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Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel

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The epithelial surface of the human small intestine is a barrier between the host and the microbial environment of the lumen. A human small intestine cDNA clone was found to encode a new member of the defensin family of antimicrobial peptides, named human defensin-6. Tissue expression of this mRNA is specific for the small intestine as determined by Northern blot analysis and polymerase chain reaction analysis. In situ hybridization demonstrated that human defensin-6 mRNA localizes to Paneth cells in the crypts of Lieberkühn. The finding of an abundant defensin mRNA in human Paneth cells supports the notion that these epithelial cells may play a key role in host defense of the human bowel. The results also strengthen the hypothesis that peptide-based host defenses are prevalent at mucosal surfaces in mammals.

cDNA; Epithelium; Crypt; Intestine; Mucosa

1. INTRODUCTION

The mucosal epithelium of the mammalian small intestine consists of four main cell types: the enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. These cells arise from a common progenitor cell in the crypts of the small intestine and are perpetually renewed throughout the life of the organism [1]. The epithelial surface formed by these cells is essential for nutrient absorbtion and constitutes a barrier between the body and the external environment of the lumen. Colonization and growth of microbes in the small intestinal lumen are a potential threat to the host and interest has focused on local antimicrobial defense mechanisms of this mucosal surface [2]. Antimicrobial peptides are proving to be a prevalent mechanism of host defense (for reviews see: [3–5]) and several peptides with antibacterial activity have been recently isolated from intestinal extracts [6–10].

Paneth cells have been implicated in intestinal host defense by various studies. Paneth cells, located at the base of the crypts of Lieberkühn throughout the small intestine and proximal colon, are most abundant in the region of the ileum [11]. These cells contain numerous apically-located eosinophilic secretory granules, and both an extensive endoplasmic reticulum and golgi network [12]. Proteins related to host defense, including lysozyme, have been localized to the Paneth cell granules, and data from several studies indicate that Paneth cells degranulate in response to live and heat killed bacteria in the intestinal lumen ([13] and references therein). Paneth cells have been shown to also contain high levels of messenger RNA (mRNA) encoding the putative preprodefensin peptides, named cryptdin in the mouse [14] and human defensin-5 (HD-5) in the human [15].

Defensins are a family of small cationic peptides originally isolated as a component of the non-oxidative antimicrobial activity of rabbit lung macrophages [16]. They have since been shown to be a major component of granular leukocytes of several species and constitute 5–10% of protein in human neutrophils [3,4]. These peptides are 30–35 amino acids in length and are distinguished by a conserved cysteine motif [3]. Defensins are membrane active [17–19] and of the nearly twenty defensin peptides characterized to date, all have microbicidal activity towards an impressive range microorganisms in vitro [3].

Mature defensin peptides have recently been isolated from the murine small bowel epithelium [9,10]. Several related peptides were isolated and the most abundant, cryptdin-1, corresponds to the previously mentioned cryptdin mRNA of the mouse Paneth cells. This peptide shows antimicrobial activity towards a strain of *Salmonella typhimurium* which has a mutation in the *pho* P virulence locus. The wild type strain of *S. typhimurium*

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Abbreviations: cDNA, complementary DNA; HD-5, human defensin-5; HD-6, human defensin-6; HNP, human neutrophil antimicrobial peptide; mRNA, messenger RNA; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate (1 × concentration: 0.15 M NaCl, 0.15 M Na-citrate pH 7.0).

with an intact *pho* P locus is resistant to this peptide [10]. The identification of small intestinal defensins, and the demonstration that for at least one enteric pathogen both in vitro resistance to defensin and in vivo virulence map to a single genetic locus, intimates their role in intestinal host defense.

In this paper we describe an abundant mRNA species of the human small intestine encoding a novel putative preprodefensin. We have localized this mRNA to the Paneth cells at the base of the crypts of Lieberkühn. Our findings not only further support the hypothesis that defensin peptides play a role in the defense of the small intestine mucosal epithelium, but also that the Paneth cell of the intestine may be a key component of the host's response to the intestinal microbial milieu.

