Inheritance of Unequal Numbers of the Genes Encoding the Human Neutrophil Defensins HP-1 and HP-3*

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It is unclear whether the six known human defensin peptides are all encoded by separate genes or whether some of them are allelic. Three of the peptides, HP-1, HP-2, and HP-3, differ by only one amino acid, and it is thought that HP-2 may represent a proteolytic product of HP-1 and/or HP-3. To help determine the relationship of these three proteins, we isolated a nearly full-length cDNA encoding HP-1 with a sequence very similar to, but different from, the previously isolated HP-1 and -3 cDNAs. Gene copy number experiments established that there were at least two but fewer than five defensin genes with a high level of similarity to the HP-1 cDNA (HP-1/3-like). Three genomic clones were isolated that contained two different configurations of the HP-1/3-like sequences. Sequencing established that one encoded the HP-1 peptide, whereas the other encoded HP-3. Analysis of DNAs obtained from 18 unrelated individuals by Southern blot analysis revealed the expected fragments as well as additional fragments that were not present in the genomic clones. This suggested the possibility of alleles; however, when DNAs from families were examined, these fragments did not segregate in an obvious Mendelian fashion. The HP-1/3-like defensin genes are on human chromosome 8. Surprisingly, somatic cell hybrid mapping showed that the number of HP-1/3-like genes on isolated copies of chromosome 8 was variable. We conclude that individuals can inherit versions of chromosome 8 harboring either two or three copies of the genes that encode the HP-1, HP-2, and/or HP-3 peptides.

Corticostatins, defensins, and cryptdins are members of an unusual family of peptides that have been isolated from the alveolar macrophages, neutrophils, bone marrow leukocytes, and/or intestinal Paneth cells of rabbits, guinea pigs, mice, rats, and/or humans (1–13). These proteins are small, often cationic peptides (29–35 amino acids) with a characteristic cysteine-rich structure and were originally isolated because of their anti-adrenocorticotropic hormone (corticostatic) (6, 7) or antimicrobial (defensin) activities (1–4). Subsequently, sequencing analysis of a cDNA clone revealed that cryptdin, from mouse intestinal Paneth cells, is a family member (13). Recently, Paneth cell sequences of human origin have also been identified (14, 15). Presently, the family consists of six human, six rabbit, seven guinea pig, and six mouse sequences.

Three of the human peptides, HP-1, HP-2, and HP-3, are almost identical in sequence. HP-1 and HP-3 are 30 amino acids long and differ only at the first amino acid, which is Ala in HP-1 and Asp in HP-3. HP-2 is 29 amino acids in length and may simply be a proteolytic product of one or both of the other two peptides as it begins with the second amino acid (Cys) of HP-1 and HP-3. In contrast, sequences of HP-4, HP-5, and HP-6 are very different, sharing only 10 amino acids with each other and the other HP peptides.

The functions of the human peptides have been studied in some detail. At higher concentrations (25–200 μg/ml range), HP-1, HP-2, HP-3, and HP-4 exhibit varying degrees of antimicrobial and/or viridical properties in vitro (2, 17, 18). In general, HP-4 seems to have the greatest defensin activity (19), whereas HP-3 has the least (2), being completely inert in assays against Candida albicans (18). At lower concentrations (1–25 μg/ml range), a mixture of HP-1, HP-2, and HP-3 affects mammalian cell growth in that it is cytotoxic against selected cell lines and promotes the growth of others (7, 20–22). Curiously, HP-1 and HP-2 are chemotactic for monocytes, whereas HP-3 is not (23). All three proteins can inhibit protein kinase C in vitro, with HP-2 displaying the greatest inhibitory effect (24). HP-4 has corticostatic activity, whereas HP-1, HP-2, and HP-3 do not (7, 20). As HP-5 and HP-6 are known only from their DNA sequences (15, 16), their functions are unknown; however, it is likely that these peptides have defensin-like activities, similar to their mouse counterparts (10, 12).

