Identification of a new murine eosinophil major basic protein (mMBP) gene: cloning and characterization of mMBP-2

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Abstract: We have identified a new eosinophil major basic protein gene family member in the mouse and have given it the designation murine major basic protein-2 (mMBP-2). The gene was initially characterized as a unique expressed sequence tag (EST) clone having significant identity to the previously recognized member of this gene family, mMBP-1. The EST was used to screen and isolate mMBP-2 from a bone marrow cDNA library. In addition, a genomic clone of mMBP-2 was isolated and this gene was shown to be physically linked to within 100 kb of mMBP-1 on the central region of mouse chromosome 2. Progressive similarity alignment of the deduced mMBP-2 open reading frame demonstrates the apparent conservation of the ''pre-pro-mature'' protein structure found in the other known mammalian MBPs. Mature mMBP-2 maintains the cationic nature associated with these proteins with a predicted p*I* of 9.95. However, unlike the human MBPs, which display a three orders of magnitude charge difference [hMBP-1 (p*I* 11.4) vs. hMBP-2 (p*I* 8.7)], mMBP-2 is only slightly less cationic than mMBP-1 (p*I* 10.5). Expression studies demonstrate that transcription of the mMBP-2 gene parallels mMBP-1 and is confined to hematopoietic compartments engaged in eosinophilopoiesis. Moreover, using mMBP-1 knockout mice and immunohistochemistry with an antisera that recognizes both mMBP-1 and -2, we demonstrate that mMBP-2 protein expression is restricted to eosinophil lineage-committed cells. *J. Leukoc. Biol.* 67: 567–576; 2000.

Key Words: *mouse · granule protein · cDNA · expressed sequence tag*

INTRODUCTION

Eosinophils are leukocytes of the myeloid lineage whose effector functions are linked with defensive mechanisms associated with the innate immune system (e.g., anti-helminth activities [1]). Although the homeostatic level of eosinophils is only 0–4% of peripheral white blood cells in humans, during the Th2-driven inflammatory responses accompanying diseases such as bronchial asthma, atopic dermatitis, and parasitic infestation, eosinophil numbers dramatically increase and accumulate in a tissue-specific manner [2, 3]. Once recruited to the site(s) of action, eosinophils execute multiple effector functions that include the production of cytokines/chemokines [4, 5], the secretion of small-molecule mediators of inflammation (e.g., leukotrienes [6]), and the release/deposition of several cationic proteins accumulated in lipid-bound granules within these cells [7]. The recruitment of eosinophils and the execution of these effector functions closely correlate with the pathological changes and tissue damage in Th2-associated disease states.

The cationic proteins of the eosinophil secondary granule possess cytotoxic [8], as well as non-cytotoxic pro-inflammatory activities [9, for review see ref. 10]. In human (h) eosinophils, the dominant protein, on a molar basis within the secondary granules, is major basic protein-1 (hMBP-1) [11, 12]. hMBP-1 is a 13.8-kDa cationic protein (calculated p*I* 11.4) initially synthesized as a ''pre-pro-mature'' secreted protein that is proteolytically cleaved to the mature hMBP-1 that is stored in the granule. This protein has been localized by immunoelectron microscopy to the electron-dense crystalloid cores of these granules [13]. Gene knockout mice (m) deficient for the murine ortholog, mMBP-1, show no evidence of this granule structure, suggesting that mMBP-1 is at least necessary for its formation [K. L. Denzler, S. Farmer, J. Crosby, M. Borchers, K. Larson, N. Lee, J. Lee, unpublished results]. In addition to lethal effects on a variety of helminth parasites [14], hMBP-1 is toxic toward host tissues and can lead to cell death with the loss of organ/tissue integrity [15]. Moreover, hMBP-1 has agonist affects on other inflammatory cell types, including induction of neutrophil superoxide production [16], and basophil and mast cell histamine release [17]. Collectively, these eosinophil-derived effects mediate pathophysiological changes during disease and can, in some cases, be directly linked to symptoms (e.g., the induction of airway hyperresponsiveness in asthma patients [18, 19]). It has also been observed that hMBP-1 is produced by fetus-derived placental trophoblasts during pregnancy [20]. Although the role(s) of hMBP-1 during gestation is

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Received September 7, 1999; revised November 14, 1999; accepted November 15, 1999.

not understood, plasma levels of the unprocessed precursor form of hMBP-1 increase dramatically during pregnancy and have been correlated to the onset of labor [21].

MBP-1 orthologs have been cloned from human [22], mouse [23], guinea pig (gp) [24], and rat (r) [25]. In addition, Aoki and colleagues [26] demonstrated the existence of two MBP genes in the guinea pig (gpMBP-1 and gpMBP-2) through the use of amino acid sequencing data from proteins recovered by reverse-phase high-performance liquid chromatography (HPLC) of guinea pig eosinophil granule protein fractions. Comparison of the primary amino acid sequences of the guinea pig MBPs to the human and mouse MBP-1 proteins showed that notable sequence identity was present only in the pre (signal sequence) region and the cationic mature proteins. Conservation within the proteolytically cleaved pro regions was limited to the maintenance of the overall negative charge of these regions through the retention of acidic amino acids [23]. Larson and colleagues presented data suggesting the existence of two MBP genes in both humans and mice [23] and Plager et al. [27, 28] have since reported the cloning/characterization of a second MBP gene/protein in humans (hMBP-2). This second hMBP family member was shown to be expressed exclusively in the eosinophil and was isolated from the secondary granules of these cells. Although a positively charged protein (p*I* 8.7), the cationic character of hMBP-2 is almost three orders of magnitude less than hMBP-1 (i.e., 8.7 vs. 11.4, respectively). Despite this charge difference, assessments of biological effector functions *in vitro* have shown that hMBP-2 and hMBP-1 have similar activities (e.g., cell killing, induction of neutrophil superoxide anion production, and interleukin-8 release, and basophil histamine and leukotriene C_4 release); however, the biological effects mediated by hMBP-2 were generally smaller than the hMBP-1-induced effects.

We demonstrated the likely existence of MBP-2 in the mouse by low-stringency genomic Southern blot using a mMBP-1 DNA fragment as a heterologous probe [23]. These results prompted our current investigations of additional MBP gene family member(s) in the mouse. The studies reported here describe the cloning and characterization of mMBP-2, the murine ortholog of the hMBP-2 gene. Alignment of the mammalian MBPs show that mMBP-2 and hMBP-2 form a unique clade based on sequence conservation, suggesting that the availability of multiple, yet unique, MBPs is selectively advantageous to eosinophil effector function.

