The Expanded Human Kallikrein Gene Family: Locus Characterization and Molecular Cloning of a New Member, KLK-L3 (KLK9)

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In rodents, kallikreins are encoded by a large multigene family but in humans, only three kallikrein genes were thought to exist. Based on the homology between the human and the rodent kallikrein loci, we defined a 300-kb human kallikrein gene region on chromosome 19q13.3-q13.4. By using linear sequence information, restriction analysis, PCR, and blotting techniques, we were able to construct the first detailed map of the human kallikrein gene locus. Comparative analysis of genes located in this area enabled us to expand the human kallikrein multigene family with some recently identified serine proteases and establish common structural features. We further identified a new kallikrein-like gene, named kallikrein-like gene 3 (KLK-L3; HGMW-approved symbol KLK9). We describe the structural characterization of the KLK-L3 gene, together with its precise chromosomal localization in relation to other kallikreins and its tissue expression pattern and hormonal regulation. © 2000 Academic Press

INTRODUCTION

The progressive development of genetic tools, together with the availability of powerful computer programs and the large amount of sequence information available from the Human Genome Project, has enabled the mapping of several loci responsible for genetic disease or the identification of areas where certain gene families are clustered.

Positional candidate cloning is a new approach for gene discovery that combines the knowledge of map position with the increasingly dense human transcript maps, the available expressed sequence tags (ESTs),²

and the availability of candidate genes mapped to the same region (Ballabio, 1993). This method greatly expedites the search process and is already one of the predominant methods of gene discovery (Collins, 1995).

Serine proteases are a group of protein cleaving enzymes that contain a serine residue in their active site. Kallikreins are a subfamily of serine proteases, originally defined as enzymes cleaving vasoactive peptides (kinins) from kininogen (Schachter, 1980). This enzyme family primarily consists of plasma kallikrein and tissue or glandular kallikreins. Plasma kallikrein is encoded by a single gene that is structurally different from genes encoding tissue kallikreins (Clements, 1997). The tissue or glandular kallikreins are involved in posttranslational modification of many polypeptides and are central to many biological processes (Rittenhouse *et al.*, 1998).

In mouse and rat, the kallikreins are encoded by large multigene families. In the mouse genome, at least 24 genes have been identified (Evans et al., 1987). The rat kallikrein gene family has been estimated to contain 20 members (Ashley and MacDonald, 1985). In humans, the kallikrein gene family locus is on chromosome 19q13.3-q13.4 (Riegman et al., 1989a, 1992; Richards et al., 1991), a region that is syntenic to the mouse kallikrein gene family locus on chromosome 7 (Nadeau et al., 1991). The human kallikrein gene family is thought to have only three members: the tissue (pancreatic-renal) kallikrein (KLK1) (Evans et al., 1988), the human glandular kallikrein (KLK2) (Schedlich et al., 1987), and prostate-specific antigen (PSA or KLK3) (Riegman et al., 1989b; Diamandis, 1998). Several early estimations of the size of this

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² Abbreviations used: KLK, kallikrein; KLK-L, kallikrein-like; PCR, polymerase chain reaction; PSA, prostate-specific antigen;

dNTPS, deoxynucleoside triphosphates; LLNL, Lawrence Livermore National Laboratory; RT, reverse transcription; EST, expressed sequence tag; TLSP, trypsin-like serine protease; HSCCE, human stratum corneum chymotryptic enzyme; NES1, normal epithelial cellspecific 1 gene; BAC, bacterial artificial chromosome; PAC, P1derived artificial chromosome; RACE, rapid amplification of cDNA ends; BCM, Baylor College of Medicine; FISH, fluorescence *in situ* hybridization.

family, using Southern blot analysis, predicted just 3–4 genes (Riegman *et al.*, 1989a; Schedlich *et al.*, 1987; Fukushima *et al.*, 1985; Baker and Shine, 1985). However, a Southern blot analysis using monkey cDNA suggested that it might contain as many as 19 genes (Murray *et al.*, 1990). Recently, a number of novel serine proteases were discovered and found to have significant similarities with the classical kallikrein genes. Also, a fragment of a putative human kallikrein gene family might be larger than originally expected.

Based on mapping of the rodent kallikrein genes and the documented strong conservation between human chromosome 19q13.3-q13.4 and the 17 loci in a 20-cM proximal part of mouse chromosome 7 (Nadeau et al., 1991; Saunders and Seldin, 1990), we identified a candidate genomic region for further analysis of the human kallikrein locus. In this paper, we describe the construction of the first detailed map of the human kallikrein gene locus and provide strong evidence that kallikreins comprise a large multigene family in humans. We further suggest that some of the recently cloned serine proteases can be considered members of this family. Also, we describe the cloning and characterization of a new kallikrein-like gene named kallikrein-like gene 3 (KLK-L3)³ and present its precise chromosomal localization, tissue expression pattern, and hormonal regulation.

