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Structural, functional analysis and localization of the human CAP18 gene

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Abstract CAP18 is an antimicrobial protein found in specific granules of PMNs. The human CAP18 (HCAP18) gene was cloned from a human genomic phage library. Sequence analysis revealed the HCAP18 gene to have 4 exons spanning 3 kb, including 700 bp of upstream DNA. Using 3′ RACE no homologs of human HCAP18 were found in human bone marrow or leukocyte populations. By PCR analysis of a somatic cell mapping panel and fluorescence in situ hybridization of a genomic clone to metaphase chromosomes the gene was mapped to chromosome band 3p21.3. Like several other genes expressed late in PMN development the CAP18 gene did not contain typical TATA box or CCAAT sequences. Expression in Cos 7 cells permitted limited mapping of the promoter function in upstream fragments of the HCAP18 gene. Western blot, Northern blot and RT-PCR analysis show HCAP18 to be produced specifically in granulocytes. This work forms the groundwork for future analysis of the genetic regulation of this antimicrobial protein during PMN differentiation.

Key words: CAP18; Granulocyte; Chromosome 3; Antimicrobial protein

1. Introduction

Since the discovery of penicillin by Fleming in the 1920s several hundred antibiotics have been identified and used clinically. Misuse of these miracle drugs has selected a plethora of drug resistant microbial strains and the search for novel antibiotics continues. The identification of vertebrate peptides with broad spectrum antimicrobial activity has opened a new era of 'endobiotic therapy' for infectious diseases [1]. Among the most promising are those antimicrobial peptides identified in mammalian PMNs. These proteins include the defensins [2,3], BPI [4,5], CAP37 [6] and members of the CAP18 family [7].

CAP18 (18-kDa cationic antimicrobial protein) was first identified, purified and cloned from rabbit leukocytes [8]. CAP18 and derivative peptides possess a number of functions important for host defense. These include: binding to and attenuation of many of the biological activities of lipopolysaccharides of diverse origin; cytotoxicity for Gram-positive and Gram-negative bacteria and modulation of the clotting cascade.

The family of CAP18 antimicrobial proteins share a similar overall structure: signal sequence, conserved N-terminal prosequence of unknown function and a C-terminal antimicrobial domain. The CAP18 mRNA is expressed predominantly in neutrophils, where the protein accumulates in the specific granules. However, the mechanisms of tissue-specific expression and protein processing are not well understood. To answer these questions, we cloned the genomic DNA for human CAP18, studied the structure of its promoter, and examined CAP18 tissue-specific expression using Western blot analysis, RT-PCR and Northern blot analysis. Although the human CAP18 cDNA was previously identified by us [9] and others [10,11], the chromosomal location and gene structure had not been determined. We report here that the HCAP18 gene is located on chromosome band 3p21.3 and is comprised of 4 exons spanning 3 kb including 700 bp of upstream DNA. Some unusual features of the HCAP18 promoter are discussed.

2. Materials and methods

2.1. Reagents

Buffy coats were obtained from the Stanford University Blood Bank. Ficoll-hypaque was purchased from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes and DNA modifying enzymes were purchased from Promega (Madison, WI). Bacterial culture media, cell culture media, lipofectamine, biochemical reagents, and antibiotics were supplied by Life Technologies (Gaithersburg, MD). Human genomic libraries, labeling kits and Northern blots were purchased from Clontech (Palo Alto, CA). Protein gels, kits for purification of mRNAs and Western blot kits were supplied by Novagen (Madison, WI).

2.2. Cloning and sequencing of HCAP18 genomic DNA

A human genomic DNA library constructed in lambda EMBL3 (Clontech) was screened by hybridization with a labeled probe prepared by PCR using HCAP18 specific primers designed from the human CAP18 cDNA [9] (HUCAP no. 1: 5′-CTCAGCTACAAGGAAGCTGTGCTTCGTGC-3′; and HUCAP no. 2: 5′-TGTCTCCTTCACTGTGAAGCTCACAGG-3′). HCAP18 cDNA was amplified by PCR using the following thermal cycling parameters: 95°C, 5 min; then 20 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min; followed by 72°C, 7 min. Hybridization and washing methods followed the Clontech manual. For restriction mapping, lambda clones were purified with phage purification kit (Qiagen, Chatsworth, CA) and digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Nco*I, and *Sac*I. The HCAP18 sequences are deposited in the GenBank Database, accession number U19970 (cDNA) and U48795 (genomic).