MATERIALS AND METHODS

Screening of a wild-type mouse bone marrow cDNA library

A random-primed cDNA library was constructed from bone marrow RNA collected from the hindlimbs of 40 C57BL/6J mice as described by Horton et al. [29]. Briefly, polyadenylated RNA was purified, and random primers used for first-strand synthesis. cDNAs were then directionally cloned into a plasmid vector pBluescript/SK$^+$ (Stratagene, La Jolla, CA). To screen the library by Southern blot, 24 individual pools of plasmid DNA (representing ~240,000 clones) were digested with SalI and NotI to release the inserts and electrophoretically separated on a 1% agarose/TBE gel. DNA was transferred to nylon-supported nitrocellulose membrane (Schleicher & Schuell, Keene, NH), which was subsequently prehybridized at 42°C in 50% formamide, 5× saline sodium citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 5× Denhardt's (1×: 0.02% w/v each Ficoll 400, bovine serum albumin, polyvinylpyrrolidone), and 0.150 mg/mL calf thymus DNA for ≥4 h. The blot was hybridized overnight in fresh buffer containing ~2 ng/ml of a ^{32}P-labeled random-primed probe [~500-bp insert of expressed sequence tag (EST) I.M.A.G.E. clone ID 354861 (Research Genetics, Huntsville, AL), GenBank Accession No. W45834]. This EST clone was initially identified in a database search for sequences with MBP-like similarity. The hybridized filter was washed at 65°C with 0.1× SSC/0.2% SDS, then exposed to Kodak XAR film at −80°C with an intensifying screen. Positive DNA pools were identified, retransformed into *Escherichia coli*, and individual clones hybridizing to the EST probe were identified by colony hybridization [29]. Plasmid DNA was isolated from positive clones through the use of cesium chloride-ethidium bromide equilibrium centrifugation gradients [30].

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from mouse embryos and placenta at various time points during development (11.5, 13.5, 15.5, and 17.5 days post coitum) using a lithium chloride/urea lysis protocol [31]. Total RNA was isolated from adult C57BL/6J mouse tissues (bone marrow, lung, liver, spleen) by immediate homogenization (Polytron, Kinematika, Switzerland) in Tri Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. RNA samples were DNase-treated with amplification-grade DNaseI (GIBCO-BRL, Grand Island, NY) for 15 min at 37°C, extracted with phenol and chloroform, and ethanol precipitated. Total RNA (1.25 µg) was reverse transcribed in a 40-µL volume for 1 h at 42°C using an anchored oligo(dT)-mixture of dT16VN, where V = A, C, or G and N = A, C, G, or T. Oligo pairs used for PCR were as follows: EST8/EST7, 5′-GAGTCTGCAGGAGATGCTACCG-3′/5′-GCTGTGGAAAGCCTGACTTTATGAC-3′; mMBPex3/mMBP3ex5, 5′-GACTCTGGATGCAAGACCTG-3′/5′-CAAGAACTTCCATCAACCCATC-3′; mEPO1/mEPO2, 5-AACTTGGCCCAGCTTAGTCG-3′/5′-GCTGATAGGTTCAACTTGGG-3′; and actin5′/actin3′, 5′-GTGGGCCGCTCTAGGCACCA-3′/5′-TGGCCTTAGGGTTCAGGGGG-3′.

Standard PCR amplifications in a 50-µL volume included cDNA derived from 30 ng of RNA, 200 µM of each dNTP (Boehringer Mannheim, Indianapolis, IN), 80 nmol of each primer, 5% dimethyl sulfoxide (DMSO), 2 units Taq Polymerase (Boerhinger Mannheim), and 1× PCR buffer supplied with the polymerase. Cycling reactions were performed with a Perkin-Elmer Gene Amp 9700 instrument using the following program: 94°C, 5 min; followed by 30 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min; and 72°C for 7 min. The PCR products were size-fractionated by electrophoresis through 1% agarose/TBE gels and visualized with ethidium bromide.

Nucleotide and protein sequence analysis

Nucleotide sequences from purified plasmid DNA templates or reamplified RT-PCR products (PCR Product Purification, Qiagen, Santa Clarita, CA) were obtained by cycle sequencing using an Applied Biosystems model 373A automated DNA sequencer. Sequences were assembled and compared using AssemblyLIGN software (Kodak, Scientific Imaging Systems, New Haven, CT) and analyzed for potential open reading frames using MacVector version 4.5.3 software (Kodak, Scientific Imaging Systems). The Diverge program of the Wisconsin Genetics Computer Group (GCG) suite of programs (version 10) was used to calculate K_a and K_s values for paired sequences to determine the non-synonymous vs. synonymous rates of mutation, respectively. Isoelectric points were calculated from deduced primary amino acid sequences using the GCG Isoelectric program. The progressive similarity alignments were produced using the algorithm of Feng and Doolittle [32]. Multiple alignment data were plotted by a Treeplot program to display the phylogenetic data in the form of a cladogram. Bootstrap analysis was performed by substituting the program BTREE for TREE. The best branching order and all positive branch lengths from at least 100 trials were then saved. The results are expressed as percentages of times that a cluster around a node is the same as in the original tree and the value placed at the nodes [33].

Chromosome localization

Linkage analysis for the mMBP-2 gene locus was performed using the Jackson Laboratory BSS Backcross DNA panel (Jackson Laboratory, Bar Harbor, ME)

(C57BL/6JEi × SPRET/Ei)F1 × SPRET/Ei [34]. For Southern blot analyses, a specific probe for mMBP-2 was prepared by RT-PCR of RNA isolated from the long bones (i.e., femurs and tibias) of the hind limbs of C57BL/6J mice. Restriction enzyme digestion of genomic DNA from C57BL/6J and *Mus spretus* mice was analyzed by Southern blot to identify an informative restriction fragment length polymorphism (RFLP). An EcoRI RFLP was detected after hybridization (0.8 M [Na^+], 50% formamide, 42°C) and a high-stringency wash (0.1× SSC/0.2%SDS at 65°C); i.e., a single ~14-kb band was observed for C57BL/6J DNA and a 7-kb band was observed for *M. spretus* DNA. The BSS backcross DNA panel was then scored for the presence or absence of the C57BL/6J-specific band. The allele pattern was compared to those of ~4300 other loci already mapped in the same cross to reveal the linkage to Chr 2. The raw data was analyzed and a linkage map generated using Map Manager [35].

Identification of a genomic clone from a bacteriophage P1 mouse genomic library

A specific PCR primer set was selected for screening a P1 bacteriophage mouse genomic library (Genome Systems, St. Louis, MO) to isolate a mMBP-2 genomic clone. The mMBP-2 primers were designed to span a potential intron by aligning the conserved mMBP-2 cDNA sequence with the genomic sequence of the paralogous mMBP-1 locus [23]. PCR amplification of C57BL/6J DNA was performed using standard PCR conditions described above and the primer set EST5/EST2: 5′-CACTGTGTGACTCTGTGTACC-3′/5′-CAAGTAGGATCTAG-CTCCATC-3′. A single 710-bp PCR product was generated and sequenced with both EST5 and EST2 oligos to confirm mMBP-2 identity.