MATERIALS AND METHODS

Strategy for new gene discovery. We have obtained sequencing data for approximately 300 kb around chromosome 19q13.3–q13.4, from the Web site of the Lawrence Livermore National Laboratory (LLNL) (http://www-bio.llnl.gov/genome/genome.html). Different computer programs were used for putative new gene prediction, as previously described (Yousef *et al.*, 1999a).

Reverse transcriptase-polymerase chain reaction (RT-PCR) for KLK-L3 cDNA. Total RNA isolated from 26 different human tissues was purchased from Clontech (Palo Alto, CA). cDNA was prepared as described below and used for PCR amplification. A primer set (L3-F1 and L3-R1) was used to identify the presence of the gene in tissues, and the reverse primer (L3-R1) was used with another primer (L3-F2) to amplify and clone the full cDNA of the gene. These primer sequences are shown in Table 1. Tissue cDNAs were amplified at various dilutions so that different levels of expression of various tissues could be assessed semiquantitatively.

Two micrograms of total RNA was reverse-transcribed into firststrand cDNA using the Superscript preamplification system (Gibco BRL, Gaithersburg, MD). The final volume was 20 μ l. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequence (please see Results), two gene-specific primers (L3-F1 and L3-R1) were designed (Table 1), and PCR was carried out in a reaction mixture containing 1 μ l of cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTPs (deoxynucleoside triphosphates), 150 ng of primers, and

Primers Used for RT-PCR Analysis

Gene	Primer name	Sequence ^a
KLK-L3	L3-F1	CATGCAGTGTCTCATCTCAG
	L3-F2	CATGGAGGAGGAAGGAGATG
	L3-R1	CTTCGGCCTCTCTTGGTCTT
PSA	PSAS	TGCGCAAGTTCACCCTCA
	PSAAS	CCCTCTCCTTACTTCATCC
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT
	ACTINAS	TCTCCTTAATGTCACGCACGA

 a All nucleotide sequences are given in the 5' \rightarrow 3' orientation.

2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ) on a Perkin–Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 min, followed by 43 cycles of 94°C for 30 s, 63°C for 1 min, and a final extension at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least two exons to avoid contamination by genomic DNA.

Breast cancer cell line and hormonal stimulation experiments. The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in RPMI medium (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics, and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. Twenty-four hours before the experiments, the culture medium was changed into phenol red-free medium containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture medium, at a final concentration of 10^{-8} M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 h and then harvested for total RNA extraction by the Trizol method (Gibco BRL). cDNA was prepared and amplified as described above. Control genes (PSA, pS2, and actin) were amplified as previously described (Yousef et al., 1999c).

Cloning and sequencing of the PCR products. To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

Identification of positive PAC and BAC genomic clones from human genomic DNA libraries. The PCR product generated with primer set Z1S and Z1AS (Table 2) was purified and then labeled with ³²P by the random primer method (Sambrook et al., 1989) and used as a probe to screen a human genomic DNA BAC library, spotted in duplicate on nylon membranes, for identification of positive clones. The filters were hybridized in 15% formamide, 500 mM Na₂HPO₄, 7% SDS, 1% BSA (w/v) at 65°C overnight, washed sequentially with $2 \times$ SSC, $1 \times$ SSC, $0.2 \times$ SSC, containing 0.1% SDS at 65°C, and then exposed to X-ray film as described (Sambrook et al., 1989). Positive clones were obtained and plated on selective LB medium, and then a single colony was transferred into LB broth for overnight cultures. A PAC clone positive for NES1 was identified by a similar methodology as described elsewhere (Luo et al., 1998). PAC and BAC libraries were constructed by de Jong and associates (see Osoegawa et al., 1999). Purification of BAC and PAC DNA was performed by a rapid alkaline lysis miniprep method, which is a modification of the standard Qiagen-Tip method. Positive clones were further confirmed by Southern blot analysis as described (Sambrook et al., 1989).

5' Rapid amplification of cDNA ends (5' RACE). According to the EST sequences and the computer-predicted structure of the

³ The HGMW-approved symbol for the gene described in this paper is KLK9.

