Mouse 230-kDa Bullous Pemphigoid Antigen Gene: Structural and Functional Characterization of the 5'-Flanking Region and Interspecies Conservation of the Deduced Amino-Terminal Peptide Sequence of the Protein

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The 230-kDa bullous pemphigoid antigen is a hemidesmosomal protein of the cutaneous basement membrane zone. The primary sequences deduced from full-length human cDNAs predict that this molecule consists of a central rod region and flanking globular domains. To get insight into regulation of the 230-kDa bullous pemphigoid antigen gene (BPAG1), and to evaluate evolutionary conservation of the amino-terminus of the protein, we screened a mouse genomic DNA library with a 0.3-kb cDNA corresponding to the 5' end of the human 230-kDa bullous pemphigoid antigen cDNA. A positive clone was isolated, and Southern analysis of the clone with the 0.3-kb cDNA allowed isolation of a 3.0-kb Hind III fragment containing the 5' end of the coding sequence. Alignment of the sequences of this subclone and human BPAG1 sequences revealed that this fragment contained 2466 bp of 5'-flanking DNA, upstream from the ATG translation initiation site, and 258 bp of translatable sequences that encode a putative polypeptide of 86 amino acids at the amino-terminus of the protein. This deduced polypeptide showed 91% homology with the corresponding human sequence. The TATAAA and CCAAT consensus sequences, as well as several putative cis-regulatory elements, were identified in the 5'-flanking region of the mouse DNA. To test the functional promoter activity of the 5'-flanking DNA, three mouse BPAG1 promoter/CAT reporter gene constructs, with the promoter segments spanning from -1133 to -25, and -213 to -1, were developed. Transient transfections of mouse transformed keratinocytes (Pam 212 cells) with these constructs revealed clearly detectable CAT activities, indicating that the 5'-flanking region contains a functional promoter. Furthermore, these experiments suggested that the upstream sequences contain upregulatory elements, as well as elements that confer, at least in part, tissue specificity to the expression of the mouse 230-kDa BPA gene. Key words: basement membrane zone/blistering skin diseases. J Invest Dermatol 103:651–655, 1994

Bullous pemphigoid (BP) is a cutaneous blistering disease characterized by the presence of circulating immunoglobulin (Ig)G autoantibodies in the patients’ sera [1–3]. Western blot analyses of BP patients’ sera have recognized at least two distinct proteins, bullous pemphigoid antigens (BPAs), that serve as autoantigens in this disease. The major antigen recognized by most patients’ sera is a 230-kDa protein, whereas another protein recognized by relatively few patients’ sera has a molecular weight of 180 kDa [4–8]. Immunoelectron microscopic studies have demonstrated that these autoantigens are localized to the hemidesmosomes in the basal keratinocyte/cutaneous basement membrane interphase [9–11].

A human cDNA clone corresponding to the 230-kDa BPA sequences was first isolated by Stanley et al [12], and cloning of a mouse cDNA was first reported by Amagai et al [13]. We have subsequently isolated a composite of cDNA clones that encode the entire human 230-kDa BPA [14–16]. These cDNAs were used for chromosomal mapping of the human 230-kDa BPA gene (BPAG1) to chromosome 6 [14], and for examination of the expression of the gene in cultured cells [15].

Recent data by Liu et al [17] have implicated antibodies recognizing the 180-kD BPA in the pathogenesis of BP. However, the precise role of 230-kDa BPA autoantibodies in the pathogenesis of BP is currently unknown. The 230-kDa BPA has been suggested to interact with intracellular intermediate filaments [18,19], and the striking similarity of deduced peptide sequences between the 230-kDa BPA and desmoplakin I suggests that these molecules may belong to the same family of cell adhesion junction plaque proteins [20]. Thus, the 230-kDa BPA is thought to be involved, by a yet-unknown mechanism, in stabilization of the cutaneous basement membrane zone.