Peritoneal cavity eosinophilia and immunohistochemistry

A peritoneal cavity eosinophilia was induced in both wild-type C57BL/6J mice and homozygous mMBP-1 knockout mice [K. L. Denzler, S. Farmer, J. Crosby, M. Borchers, K. Larson, N. Lee, and J. Lee, unpublished results] by injection of a protein extract from the helminth parasite, *Mesocestoides corti* [23]. The peritoneal exudate cells were collected by lavage and ~10^4 cells were cytocentrifuged (Cytospin3, Shandon Scientific Ltd., Cheshire, UK) per slide. Slides were air dried and then immediately fixed in 100% ethanol for 10 min. Immunohistochemistry with a polyclonal antisera was performed to detect cells expressing mMBP proteins. We used 100 µL of 1:250 dilutions of either rabbit polyclonal antiserum against mMBP-1 [36] as the primary antibody or rabbit pre-immune serum as a negative control. After a 2-h incubation at room temperature (RT) in a humidified chamber, slides were washed for 5 min in 0.05 M TBS (50 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4). One hundred microliters of a 1:200 dilution of 2° antibody, biotinylated goat anti-rabbit IgG (DAKO, Carpenteria, CA), was incubated for 30 min at RT. After several washes in 0.05 M TBS, ~100 µL of the 3° reagent, alkaline phosphatase-conjugated streptavidin (BioGenex, San Ramon, CA), was applied to each sample and incubated for 30 min at RT. Slides were again washed in 0.05 M TBS and 100 µL of Vector Red substrate (Vector Labs, Burlingame, CA) containing 1.25 mM levamisole was added to each sample and incubated for 30 min in the dark. Color development was terminated with a tap water rinse. Slides were immediately counterstained with Mayer's Hematoxylin (Zymed Laboratories Inc., S. San Francisco, CA) for 1 min at RT to allow the examination of cell and nuclear morphology. Washed slides were dehydrated in an increasing ethanol series ending with xylene. Coverslips were applied with Consulmount (Shandon, Pittsburgh, PA). Photomicrographs were taken with a Zeiss Axiophot (Carl Zeiss, Inc., Thornwood, NY) and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

RESULTS

Identification of a new eosinophil MBP gene in the mouse

Genomic Southern blot data had suggested that in addition to mMBP-1 the murine genome appeared to contain an additional MBP-related gene family member [23]. Moreover, the likelihood of a second divergent MBP gene in the mouse (order Rodentia) was supported by the confirmed existence of multiple MBP genes in species from other extant mammalian orders, humans (order Primata), and guinea pigs (order undetermined [37]). mMBP-2 was initially identified as an EST (GenBank accession W45834) in a Soares mouse embryo cDNA library (NbME13.5–14.5 day library). Sequence analysis of this clone showed that the EST displayed a high degree of sequence identity with a segment of the mMBP-1 gene as well as the other known mammalian MBP genes. Preliminary RT-PCR assays using bone marrow total RNA and specific oligonucleotide primers derived from the EST demonstrated the presence of mMBP-2 transcripts in this hematopoietic compartment (data not shown). To characterize these mMBP-2 transcripts, the EST was used as a probe in a high-criterion screen (i.e., hybridization, 50% formamide, 0.75 M [Na^+], 42°C; final wash, 0.015 M [Na^+], 65°C) of a random-primed mouse bone marrow cDNA library [29]. Several hybridizing clones were recovered and the complete open reading frame of mMBP-2 (222 amino acids) was assembled from two overlapping clones (**Fig. 1**). Consistent with the other identified mammalian MBPs, mMBP-2 appears to be translated as a larger pre-pro-mature polypeptide proteolytically cleaved during the posttranslational processing that yields mature mMBP-2.

Eosinophil MBPs: structure and evolutionary conservation

Alignment of the entire mMBP-2 open reading frame with either mMBP-1 or hMBP-2 showed that, although the murine paralogs shared 53% amino acid identity, the degree of sequence identity increased when mMBP-2 was compared with hMBP-2 (63% amino acid identity, **Fig. 2A**). This alignment data demonstrates that sequence identity between murine and human MBP-2 occurs in all of the regions of the precursor molecule: amino-terminal secretory signal sequences (63%), the proteolytically cleaved pro regions (58%), and the mature polypeptides (66%). This sequence identity is reflected in the conservation of ionic character associated with different regions of these mammalian orthologs. However, of interest is the relative difference in cationic character between MBP-1 and -2 when comparing paralogs within each species. Mature hMBP-2 has a p*I* that is nearly three orders of magnitude lower than hMBP-1 (8.7 vs. 11.4), whereas mature mMBP-2 maintains the strong cationic nature of mMBP-1 (9.95 vs. 10.5). The differences in charge between the MBP paralogs within these species are provocative in light of the unusually high cationic charge of both MBP paralogs in guinea pigs (p*I*s of 11.7 and 11.3), a species that may represent a separate mammalian order. These data suggest that if charge is representative of one or more effector functions, then the role(s) of MBP-2 vary in different mammals.

To evaluate regions of identity and the degree of conservation among the MBPs, the deduced primary amino acid sequence for mMBP-2 was compared to the six known mammalian MBPs (Fig. 2B). These analyses demonstrate that the mature region of mMBP-2 displays a high degree of sequence identity with the collective group of other mammalian MBPs, suggesting a commonality of effector function. Sequence identity was limited to mature mMBP-2 because the primary amino acid sequence