2.3. Characterization of 5′ upstream region

Luciferase and β-galactosidase were used as a reporter genes. The 5′ upstream regions constructed by PCR were subcloned into pGL3 and pGal (Promega) by PCR with *Pfu* polymerase (Stratagene, La Jolla, CA) using the following primers:

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5P1: 5′-GACCGAATTCCACCCTGGCAGGCAGCCACGG-3′;
5P2: 5′-GAGCGAATTCTGAAGAACCCACTTCCTCATC-3′;
5P3: 5′-CTGCGAATTCGTCGCCTCGGCACCTGGCTCTC-3′;
5P4: 5′-CTGCGAATTCCTTAATCGATGCCTACAGGGTG-3′;
5P5: 5′-CGTCGAATTCGTCTCGAACTCCTTATCTCAGG-3′;
5P7: 5′-CGTCGAGCTCTCATACTGAGTCTCACTCTG-3′;
3P 5′-CGTGGGCTCCGGTCCCCATGTCTGCCTCCCTCTAG-3′.

All of the subclones were confirmed by sequencing. Each clone (2 μg) was transfected into Cos 7 cells using Gibco-BRL Lipofectamine (6 μl) and analyzed at 24 h and transfected into K562, U937, and HL60 cell lines using the Bio-Rad Gene Pulse II (BioRad, Richmond, CA). The conditions used were: 960 μF, 0.2 kV in a 0.5 ml cuvette. The medium was changed with DMEM containing 10% FBS. After 24–48 h incubation, transfected cells were extracted and assayed for luciferase activity in a scintillation counter (Packard Instruments, Downer's Grove, IL) using kits from Promega. To measure histochemically, expression of the β-galactosidase gene was detected by staining with X-gal substrate after fixing the transfected cells.

2.6. Somatic cell hybrid panel and PCR amplifications

The HCAP18 gene was assigned to a human chromosome by using a mapping panel containing 15 human×Chinese hamster somatic hybrid cell lines derived from several independent fusion experiments and contain defined subsets of human chromosomes (summarized in [13]). HCAP18-specific primers were designed from prosequences of the gene. The primers were HUCAP no. 1 and HUCAP no. 2 shown above. The genomic DNA samples from the panel were amplified by PCR using the conditions: 95°C, 5 min; then 35 cycles of 94°C, 1 min; 58°C, 1 min; 72°C, 1 min; followed by 72°C, 7 min. The PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

2.7. Fluorescence in situ hybridization

Fluorescence chromosomal in situ hybridization (FISH) was performed as an independent approach to define further the chromosomal localization of the HCAP18 gene according to a protocol as described [13]. Briefly, a genomic clone of HCAP18 with an 8 kb insert was labeled with biotin-16-dUTP by nick-translation using commercial reagents (Boehringer Mannheim, Indianapolis, IN). The lambda clone was isolated from the Clontech human genomic library and subcloned into pUC18 after restriction mapping. Labeled probe was annealed to cold competitor human placental DNA in the presence of salmon sperm DNA as carrier and hybridized at a concentration of 100–200 ng/25 μl per slide to pre-treated and denatured metaphase (as described recently [14]) chromosomes from a human peripheral blood lymphocyte culture. Hybridization, washing, signal detection and imaging by CCD camera were carried out as described. Black-white photographs were generated from PICT files.

2.8. 5′ and 3′ RACE

Human bone marrow mRNAs and peripheral blood leukocyte mRNAs (Clontech) were used for the template. The 5′ RACE and 3′ RACE systems of Life Technologies were used. In 5′ RACE, HC3 (5′-TCTCCTTCACTGTGAAGCTCACAGGAAAAA-3′) for cDNA synthesis and UAP and HUCAP no. 1 (5′-GCTTTGGCGTGTCTGGGTCCCC-3′) for PCR primers were used (conditions of PCR: 94°C×1 min, 55°C×1 min, 72°C×2 min, 35 cycles). PCR products were cloned into a TA cloning vector (Invitrogen). After isolation of clones, sequencing analysis was performed using M13 reverse and forward primers. The initiation site was based on the ends of the sequence obtained.