The BP antigens are expressed exclusively in the stratified squa-
mous epithelia [2,21]. Thus, it is plausible that the BPAG1 promoter plays a critical role in the regulation of tissue-specific expression of this gene. We have recently cloned the entire human BPAG1 gene, including up to ~3 kb of the 5'-flanking DNA [22,23]. To understand the molecular mechanisms underlying the regulation of BPAG1 gene expression in further detail, we have characterized the 5'-flanking region of the mouse gene and developed BPAG1 promoter/reporter gene constructs. This study shows evidence for transcriptional regulation in the tissue-specific expression of this gene.

MATERIALS AND METHODS

Isolation and Characterization of Mouse 230-kDa BPA Genomic Clones A mouse fibroblast genomic library in pFIX plasmid vector (Stratagene) was screened with a 0.3-kb cDNA corresponding to the 5' end of human BPA cDNA sequences (nucleotide positions +1 to 250; see [16]), synthesized by polymerase chain reaction (PCR) amplification. The genomic clones isolated here were purified, and the inserts were characterized by restriction enzyme digests and Southern analysis with the 0.3-kb cDNA [24]. The restriction endonuclease digestion products were fractionated by electrophoresis on 1% agarose gels, and the sizes of the DNA fragments were estimated by comparison with standard DNA markers. Appropriate DNA fragments were isolated by gel electrophoresis and subcloned into the Bluescript vector (Stratagene) for sequencing [24]. Nucleotide sequencing was performed on both strands using the dideoxynucleotide chain termination method, utilizing universal and reverse primers or with synthetic oligonucleotide primers [25]. The computer search for comparison with human 230-kDa BPA sequences and for consensus sequences, including the putative signal peptide, was performed by Genetix (Hitachi) and by pc/Gene (IntelliGenetics) programs.

Construction of Mouse 230-kDa BPA Promoter/Chloramphenicol Acetyl Transferase (CAT) Reporter Gene Plasmids One genomic clone was found to contain the 5'-flanking DNA sequences of the mouse BPAG1 gene (see Results). To develop BPAG1 promoter/reporter gene chimeric constructs, double-stranded genomic DNA fragments were generated by PCR amplification. The primers used for PCR contained an XhoI site in the 5' oligomer and a HindIII site in the 3' oligomer, which enabled direct cloning of the PCR products into pBSOCAT, a promoterless construct containing the entire CAT gene, small t intron, and the polyadenylation signal in plasmid Bluescript (pBSK) [26].

Transient Cell Transfections and CAT Assays The reporter gene/CAT constructs, as well as pBSOCAT (a negative control), 20 μg each per 20 cm dish, were used in a transfection of a SV2-β-Gal transfectant of mouse keratinocytes (Pam 212 cells) [27], mouse melanoma cells (B16 cells) [28], and mouse colon carcinoma cells (CT 26) [29] cultured in modified Eagle's medium (MEM) with 10% fetal calf serum (FCS), and of mouse fibroblasts (NIH 3T3 cells), obtained from the American Type Culture Collection (ATCC) cell repository, cultured in MEM with 10% calf serum. The endogenous expression of the 230-kDa BP antigen in these four mouse cell lines was examined both at the protein and the mRNA levels. For assay at the protein level, indirect immunofluorescence of cell cultures was performed with serum from a patient with bullous pemphigoid with autoantibodies recognizing the 230-kDa BP antigen epitopes only, as determined by Western analysis. The secondary antibody was a tetramethylrhodamine mouse anti-human IgG. The immunofluorescence detection was performed with standard procedures. At the mRNA level, the BPAG1 gene expression was determined by reverse transcription (RT)-PCR. First, total RNA was isolated from the cultured mouse cells, and the synthesis of first-strand cDNA was catalyzed by avian myeloblastosis virus reverse transcriptase using the downstream primer shown in the legend to Fig 4. Subsequently, a 324-bp segment extending from positions +1801 to +2124, as indicated previously [13], was amplified by the primer pair shown in Fig 4. The PCR amplification products were examined on 1.2% agarose gel electrophoresis.

The transfections were performed with Lipofectin (Stratagene). In all transfections, the constructs were co-transfected with a SV2-β-Gal control construct, which was used as an internal control of transfection efficiency [24]. Following a 24-h incubation, CAT activity as an indicator of the promoter activity was determined by incubation with [14C]chloramphenicol, as previously described [26]. Beta-galactosidase activity was determined in the same samples, and the CAT activity values were corrected for β-galactosidase activity to allow direct comparison of the BPAG1 promoter activity in different cell cultures transfected in parallel.