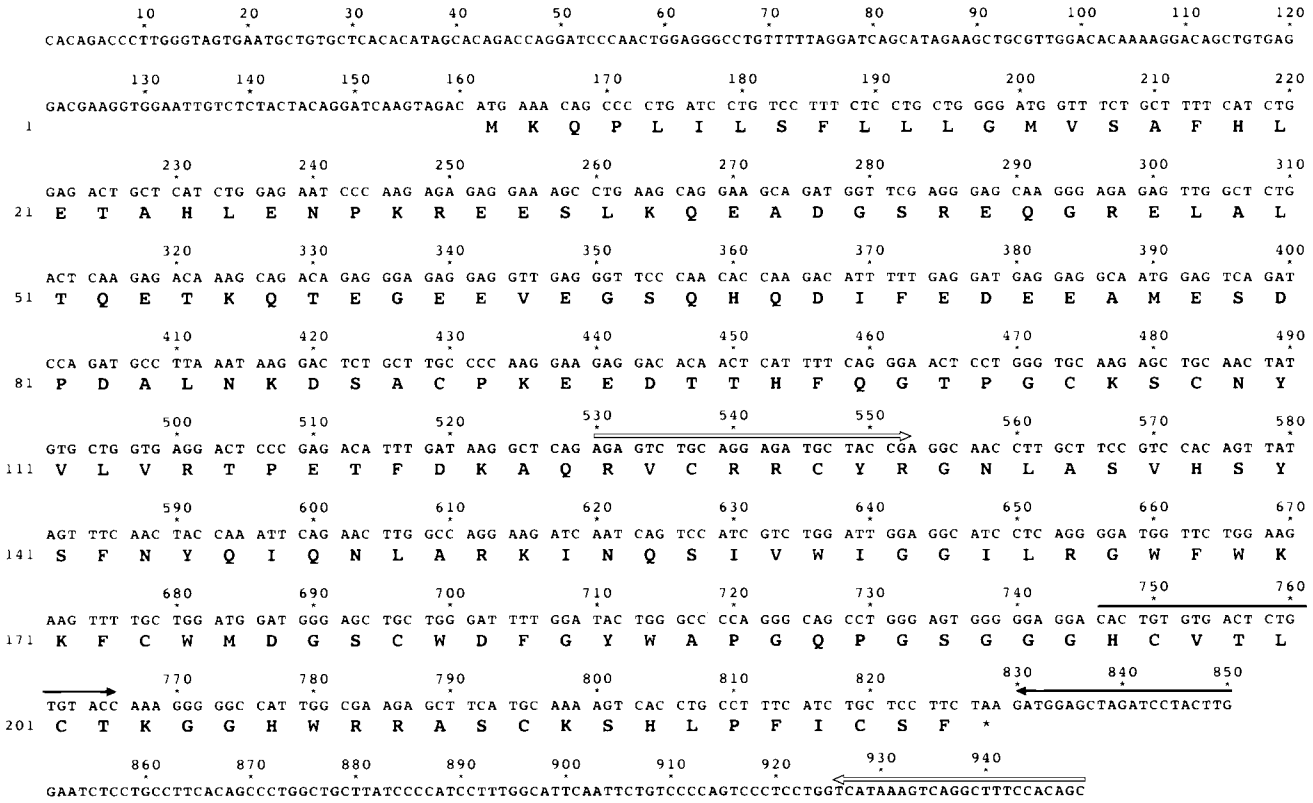

Fig. 1. cDNA and deduced amino acid sequences of murine eosinophil mMBP-2. The 949-bp cDNA sequence is displayed as the coding strand and is numbered above the nucleotides. The deduced amino acid sequence for mMBP-2 is shown below the nucleotide codons and is numbered to the left, from a methionine start codon (amino acid 1) at nucleotide (nt) 161 to an in-frame stop codon (after amino acid 222) at nt 827. Primer sets used for RT-PCR (EST8/EST7) are indicated with open arrows and for PCR (EST5/EST2) with closed arrows. GenBank accession number AF202533.

of the proteolytically cleaved pro region, as a collective group, is not well conserved. However, the pro region of mMBP-2 resembles the other pro regions by encoding a disproportionate number of negatively charged amino acids, conserving the anionic character associated with this region of the precursor molecule [38].

The evolutionary relationships of the known mature mammalian MBPs were analyzed and expressed quantitatively as an unrooted phylogenetic tree (**Fig. 3**). Construction of the tree is based on a progressive alignment strategy that produces a preliminary pairwise determination of phylogenetic order of sequences and subsequently assembles a multiple alignment and assigns evolutionary distance scores [32]. The tree topology (i.e., branching order and clusters) is a representation of the evolution of the mature MBP sequences. Bootstrap analysis of the tree was also performed [33] to estimate the statistical fluctuations inherent with the generation of this tree. The confidence levels of the tree branching order are presented as percentages derived from the bootstrap results and are shown in Figure 3 on a node by node basis. These evolutionary analyses show that the rodent and primate MBP-1 genes form unique clades relative to the MBP-2 genes. Moreover, the murine and human MBP-2 genes form a single clade that, it is interesting to note, does not include either of the guinea pig genes. The two mature guinea pig MBPs group as a separate branch, suggesting that specific selective pressures on MBP effector function exist in different mammalian orders. However, the data does not distinguish a specific orthologous relationship between the individual guinea pig genes and the other mammalian MBPs (i.e., the analyses do not identify which one of the two guinea pig MBPs is the murine/human MBP-2 ortholog).

mMBP-2 co-localizes with mMBP-1 on mouse chromosome (Chr) 2.

Linkage analysis of the mMBP-2 genomic locus was performed using the Jackson Laboratory BSS Backcross DNA panel. The BSS mouse interspecific backcross panel consists of DNA of 94 progeny from a (C57BL/6JEi × SPRET/Ei)F1 × SPRET/Ei backcross. These data demonstrated that mMBP-2 was linked with genes located on mouse chromosome 2 (**Fig. 4A**). The linkage data is presented graphically in the chromosome map shown in Figure 4B. The locus symbol for mMBP-2, *Prg3*, was approved by the International Mouse Nomenclature Committee. This linkage data demonstrated that mMBP-2 (*Prg3*) mapped to the same locus we had previously mapped mMBP-1 (locus symbol *Prg2*) with no recombination events occurring between these two genes [39].

mMBP-2 and mMBP-1 are physically linked on a single P1 genomic clone

An mMBP-2-specific primer set was created for screening a bacteriophage P1 mouse genomic library (Genome Systems Inc., St. Louis, MO). From this screen, we obtained a P1 clone with a 75- to 100-kb insert bearing the mMBP-2 gene. Both mMBP-2 and mMBP-1 were identified on this P1 clone by PCR