The probable tertiary structure of the peptides has been determined (25). They are considered to be cyclic as the first and last cysteines are linked (26). In addition, they form dimers with charged and hydrophobic molecules clustered on two different faces of the molecule, rendering them amphiphilic. Under optimal conditions, HP-1 can permeabilize the outer and inner membranes of Escherichia coli (27), forming voltage-gated ion channels (28). Furthermore, the peptides do not display viridical activity against viruses without envelopes (17), suggesting that membrane interaction is necessary for defensin-like function. The corticostatin activity of the rabbit peptides occurs via receptor interaction (29, 30), so it is likely that all of the peptides in this family exert their functions via some type of membrane interaction.

Although much is known about the function and structure of these peptides, relatively little is known about them at the DNA and RNA levels. cDNAs encoding HP-1, HP-3, HP-4, HP-5, and HP-6 have been isolated (15, 16, 31–34). The two cDNA sequences encoding HP-1 and HP-3 are almost identical.

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differing by only three nucleotides; whereas, the cDNA encoding HP-4 shares a 72% identity with the HP-1 and HP-3 cDNAs. The cDNA encoding HP-6 is also very similar to the sequences encoding HP-1, HP-3, and HP-4 in certain regions. For example, nucleotides 9 to +81 of the HP-6 sequence (16) are 84% identical with nucleotides +85 to +174 of the HP-1 sequence (31). Genes encoding HP-1, HP-3, and HP-5 have also been sequenced (15, 35), as have two of the rabbit genes (encoding MCP-1 and MCP-2) (36). Like HP-1, HP-2, and HP-3, the MCP-1 and MCP-2 peptides differ by a single amino acid. Although one might expect that the two rabbit genes are simply alleles of each other, analysis of their gene structure revealed that this is not the case. In contrast, two rat peptides that differ by a single amino acid are apparently allelic (8). Although two genomic sequences encoding the HP-1 and HP-3 peptides have been identified (35), it is unclear whether they represent two different genes or two alleles of the same gene.

We originally initiated these studies to determine whether the HP-1, HP-2, and HP-3 peptides are alleles or are encoded by different genes. Gene copy number experiments were performed that determined there is more than one gene per haploid genome. Genomic clones from a lambda phage library were isolated and characterized by restriction digest mapping. The restriction map analysis indicated that two clones were similar to the previously published genomic sequences encoding HP-1 and HP-3 (35), but a third clone was different. Sequencing determined that the former clones encoded HP-1, whereas the third encoded HP-3. To ascertain whether two different alleles or two different genes had been isolated, we performed Southern blot analyses on genomic DNAs from unrelated individuals as well as from families. Novel fragments, which were additional to the ones predicted from the genomic maps, were present in DNAs from unrelated individuals, confirming that there are multiple HP-1/3-like genes. Surprisingly, when DNAs from grandparents, parents, and children within the same family were examined, we discovered that the genes did not segregate in an obvious Mendelian fashion. To determine which fragments corresponded to which gene, DNAs from somatic cell hybrids containing single human chromosome 8s were examined. This analysis determined that the numbers of HP-1/3-like genes on the different chromosome 8s varied. Thus, individuals can inherit unequal numbers of these genes, depending upon which chromosomes they inherit from their parents.

MATERIALS AND METHODS

Genomic DNA Isolation and Southern Blot Analysis—DNA was isolated from human peripheral blood lymphocytes or lymphoblastoid cell lines. DNA samples were digested with restriction endonucleases, electrophoresed through agarose gels, transferred to nitrocellulose or nylon membranes, and hybridized to radiolabeled DNA probes as described previously (37).

Screening of a Myeloid cDNA Library—A cDNA library was prepared from leukocyte poly(A+)-RNA isolated from a patient with acute myelogenous leukemia and cloned into λgt11 (38). cDNA clones were selected from this library by screening with pC-A3, a clone containing the 3′-HP-1 sequence, and inserts were subcloned into a pUC vector for further analysis. The pC-A3 sequence encodes the mature peptide.

Nucleotide Sequencing—The nucleotide sequences were determined by the dideoxy chain termination method of Sanger et al. (39). Both strands from overlapping regions were sequenced. For the genomic clones, the region from EcoRI to BamHI was subcloned into pUC18, and only the region encoding exon 3 was sequenced. The synthetic primer ACTGCTAATCTCATCTCAA was used in one direction, and the appropriate pUC primer was used in the other.

