Novel cathelicidins in horse leukocytes[1]

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Received 2 August 1999

Abstract Cathelicidins are precursors of defense peptides of the innate immunity and are widespread in mammals. Their structure comprises a conserved prepropiece and an antimicrobial domain that is structurally varied both intra- and inter-species. We investigated the complexity of the cathelicidin family in horse by a reverse transcription-PCR-based cloning strategy of myeloid mRNA and by Southern and Western analyses. Three novel cathelicidin sequences were deduced from bone marrow mRNA and designated equine cathelicidins eCATH-1, eCATH-2 and eCATH-3. Putative antimicrobial domains of 26, 27 and 40 residues with no significant sequence homology to other peptides were inferred at the C-terminus of the sequences. Southern analysis of genomic DNA using a probe based on the cathelicidin-conserved propiece revealed a polymorphic DNA region with several hybridization-positive fragments and suggested the presence of additional genes. A null eCATH-1 allele was also demonstrated with a frequency of 0.71 in the horse population analyzed and low amounts of eCATH-1-specific mRNA were found in myeloid cells of gene-positive animals. A Western analysis using antibodies to synthetic eCATH peptides revealed the presence of eCATH-2 and eCATH-3 propeptides, but not of eCATH-1-related polypeptides, in horse neutrophil granules and in the secretions of phorbol myristate acetate-stimulated neutrophils. These results thus suggest that eCATH-2 and eCATH-3 are functional genes, whereas eCATH-1 is unable to encode a polypeptide.

Key words: Cathelicidin; Antimicrobial peptide; Short interspersed nucleotide element; Horse

1. Introduction

Animal antimicrobial peptides are believed to be an important defense mechanism of innate immunity [1,2]. A group of antimicrobial peptides that are synthesized at the C-terminus of precursors designated cathelicidins have been described in mammals [3,4]. The secretory compartment of neutrophils is a primary storage site of members of this family [3,4]. Cathelicidins display a highly conserved, cathelin-like [5] N-terminal propiece followed in most cases by a C-terminal domain of fewer than 100 residues which is characterized by a remarkable structural variety [3,4]. As shown for bovine and porcine congeners [3,4,6,7], this piece gains antimicrobial activity after the propiece has been cleaved off upon neutrophil degranulation.

Members of the cathelicidin family were first cloned in cattle from myeloid bone marrow cells mRNA [8]. 11 Genes were described in this species [9] and physically mapped to the bovine chromosome 22q24, where they cluster at a CATHL@ locus [10]. A variety of members of this family were identified in other mammalian species and found to be expressed in several other tissues in addition to myeloid-derived cells [4,11–18]. Cathelicidin genes span about 2 kb and display a conserved four exon/three intron organization. Substantial variability, however, was found in the species analyzed with respect to the number of congeners (e.g. only one described in human) and to the sequence of the exon four region encoding the C-terminal domain [4].

As far as we know, perissodactyl species have not yet been examined for the presence of cathelicidins and only two unrelated equine peptides with antimicrobial activity were formerly reported [19,20]. The widespread presence and heterogeneity of mammalian cathelicidins suggested that novel members of this family may be found in equine species. In the present study, we searched novel cathelicidins in horse by a reverse transcription (RT)-PCR-based analysis of horse myeloid mRNA and performed a Western analysis to show that the horse cathelicidins are stored in the neutrophil granules and can be secreted from intact neutrophils. We also investigated this gene family by Southern blotting and PCR analysis of genomic DNA.

