Identification on Mouse Chromosome 8 of New β-Defensin Genes with Regionally Specific Expression in the Male Reproductive Organ*

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Defensins are important elements in innate immunity that can also trigger adaptive immune responses. The defensins form a family of small cationic antimicrobial peptides with six characteristic cysteine residues, whose pairing pattern in forming three intramolecular disulfide bonds defines the α- and β-defensin subfamilies. In a search for new β-defensin genes, we performed computational analysis using the Celera mouse genome database and found exons encoding 23 different β-defensins, including the eight previously characterized members of this family. Among the new β-defensins, nine of them form two groups of phylogenetically related sequences that were characterized in greater detail. Northern blot, reverse transcription PCR, and in situ hybridization analysis showed that expression of these genes is restricted to the epididymis, with a specific regional expression pattern. One of the new β-defensins (Defb38) was chemically synthesized; in in vitro assays on Gram-positive and -negative bacterial strains, Defb38 showed the characteristic salt-dependent antimicrobial activity of β-defensins. The results demonstrate the existence of a relatively large number of β-defensins with specific expression in distinct regions of the murine epididymis and suggest complex roles for these proteins in host defense and other physiological processes of the male reproductive tract.

All defensins have the capacity to kill a specific spectrum of microorganisms in vitro and are considered to be effectors of host innate immunity (1). Recent reports also showed that defensins can have chemokine-like activities and, thus, augment adaptive immune responses. Human β-defensin 2 (HBD2) binds and activates chemokine receptor CCR6, resulting in chemotraction of CCR6-expressing cells (2). Comparison of the three-dimensional structures of HBD2 and the CCR6 chemokine ligand (CCL20) showed that, despite their distinct molecular sizes, both polypeptides have similar folded structures with a remarkable resemblance in the domain putatively responsible for CCR6 binding (3). Supporting the idea of a functional connection between chemokines and defensins, some chemokines are reported to have defensin-like antimicrobial activity (4, 5). Dendritic cell maturation resulting from defensin interaction with Toll-like receptors (6) is another way in which defensins can modulate adaptive immune responses.

Using a bioinformatic approach, we searched for new β-defensin genes in the Celera mouse genome database. Searches based on known β-defensin sequences identified three contigs on chromosome 8 harboring potential new β-defensin genes. Exhaustive analysis of the sequences using six-cysteine patterns identified putative exons encoding the secreted form of 23 different β-defensins, including the eight members of this family characterized thus far. Two groups of phylogenetically related sequences were studied in detail. Transcripts for these sequences were detected in specific regions of the epididymis, and a cDNA encoding the entire protein was characterized for seven of them. As predicted for β-defensins, a chemically synthesized peptide reproducing the predicted mature protein encoded by one of the newly identified genes showed in vitro bactericidal activity against Gram-positive and -negative bacterial strains. This study reveals a large number of murine β-defensins with a specific regional expression pattern within the epididymis, suggesting great complexity in the roles of these small proteins in host defense, as well as in other physiological processes within the male reproductive tract.

EXPERIMENTAL PROCEDURES

Bioinformatics—The TBLASTN program (7) was used to query the Celera mouse genome assembly (www.celera.com) with the protein sequences of known β-defensins. Downloaded genomic DNA sequences were searched using the FindPatterns program from Wisconsin package (Genetics Computer Group, Madison, WI). Protein sequences were mutually aligned using the ClustalX program (8), and derived dendrograms were generated with the Njplot program (9).

β-Defensin mRNA Characterization and Expression—Total RNA from mouse organs was extracted with Tri-reagent (Sigma). For single-stranded cDNA synthesis, 5 μg of total RNA were reverse-transcribed using random hexamers and 100 units of Superscript II reverse transcriptase (Invitrogen). DNA fragments corresponding to partial new β-defensin cDNAs were obtained by PCR amplification of murine cDNA (15 s at 94 °C and 60 s at 68 °C for 35 cycles). In Northern blots, 10 μg of RNA from mouse organs were electrophoresed on a denaturing formaldehyde-agarose gel and blotted onto nylon Hybond membranes (Amersham Biosciences). Prehybridization of the membranes and hybridization with β-32P-labeled DNA fragments containing partial coding sequences of the putative new β-defensin genes were carried out in Rapid-Hyb buffer (Amersham Biosciences) as recommended by the supplier.