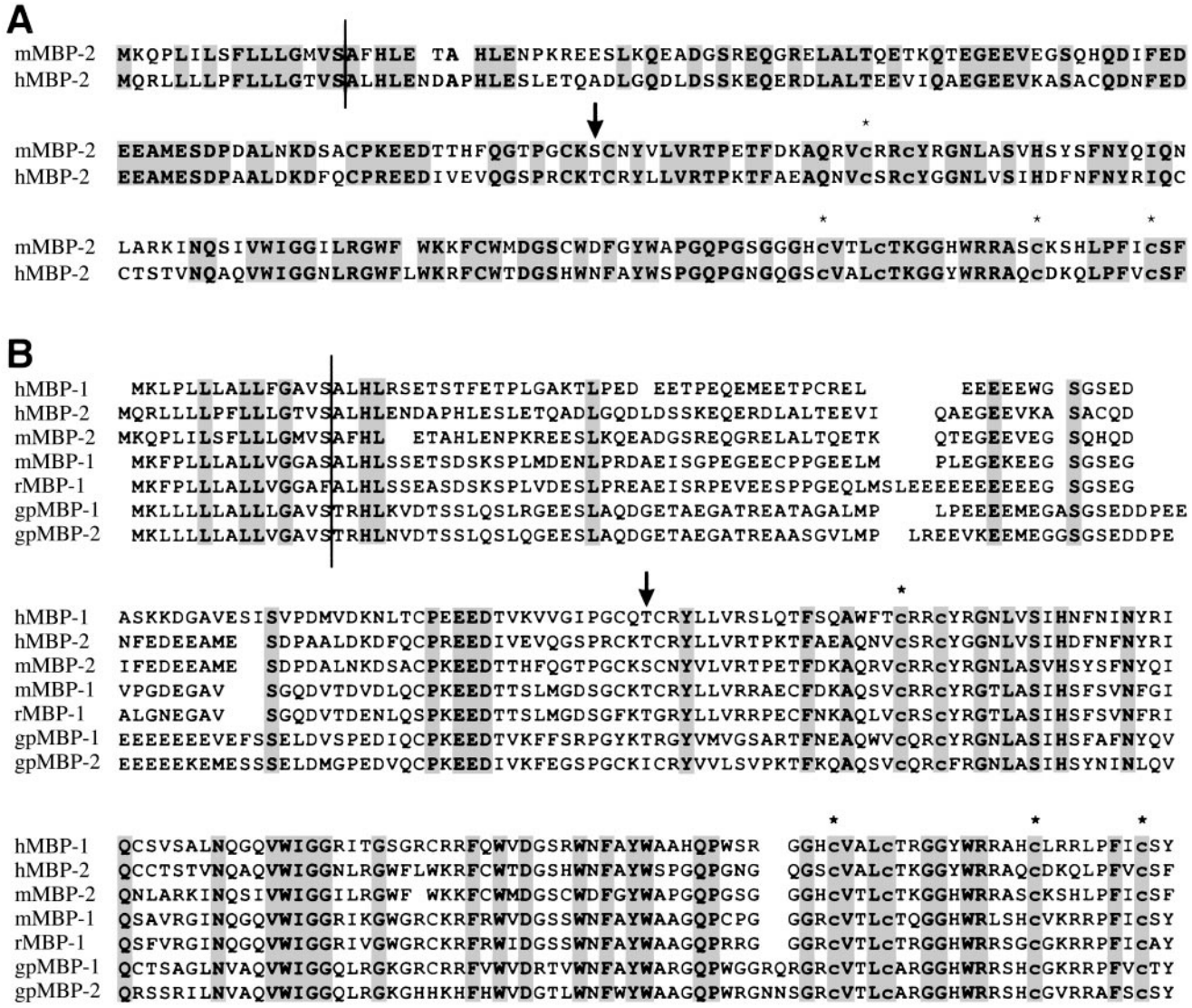

Fig. 2. Primary amino acid sequence alignment of the pre-pro-mature protein sequences of mammalian MBP gene family members. (A) The deduced amino acid sequence of mMBP-2 protein is aligned to maximize homology with hMBP-2. (B) Progressive similarity alignment of the pre-pro-mature protein of mMBP-2 and the other known mammalian MBPs using the method of Feng and Doolittle [32]. The order of sequence input was as follows: hMBP-1 (M34462), hMBP-2 (AF132209), mMBP-1 (L46768), mMBP-2 (AF202533), rMBP (D50568), gpMBP-1 (D90251), gpMBP-2 (D00817). The positions of sequence identity are enclosed in shaded boxes. The vertical line represents the proteolytic cleavage site between the pre signal peptide sequences and the acidic pro regions. The arrow indicates the amino-terminal residue of the mature form of MBP-1 isolated from eosinophil secondary granules [47]. Asterisks are positioned over the conserved half-cystines shown to be involved in forming disulfide linkages in hMBP-1. h, human; m, mouse; r, rat; gp, guinea pig.

using either an mMBP-2-specific or an mMBP-1-specific primer set (**Fig. 5**). The identity of the PCR products as either mMBP-2 or -1 was confirmed by DNA sequencing (data not shown) thus demonstrating that these genes are linked to within ~100 kb of one another.

The expression pattern of mMBP-2 is coordinate with the expression of mMBP-1

Primer sets specific for mMBP-1 and mMBP-2 were used to determine the patterns of MBP gene expression in adult mouse tissues engaging in eosinophilopoiesis (i.e., bone marrow, spleen, and thymus) as well as several non-hematopoietic adult tissues (e.g., heart, liver, and lung). RT-PCR amplification of these tissue-specific cDNAs demonstrated that the expression pattern of mMBP-2 was nearly indistinguishable from the pattern associated with mMBP-1 (**Fig. 6A**). The only exception was the detection of mMBP-1 transcripts in the lung, whereas under the same cycling conditions, expression of mMBP-2 was not detected. This observation is somewhat baffling and suggests that either a cell type in the lung differentially expresses mMBP-1 or, alternatively, the sensitivity of this PCR assay may be sufficient to detect mMBP-1 but not mMBP-2 transcripts resulting from low levels of extramedullary eosinophilopoiesis occurring in the lung.

In humans, hMBP-1 gene expression is not limited to eosinophils. *In situ* studies have shown that an additional source of hMBP-1 expression is the placenta, specifically the placental X cells and placental-site giant cells [20]. Although the function of the placental hMBP-1 is not understood, correlations have been made between elevated serum levels of

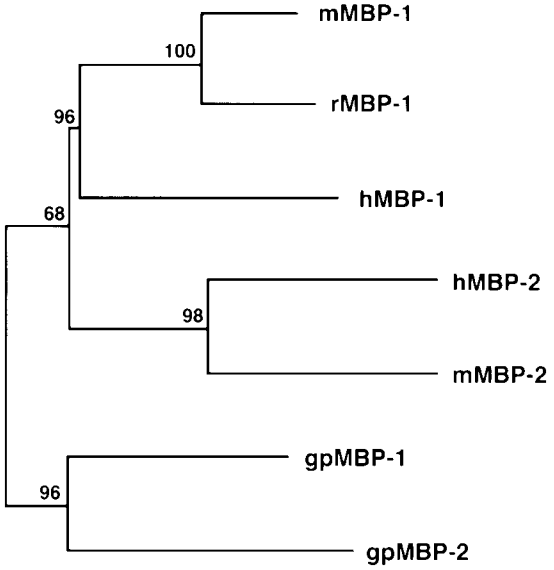

Fig. 3. An unrooted phylogenetic tree based on primary amino acid sequence homology within the mature domains of mammalian MBP family members. The phylogenetic tree was constructed using the same algorithm of Feng and Doolittle [32] that generated the multiple sequence alignment of Figure 2B. The topology of the tree provides an indication of how the mature MBP sequences group and the branch lengths provide a measure of evolutionary distance. The order of sequence input was the same as Figure 2. Bootstrap analysis results are presented as percentages on a node by node basis.