2. Materials and methods

2.1. RNA extraction, cDNA cloning and sequencing

Total RNA was extracted from bone marrow cells isolated from ribs [21] of adult horses. The 3′ and 5′ cDNA ends of equine cathelicidins (eCATHs) were amplified as reported [22]. Horse bone marrow mRNA was first reverse-transcribed using the oligo(dT) adaptor primer 5′-TCGGATCCCTCGAGAAGC$(T)_{18}$-3′. Cathelicidin-related 3′ cDNAs were then amplified by PCR using the adaptor primer 5′-TCGGATCCCTCGAGAAGCTT-3′ and one of the following sense oligonucleotide primers: 5′-ACCGAATTCAGCTACAGGGAGGCCGT-3′, 5′-CGCGAATTCTGTGAGCTTCAGGGTG-3′, 5′-ACCGAATTCAGTGTGACTTCAAGGA-3′. The three sense primers used are based on the most conserved region of cathelicidins and all contain additional bases providing restriction sites. For each horse cathelicidin, the 5′ end cDNA amplification was performed by using the sense primer 5′-CAAGAATTCGGAGACTGGGGACCATGGAGA-3′ based on the conserved 5′ untranslated sequence of previously described cathelicidins [4] and antisense primers based on sequences unique to each horse cathelicidin, i.e. 5′- CGGGATCCAGGAAGAAGAATCCGC-3′ (AS eCATH-1) complementary to nucleotides (nt) 429–445 of the eCATH-1 sequence, 5′-GCGGATCCAGAAGCCCAGCCAGAA-3′ (AS eCATH-2) complementary to nt 509–527 of the eCATH-2 sequence, 5′-CGGGATCCAGACCCTAGG-

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[1] Accession numbers: the nucleotide sequences of eCATH-1, eCATH-2 and eCATH-3 are deposited in the EMBL nucleotide sequence database under accession numbers AJ224927, AJ224928 and AJ224929.

0014-5793/99/$20.00
PII: S0014-5793(99)01097-2

AGGCTA-3′ (AS eCATH-3) complementary to nt 503–519 of the eCATH-3 sequence. Amplified cDNAs were cloned in Bluescript KS^+ vector (Stratagene, San Diego, CA, USA) and sequenced on both strands with deazaguanosine and automated fluorescent DNA sequencing (EMBL fluorescent DNA sequencer, Heidelberg, Germany). At least five clones generated from different RT-PCR reactions were completely sequenced in both directions for each different cDNA.

2.2. Northern analysis

Northern analysis was performed as described [23], using equal amounts (10 μg) of total RNA from equine bone marrow cells. Hybridization was carried out using [^{32}P]dCTP random-primed, labelled fragments corresponding to nt 377–477 of eCATH-1, nt 373–527 of eCATH-2 and nt 424–560 of eCATH-3 cDNAs. Filters were rehybridized with a probe based on the cDNA sequence of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as loading control. Slot blots containing equivalent amounts of the eCATH cDNAs were used as controls to set up the hybridization conditions and to determine the relative hybridization signals. Plasmid cDNA (1.5, 50 ng) was denatured for 10 min at 65°C in 0.4 M NaOH, applied to nylon membranes in 2×SSC using a slot blot apparatus and hybridized under the experimental conditions used for Northern blotting.

2.3. Southern blotting of genomic DNA

Genomic DNA was prepared from blood cells [24] obtained from six unrelated horses. Aliquots of 10 μg of DNA were digested with *Eco*RI, *Bam*HI, *Hin*dIII and *Xba*I restriction endonucleases and transferred onto nylon filters (Genescreen plus Du Pont, NEN Products, Boston, MA, USA) after gel electrophoresis. Filters were hybridized with a ^{32}P-labelled fragment corresponding to nt 1–344 of eCATH-3 (cathelicidin-conserved probe) or with the labelled fragments corresponding to the 3′ cDNA ends used for Northern analysis (gene-specific probes). Hybridizations were carried out for 5–6 h at 55°C (low stringency) or 65°C (high stringency) in 10 ml of QuikHyb Hybridization Solution (Stratagene) containing 100 μg/ml of denatured salmon sperm DNA. Filters were washed at room temperature with 2×SSC (0.3 NaCl, 30 mM tri-sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) and for 10–60 min at 55 or 65°C in 0.1×SSC and 0.1% SDS.

2.4. PCR amplification of genomic DNA

Genomic DNA was extracted from peripheral blood of 70 unrelated animals: 13 show-jumper, 23 Lipizzaner and 34 horses of unknown origin. The nine families were from the English Thoroughbred population. Aliquots of 30 ng genomic DNA were used for PCR amplification. The sense primer 5′-ACCGAATTCAGTGTGACTTCAAGGA-3′ derived from the conserved proregion (nt 282–299) and the antisense primers AS eCATH-1, AS eCATH-2 and AS eCATH-3, based on sequences unique to each cathelicidin, were used. 35 Cycles of amplification were carried out [22]. Amplified products were separated by gel electrophoresis, blotted onto nylon filters and hybridized under the same experimental conditions used for Southern blotting of genomic DNA.