The 5′- and 3′-ends of the new β-defensin cDNA were isolated using the SMART RACE cDNA amplification kit (Clontech). Real time-quan...
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**A**

| Defb13 | CXXNNGCQQSA-CTPTEFGKQTGCTQAN-FLCCR |
| Defb35 | CRLGRGKCR-A-CISSERIKWGGKLN-FFCCR |
| Defb15 | CSRGRGTCRAS-CLQKEEVLALCQQN-LKCC |
| Debb1 | CTYFGPQCNMCPETKKFGNCHPDDLAMCC |
| Defb9 | CKEKGCYV-PYCFSHKX1GSPCFPFRCC |
| Debb0 | CTYFGPQPCHHACGPNSAEPCHPNLCC |
| Debb2 | CTNMGYVIRAIPCEARPSPGFPEKKPC |
| Debb10 | CLQGNGKCRACCPSEMTLQCTCPIFKPNCC |
| Defb40 | CLIGNNCHQXCPWLPQVLWTCTYKUGKCC |
| Defb37 | CTIENKUTCTRLNCPMLHNVCTGYKKGCC |
| Defb39 | CQWGRGKCRAS-CLQKEEVLALCQQN-LKCC |
| Defb39 | CQWGRGKCRAS-CLQKEEVLALCQQN-LKCC |
| Defb38 | CQWGRGKCRAS-CLQKEEVLALCQQN-LKCC |
| Defb37 | CQWGRGKCRAS-CLQKEEVLALCQQN-LKCC |

**B**

Defb13 Defb15 Debb35 Debb12 Defb34 Defb15 Debb4 Debb14 Debb2 Defb4 Debb1 Debb10 Debb12 Debb3 Debb5 Defb6 Debb8 Debb7

**Fig. 1.** Amino acid sequence similarities among β-defensin proteins. A, alignment of predicted β-defensin mature protein sequences encoded on murine chromosome 8. For clarity, only the sequence between the first conserved cysteine and the amino acid following the last conserved cysteine is shown. Asterisks mark the six conserved cysteines. Top to bottom order is telomere to centromere. B, phylogenetic tree generated by the NJplot program with the sequences aligned in panel A. The names of the β-defensins characterized in this study are shown in bold. Accession numbers for newly characterized cDNAs are as follows: Ad489588, Defb37; Ad575424–28, Defb38–Defb40; Ad575427, Defb13; AJ575428, Defb15.

**Fig. 2.** Transcript expression of new β-defensin genes. Northern blot analysis of new β-defensin gene expression in male reproductive organs using probes specific for the genes indicated. Abbreviations used are as follows: Cx, epididymis caput; Cc, epididymis corpus; Cg, coagulation gland. Estimated transcript sizes in nucleotides were 830 and 530 for Defb15/ Defb34; 2900 and 1150 for Defb12/Defb35; 2900, 2050, and 1430 for Defb13; 460 for Defb37; 440 for Defb38; and 530 for Defb39.

Reflex IV equipped with the SCOUT source in positive ion reflector mode with delayed extraction (Bruker Daltonics, Bremen, Germany). Folding and disulfide bridge formation of the HPLC products were carried out as described (12). Antimicrobial Activity Assay—The colony count assay described by Harwig et al. (13) was followed with minor modifications. Mid-logarithmic phase Escherichia coli (ATCC 11303 strain), Pseudomonas aeruginosa (PA01 strain) (14), and Enterococcus faecium (CCTC 4102 strain) were suspended in 10 mM sodium phosphate buffer (15 mM sodium concentration), adjusted to a density of 5 x 10^8 colony-forming units/ml.
Identification of New β-Defensin Genes in the Mouse Genome by Bioinformatics—The genomic structure of known β-defensin genes is composed of two coding exons, a 5'-exon encoding the signal peptide and a 3'-exon encoding most of the mature protein in which the six-cysteine motif is found. To search for potential new β-defensin genes in the mouse genome, TBLASTN was run on the murine Celera genomic data base using the mature protein sequence of known β-defensins as bait. We identified three contigs covering 500 kb in chromosome 8, located in tandem in the chromosome. Given the lack of data about these β-defensin genes, we undertook a more detailed characterization.