2. MATERIALS AND METHODS

Reagents and general methodology for cloning, sequencing and PCR amplification were described [15]. The oligonucleotides HSIB262s (GCACTGTCATGGGTATTAACCACAGATTCTGCTGCCTCTGAGGGATGA) and HSIB309a (TCATCCCTCAGAGGCAGCAGAATCTGTGGTTAATACCCATGACAGTGC) were used as sense and antisense hybridization probes, respectively, under the same experimental conditions as described [15]. For Southern blot analysis the cDNA probe was constructed by a *Hin*dIII digestion of subclone pSI14 at nucleotide +93 and also in the multiple cloning site of the Bluescript SK vector. The product was then re-ligated yielding subclone pSI14-3′*Hin*dIII. The final insert product was then isolated and radioactively labelled using standard protocols as described [15].

3. RESULTS

A human small intestine complementary DNA (cDNA) library was screened with a 43 base oligonucleotide probe complementary to the conserved sequence at the 5′ end of the known human defensin mRNAs [15]. At least two classes of clones were evident based on hybridization analysis. One class, which encodes HD-5, has been described elsewhere [15]. Two independent clones of a second class were subcloned and sequenced in their entirety. The composite sequence is shown in Fig. 1. Clone pSI14 extended 10 nucleotides more 5′ than clone pSI30 to base −18 (relative to the putative start codon ATG numbered as +1, +2, +3 respectively) and ends at nucleotide +422. The overlapping sequences were identical except pSI30 had a single base difference in the 3′ untranslated region at nucleotide +372, where thymine is substituted for a cytosine. The 5′ most start codon is in an appropriate context for translation initiation [20] and an open reading frame continues for 100 codons. A polyadenylation signal is present 11 nucleotides from the polyadenylated tail [21]. The putative prepropeptide deduced from the single open reading frame has all of the structural features common to the numerous preprodefensins described to date [15,22–25], and we refer to this sequence as human defensin 6 (HD-6). Database searches yielded no significant sequence similarity other than to known defensins.

A Northern blot of 10 μg total RNA from human small intestine was hybridized to an antisense oligonucleotide probe from a unique region of the 3′ end of HD-6 and is shown in Fig. 2. An abundant message of approximately 450 bases is detected. In a separate experiment, Northern blot analysis using 2 μg of polyadenylated RNA from human pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart was negative upon 10 day exposure for all tissues under the same conditions (data not shown; subsequent hybridization to this blot with an actin probe demonstrated intact RNA for all tissues).

An antisense primer from nucleotides +397 to +367 and an upstream sense primer from nucleotides +82 to +101 were used for PCR analysis of cDNA from several tissues to determine the distribution of HD-6 mRNA (Fig. 3A). The small intestine lane shows a major PCR product of 0.3 kb in size as expected based on the selected primers and the cDNA sequence (Fig. 1). Al-

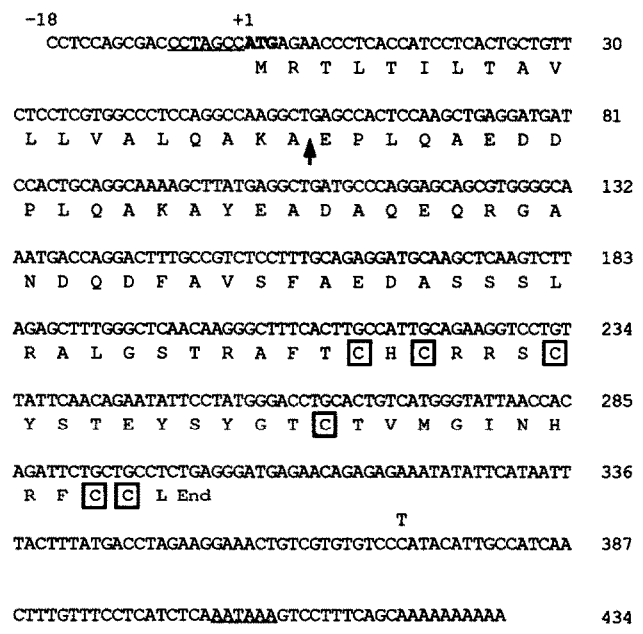

Fig. 1. Nucleotide sequence of HD-6 cDNA. The sequence presented is a composite of two overlapping cDNA clones, pSI14 and pSI30. Both clones were sequenced from both DNA strands in their entirety. The consensus sequences for translation start [20] and polyadenylation addition [21] are underlined. The putative initiating methionine codon (bold) is assigned +1 to +3. Conserved cysteine residues characteristic of defensins are boxed [3]. The arrow indicates the predicted eucaryotic signal sequence cleavage site [26]. The deduced amino acid sequence of the open reading frame is indicated in single letter code. The single letter abbreviations for the amino acids are: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. pSI14 started at the 5′ most nucleotide and ended at base +422. pSI30 which started at base −8 and included a polyadenylated tail, had a single base difference at nucleotide +372 where the base thymine is substituted for a cytosine. The Genbank accession number for the HD-6 sequence is M98331.