2.5. Sequence analysis

DNA sequence assembly and analysis and sequence homology searching were carried out using the software package of the Wisconsin Genetics Computer Group. Secondary structure prediction analysis of eCATH-1, eCATH-2 and eCATH-3 was performed using the PHD neural network [25].

2.6. Neutrophil granule preparation and neutrophil degranulation

Horse peripheral neutrophils were isolated from fresh blood [26] and total granule populations were prepared from neutrophils as reported [27]. Neutrophil degranulation was induced as described [6]. Briefly, freshly isolated neutrophils (>95% of the cells) were resuspended at 5×10^7/ml in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and incubated with 150 nM phorbol myristate acetate (PMA) at 37°C for 30 min. Supernatants were collected after centrifugation to remove cells, protein in the cell-free medium was precipitated with 10% TCA and solubilized in SDS-sample buffer.

2.7. Analytical assays and antibodies

Determination of the activity of the lactate dehydrogenase in the cell-free media, SDS-PAGE and Western blot analysis were performed as described [6]. Rabbit antisera to eCATH-1, eCATH-2 and eCATH-3 were raised by repeated injections of synthetic eCATH peptides with sequences corresponding to the diverse C-terminal regions of the deduced eCATH sequences. The IgG fractions of the antisera were obtained as reported [6] and antigen-specificity and lack of cross-reactivity were determined by Western blotting.

3. Results and discussion

3.1. Identification of cathelicidin transcripts in horse

Novel members of the cathelicidin family were searched in horse bone marrow cells by using a RT-PCR-based approach that allows for amplification of transcripts containing the conserved cathelin-like propiece [3]. Sense oligonucleotide primers based on the conserved cathelin sequence and an oligo(dT) adaptor primer were used to amplify the 3′ cDNA ends of putative congeners from total RNA isolated from bone marrow cells of a single horse. Sequence analysis of amplified products suggested the presence of three different cathelicidin sequences. The 5′ regions of the transcripts were amplified and sequenced as described in Section 2. The full length cDNA sequences, as obtained from partially overlapping 3′ and 5′ sequences, and the deduced polypeptides are aligned in Fig. 1A and B, respectively. These sequences were designated eCATH-1, eCATH-2 and eCATH-3. The expression of the eCATH genes in myeloid cells was also analyzed by Northern blotting, using aliquots of the same RNA sample used for PCR amplifications. Hybridization of the blots with probes based on the unique 3′ sequences of eCATH cDNAs indicated abundant expression of eCATH-2 and eCATH-3 genes and a significantly low level of expression of the eCATH-1 gene (Fig. 2).

3.2. Analysis of the nucleotide and deduced amino acid sequences

The cDNA sequences share 92–99% identity in the 5′ region spanning nt 1–390 and encoding the prepropiece. The highest level of identity in this region is observed between eCATH-1 and eCATH-3. The peptide coding regions (boxed in Fig. 1A) have different lengths and bear no significant sequence homology. Analysis of the 3′ untranslated region (UTR) indicated that in eCATH-1 and eCATH-2, the polyadenylation signal is comprised between positions 510–515 and 537–542, respectively (underlined in Fig. 1A). In eCATH-3, the corresponding region contains a substitution from A to G and the 3′ UTR is longer (Fig. 1A). The functional polyadenylation signal in the eCATH-3 transcript is likely contained in an $(AAAT)_9$ microsatellite (nt 736–771), 10 nt upstream of the 3′ end. A BLAST search of the eCATH-3 3′ UTR revealed a 185 bp region (in italics in Fig. 1A) with characteristic features of short interspersed nucleotide elements (SINEs) [28], including a tRNA-derived region, direct repeats flanking the 5′ and 3′ ends (dashed boxed in Fig. 1A) and a 3′ AT-rich tail (the AAAT microsatellite). Two perissodactyl-specific SINE families, ERE-1 and ERE-2, were previously reported [28,29]. The ERE-1 structure comprises $tRNA^{ser}$ subunit-subunit I-subunit II, whereas the structure of ERE-2 is subunit I-subunit III-subunit III [29]. The SINE sequence of eCATH-3, with a subunit structure $tRNA^{ser}$-I-III, contains subunits distinctive of both families, with identities ranging from 72 to 86%. BLAST searches also revealed repetitive sequences with a similar $tRNA^{ser}$-I-III structure in unrelated horse genes such as transferrin, β-lactoglobulin and prostaglandin G/H synthase-