RESULTS

Isolation of Genomic DNA Containing the 5'-Flanking Region of the Mouse BPAG1 Gene A 0.3-kb 5' fragment of human 230-kDa BPA cDNA was used to screen a mouse genomic DNA library. A positive clone, about 15 kb in size, was isolated and characterized by multiple restriction endonuclease digests, followed by Southern hybridizations. Hybridization with the 0.3-kb human cDNA allowed isolation of a 3.0-kb Hind III fragment. In Northern blot analysis with mouse keratinocyte RNA, this fragment hybridized with a ~9-kb transcript, which corresponds to the size of mouse 230-kDa BPA mRNA [13] (data not shown). This DNA fragment was subjected to nucleotide sequencing; the entire sequence of this fragment is shown in Fig 1.

Comparison of Human and Mouse Sequences Comparison of the nucleotide sequences of the Hind III fragment with human 230-kDa BPA cDNA sequences revealed that this clone consisted of sequences corresponding to the 5'-end of the cDNA and of 5'-flanking genomic sequences. Analysis of nucleotide sequences allowed us to deduce 86 amino acids at the most N-terminal end of the mouse 230-kDa BPA protein, corresponding to exons 1 and 2 on the coding region (see Fig 1). Alignment of the deduced human [16] and mouse amino acid sequences showed a high degree of homology, with an extra threonine residue being present in the human sequence (Fig 2). The overall homology in this region was 91% at the amino acid level and 85% at the nucleotide level. To detect a putative eukaryotic secretory signal sequence [30], we ana...
lyzed sequence 1-86 with a computer program. The program predicted that the mouse polypeptide contains a putative signal sequence of 42 amino acids (Fig 2).

Sequence Analysis of the 5'-Flanking DNA The nucleotide sequence of the 2466-bp segment upstream from the translation initiation codon ATG (nucleotide position +1 to +3) revealed several features suggestive of a promoter. Specifically, a canonical CCAAT motif was located in the position −247 to 243 and a variant of the consensus TATA(A/T)A sequence, TATTATA, was identified in the position −100 to 95. These sequences are well conserved between the mouse and human BPAG1 5'-flanking region, and specifically, the corresponding consensus sequences are present in the human gene in nucleotide positions −283 to 279 and −133 to 128, respectively [22]. In addition to these motifs, eight putative AP-1 binding sites [31], four putative AP-2 binding sites [32], five putative glucocorticoid responsive elements [33,34], and a putative cAMP responsive element [35] were identified in the mouse sequence (Fig 1). Also, two putative CK-8imer sequences, AARCCAAA, which have been suggested to confer tissue-specific expression on several genes in epithelial cells [36–38], were identified in the positions −1184 to 1177 and −645 to 638 (Fig 1). A similar consensus sequence was also found in the human BPAG1 promoter region [22], suggesting functional importance. Direct comparison of the mouse and human nucleotide sequences within −1 to −500 indicated 63.4% identity. The above data suggested that −2.5 kb of 5'-flanking DNA is representative of the promoter region of the mouse BPAG1 gene.

Expression of Mouse BPAG1 Promoter/CAT Reporter Gene Constructs in Cultured Cells To test the functional promoter activity of the 5'-flanking region, three chimeric promoter/CAT reporter gene plasmids were developed by inserting segments extending from −1133 to −1 (construct pMBPCAT 1133), from −525 to −1 (construct pMBPCAT 525), and from −213 to −1 (construct pMBPCAT 213) into pBSCAT, a promoterless vector [26]. These constructs were then tested in transient transfections of several mouse cell lines. All transfections were performed in parallel with pSV2CAT (a positive control) and with pBSCAT (a negative control).