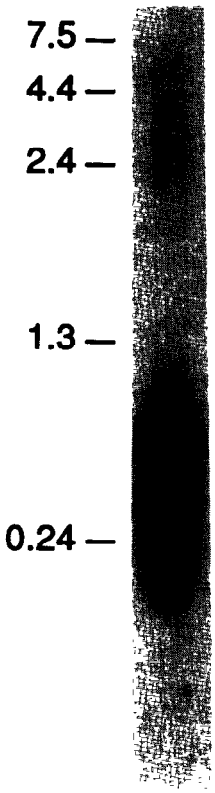

Fig. 2. Northern blot hybridization of human small intestine RNA with a HD-6 probe. Total RNA (10 μg) from adult human small intestine was size fractionated in a standard formaldehyde/agarose gel, capillary blotted to a nylon membrane and hybridized with a HD-6 oligonucleotide probe (HSIB309a). Hybridization conditions were 50% formamide/5 × SSC/5 × Denhardt's (1 × Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin in aqueous solution)/1% SDS (sodium dodecyl sulfate) at 37°C overnight and the highest stringency wash conditions were 1 × SSC at 55°C for 30 minutes.

though lung, mammary gland, and salivary gland show a trace product of similar size, hybridization experiments indicate that only the small intestine and genomic PCR products are HD-6 related sequences (data not shown). A product of approximately 1100 base pairs is seen in the human genomic lane (Fig. 3A). This result suggests the presence of an intron of about 800 base pairs located between the oligonucleotide primers, a result expected if the HD-6 gene is similar in structure to the HD-5 gene [15]. A similar PCR analysis of these same samples using oligonucleotide primers from cDNA sequences common to the hematopoietic defensins, human neutrophil antimicrobial peptide-1 (HNP-1) and HNP-3 [22], yields a product from the bone marrow cDNA and genomic DNA (Fig. 3B). This result is consistent with the known site of hematopoietic defensin mRNA expression [22] and highlights the tissue specificity for individual members of the defensin family. A control amplification experiment using alpha tubulin primers demonstrates the presence of adequate cDNA template in all samples (Fig. 3C).

The same antisense oligonucleotide probe that was used in the Northern blot analysis was used for in situ hybridization experiments to identify the cell types within the small intestine that express HD-6 mRNA. A strong positive signal is seen in cells at the base of the crypts in sections of human small intestine (Fig. 4A and B, arrows). The experiment clearly shows that the message is localized to the Paneth cells. Serial sections hybridized with an HD-5 antisense oligonucleotide probe [15] show signal in the same cells, and the amount of mRNA detected by this technique is roughly equivalent for the two defensin probes (unpublished observations). Treatment of a serial tissue section with ribonuclease prior to probe hybridization eliminates the positive signal (Fig. 4C). No signal is detected with a complementary sense strand probe (Fig. 4D). These two control experiments demonstrate that the signal detected with

