duplicated α-globin gene [39].

Studies of Adh gene expression suggest that promoter switching during development of *D. melanogaster* allows higher-level synthesis of alcohol dehydrogenase in the adult. In contrast, for MBP expression, promoter switching during eosinophil differentiation may be used to reduce protein synthesis. MBP, which is toxic to cells, is synthesized in precursor cells as a pre-proprotein and processed. The mature form is stored in the granules of fully differentiated mature eosinophils. Once stored in this stable form, terminally differentiated eosinophils may reduce their MBP expression, using the P2 to P1 promoter switch. This hypothesis could be tested by appropriate studies on the relative strengths of the P1 and P2 promoters. Such experiments will be important in ruling out another possibility that differences in MBP transcript levels somehow arise from differences in stabilities of the two mRNAs.

In summary, this study has shown that the human MBP gene

was transcribed from two promoters. Activation of the upstream promoter and repression of the downstream promoter may take place during eosinophil differentiation. This raises the possibility that MBP biosynthesis is regulated by promoter interference during eosinophil development. Alternatively, the activity of the two MBP promoters may depend on the availability of other factors, the expression of which is subject to developmental control. It remains to be determined whether the expression of other eosinophilic granule protein genes is also similarly regulated during differentiation through the use of alternative promoters and differential splicing.

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