For 3′ RACE, an adaptor primer for cDNA synthesis and UAP and a specific 3′ RACE primer (sequence: 5′-AGCTTCACATGTAGGAGACAG-3′) for PCR primers were used (conditions of PCR: 94°C×1 min, 45–55°C×1 min, 72°C×2 min, 35 cycles). The 3′ RACE primer sequence was designed from a consensus of sequences which have wide homology among the CAP18 family. PCR products were cloned into a TA cloning vector (Invitrogen). After isolation of clones, sequencing analysis was performed using the M13 reverse and forward primers.

2.9. Tissue-specific expression of HCAP18 in PBMC by Western Blot and RT-PCR analysis

Granulocytes were separated from monocytes/platelets using Ficoll gradient centrifugation with Histopaque-1119 and Histopaque-1077. After resuspension of the cells in an appropriate volume of PBS, one part of the cells was used for the purification of mRNA using the Novagen (Madison, WI) mRNA purification kit and the other part of the cells was extracted for Western blot analysis by boiling after resuspension of the cells with 2×SDS sample buffer. The samples were loaded onto 4–20% SDS PAGE, and transferred to nitrocellulose membrane after electrophoresis. Titered polyclonal rabbit antibody (diluted 1:500) prepared against HCAP18 prosequences (amino acids 1–105) was applied to the blot. After washing, blots were exposed to preabsorbed polyclonal goat anti-rabbit heavy chain antibody (diluted 1:200) followed by use of the enhanced chemiluminescence Western blot analysis system (Amersham). The mRNAs from monocytes and granulocytes were reverse-transcribed with random hexamer primers using StrataScript reverse transcriptase (Stratagene) for the synthesis of cDNA. The primers HUCAP no. 1 and HUCAP no. 2 primers (sequences above) were used to amplify DNA under the following PCR conditions: 95°C, 5 min; then 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min; followed by 72°C, 7 min. The PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining. The gel was transferred to a nitrocellulose membrane and hybridized with an internal HCAP18 specific primer (HC4: 5′-CCCACGATGGATGGGGACCCAGAC-3′). For a control, the human β-actin control amplimer set was used.

3. Results

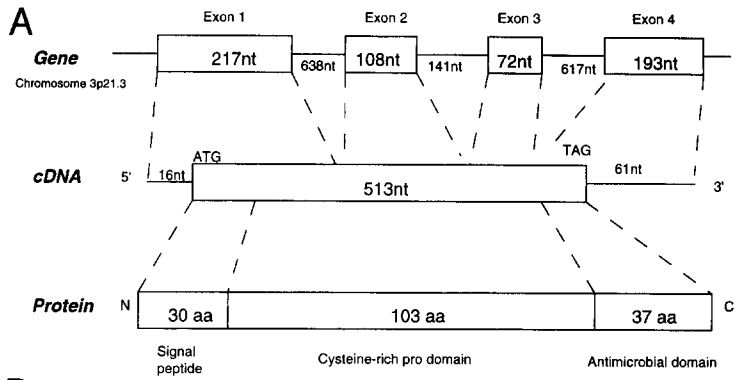
3.1. Genomic structure of HCAP18 gene

A human genomic DNA library constructed in lambda EMBL3 was screened by hybridization with an HCAP18 specific probe prepared by PCR. Seven positive plaques were detected out of 3×10^6 plaques screened. All of the clones had the same restriction map (data not shown). A representative clone (no. 12) was subjected to further analysis. An 8 kilobase size *Eco*RI fragment was subcloned into pUC18 and sequenced bidirectionally. Fig. 1A presents a schematic representation of the HCAP18 gene, cDNA and protein. Fig. 1B presents the entire sequence of the HCAP18 gene. The HCAP18 coding sequence is assembled from 4 exons covering 2 kb of DNA. The transcription initiation site is located 16 nucleotides upstream from the first ATG. The 5′ upstream DNA does not contain typical TATA box or CCAAT sequences. Inspection of the sequence revealed that exon 4 contains the entire C-terminal antimicrobial domain. However, in addition to encoding the known antimicrobial peptide this exon also encodes an additional six residues located on the N-terminal end of the antimicrobial peptide.