hMBP-1 and the onset of labor [21]. The potential relevance of placental MBP expression to gestation was assessed in the mouse. Total RNA was isolated from mouse embryos (including the visceral yolk sac and amnion) and placentas at several developmental time points. The presence of transcripts was determined either by Northern blot (data not shown) or RT-PCR (Fig. 6B). These data demonstrate that at each gestational age, the presence of either mMBP-2 or mMBP-1 transcripts was limited to the embryos (i.e., transcripts from neither mMBP gene could be detected in the placenta despite the sensitivity of this PCR-based assay). PCR reactions using a primer pair specific for another eosinophil-specific granule protein gene, eosinophil peroxidase (mEPO), were performed as an independent measure of eosinophil-derived gene expression. The pattern of mEPO gene expression was identical to both mMBP-1 and -2, suggesting that fetal eosinophilopoiesis is the source of the embryonic mMBP transcripts and the original EST that we identified as mMBP-2.

mMBP-2 expression is localized to mouse eosinophils

Although the expression of mMBP-2 was restricted to tissues engaging in eosinophilopoiesis, the specific cellular origin of these transcripts remained unknown. The specificity of mMBP-2 gene activity was investigated further to determine the hematopoietic cell type(s) expressing this protein through the use of two novel reagents. As suggested by the strong sequence identity between mMBP-1 and -2, Western blots using protein derived from *in vitro* translation of mMBP-2 transcripts demonstrated that a rabbit polyclonal antisera raised against mMBP-1 [36] cross-reacts with mMBP-2 (data not shown). Moreover, our laboratory has recently developed an mMBP-1 knockout mouse homozygous for a targeted disruption of the mMBP-1 gene [K. L. Denzler, S. Farmer, J. Crosby, M. Borchers, K. Larson, N. Lee, and J. Lee, unpublished results]. These mice are unable to express mMBP-1 and are devoid of mMBP-1 protein. Immunohistochemical staining of white blood cells from these knockout animals should clearly demonstrate the presence and cell-specificity of mMBP-2 expression (i.e., our cross-reactive mMBP-1 polyclonal antisera will only detect mMBP-2 in an mMBP-1-deficient mouse). A peritoneal cavity eosinophilia was induced in wild-type and mMBP-1 null mice by immunization and challenge with an acetone protein extract from the helminth parasite *Mesocestoides corti*. Cytospin preparations of peritoneal cavity cells from these sensitized/challenged mice (~25% eosinophils) were stained using the rabbit mMBP-1 polyclonal antisera, and representative photomicrographs of the immunohistochemical staining are shown in **Figure 7**. Preimmune control serum was used as a negative control and did

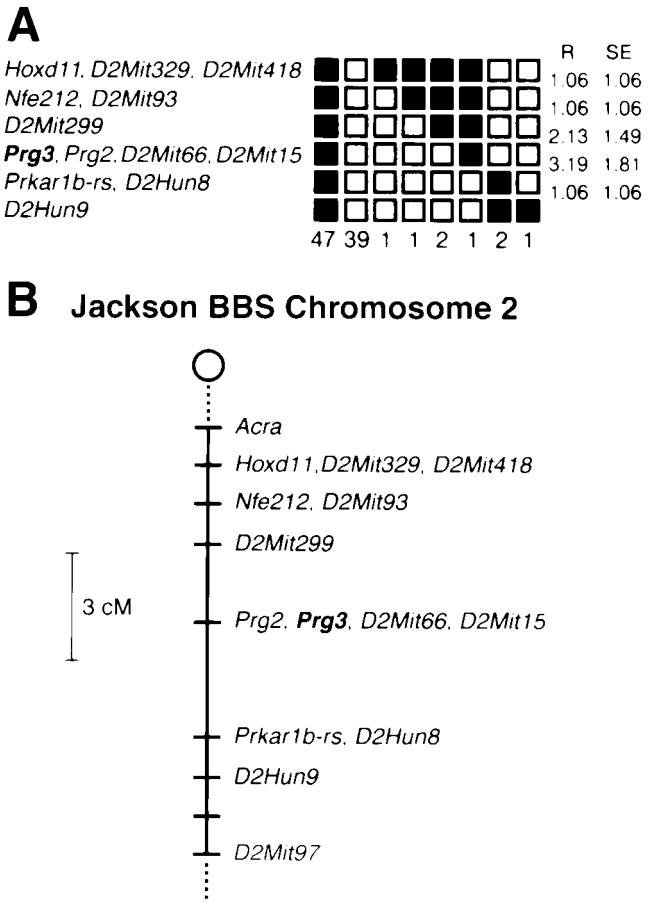

Fig. 4. Localization of the mMBP-2 gene (locus symbol *Prg 3*) on mouse chromosome 2 (Chr2) using a Jackson BSS interspecific backcross panel. (A) Haplotype figure from the region of the BSS Chr 2 surrounding *Prg 3*. Filled boxes indicate the C57BL/6J allele. Open boxes indicate the SPRET/Ei allele. Numbers at the bottom of each column of boxes are the number of animals with that haplotype from the 94-progeny backcross panel. For each interval, the frequency of recombination (R), equivalent to centimorgan (cM), and the standard error (SE) are given to the right. (B) Map figure from the Jackson backcross showing part of Chr 2, with the centromere toward the top. The chromosomal order for the loci with map distances in cM ± the standard error, D2Mit299- (2.13 ± 1.49) -Prg3, Prg3- (3.19 ± 1.81) -Prkar1b-rs. A 3-cM scale bar is shown to the left. Loci mapping to the same position are shown in arbitrary order.

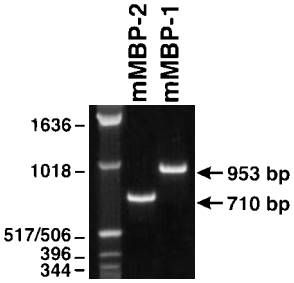

Fig. 5. Localization of both murine MBP-2 and MBP-1 genes to a 75- to 100-kb pair mouse genomic DNA fragment. PCR of a single bacteriophage P1 mouse genomic clone (Genome Systems) using specific primer sets that span intronic sequences of mMBP-2 and mMBP-1. The predicted PCR product sizes are listed to the right of the gel.

not exhibit staining of any cell type from either wild-type or mMBP-1 null mice (Fig. 7, A and C). As expected, wild-type mice displayed intense eosinophil-specific staining with the anti-mMBP-1 antisera, reflecting the abundance of this protein in the eosinophil secondary granule (Fig. 7B). This staining intensity was dramatically reduced in mMBP-1 null mice, however, we nonetheless observed reproducible staining with this antisera only in eosinophils (Fig. 7D). The weakness of the positive staining compared to the mMBP-1 wild-type cells may result from the limited efficiency of antibody cross-reactivity and/or lower protein expression levels of mMBP-2 (i.e., relative to mMBP-1) per eosinophil. Regardless of the cause of lower staining intensity, these data unambiguously demonstrate that mMBP-2 expression is limited to the eosinophil in the mouse.