New β-Defensin Genes with Specific, Regionally Regulated Expression in Epididymis—We analyzed the new β-defensin sequences to determine whether they were bona fide genes by identifying the putative 5'-exons encoding the corresponding signal peptides, although TBLASTN with signal peptides from known β-defensins failed to identify potential candidates. Sequences upstream of each exon were translated in the three possible reading frames and analyzed for the hydrophobic signal peptides, although TBLASTN with signal peptides from known β-defensins failed to identify potential candidates. Sequences upstream of each exon were translated in the three possible reading frames and analyzed for the hydrophobic signal peptides, although TBLASTN with signal peptides from known β-defensins failed to identify potential candidates. 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The regions selected were then scanned for classical exon-intron boundaries, and potential 5'-exons were identified in all cases, except for Defb34 and Defb35. An additional criterion was mRNA expression analysis. To test whether the predicted new β-defensin sequences were transcribed, oligonucleotide pairing in both exons was designed and used to amplify cDNA from a variety of tissues by PCR. Bands of the predicted size were detected mainly in amplifications of epididymis cDNA. The nucleotide sequence of each product was determined, and the predicted amino acid sequences predicted by the nucleotide sequences identified, as well as their relative localization along mouse chromosome 8. The genes identified and characterized here are designated according to the official names given by the HUGO Gene Nomenclature Committee and Mouse Genomic Nomenclature Committee (www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl).

Based on this alignment (Fig. 1A), we analyzed the phylogenetic relationship among the different β-defensins based on the predicted amino acid sequence of the second exon of the genes. The results showed a high degree of sequence similarity between some of the newly identified β-defensins, which were grouped into two separate branches of the dendrogram (Fig. 1B). One branch was formed by Defb37, Defb38, and Defb40, and the other was formed by Defb12, Defb13, Defb15, Defb34, and Defb35. In each branch, β-defensin sequences are located in tandem in the chromosome. Given the lack of data about these β-defensin genes, we undertook a more detailed characterization.
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Fig. 4. New β-defensin genes with regionally regulated expression in the epididymis. Sagittal cryosections from BALB/c mouse epididymis were prepared and in situ hybridized with antisense RNA probes. The expression of Defb12 in the distal half of the caput (A) and Defb38 in the proximal half of the corpus (B) is shown. C, control hybridization with a sense RNA Defb12 probe.

Fig. 5. Characterization of chemically synthesized Defb38. The predicted sequence of mature murine Defb38 protein was synthesized by Fmoc chemistry, purified by HPLC on a C-18 Nucleosil 120 column (A), and analyzed by mass spectrometry (B). Expected mass for the synthesized protein is 4825.64 Da, a.i., arbitrary intensity.

### Table II

| Amino acid analysis of the chemically synthesized Defb38 peptide |
|---------------------------------------------------------------|
|                  | Experimental | Predicted |
| Asx               | 2.05         | 2         |
| Thr               | 1.6          | 2         |
| Ser               | 0.95         | 1         |
| Glx               | 3            | 3         |
| Gly               | 4.4          | 4         |
| Ala               | 0.9          | 1         |
| Val               | 1.5          | 2         |
| Ile               | 0.8          | 1         |
| Leu               | 0.95         | 1         |
| Tyr               | 4.9          | 5         |
| Phe               | 0.85         | 1         |
| His               | 1.2          | 1         |
| Lys               | 6.5          | 6         |
| Arg               | 1.5          | 2         |
| Pro               | 1.8          | 2         |

exon structure was confirmed. Rapid amplification of cDNA ends (RACE) was then performed on both cDNA ends, except on Defb12, for which an expressed sequence tag encoding the entire protein existed (accession number AK020311), and the full coding sequences of Defb13, Defb15, Defb39, and Defb40 were determined.