3.2. Assignment of the HCAP18 gene to human chromosome 3, band p21.3

PCR amplification with HCAP18-specific primers generated a 177 bp fragment from human DNA as expected and no product from Chinese hamster DNA. Amplification of the human×Chinese hamster mapping panel gave positive results only in those hybrid cell lines that had retained human chromosome 3. All other human chromosomes were excluded by the presence of discordant hybrids (Table 1). Furthermore, specific PCR amplification was obtained from a hybrid cell line (XII-2D-2a aza) containing two copies of the short arm of chromosome 3, but not from another hybrid cell line (XVIII-7B-3a aza) retaining only the long arm of chromosome 3. These results indicated that the HCAP18 gene is located on the short arm of chromosome 3.

Fluorescence in situ hybridization independently confirmed the result from the somatic cell hybrid mapping panel. Of 24 metaphase spreads analyzed, 20 exhibited a specific fluores-



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  23  ACCCAAAGGGATGGCCACTCCCTGGGGCGGTGGTCACTGGTGCTCCTGCTGCTGGGCCTG
  83  GTGATCGCTCTGGCCATCATTGCCCAGGTCCTCAGCTACAAGGAAGCTGTGCTTCGTGCT
 143  ATAGATGGCATCAACCAGCGGTCCTCGGATGCTAACCTCTACCGCCTCCTGGACCTGGAC
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1703  cgaagaggggcgtctaggtggggagggtcttggattgaccctgggtacatccccgacaa
1763  ggaacctgtttcttcctgtacacaaccccagGATAACAAGAGATTTGCCCTGCTGGGTGA
1823  TTTCTTCCGGAAATCTAAAGAGAAGATTGGCAAAGAGTTTAAAAGAATTGTCCAGAGAAT
1883  CTAGGATTTTTTGCGGAATCTTGTACCCAGGACAGAGTCC**TAG**TGTGTGCCCTACCCTG
1943  GCTCAGGCTTCTGGGCTCTGAGAAATAAACTATGAGAGCAATTTCctcaggcttcagtct
2003  cacttgttttgcctcctctctctcaccacaactgagcccttagctcagggagtccacgtg
2063  tgagtgtgagtgtgtgtgagtgtgacacagaggtggcgagggcagtgttccatccaggag
2123  gacacagggtaa
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Fig. 1. (A) Schematic structure of the human CAP18 gene. (B) Sequence of the human CAP18 gene. The HCAP18 gene is comprised of 4 exons (capital letters) spread over approx. 3 kb including 700 bases of 5′ upstream sequence from the transcription initiation site. The borders of the three introns do not interrupt codons. Exon 4 contains the entire C-terminal antimicrobial domain with an additional six amino acids at its N-terminus. Initiation and termination codons are shown in bold.