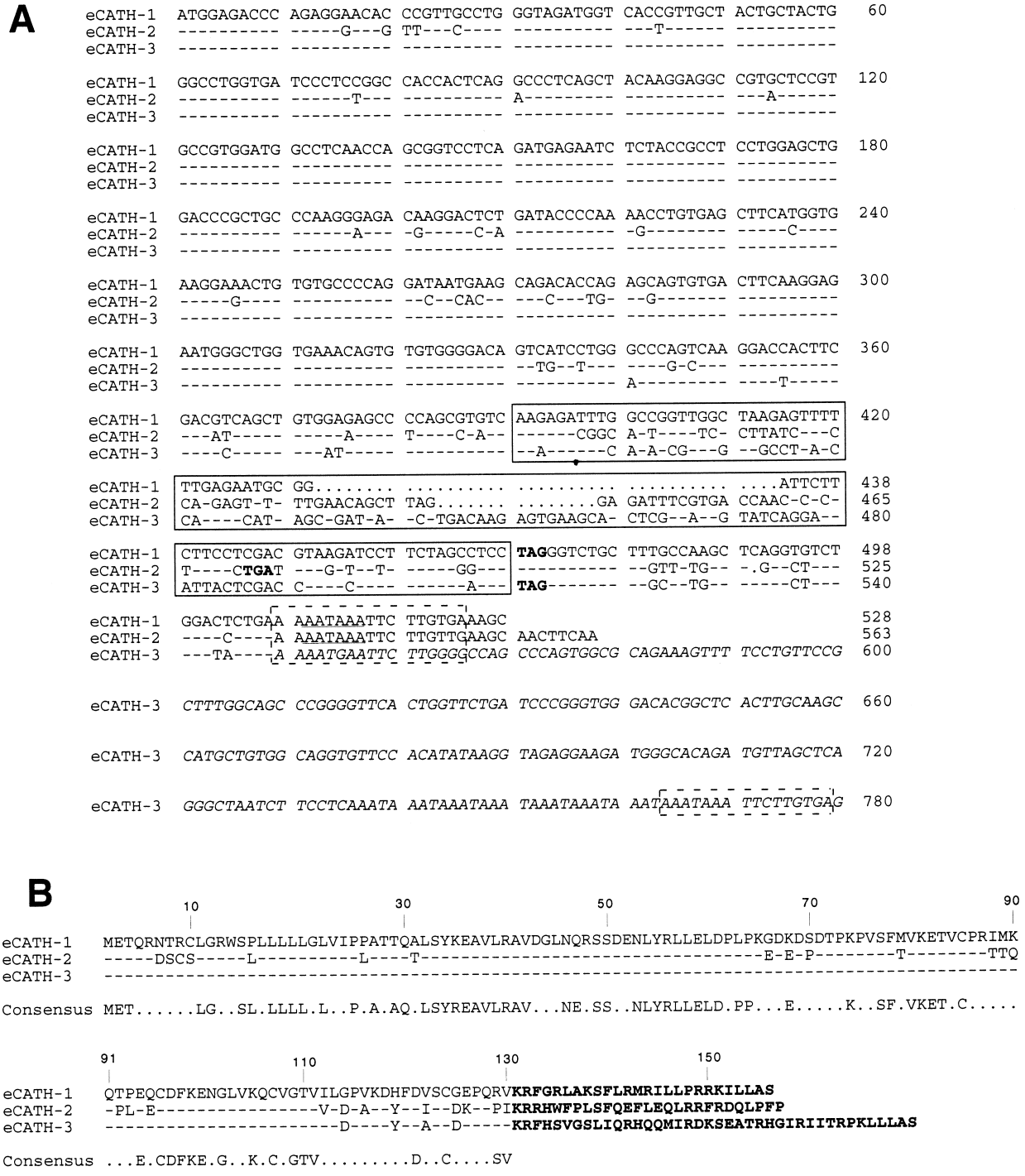

Fig. 1. A: Alignment of the cDNA sequences of the horse cathelicidins eCATH-1, eCATH-2 and eCATH-3. Sequences putatively encoding the antimicrobial domains are boxed. Stop codons are in bold face and polyadenylation signals are underlined. The SINE region of the eCATH-3 sequence is in italics and conserved direct repeats are dashed boxed. Dashes denote identical residues, dots denote gaps. Numbering is on the right. B: Alignment of the deduced amino acid sequences. Putative antimicrobial domains are in bold face. A consensus amino acid sequence has been deduced from the known cathelicidin sequences and comprises amino acid residues that are present at corresponding positions in at least 90% of the known sequences.

2. The presence of a retroposon and of the associated AAAT uninterrupted microsatellite in the eCATH-3 sequence suggests that the corresponding genomic region is unstable and may account for the generation of genetic variation [30]. This region was probably generated by insertion of the retroposon within an ancestral 16 bp target sequence containing the polyadenylation signal. At the 3′ end of the inserted sequence, target site duplication gave rise to an $(AAAT)_2$ simple repeat, which was then amplified and generated the $(AAAT)_9$ microsatellite. A previously reported cathelicidin gene in which SINEs have been observed is the human FALL39. In particular, Alu sequences have been found in the promoter region and downstream of this gene [31].

Analysis of the deduced amino acid sequences indicated