Northern blot using specific probes was used to study mRNA expression of the newly identified β-defensins. Analysis of a broad variety of tissues showed that all off the new β-defensins are expressed exclusively in the epididymis (not shown), a tubular male sex structure that lies on and around each testicle, connecting it with the vas deferens. Because the epididymis consists of three distinct regions forming the head (caput), body (corpus), and tail (cauda), a more detailed analysis was carried out of β-defensin mRNA expression in the epididymis regions, as well as in other organs of the male reproductive system such as whole testis, seminal vesicle, prostate, and coagulation gland. The results showed that, although always restricted to the epididymis, the putative new β-defensins had differential expression patterns (Fig. 2). Because of the similarity between their nucleotide sequences in the second exon in the case of the Defb15/Defb34 (87% identity) and Defb12/Defb35 (97%) pairs, the probes used were able to hybridize with transcripts corresponding to each member of the pair. Defb40 transcripts were not detectable by Northern blot analysis.

Real time quantitative RT-PCR analysis was performed to study the specific epididymis expression corresponding to Defb12, Def15, Defb34, Defb35, Defb39, and Defb40. Specific oligonucleotides pairing in the second exon were used to amplify cDNA from DNase I-treated RNA (Fig. 3); genomic DNA was also amplified and used as a reference to correct differences in amplification efficiency. Defb40 is expressed in the corpus at a level similar to that of Defb39; it is also detectable in the cauda and is at least 200 times less abundant than Defb39 in the caput (Table I). Quantitative analysis for Defb15/Defb34 and Defb12/Defb35 shows that all four sequences are transcribed, although Defb34 and Defb35 are less expressed in the corpus than their counterparts. Although both β-defensins have a similar expression pattern, Defb34 expression in the caput is at least 100 times lower than that of Defb15. The Defb35 expression level in the caput is ~30 times lower than that of Defb12, although it is detected at a level similar to that of Defb12 in other epididymis regions. The PCR data indicate that the bands detected in Northern blot would correspond mainly to Defb12 and Defb15 transcripts. The expression analysis thus shows that transcripts for Defb12/Defb35, Defb15/Defb34, and Defb13, whose proteins were phylogenetically grouped in one of the branches, were expressed mainly in the caput, although they were also detected by RT-PCR in the corpus and in the cauda. Defb37, Defb38, and Defb39 transcripts, all grouped in the second branch, were expressed maximally in the corpus, weakly in the cauda, and only in the case of Defb39 was the transcript clearly present in the caput.

Specific regional β-defensin expression within the epididymis was confirmed by in situ hybridization analysis using Defb12- and Defb38-specific probes. Consistent with the results from the Northern blot analysis, Defb12 and Defb38 transcripts were detected in the caput and the corpus, respectively (Fig. 4).
In the case of Defb12, mRNA expression was confined mainly to the distal part of the caput, proximal to the corpus, with a sharp border separating Defb12-expressing and non-expressing cells.