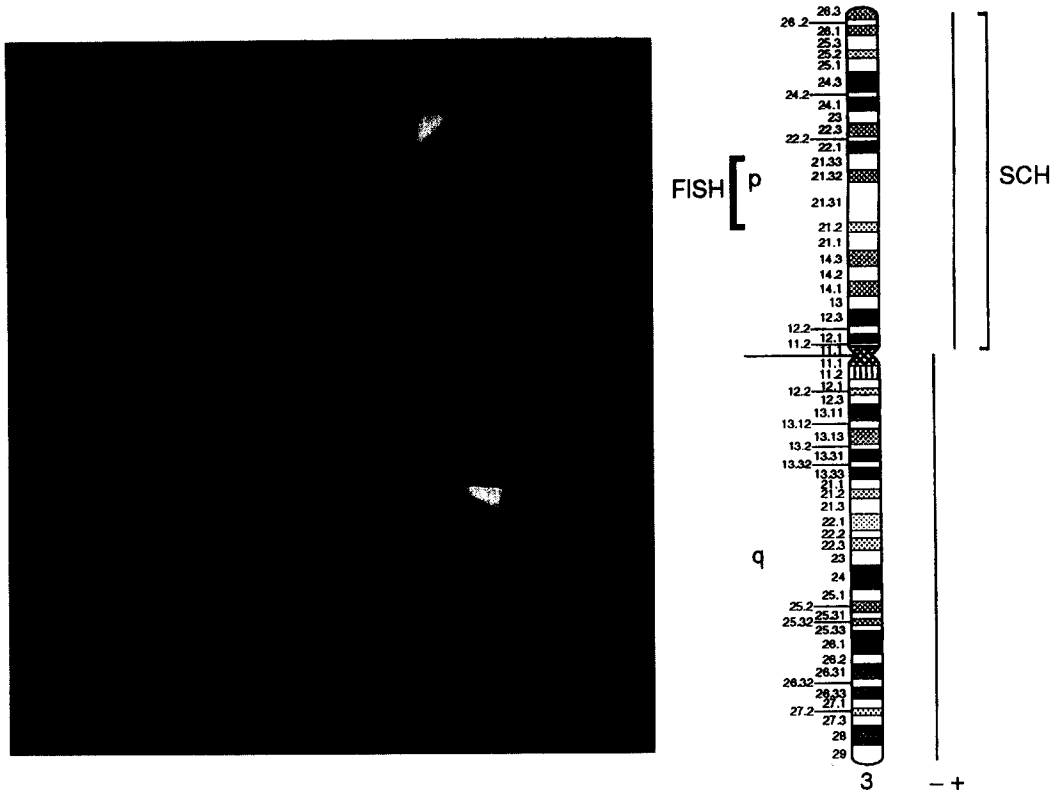

Fig. 2. (Left) Chromosomal localization of the human HCAP18 gene by fluorescence in situ hybridization (FISH) of a gene-specific lambda clone. Specific signals (indicated by arrowheads) are present on human chromosome 3 at bands p21.3. (Right) Ideogram of human chromosome 3 [35] illustrates the somatic cell hybrid (SCH) and the FISH localization of HCAP18 gene. The vertical bars represent the portion of chromosome 3 retained in two somatic hybrid cell lines and the plus and minus signs symbolize the positive and negative PCR amplifications using HCAP18-specific primers.

cent signal on both chromatids of a single chromosome 3, and 13 of these had signals on both chromosome 3 homologs. The chromosomes were identified based on an R banding pattern produced by BrdU incorporation in late S-phase after synchronization of the cells. The localization of the signal corresponds to band 3p21.3 (Fig. 2). No other chromosomal site was labeled above background.

3.2. Characterization of 5′ upstream region

To identify sequences important to promoter activity, 5′-truncations were introduced into the pGL3 reporter vector containing a luciferase reporter gene. The promoterless luciferase plasmid (pGL3-Basic) and the luciferase plasmid under the control of the cytomegalovirus enhancer promoter (pGL3-Control) were used as negative and positive transfection control, respectively. After each construct was transfected into Cos 7 or K562 cells for 2 days, the cells were harvested and lysates assayed for luciferase activity using a scintillation counter. Each luciferase construct was co-transfected with a pCMV-β-galactosidase vector, an internal standard for the

Table 1
Comparison of HCAP18 PCR product with human chromosomes in human×Chinese hamster somatic cell hybrids

PCR product/ Chromosome	Human chromosome number																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Discordant hybrids																							
+/−	6	6	0	5	8	3	8	5	7	8	5	4	4	2	5	3	10	6	4	6	3	5	3
−/+	3	0	0	2	2	3	1	3	1	0	2	2	2	3	3	2	2	4	5	3	3	4	2
Concordant hybrids																							
+/+	3	4	9	4	2	6	1	5	2	2	5	6	6	8	5	5	0	4	5	4	7	5	1
−/−	2	4	4	2	3	2	3	2	3	5	1	3	2	2	2	3	3	1	0	2	1	0	1
Informative hybrids	14	14	13	13	15	14	13	15	13	15	13	15	14	15	15	13	15	15	14	15	14	14	7
% discordance	64	42	0	53	67	43	69	53	61	53	54	40	43	33	53	38	80	67	64	60	43	64	71

Data for chromosomes with rearrangements or present at low copy number (< 0.1) were excluded. For each chromosome the number of hybrid cell lines are listed in 4 categories defined by the presence (+) or absence (−) of the HCAP18-specific PCR product and the presence (+) or absence (−) of the respective chromosome. The % discordance is calculated as the sum of discordant over total informative hybrids for each chromosome.