DISCUSSION

Evidence was presented for the existence of a second mMBP gene family member in the mouse. The data show that these two MBP gene family members are physically linked and are in close proximity, as has been determined for the two human MBP gene family members [G. J. Gleich, personal communication]. This suggests that MBP-1 and MBP-2 arose by a recombination-mediated duplication event on a chromosomal segment that was syntenic to mouse Chr 2 and human Chr 11 [40]. The identification of two eosinophil MBP genes in potentially three extant mammalian orders suggests that the duplication event giving rise to these genes occurred before the major radiation of mammalian orders 75–100 million years ago [41]. Implicit in this view is that all mammals would possess two MBP genes. We would argue that the evolutionary conservation of two eosinophil MBP genes in different mammalian orders is not serendipitous, and that a selective advantage exists by having these MBP variants.

The conservation of structural features among the mammalian MBPs suggests a commonality of function. However, this high amino acid sequence identity among the mature proteins has not led to the identification of an enzymatic function associated with the MBPs. Indeed, the only suggested functions of MBPs are associated with their strong cationic character [42–44], although this cationic character varies dramatically. That is, despite the evolution of difference in cationic character (i.e., p*I*) between the mammalian MBPs there appears to be a selective advantage to maintain amino acid identity between MBP-1 and MBP-2. This selective advantage is exemplified by calculations of K_a/K_s ratios [where K_s is the number of synonymous (i.e., silent) substitutions per synonymous site and K_a is the number of non-synonymous substitutions per non-synonymous site] between MBP-1 and MBP-2 in mice, humans, and guinea pigs. These calculations consistently yield values less than one (i.e., a preponderance of silent nucleotide substitutions). For example, the K_a/K_s ratio comparing the mature domains of mMBP-1 and mMBP-2, is 0.238; for hMBP-1 and hMBP-2, K_a/K_s is 0.219; and for gpMBP-1 and gpMBP-2, K_a/K_s is 0.612.

Murine and guinea pig MBP-1 and MBP-2 proteins have evolved such that each paralogous pair (i.e., MBP-1/MBP-2) is similar in charge. However, hMBP-1 is three orders of magni-

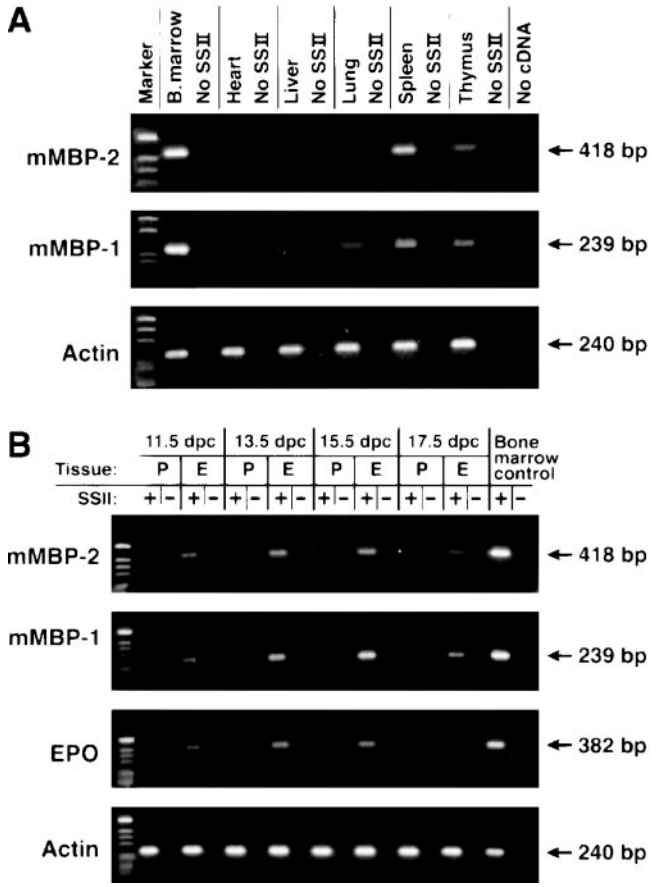

Fig. 6. mMBP-2 RNA is expressed at sites of eosinophilopoiesis. (A) RT-PCR assays of adult mouse tissues for eosinophil gene expression. Primer sets specific for mMBP-2 or mMBP-1 were used to determine the pattern of gene expression. (B) mMBP-2 expression parallels mMBP-1 expression in RT-PCR assays of murine embryonic and placenta RNA. Total RNA was prepared from the placenta (P) and embryo (E) at the time points indicated as days post-coitum (dpc) and analyzed by RT-PCR using primers specific for mMBP-2, mMBP-1, and EPO. The RT-PCR primer sets used are listed to the left of the gels and predicted product sizes are listed to the right. The DNA size standard used in the far left lanes of each panel is the GIBCO-BRL 1-kb Ladder. An actin primer set was used as a positive control for sample preparations. +SSII lanes indicate reverse-transcribed RNA samples, and No SSII and -SSII lanes are RNA samples that did not receive reverse transcriptase and serve as negative controls for DNA contamination. Adult mouse bone marrow total RNA was used as a positive control for each primer set.