Gene Copy Number Determination—Using a previously described protocol (40), we digested genomic DNA with EcoRI, electrophoresed it through a 1% agarose gel, and transferred it to nitrocellulose. BamHI-linearized pC-A3 DNA corresponding to one, two, and five copies of HP-1 per haploid genome were electrophoresed and transferred with the genomic digests. The filters were prepared as described above and probed with a 32P-labeled subclone of pC-A3 in an M13 vector that does not cross-hybridize with pBR322. Hybridization and washes were performed using stringent conditions (hybridization was at 68 °C in 0.1× NaCl, 0.009 M Na3 citrate, 0.04% each of Ficoll-400, polyvinylpyrrolidone, and bovine serum albumin, 1 m EDTA, and 0.05% SDS, pH 7.0; washes were at 68 °C for 1 h each in 0.1× NaCl, 0.015 M Na3 citrate, 0.5% SDS, pH 7.0). The resulting autoradiographs were analyzed on a Molecular Dynamics Bioimage scanner.

RESULTS

Isolation of a Full-length HP-1 cDNA—To facilitate studies of the HP-1/3-like gene structure(s), we decided to isolate a full-length cDNA encoding an HP-1 (or HP-3) peptide. Previously, we had determined the HP-1 sequence by analyzing overlapping cDNAs (pH-E2 and pC-A3, containing sequences from the HP-1 5′- and 3′-ends, respectively) (31). An identical HP-1 cDNA sequence was also determined independently using overlapping sequences (32); however, since the HP-1 and HP-3 cDNAs are extremely similar, isolation of a full-length cDNA seemed essential to avoid potential problems. A cDNA library made from a patient with acute myelogenous leukemia (38) was screened using pC-A3. A new clone was selected, subcloned (pUC4A), and sequenced (Fig. 1). Comparison of pUC4A with the previously reported HP-1 and HP-3 cDNA sequences (31–33) showed that pUC4A encodes the HP-1 peptide (Table I). Although it is nearly identical to the previously reported sequences (Table I), it differs from the earlier HP-1 and HP-3 cDNAs.

Gene Copy Number Determination—As a first step toward determining whether HP-1, HP-2, and HP-3 represent alleles of a single peptide or are peptides encoded by separate genes, we performed gene copy number experiments (40). Genomic DNAs from several different individuals were digested and compared with titrated quantities of a standard representing the HP-1/3-like genes. Densitometric analyses of the autoradiograms indicated the presence of at least two and less than five HP-1/3-like defensin genes per haploid genome (see Fig. 2), with a copy number close to two.
Isolation of HP-1/3-like Genes—To further characterize the genes encoding these peptides, genomic clones were isolated from a human leukocyte DNA library by using pC-A3 as a probe. Three different recombinant phage were isolated and mapped by restriction enzyme analyses. Two of the clones contained overlapping regions of DNA spanning approximately 17 kb with a restriction map that was consistent with the previously published HP-1 and HP-3 genomic sequences (35), with the exception of an additional BglII site between exons I and II. Sequencing indicated these clones encoded the HP-1 peptide (not shown). The third clone was very similar but distinct and contained 16 kb of DNA. Sequencing revealed that this clone encoded the HP-3 peptide (not shown). In all three phages, the gene itself was confined to a region less than 3 kb (Fig. 3).

Detection of Multiple Genomic Sequences with Similarity to the 5'-End of HP-1—To confirm the gene copy number data and to determine whether the HP-1 and HP-3 sequences represented different alleles or different genes, we digested DNA samples from 18 unrelated individuals with BamHI, EcoRI, and HindIII, subjected the samples to Southern blot analysis, and hybridized the filters with radiolabeled pC-A3. The patterns obtained using all three enzymes were consistent with the genomic clone data and suggested that either pC-A3 hybridized to sequences from a single gene or, as the gene copy number experiment suggested, there were two or more HP-1/3-like genes with indistinguishable restriction fragment patterns using these experimental conditions. When DNAs were digested with BglII and probed with pUC4A, containing the entire HP-1 cDNA sequence, multiple bands were detected in addition to those that could be explained by the maps of our genomic clones (data not shown). Thus, we determined that the 5'-cDNA sequences present in pUC4A hybridize to a minimum of two genes; however, it was still unclear whether these were all HP-1/3-like genes or whether there was cross-hybridization with the related HP-4, HP-5, and HP-6 sequences (15, 16, 34).