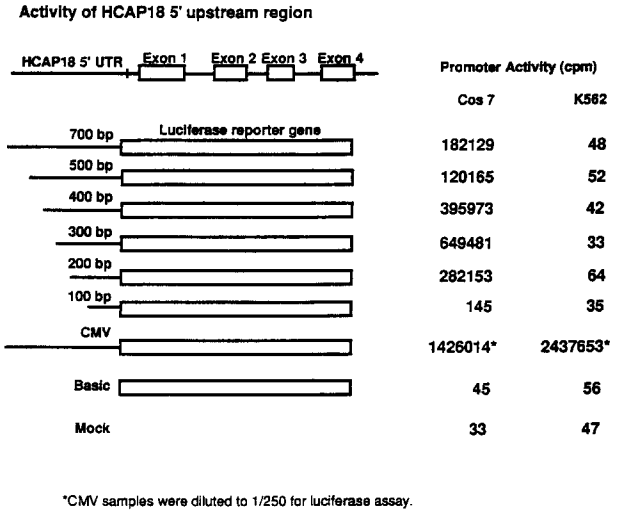

Fig. 3. Promoter activity of HCAP18 5′ upstream region. 5′-truncations of the HCAP18 promoter constructed using PCR were introduced into the luciferase reporter plasmid. The promoterless luciferase plasmid and the luciferase plasmid under the control of the cytomegalovirus enhancer promoter were used as negative and positive transfection controls, respectively. Cos 7 cells (5×10^5) were transfected with 2 μg of each construct. The amount of luciferase was determined after 48 h by scintillation counter, and the results were normalized for protein content and β-galactosidase activity (measured from a β-galactosidase cotransfection). Values represent the mean of three representative experiments. CMV samples were diluted to 250-fold.

transfection efficiency, using Lipofectamine as described above.

The luciferase activities of these constructs in Cos 7 cells are shown in Fig. 3. HCAP18 promoters containing greater than 300 nucleotides of upstream sequence reproducibly express greater than 3000-fold more luciferase activity than the promoterless vector with maximum activity demonstrated by the 300 bp construct (649 000 cpm). However, in Cos 7 cells the CMV promoter is approx. 2000 times stronger than the strongest HCAP18 promoter construct. Among the HCAP18 promoter constructs, the 300 and 400 bp constructs have the highest activity. No HCAP18 reporter activity was detected in K562 cells. These results were confirmed by visual observation of β-galactosidase staining (revealed by the X-gal substrate) of Cos 7 cells transfected with the 300 bp HCAP18-promoter driving β-galactosidase (data not shown).

3.4. *Attempt to find novel HCAP18 family members by 3′ RACE*

Previously, several laboratories demonstrated a family of porcine proteins called protegrins shown to be homologous to CAP18 [15–17] and related bovine proteins [18]. Although these proteins have 60–70% amino acid identity between human and porcine species in the conserved cysteine-rich prosequence, there is considerable divergence in the amino acid sequences of the C-terminal domains (see table in [1]). Therefore, an effort was made to identify other HCAP18 family members. For this purpose mRNA from human bone marrow and peripheral blood leukocytes was subjected to 3′ RACE. No novel mRNAs were identified from among 12 bone marrow clones and 5 leukocyte clones. The polyadenylation site was not precisely conserved among these clones: several clones had $TTC(A)_n$ whereas others had $TT(A)_n$ (data not shown). Further studies will be required to confirm the absence of multiple human CAP18 peptides.

3.5. *Granulocyte specific expression of HCAP18 in PBMC by Western blot, RT-PCR analysis and Northern blot*

Northern blots revealed the HCAP18 mRNA to be approx. 800 bp in length similar to that previously reported for the rabbit [1]. Northern analysis of a large number of tissues showed that only bone marrow contains HCAP18 transcripts (Fig. 4A). Among the various subsets of cells present in peripheral blood, only granulocytes expressed the HCAP18 gene (see Fig. 4B). In addition using RT-PCR and Western blot methods, HCAP18 was not detected in various leukocyte cell lines including K562, HL60 and U937.