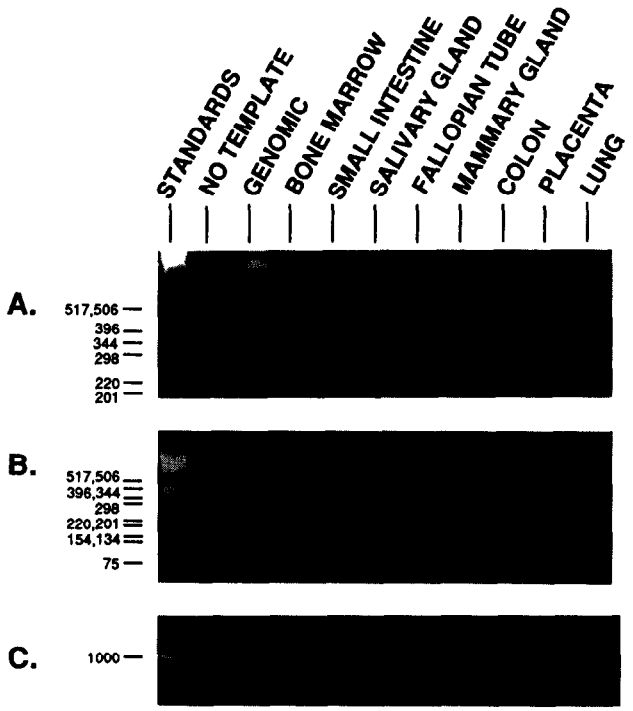

Fig. 3. PCR analysis of tissue expression of HD-6. Pools of lambda phage cDNA from indicated human tissue for use as PCR templates were prepared as described [15]. Human genomic DNA was used as a control template. Amplification products were size fractionated in a 3% agarose gel and visualized by ethidium bromide staining. (A) PCR amplification using HD-6 primers. A downstream antisense primer spanning bases +397 to +367 (HSIB387a: TTGATGGCAATGTATGGGAC) and an upstream sense primer spanning bases +82 to +101 (HSIB082s: CCACTGCAGGCAAAAGCTTA) were used in a standard amplification protocol [15]. (B) PCR amplification using oligonucleotide primers common to HNP-1 and HNP-3 [22]. An antisense primer spanning bases +388 to +365 (HNP367a: TTCCCTGTAGCTCTCAAAGCAAAT) and a sense primer spanning bases +18 to +38 (HNP19s: CCCTGCCTAGCTAGAGGATTT) were used in an amplification as in (A). (C) Control PCR amplification with primers for human tubulin as previously described [15].