HP-1/3-like Alleles Do Not Segregate in an Obvious Mendelian Fashion. To simplify our analysis, we decided to probe with a genomic fragment rather than a cDNA. Since the genomic clone restriction mapping indicated that the 5' regions of the genes were different, a 1.6-kb EcoRI fragment from the genomic clone representing the HP-1 gene (Fig. 3) was subcloned into a pUC vector for further analysis. Genomic DNAs from unrelated individuals were digested with either Asp718 or BamHI and subjected to Southern blot analyses (Fig. 4). In accordance with the genomic clone data, a single, intensely hybridizing band was detected in the BamHI digests of DNAs from nine unrelated individuals, consistent with the interpretation that HP-1 and HP-3 are either alleles or they represent two (or more) more highly related genes. In contrast, either one or three bands were noted in the Asp718 digests. The sizes suggested that the two lower bands might be the products of the upper band, i.e. the EcoRI fragment was detecting a polymorphism, which would indicate that HP-1 and HP-3 were alleles rather than separate genes.

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**Table I**

| cDNA         | Nucleotide position | Reference |
|--------------|---------------------|-----------|
| pC-A3/pH-E2  | 22                  | 31        |
| HNP1A/HNP1B  | 287                 | 32        |
| pUC4A        | 444                 | This work |
| HNP-3        | 476                 | 32        |
| pCG14        | 492                 | 33        |

* Nucleotide positions are based upon the previously reported numbering system (31), with a "—" indicating that the reported sequence did not extend that far.
* cDNAs with C in this position encode HP-1, whereas those with A encode HP-3.
Unequal Numbers of HP-1/3-like Genes—The genes encoding the neutrophil defensins have been mapped to human chromosome 8 (31, 44). Therefore, to help determine the source of the variation in this gene family, we obtained multiple DNA samples from somatic cell hybrids that were prepared using cells from a single individual and that contained either one or both copies of chromosome 8 (45). The hybrid DNAs were digested with BglI, and Southern blot analysis was performed using the 1.6-kb EcoRI genomic fragment from the 5’-end of the gene, we were unable to detect alleles that segregated in a typical Mendelian fashion (data not shown).

To determine whether the Asp718 bands were indeed alleles, 23 DNAs from a three-generation pedigree were examined by digesting with Asp718 and probing with the pUC4A cDNA. Surprisingly, using this approach we were able to show that the two smaller Asp718 bands were not the products of the larger band, as many family members had only one, rather than both, of the two smaller bands (Fig. 5). Furthermore, the combined data suggested that our probe was probably detecting two (or more) highly related genes with alleles that were comigrating and therefore cohybridizing. An alternative but less likely explanation of the data was that at the DNA level we were detecting a gene (or genes) that did not have a corresponding allele (or alleles) on the sister chromosome. To help resolve the situation, additional experiments were performed using BglI digests of DNAs from several smaller pedigrees. Again, after probing with the 1.6-kb EcoRI genomic fragment from the 5’-end of the gene, we were unable to detect alleles that segregated in a typical Mendelian fashion (data not shown).

Fig. 4. Southern blot analysis of the 5’-end of the HP-1/3-like genes in genomic DNAs from unrelated individuals. Genomic DNAs from nine unrelated individuals were digested with Asp718 or BamHI and subjected to Southern blot analysis. To maximize resolution, field inversion gel electrophoresis was used to separate the DNA fragments. The probe was the 1.6-kb EcoRI fragment from the 5’-end of the HP-1 gene (see Fig. 3).

FIG. 4. Southern blot analysis of the 5’-end of the HP-1/3-like genes in genomic DNAs from unrelated individuals. Genomic DNAs from nine unrelated individuals were digested with Asp718 or BamHI and subjected to Southern blot analysis. To maximize resolution, field inversion gel electrophoresis was used to separate the DNA fragments. The probe was the 1.6-kb EcoRI fragment from the 5’-end of the HP-1 gene (see Fig. 3).