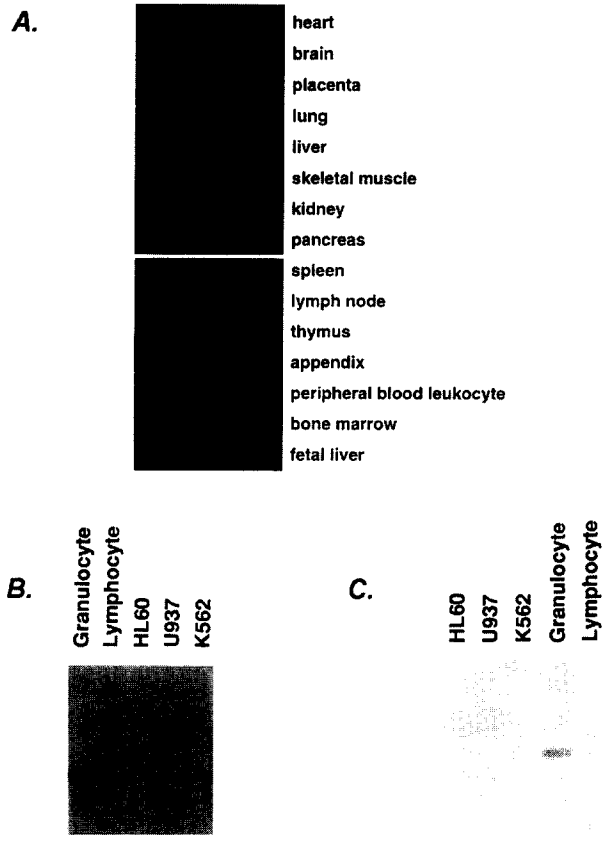

Fig. 4. (A) Northern blot analysis demonstrates HCAP18 expression in bone marrow tissue. Northern blots from 15 different tissues were purchased from Clontech. Blots were probed with an HCAP18-specific probe (HC4, 10^7 cpm) prepared form the HCAP18 cDNA using a random labelling kit from Promega. The major band is at 800 bp (markers not shown). (B) Specific expression of HCAP18 mRNA in granulocytes: RT-PCR analysis. Approx. 2×10^7 cells were isolated from a human buffy coat. RNA was purified, and RT-PCR carried out as described in methods. An end-labelled internal primer was used to detect amplified DNA. Holo-HCAP18 is specifically amplified only in purified granulocytes. The amplified band is 450 bp (markers not shown). (C) Specific expression of HCAP18 protein in granulocytes: Western blot analysis. Samples prepared from approx. 2×10^7 cells (see Section 2) were electrophoresed on a 4–20% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The membrane was immunoblotted with a titered antibody against the HCAP18 prodomain according to methods. Holo HCAP18 is found only in purified granulocytes. The positive band is located at a molecular mass of approx. 16.5 kDa (markers not shown).

4. Discussion

CAP18 is one of the recently identified PMN antimicrobial proteins [1]. Holo-CAP18 is comprised of two functional domains. The C-terminal domain of 37 amino acids forms [19,20] an amphipathic α-helix to bind LPS and mediates anti-bacterial activity against both Gram-positive and Gram-negative organisms [21,22]. In vivo studies indicate that the anti-microbial domain of CAP18 has protective activity in a pig model of endotoxemia [23].

Although the amino acid sequence and pattern (four conserved cysteines within a sequence of approx. 100 amino acids) of the N-terminal domain are more conserved than the quite diverse C-terminal fragments, the function of this cysteine rich pro-sequence is not known. Perhaps it serves to protect and localize CAP18 to its subcellular location in the specific granules of mature neutrophils [24].