The constructs pMBPCAT 1133, pMBPCAT 525, and pMBPCAT 213 were utilized in transient transfections in cultures of Pam 212 keratinocyte, B16 melanoma, CT26 colon carcinoma, and NIH3T3 fibroblast cell lines. To examine whether these cells express the BPAG1 gene, two types of experiments were performed. First, indirect immunofluorescence with serum from a patient with BP, which by Western blot analysis was shown to react only with the 230-kD BPA, was performed. Bright immunostaining was noted on cultured Pam 212 cells (Fig 3A). In contrast, three other mouse cell lines used in this study, B16 melanoma cell line, CT26 colon carcinoma cell line, and NIH 3T3 fibroblast line, gave an essentially negative staining reaction (Fig 3B–D). Secondly, RT-PCR of mRNA isolated from these four different cell lines was performed. Following reverse transcription, a segment corresponding to nucleotides +1801 to +2124 was amplified by PCR using primers as described in Materials and Methods. An amplification product of the expected size, 324 bp, was noted only when Pam 212 cell mRNA was used as the template (Fig 4, lane A). In contrast, no band of similar size was obtained in mRNA products from the three other cell lines tested (Fig 4, lanes B–D). Thus, significant expression of the mouse BPAG1 gene could be detected only in Pam 212 cells, as determined at the mRNA and protein levels.

The same mouse cell lines tested above were then utilized in transient transfections with the PBAG1 promoter/CAT constructs. Induction CAT activity in the cells transfected with the pMBPCAT 1133 construct revealed a strong signal in Pam 212 cells and a low level of expression in NIH3T3 cells, whereas essentially no activity was detected in B16 or CT26 cells (Fig 5A). Quantitation of the

Figure 2. Comparison of deduced amino acid sequences at the amino-terminal end of mouse and human 230-kDa bullous pemphigoid antigens. The presence of the putative signal peptidase cleavage site, as predicted by computer analysis, is indicated (solid triangle). However, as discussed in the text, this computer prediction may not indicate biologic functionality. Double dots, identical amino acids; single dots, conserved amino acids. Note the presence of an extra threonine residue (T) inserted into position +18 of the human sequence (solid circle). The overall homology of these sequences is 91%.
relative activity by counting the radioactivity in the acetylated form of [14C] chloramphenicol. The values are mean ± SD of three separate experiments.

Figure 5. Assay of CAT activity. Cultures of different cell types were transfected with the pMBPCAT 1133 construct, which contains the segment from −1133 to −1 of the 5′-flanking DNA of the BPAG1 gene. Parallel transient transfections were made with the promoterless pBSOCAT construct as a negative control. A) Lane 1, Pam 212 cells transfected with pBSOCAT (negative control); lane 2, Pam 212 cells; lane 3, B16 melanoma cells; lane 4, CT26 colon carcinoma cells, and lane 5, NIH3T3 cells, all transfected in parallel with the construct pMBPCAT 1133. B) Quantitation of CAT assays similar to those shown in (A). The relative values are expressed as radioactivity in the acetylated form of [14C] chloramphenicol. The values are mean ± SD of three separate experiments.

DISCUSSION

The screening of the mouse genomic DNA library with a human cDNA clone resulted in isolation of one positive, 15 kb, lambda phage clone that was shown to correspond to the 5′-end of the gene and to contain upstream flanking sequences. Specifically, sequence analysis of a 3-kb Hind III subclone revealed sequences with a high degree of homology with those of human BP antigen cDNA, and Northern blot analysis with this genomic fragment recognized 9 kb mRNA, which corresponds to the size of the mouse 230-kDa BP transcript [13]. These results indicated that we had isolated a genomic clone that contains sequences corresponding to the mouse 230-kDa BPAG1 gene.

We have recently elucidated the full-length primary sequence of the human 230-kDa BPAG1 by cloning overlapping cDNAs [16]. Analysis of deduced amino acid sequences, using a computer program, predicted a putative signal peptide of 43 amino acids and the presence of a membrane-associated sequence of 17 amino acids. The latter computer prediction was taken to suggest that the 230-kDa BP may be a membrane-associated protein [16]. Search for a signal sequence in the mouse gene also identified a putative signal peptide cleavage site, a Ser-Cys bond at position 42–43. However, several lines of evidence, based on immunofluorescence and immunoelectron microscopic data, have strongly suggested that the 230-kDa BP antigen is an intracellular protein [9,10,39,40]. Thus, the computer prediction of a signal peptide, suggesting secretion of the protein, may not be indicative of biologic function. However, it remains to be seen whether the 230-kDa BP antigen, which apparently forms the intracellular hemidesmosomal plaque, is associated with the inner portion of the plasma membrane.