Fig. 5. Southern blot analysis of family DNAs. Genomic DNAs from the mother, father, and three of their offspring were digested with Asp718 and subjected to Southern blot analysis. The filter was probed with pUC4A, representing the HP-1 cDNA (see Fig. 1). Lanes 1 and 2 contain the parental DNAs, whereas lanes 3-5 contain the children’s DNAs.

Fig. 6. Southern blot analysis of DNAs from somatic cell hybrids containing individual chromosome 8s. DNAs from somatic cell hybrids containing either one or two human chromosome 8s were digested with BglI and subjected to Southern blot analysis using a 1.6-kb EcoRI fragment from the HP-1 gene (see Fig. 3). This probe does not cross-react with hamster DNA. Hybrids in lanes 1-4, 8-10, 12, and 13 contain the same copy of chromosome 8 from a single individual, whereas the hybrids in lanes 5, 6, and 14-16 contain the other chromosome 8 from a different individual. The hybrid in lane 7 contains both copies of chromosome 8 from that individual, whereas the hybrid in lane 11 contains a single chromosome 8 from a different individual. Lanes 1-16 correspond to hybrids 1HL3, 1HL26, 1HL27, 1HL33, 1HL35, 1HL19, 1HL30, 1SHL1, 1SHL12, 1SHL15, 1HL12, 1SHL3, 1SHL27, 1SHL7, 1SHL30, and 1SHL32, respectively (45).

Unequal Numbers of HP-1/3-like Genes—The genes encoding the neutrophil defensins have been mapped to human chromosome 8 (31, 44). Therefore, to help determine the source of the variation in this gene family, we obtained multiple DNA samples from somatic cell hybrids that were prepared using cells from a single individual and that contained either one or both copies of chromosome 8 (45). The hybrid DNAs were digested with BglI, and Southern blot analysis was performed using the 1.6-kb EcoRI fragment. To our surprise, unequal numbers of DNA fragments corresponding to the HP-1/3-like genes were detected in the different hybrids. Either two bands (1.7 and 2.3 kb) or three bands (1.2, 2.3, and 2.9 kb) of equal intensity were observed (Fig. 6), depending upon which chromosome 8 was present. This suggested that either two genes or three genes, respectively, were present on the chromosome that was present in the hybrid. In addition, a hybrid containing a single chromosome 8 from a second individual had a single, strongly hybridizing band. At first it appeared as though this might represent a single gene; however, when the intensity of the signal from this blot was compared with the intensity of a control probe from chromosome 8 (pSW50, which hybridizes to the D8S7 locus (46)) that was rehybridized to the same blot, scanning densitometric analysis showed that this band actually represented two genes (data not shown). As the D8S7 locus is polymorphic, pSW50 also served to confirm the identity of the individual chromosome 8s within the hybrids. It is important to note that the 1.6-kb probe does not cross-hybridize with hamster DNA, so the only bands detected were human in origin. Furthermore, although the hybrids did contain other human chromosomes, there was no correlation of the hybridization pattern with any other human chromosomes.

Our somatic cell hybrid data showed that individuals can inherit copies of chromosome 8 with either two or three highly related loci corresponding to the 5’ regions of the HP-1/3-like genes encoding the neutrophil defensins.
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genes. To confirm that all of these bands actually contained 5'-coding regions in addition to the genomic sequences, the somatic cell hybrid blot was rehybridized with the pUC4A cDNA. As expected, the same bands were detected by pUC4A along with additional bands (data not shown).

**DISCUSSION**

We were interested in determining whether the peptides HP-1, HP-2, and HP-3 originate from a single gene or alternatively, whether they represent the products of almost identical, but separate genes. By a combination of studies such as gene copy number experiments and Southern blot analysis, we showed that multiple HP-1/3-like genes exist; however, using this approach we were still unable to distinguish whether the HP-1 and HP-3 peptides arose from separate genes or whether they were allelic variants of a single gene. We reasoned that by analyzing DNAs from somatic cell hybrids containing opposing copies of chromosome 8 from a single individual, we would be able to compare the band patterns on the individual chromosomes and distinguish between the two possibilities. To our surprise, we discovered that the chromosomes harbored unequal numbers of the HP-1/3-like genes, showing unequal numbers of these genes can be inherited.