Human CAP18 maps to chromosome 3 band p21.3 in the vicinity of a number of other genes, including macrophage-stimulating factor-1 or hepatocyte growth factor like-protein (MST1, HGF), guanine nucleotide binding protein alpha inhibitory subunit 2 (GANI2), myosin light chain 3 (MYL3), galactosidase beta-1 (GLB1), collagen VII alpha-1 polypeptide (COL7A1), cholecystokinin (CCK), and natural killer triggering receptor (NKTR). The mouse counterparts of these genes have been mapped to a conserved syntenic region on distal mouse chromosome 9 (data from [25,26],). Therefore, we predict that the mouse homolog of HCAP18 is also located on mouse chromosome 9. At the present time, neither human region 3p21.3 nor the homologous mouse chromosome 9 region is known to carry genes for any inherited disorders or phenotypic trait suggestive of HCAP18 involvement.

Several families of functionally related proteins with lipopolysaccharide binding and antimicrobial activity are found to be clustered in the human genome. Both LPS binding protein (LBP) and bactericidal permeability increasing protein (BPI/CAP57) are located on chromosome 20q11.12-q23 [27], while azurocidin (CAP37) resides on chromosome 19p13.3, together with at least two other family members, neutrophil elastase and proteinase 3 [28]. HCAP18 shares a significant homologous sequence at its N-terminal domain with a similar protein from pig [29] and sheep [30] myeloid cells, the indolicidin protein from cows [31], and an antibacterial 15 kDa protein (p 5s, p15H) from human leukocytes [32], suggesting a novel multigene family. It is likely that HCAP18 is also located in a cluster with other members of this family of related genes.

The cloning of genomic DNA for HCAP18 forms the groundwork for analysis of the genetic regulation of this antimicrobial protein during differentiation. The HCAP18 gene is comprised of 4 exons spanning 3 kb including 700 bp of 5′ upstream DNA. Like the previously characterized protegrin gene, the HCAP18 gene has a short 5′ untranslated region (16 nt and a promoter lacking typical TATA box or CCAAT sequences. Several granulocyte-specific proteases (azurocidin, protease 3, neutrophil elastrase, cathepsin G, and myeloperoxidase) are expressed at the promyelocytic or promonocytic stage of myeloid differentiation. The promoters of these five genes share TATA-like elements, a CCAAT box, a PU.1 binding site and a CT-rich element within the first 200 bp of the major transcriptional start site. In contrast, the promoters of myeloid genes expressed at later stages of granulocyte/macrophage maturation, such as CD22B, CD18, and CD11C, lack the 'TATA' box as well as the Ets/PU.1 binding site. Because the HCAP18 promoter structure is more like this later group of proteins, HCAP18 gene expression may only occur during later stages of granulocyte maturation. In fact, the RT-PCR experiments on mature granulocytes support the idea that HCAP18 is expressed during a late stage of differentiation (Fig. 4B). Failure to find expression of HCAP18 (by RT-PCR or Western blot) in HL60 (promyelocytic cell line) and U937 (monocytic lineage), both undifferentiated hematopoietic cell lines, also supports this idea. It is probable that the positive HCAP18 Northern blot of bone marrow is the result of contaminating mature PMNs. In other work CAP18 mRNA was found in testis [32]. Thus, like lactoferrin [33], HCAP18 may be a useful marker to characterize disorders of granulocyte terminal differentiation, such as acute leukemia and the myelodysplastic syndrome.

Technical problems thwarted the facile characterization of the HCAP18 promoter. Efforts to transfect various promoter constructs into HL60 and U937 cells were not successful. Crystal's laboratory [34] also noted difficulty in transfecting HL60 cells. Our attempts to transfect HL60 cells with plasmid vectors were also unsuccessful despite trying a variety of available methods. Sequences of the HCAP18 promoter spanning 300–400 bp upstream can be demonstrated to have modest promoter activity in Cos 7 cells, albeit at a rather low level compared to a standard CMV driven reporter gene. Since physiologically relevant CAP18 expression is not expected in such cells, it is possible that the observed activity represents the basal activity of the 'core' promoter. However, none of the 5′ upstream fragments showed even this basal activity in the erythromyeloid line, K562, suggesting that HCAP18 gene expression occurs only in the later stages of neutrophil maturation and is under tight control. Future studies will be required to delineate the precise nature of this fascinating stage and tissue-specific regulation.

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