We previously compared human amino acid sequences deduced from a 2.3-kb BPA cDNA at the carboxyl terminal portion of the protein [15] with corresponding mouse sequences [13]. The homology in this area between mouse and human sequences was 83% at the amino acid level and 80% at the nucleotide level. In the present study we showed that the deduced 86 amino-acid segment at the N-terminus of the mouse polyepptide had 91% homology with the corresponding human 230-kDa BP antigen sequence. These results indicate that both the N-terminus and C-terminus of the 230-kDa BP antigen appear to be well-conserved between mouse and human, suggesting functional importance for these segments of the protein.

Sequence analysis of the 5′-flanking region of the mouse BPAG1 gene revealed the presence of several sequences that were homologous to the sequence elements thought to play a role in the regulation of the human gene. To test the functional promoter activity of the 5′-flanking region of the mouse gene, we performed transient transfections with three constructs, pMBPCAT 1133, pMBPCAT 525, and pMBPCAT 213, first in Pam 212 cell cultures. We were able to detect strong CAT activity with all three constructs, suggesting that these fragments contained 230-kDa BPA promoter.

Furthermore, these experiments revealed that the CAT activities with pMBPCAT 1133 and pMBPCAT 525 were clearly higher than that obtained with pMBPCAT 213. This finding suggests that the 312-bp segment between positions −525 and −213 contains some cis-elements that confer upregulation of the expression in keratinocytes. Careful examination of this upstream sequence identified a canonical CCAAT motif at −[247 to 243], which is possibly responsible for the difference in the CAT activities between the shortest and the two longer constructs.

The 230-kDa BP antigen is expressed in the stratified squamous epithelia and possibly in a few other tissues [2,21]. Previous studies on the human BPAG1 gene have indicated that the promoter region of the gene contains cis-regulatory elements that are responsible for tissue-specific expression of this gene. Within the 5′-flanking region of the BPAG1 gene, we found sequences highly homologous with the human BPAG1 5′-flanking DNA. The CAT activities were determined as shown in Fig 5A,B. The relative values are expressed as radioactivity in the acetylated form of [14C] chloramphenicol. The values are mean ± SD of three separate experiments.
to CK-8mer and AP2 sequences, which have been suggested to be involved in tissue-specific expression of epidermal genes [36–43]. These sequences are found both in human and mouse BPAG1 promoter regions, and a specific AP-2-like cis-element, KRE2, has been shown to contribute to the tissue-specific expression of the human BPAG1 gene in keratinocytes [22,23]. To examine the possibility that the mouse gene may display similar tissue specificity, transient transfections using Pam 212, NIH 3T3, B16, or CT26 cells were performed with the construct pMBPCAT 1133. Indirect immunofluorescence analyses and RT-PCR amplification of BPAG1 mRNA sequences revealed that this gene was expressed at significant levels only in Pam 212 cells, whereas the B16, CT26, and NIH3T3 cells showed minimal expression. High levels of CAT activity were observed only in Pam cells (Fig 5). Significantly lower expression was detected in NIH3T3 cells, whereas B16 and CT26 cells showed no activity. The reasons for the low, yet detectable, level of expression in NIH3T3 cells are not entirely clear, but the results may suggest that additional factors outside of the promotor region may be required for absolute tissue specificity. Such elements could reside in upstream regions of the gene, within the first intron, as suggested, e.g., in the case of type I collagen [44,45] or in the 3'-end of the gene (as suggested in the case of keratin 1 [46]). Thus, the 1.1-kb segment of 5'-flanking sequences of the mouse BPAG1 gene appears to contain cis-acting elements that confer, at least in part, tissue specificity to the expression of the BPAG1 gene.

We thank Tamara Alexander for skillful secretarial assistance. This work was supported in part by a grant from the Rare and Intractable Disease Research Committee of the Ministry of Welfare of Japan; by a grant (05670718) from the Ministry of Education, Japan; and by USPHS, NIH grant PO1-AR38923.

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