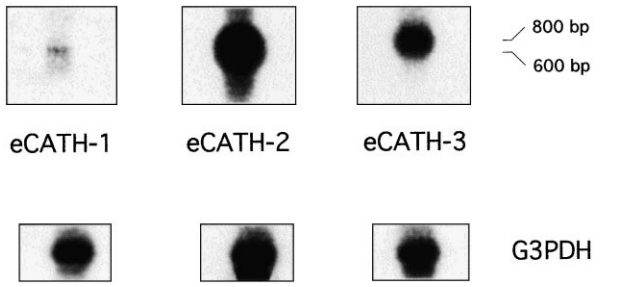

Fig. 2. Comparative expression of eCATH-1, eCATH-2 and eCATH-3 genes in bone marrow cells. Northern analysis was performed using equal amounts of total horse bone marrow RNA, probed with cDNA fragments corresponding to unique 3′ sequences of eCATH-1, eCATH-2 or eCATH-3 (gene-specific probes). The same filters were re-hybridized with a G3PDH cDNA probe as loading control.

eCATH polypeptides (Fig. 2B) with mol weights of 17 647, 18 153 and 19 299 and pI values of 10.2, 4.8 and 8.9, respectively. Further attempts to amplify additional members of this gene family from bone marrow RNA resulted in identification of a slightly different form of the eCATH-3 transcript (not shown), with base substitutions that change amino acid residues in the signal sequence region, at positions 6 (from N to D), 7 (from T to S), 8 (from R to C), 9 (from C to S) and in the prosequence region, at position 69 (from D to A), but not in the peptide coding sequence. The three congeners shown in Fig. 1B share 80–97% identity in the prepropieces (residues 1–130). When compared with corresponding prepropieces of previously described cathelicidins from other species, these regions display a 58–72% sequence identity. The deduced C-terminal domains following the putative split sites are 26, 27 and 40 residues long. No sequence homology to other peptides was found. Their cationic character, however, and the possibility for them to assume an α-helical conformation (not shown), as predicted by the PHD neural network [25], are features common to many antimicrobial peptides.