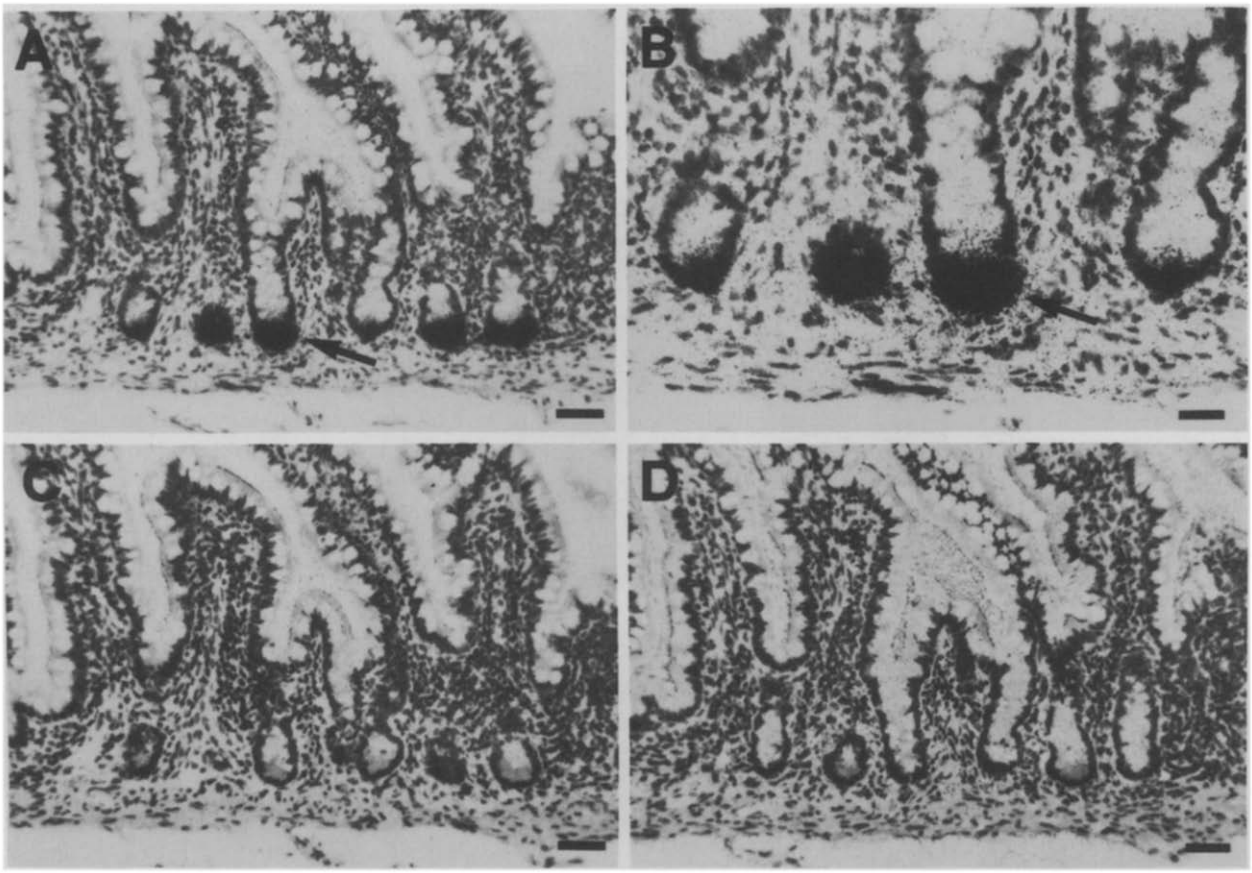

Fig. 4. Detection of HD-6 mRNA in the Paneth cells of the small intestine by in situ hybridization. Paraffin embedded sections of adult ileum were hybridized with HD-6 oligonucleotide probes end-labelled with [^{35}S]dATP, washed under high stringency, exposed to photographic emulsion and then stained with hematoxylin and eosin as previously described [15]. (A) Low power view of sections hybridized with a HD-6 antisense oligonucleotide probe (HSIB309a). (B) High power view of same section. (C) Low power view of a serial section treated with 10 μg/ml of RNase A for 15 min at room temperature prior to hybridization with HSIB309a as in (A). (D) Low power view of a serial section hybridized with a control sense strand oligonucleotide probe (HSIB261s). The arrows in (A) and (B) indicate examples of positive signal at the base of a crypt. The bar in (A), (C) and (D) equals 50 μm; in (B) it equals 25 μm.

the antisense probe is due to the presence of specific RNA in these cells.

A Southern blot of human genomic DNA digested with four restriction enzymes was hybridized with a cDNA probe from the 3′ region of the HD-6 cDNA (Fig. 5). A single band is present in all lanes. This result is consistent with the presence of a single copy of the HD-6 gene in the human genome. The single nucleotide difference in the 3′ untranslated region between pSI14 and pSI30 (Fig. 1) is therefore most probably a sequence polymorphism.

4. DISCUSSION

In this paper we describe HD-6, a new member of the human defensin family. The cDNA clone encoding this defensin was identified within a human small intestine library by its conserved 5′ nucleotide sequence common to previously characterized defensin cDNA's. Northern blot analysis (Fig. 2) demonstrates the presence of abundant HD-6 mRNA in the human small intestine, and PCR analysis (Fig. 3) indicates that the tissue expression of the mRNA is specific for the small intestine. In situ hybridization (Fig. 4) shows that the abundant mRNA localizes to the Paneth cells at the base of the crypts of Lieberkühn. Southern blot analysis (Fig. 5) indicates that HD-6 is present as a single copy gene in the human genome. This is the second human small intestinal defensin message detected in the Paneth cell. The other, HD-5, was previously reported by our group [15].

The primary structure of the prepropeptide derived from the HD-6 cDNA sequence shows similarity to the previously described defensins. The putative prepropeptide has a highly conserved sequence at the amino-terminus compared with previously described defensins [15,22–25], followed immediately by a eucaryotic signal sequence cleavage site [26] between amino acids 19 and 20 (Fig. 1, arrow). There is a six cysteine motif at the carboxyl-terminus, characteristic of the mature defensin

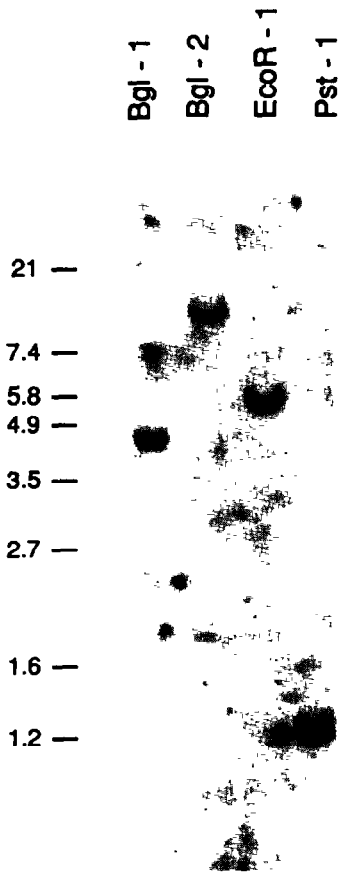

Fig. 5. Southern blot hybridization of human genomic DNA with a HD-6 probe (pSI14–3′*Hin*dIII). Genomic DNA was digested with the indicated restriction enzymes, size fractionated on a 1% agarose gel and then capillary blotted to a nylon membrane. The blot was hybridized with the probe in 50% formamide/5 × SSC/1 × Denhardt's/1% SDS at 42°C overnight and the high stringency wash conditions were 0.1 × SSC at 65°C for 30 min.