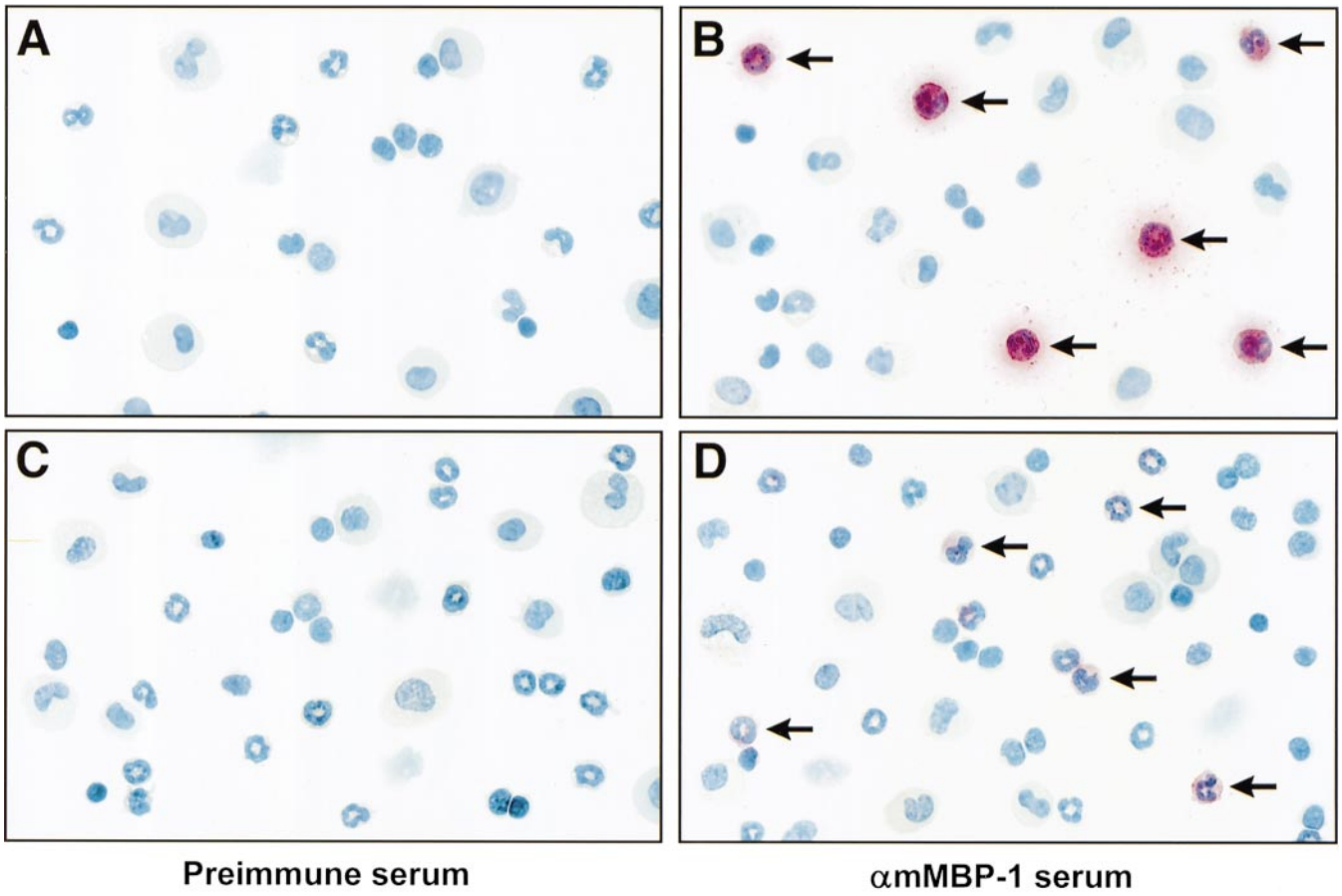

Fig. 7. Eosinophil-restricted expression of mMBP-2. Wild-type and mMBP-1 knockout mice [K. L. Denzler et al., unpublished results] were immunized and challenged with antigen from the helminthic parasite *M. corti* [23] to induce a peritoneal eosinophilia. Cell differentials of the peritoneal lavage cells show eosinophils to be the predominant infiltrating granulocyte in both wild-type and knockout samples. Wild-type (A, B) and mMBP-1 knockout mice (C, D) extravascular peritoneal cavity cells were collected and cytocentrifuged onto slides. For immunohistochemistry, cell samples were stained with either rabbit anti-mMBP-1 serum or rabbit preimmune serum (negative control) as the primary antibody. Examples of positively stained cells are indicated with arrows. Photomicrographs were taken at ×630 magnification.

tude more basic than hMBP-2. If charge is a critical determinant of MBP effector function, the differences in charge suggest the two human MBPs possess unique functions. This charge difference, and potential functional difference between the human proteins, may explain in part the observation that only hMBP-1 is expressed in the placenta during pregnancy. Perhaps an unknown function of hMBP-1 during pregnancy provides selective pressures affecting the evolution of this protein that is not occurring for the eosinophil-restricted MBPs of mice and guinea pigs.

In human and guinea pig eosinophils, MBP-1 and MBP-2 are found as mature proteins in the secondary granules, with MBP-1 apparently being necessary for the formation of the distinctive electron-dense core structure of the granule [45, 46]. We have demonstrated in the mouse that expression of mMBP-2 RNA parallels that of mMBP-1 and is associated with sites of eosinophilopoiesis. This interpretation is supported by our immunohistochemistry data, which localizes mMBP-2 protein expression specifically to eosinophils. If both MBPs are localized to the secondary granules, the possibility that mMBP-2 contributes structurally to the electron-dense crystalline core must be considered. Furthermore, if mMBP-2 is a granule component, we would predict that tissue deposition of mMBP-2 contributes functionally to activities associated with eosinophils in chronic inflammatory diseases.

Co-localization of the murine MBP genes to Chr 2 and subsequent identification of a DNA fragment (75-100 kb) that encodes both mMBP genes allowed us to undertake studies of the genomic organization and regulation of these transcription units. In the mouse, mMBP-2 and mMBP-1 genes appear to be coordinately expressed and both are specifically restricted to eosinophils (i.e., in contrast to humans, neither mMBP gene is expressed in fetally derived placental cells). It is likely that the genes share transcriptional regulatory features, however, the expression of these two loci do not appear to be linked because mMBP-1 knockout mice are still capable of expressing mMBP-2.

The conservation of homologous MBP gene pairs in multiple mammalian orders suggests that the presence of two MBPs within a given species has functional implications. Moreover, this conservation of sequence identity further suggests that these protein pairs have similar effector functions, although it does not preclude the possibility that MBP-1 and MBP-2 may also have unique non-overlapping activities. To date, the specific nature of MBP effector functions remains elusive. However, the availability of protein purification strategies and gene transfer/knockout technologies in the mouse will permit

the unambiguous determination of specific effector functions attributable to each major basic protein.

ACKNOWLEDGMENTS

This work was supported by funding from the Mayo Foundation and a postdoctoral fellowship (M. P. Macias) from the National Heart, Lung, and Blood Institute (F32 HL10105). We gratefully acknowledge the assistance and personal communications of Drs. Russell Doolittle for construction of the MBP progressive similarity alignments, phylogenetic tree, and bootstrap analysis, Lucy Rowe for the chromosome localization analyses, Michael P. McGarry for production of the anti-mMBP-1 polyclonal serum, and Eric Pearlman for assistance with immunohistochemistry. We thank Dr. Gerald Gleich for sharing data with us prior to publication. We would also like to thank the Mayo Clinic Scottsdale Graphic Artists, Marv Ruona and Julie Jensen, for figure preparation, and Jill Martin at the Mayo Clinic Scottsdale Molecular Biology Core Facility for oligonucleotide syntheses.

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