3.3. Analysis of the gene family

Analysis at the DNA level was performed by hybridizing genomic transfers from six unrelated horses with probes derived from the cloned cDNAs. A 344 bp restriction fragment corresponding to the 5′ cDNA sequence of eCATH-3 (cathelicidin-conserved probe) was used to reveal fragments containing the conserved preprosequence. As shown in Fig. 3, some of the hybridizing fragments were present in all individuals, while the size of other fragments was variable. The restriction fragment length polymorphism observed may be due to point mutations within the restriction sites or due to rearrangements involving more extended regions. The 3′ cDNA probes corresponding to each peptide domain (gene-specific probes) were then hybridized with the genomic blots. In the six horses, eCATH-2- and eCATH-3-specific probes were homologous to single restriction fragments (Fig. 4), suggesting that the genomic regions encoding these polypeptides are not polymorphic. However, when using the eCATH-1-specific probe, hybridization-positive fragments were only observed in three (d–f) out of six DNA samples and no signal was detected in samples a–c, even after hybridization under low stringency conditions. This observation is consistent with either absence or extensive rearrangement of the eCATH-1 gene

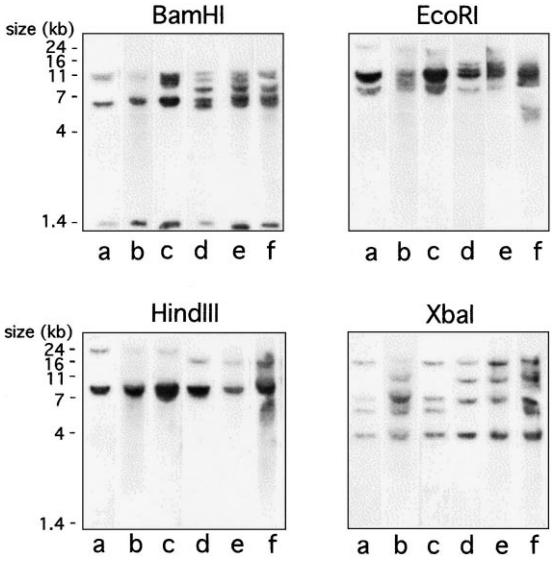

Fig. 3. Southern blot analysis of horse genomic DNA. Each blot contains DNA samples (denoted a–f) obtained from six horses and digested with one of the restriction endonucleases indicated in the figure. Blots were hybridized with a ^{32}P-labelled fragment corresponding to the conserved 5′ cDNA sequence of eCATH-3 (cathelicidin-conserved probe). Size markers are indicated on the left.

in individuals a–c and with the presence in the horse population of a null eCATH-1 variant. To investigate the frequency and transmission of positive and null eCATH-1 variants, genomic DNA from a larger sample of horses was amplified by PCR using the gene-specific primers that amplify the 3′ region of eCATH-1. The eCATH-1-positive variant was observed in 34 out of 70 unrelated horses (49% of the population analyzed). The product of nine crosses was then analyzed. No eCATH-1-specific amplification was observed in the six foals generated by five crosses where both parents had the null variant. These results suggest that positive and null variants are allelic forms. The null allele is transmitted as autosomal recessive trait and is present with a frequency of 0.71 in the horse population analyzed in this study. It is tempting to speculate that the null allele is the result of recombination events involving an unstable SINE-containing genomic region similar to that observed in the eCATH-3 sequence, which may

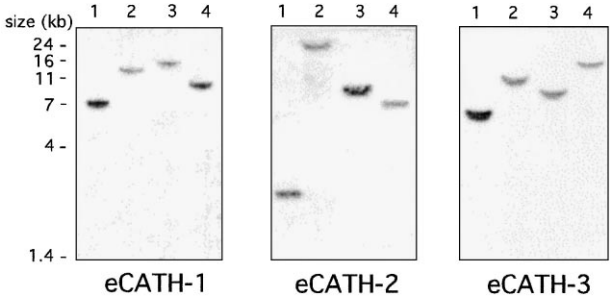

Fig. 4. Southern blot analysis of horse genomic DNA digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3) or *Xba*I (lane 4). Each blot was hybridized with a gene-specific probe based on the 3′ cDNA sequence of eCATH-1, eCATH-2 or eCATH-3, as indicated. In the case of eCATH-2 and eCATH-3, the hybridization patterns displayed in the figure are representative of the results obtained from DNA samples a–f, whereas the CATH-1 pattern is representative of DNA samples d–f.