peptides (Fig. 6). The amino-terminus of the putative mature HD-5 and HD-6 cannot be predicted with certainty. Therefore, several residues of the putative precursor molecules immediately adjacent to the first cysteine are shown in lower case notation (Fig. 6). HD-6 has a single amino acid omission between the second and third cysteine residues. Variation in defensin structure between these two cysteines has been previously noted in rabbit NP-3b, which has an additional amino acid in this region [27]. The conservation of the cysteines and other residues found in mature defensins, including arginine-6, glutamic acid-14, glycine-18, and glycine-24, suggest that HD-6 has a similar tertiary structure as the hematopoietic human defensin HNP-3 as described by Hill et al. [28]. These structural features therefore suggest that HD-6 has similar bioactivity as previously characterized defensins [3]. This supposition then supports the probable role of HD-6 as an antimicrobial factor in the small intestine. However, the HD-6 peptide itself has yet to be isolated and its activity tested. Although the size of the putative mature HD-6 peptide would make chemical synthesis tenable, the presence of six potential half-cystines and the uncertainty of the amino-terminus renders chemical synthesis beyond the scope of this work.

Defensins belong to an expanding group of antimicrobial peptide families [4,5,29]. Members of several of these families have been identified in vertebrate gastrointestinal [6–10,14] and respiratory systems [16,30]. The finding of antimicrobial peptides associated with non-hematopoietic tissues alludes to a widespread mechanism of peptide-based mucosal defense. The detection of enteric defensins in the mouse Paneth cells [9,10,14,24], together with the detection of abundant mRNA encoding the putative enteric defensins HD-5 [15] and now HD-6 in human Paneth cells, indicates that these specialized epithelial cells play a key role in host defense of the bowel.

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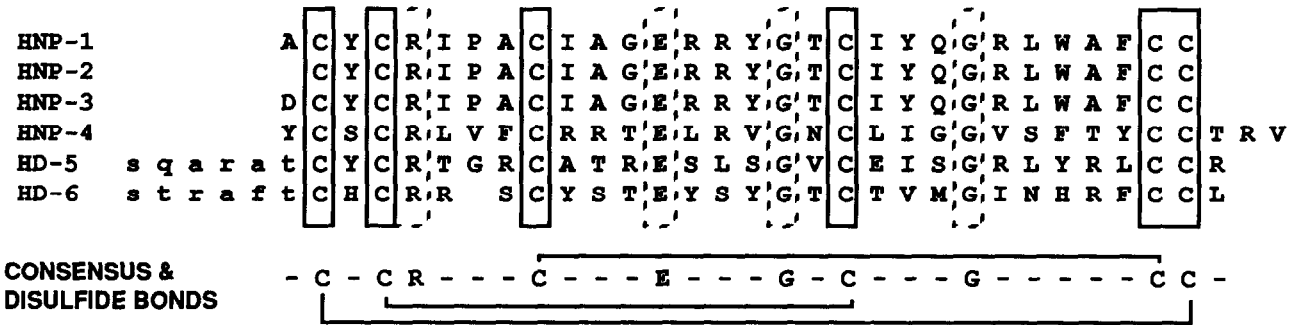

Fig. 6. A comparison of deduced human small intestinal defensin sequences with primary structure of isolated human defensin peptides. The HNP-1 through −4 peptide sequences and the HD-5 deduced sequences were previously reported [15,31,32]. Solid boxes highlight the six conserved cysteine residues and broken boxes highlight four other conserved amino acids: arginine-6, glutamic acid-14, glycine-18, and glycine-24 (based on the numbering scheme by Hill et al. [28]). The amino-terminal amino acid of the putative mature peptides HD-5 and HD-6 are not known and six adjacent residues of the precursor are shown in lower case notation to emphasize this fact. HD-6 is shown with a gap between the second and third cysteine residues to maximize alignment.

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NOTE ADDED IN PROOF

Reference 15 (Jones, D.E. and Bevins, C.L., in press) has now been published: Jones, D.E. and Bevins, C.L. (1992) J. Biol. Chem. 267, 23216–23225.

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