Our somatic cell hybrid data demonstrates that the HP-1 and HP-3 sequences we isolated represent two genes rather than two alleles. The 1.2- and 2.9-kb BglI bands in the somatic cell hybrid DNAs (see Fig. 6), found on a single chromosome 8, correspond with the expected HP-1 and HP-3 fragments, respectively, as seen in Fig. 3. The 2.3-kb band, which was present in all the somatic cell hybrid DNAs, corresponds with the expected band from the published HP-1/HP-3 gene sequence (35) and spans the BglI site just 5' of exon I to the BglI site inside exon II. In all of the hybrids, we also detected a small (500-600-base pair) BglI fragment with the genomic probe, which did not hybridize with the cDNA (not shown). This fragment represents the end 5'-BglI site depicted in Fig. 3 to the first internal BglI site in HP-1. The most likely origin of the 1.7-kb band detected in the hybrids would be a segment that spans the outer BglI site to the site between exons I and II. It is presently unclear whether the 1.7-kb fragment represents an allele of HP-1 or HP-3 or it encodes a third HP-1/3-like gene (such as HP-2) or pseudogene.

Given the similarities of the HP-4, HP-5, and HP-6 sequences (15, 16, 34) with the HP-1/3-like sequences in the 5'-transcribed regions, it might seem reasonable to suspect that one of the strongly hybridizing bands we detected represents one of these other genes; however, comparison of our data with the published data shows that this is probably not the case. For example, the genomic EcoRI fragment, which we used as a probe, is 5' to the region of shared similarity with HP-5 (15) and is not necessarily present upstream of the HP-5 gene. Still, the EcoRI fragment did detect three other larger, much weaker hybridizing bands in the somatic cell hybrids digested with BglI, which cross-hybridized with the pUC4A cDNA (data not shown) and two smaller (6.0 and 7.0 kb), weaker bands in the BamHI digests (Fig. 4). It is possible that these weaker bands represent minor regions of shared similarity with other genes such as HP-4, HP-5, and HP-6.

Given our inability to demonstrate typical Mendelian inheritance within families combined with our somatic cell hybrid data, we propose that variable numbers of the HP-1/3-like genes are transmitted to individuals within the general population. It is probable that the genes are physically linked on the individual chromosomes, as preliminary data using field inversion gel electrophoresis showed a single hybridizing Eagl band of 300 kb by Southern blot analysis (data not shown). Thus, one would inherit a block of genes (most likely two or three) from each parent, the number being determined by the number on the parental chromosome 8. In agreement with our hypothesis is the finding that approximately 10% of the population lacks the HP-3 peptide (47). Interestingly, the 4.0-kb Asp718 band we observed was present in DNAs from 16 of 18 unrelated individuals (89%). This Asp718 fragment was present in our complete HP-3 genomic clone (data not shown), which had the 2.9-kb BglI fragment. As the 2.9-kb BglI fragment was a third HP-1/3-like fragment in the somatic cell hybrid, it is intriguing to speculate that this configuration of the HP-3 gene encodes the HP-3 protein present in 90% of the population. In fact, although this was the fragment that did not display the hypothesized Mendelian inheritance when we examined our three-generation pedigree (Fig. 5), analysis of the familial data shows proper segregation when this allele is used as a haplo-type marker. Still, there is a problem with our hypothesis since a second genomic sequence encoding HP-3 has been reported (35), and its role would be left unexplained.

In summary, we detected a second cDNA sequence (pUC4A) that encodes the HP-1 peptide and showed that the numbers of the defensin HP-1/3-like genes vary from individual to individual. Furthermore, we proved that HP-1 and HP-3 are encoded by two different genes on the same chromosome. At present, no sequence has been detected that is known to specifically encode HP-2, and HP-2 may be a proteolytic product of the HP-1 or HP-3 peptides (or both). One interesting possibility is that one of the mRNAs thought to encode HP-1 may actually encode HP-2. For example, subtle differences in the mRNAs might be indicative of cell-specific expression. The final, processed peptide would then reflect the parental cell type in which it was originally transcribed. It is not unrealistic to believe that a specific HP-2 gene exists since we have shown that there can be three configurations of the HP-1/3-like genes on a single chromosome. Further studies will be necessary to elucidate the exact relationship between the genomic structure, expression, and translation of this interesting family of peptides.

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