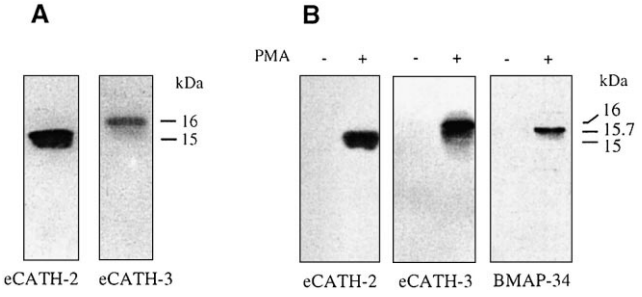

Fig. 5. (A) Immunodetection of eCATH propeptides in horse neutrophil granule lysates. Blots of neutrophil granule lysates were probed with the IgG fraction of anti-eCATH-2 and anti-eCATH-3 antisera. Molecular masses are indicated on the right. (B) Immunodetection of eCATH-2 and eCATH-3 in horse and of BMAP-34 in bovine neutrophil secretions. Equine or bovine neutrophils were incubated in the absence (−) or presence (+) of 150 nM PMA. Protein was TCA-precipitated from the cell-free media and subjected to SDS-PAGE and immunoblotting. Blots corresponding to cell-free media of horse neutrophils were probed with the IgG fraction of anti-eCATH-2 and anti-eCATH-3 antisera, blots corresponding to cell-free media of bovine neutrophils were probed with anti-BMAP-34 antisera.

be found downstream of the polyadenylation sequence. Further grounds to support this hypothesis may come from sequencing the flanking regions of the gene.

3.4. Storage of the propeptides in neutrophil granules and release from stimulated neutrophils

The genomic analysis demonstrated the existence of null and positive eCATH-1 variants. The possibility that the positive allele was non-functional remained open and was supported by low abundance eCATH-1 mRNA in myeloid cells of the gene-positive horses (Fig. 2). We tested the possibility that the positive allele was non-functional by investigating the presence of the putative eCATH-1 polypeptide in peripheral neutrophils of gene-positive animals. Neutrophil granule lysates from several horses were analyzed by Western blotting and antibodies against synthetic eCATH peptides were used to reveal eCATH polypeptides. Anti-eCATH-2 and anti-eCATH-3 antibodies revealed eCATH bands corresponding to propeptides (14 969 and 16 066 kDa, respectively) in all the horses analyzed (Fig. 5A). By contrast, blots probed with anti-eCATH-1 antibodies were all negative (not shown). eCATH-1-specific antibodies failed to reveal eCATH-1-related bands both in neutrophils and in neutrophil granule lysates. The results thus suggest that the eCATH-1 gene is unable to encode a protein.

The ability of horse neutrophils to secrete eCATH proteins was then tested. Intact horse neutrophils were treated with the secretagogue PMA, which was previously shown to induce release of cathelicidins from cattle [6] and pig [7] neutrophils. After incubation with PMA for 30 min, cells were removed by centrifugation and their integrity was evaluated by assaying the activity of the cytosolic lactate dehydrogenase in the cell-free medium. This was never found to exceed 4% of the total cell activity. Protein in the cell-free medium was TCA-precipitated, solubilized in SDS-sample buffer and analyzed by Western blotting. This analysis consistently revealed the presence of eCATH-2 and eCATH-3 propeptides in the secretions of PMA-treated neutrophils (Fig. 5B). In keeping with these results, antibodies against the bovine cathelicidin BMAP-34 [32] detected BMAP-34 propeptide in the cell-free medium of PMA-stimulated bovine neutrophils (Fig. 5B). While these data indicate that the horse cathelicidins are released upon neutrophil stimulation, the absence of eCATH-1-related bands in blots corresponding to PMA-induced secretions (not shown) further supports the hypothesis that the eCATH-1 gene is non-functional.

This analysis thus demonstrates that eCATH-2 and eCATH-3 are functional genes. The results also demonstrate that the horse cathelicidins are stored in the secretory granules of neutrophils in unprocessed forms and can be released upon neutrophil activation, as already shown for other members of this family in different mammalian species [3,4].

Acknowledgements: We are grateful to Twink Allen, Antonio Pilla and Eric Cizeron for providing us with horse blood samples. This work was supported by grants from the Italian Ministry for University and Research (P.R.I.N. Cofin. 1997) and from the Istituto Superiore Sanità, Programma Nazionale di Ricerca sull'AIDS.

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