The Newly Identified β-Defensin Defb38 Has Antimicrobial Activity in Vitro—The broad spectrum of in vitro antimicrobial activities shown by defensins (16), as well as the protection against salmonellosis observed in transgenic mice expressing a human intestinal defensin (17), suggests that β-defensin expression in specific epididymis zones may have a role in preventing infection-mediated epididymitis, a pathology that can result in male infertility. To test the antimicrobial potential of the new murine β-defensins, we chemically synthesized a trial polypeptide corresponding to the predicted amino acid sequence of Defb38, a β-defensin with high corpus expression. The quality of the synthetic Defb38 was confirmed by HPLC and mass spectrometry analysis (Fig. 5), as well as by amino acid composition (Table II) before assaying its antibacterial activity against several bacterial strains. Synthetic Defb38 killed both Gram-negative (E. coli and P. aeruginosa) and Gram-positive bacteria (E. faecium) (Fig. 6A) in a dose-dependent manner, as was also observed with magainin, a peptide with known antimicrobial activity (Fig. 5). Some studies have demonstrated that the microbicidal activity of defensins can be blocked by increasing the ionic strength of the incubation medium (18, 19). Concurring with this, Defb38 antibacterial activity diminished progressively in the presence of increasing NaCl concentrations (Fig. 5C), as was also observed with trypsin treatment (Fig. 5D). These data indicate that Defb38 is a bona fide member of the β-defensin family and support its possible role in epididymis immune defense.

A cluster of five β-defensin genes was recently reported on human chromosome 20 (20). Because expression of these β-defensins was highest in the male genital tract, with a distribution in functionally different epididymal segments, a role in sperm maturation was suggested (20). According to sequence similarities and chromosomal localization (15, 21), the β-defensins identified here are not the murine counterparts of those reported by Rodriguez-Jimenez et al. (20). The parallels in the expression pattern within the epididymis nonetheless allow speculation that these murine β-defensins might also have a role in the sperm maturation process.

Bin1b is a rat β-defensin homologous to murine Ep2c (now called Defb45), and is expressed specifically in the epididymis caput (22). In addition to its microbicidal activity, a role for Bin1b in epididymis function and fertility was proposed based on its regulated expression, which is maximal during the sexually mature period in rat (22). We performed a preliminary analysis of β-defensin expression in 1-, 3-, 6-, and 9-month-old mouse epididymis using real time quantitative RT-PCR. Although some modulation was observed in Defb12, 13, 15, 38, and 39 expression, the data do not support a role for these murine β-defensins in epididymis function (not shown).

Evolutionary Relationships between β-Defensins—The high degree of sequence conservation and shared specific expression in the epididymis suggest a common origin for the nine new β-defensin genes characterized here. Phylogenetic analysis based on the predicted amino acid sequence of the first exon of these genes showed their separation into two main branches, one with Defb12, Defb13, and Defb15, and the other with Defb37, Defb38, Defb39, and Defb40 (Fig. 7). This confirms the results obtained using the second exon-predicted sequence (Fig. 1B) and indicates that new defensin genes arose by genomic duplications that involved entire genes. After gene duplication, mutations affecting gene control regions could have led to the distinct constitutive expression levels observed. The paradigmatic example of this is the Defb37–Defb40 cluster. All members are expressed mainly in the corpus and are ex-
pressed differentially in other epididymis regions. The most similar expression patterns are those of β-defensins with similar sequences, as is the case for Defb38 and Defb40. The situation is slightly different in the other β-defensin phylogenetic branch analyzed. The sequence similarity between the DNA regions encompassing Defb15/Defb35 and Defb12/Defb34 indicates a relatively recent duplication event. As the Defb15 and Defb12 expression pattern is very similar, independent mutations in Defb35 and Defb34 must have occurred after duplication, reducing the expression level and disturbing the spatial control along the epididymis. Characterization of the promoter and control regions of these genes would aid to identify specific sequences and factors that direct epididymal and subepididymal expression.

Despite the overall β-defensin genomic conservation between man and mouse, there seems to be no human counterpart for Defb12. Yamaguchi et al. reported no experimental characterization of these antimicrobial peptides in the innate and adaptive host defense of the male reproductive tract.

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While this study was in progress, Schutte et al. reported new human and murine β-defensin genes, including the murine genes described here, although their data were based entirely on computational analysis and provided no experimental characterization (21). Yamaguchi et al. also reported epididymis-specific expression of Defb12 and Defb15 (formerly Defb11) (15). Our work substantially increases the number of murine β-defensins identified with a specific regional expression pattern within epididymis, revealing further complexity in the role of these antimicrobial peptides in the innate and adaptive host defense of the male reproductive tract.

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