TABLE 2

Primer name	Sequence ^a	Coordinates	GenBank Accession No.	Gene name
Z1S Z1AS	GACCCTGACATGTGACATCTA GCCACTGCCTGATGGAGACTG	979–999 1,422–1,402	U62801	Zyme
GL3-F1 LL3-R1	AACATCAGCATCCTGGAGAA CTTCGGCCTCTCTTGGTCTT	7,324-7,343 8,051-8,060	AF135026	KLK-L3
L2-1 L2-2	GGGTCAGAGCTGCAGAGAAG GGGCCTGTCGTCTGCAATGG	$\begin{array}{c} 11,104{-}11,123\\ 11,522{-}11,541\end{array}$	AF135028	KLK-L2
KLK-L1	ATGGCCACAGCAGGAAATCC GGTCACTTGTCTGCGCAGAC	1,411–1,430 1,990–2,019	AF113141	KLK-L1
PS PAS	CCCAACCCTGTGTTTTTCTC GGCCCTCCTCCCTCAGA	3,634-3,653 4,143-4,118	M33105	PSA^b
K1S K1AS	ATCCCTCCATTCCCATCTTT CACATACAATTCTCTGGTTC	2–22 324–305	M18157	KLK1 ^c
K2S K2AS	AGTGACACTGTCTCAGAATT CCCCAATCTCACCAGTGCAC	$\frac{131 - 150}{580 - 561}$	AF024605	$KLK2^{d}$
NS NAS	GCTTCCCTACCGCTGTGCT CACTCTGGCAAGGGTCCTG	552-570 763-744	AF055481	NES1 ^e

Primers Used for Gene-Specific PCR Amplification of the Kallikrein Genes Using DNA as a Template

^{*a*} All nucleotide sequences are given in the $5' \rightarrow 3'$ orientation.

^b Prostate-specific antigen.

^{*c*} Human renal kallikrein.

^{*d*} Human glandular kallikrein.

^e Normal epithelia cell-specific 1 gene.

KLK-L3 gene, two gene-specific primers were designed. Two rounds of RACE reactions (nested PCR) were performed with 5 μ l Marathon Ready cDNA of human testis (Clontech) as a template. The reaction mix and PCR conditions were selected according to the manufacturer's recommendations. Positive bands were gelpurified using Qiagen Gel Purification kits according to the manufacturer's recommendations.

Gene-specific amplification of other genes from genomic DNA. According to the published sequence of PSA, KLK1, KLK2, normal epithelial cell-specific 1gene (NES1), KLK-L1, KLK-L2 and zyme genes, we designed gene-specific primers for each of these genes (Table 2) and developed polymerase chain reaction (PCR)-based amplification protocols that allowed us to generate specific PCR products with genomic DNA as a template. The PCRs were carried out as described above but using an annealing/extension temperature of 65°C.

Structure analysis studies. Multiple alignment was performed using the Clustal X software package available at ftp://ftp.ebi.ac.uk/pub/ software/dos/clustalw/clustalx/(clustalx1.64b.msw.exe) and the multiple alignment program available from the Baylor College of Medicine (BCM) search launcher (kiwi.imgen.bcm.tmc.edu:8808/searchlauncher/launcher/html). Phylogenetic studies were performed using the Phylip software package available from http://evolution.genetics. washington.edu/phylip/getme.html. Distance matrix analysis was performed using the Neighbor-Joining/UPGMA program, and parsimony analysis was performed using the Prompters program. The hydrophobicity study was performed using the BCM search launcher programs (http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html). Signal peptide was predicted using the SignalP WWW server (http:// www.cbs.dtu.dk/services/signal). Protein structure analysis was performed with the SAPS (structural analysis of protein sequence) program (http://dot.imgen.bcm.tmc.edu:9331/seq-search/strucpredict.html).

RESULTS

Construction of a Contiguous Map of the Human Kallikrein Locus on Chromosome 19q13.3–q13.4

Sequence information around the human chromosome 19q13.3-q13.4 locus (the proposed kallikrein locus) is available at the Lawrence Livermore National Laboratory Web site. We have obtained sequences of approximately 300 kb in length. These sequences were in the form of contigs of different lengths. A restriction analysis study of the contigs was performed using various computer programs. With the aid of the *Eco*RI restriction map of this area, which is also available at the LLNL Web site, we were able to define the relative positions of these contigs in relation to one another. Some contigs were overlapping, enabling us to construct a contiguous segment; however, three gaps were present. BLAST analysis of these segments against the GenBank database (Altschul et al., 1997) enabled us to define the precise location of two classical kallikreins, namely PSA and KLK2. We also localized other newly discovered serine proteases that are homologous with the kallikrein genes, namely protease M/zyme/neurosin (Anisowicz et al., 1996; Little et al., 1997; Yamashiro et al., 1997), human stratum corneum chymotryptic enzyme (HSSCE) (Hansson et al., 1994), neuropsin (Yoshida et al., 1998), normal epithelial cellspecific 1 gene (NES1) (Liu et al., 1996), trypsin-like serine protease (TLSP) (GenBank Accession No.

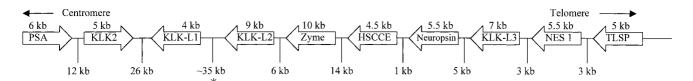


FIG. 1. An approximately 300-kb region of almost contiguous genomic sequence around chromosome 19q13.3–q13.4. Genes are represented by horizontal arrows denoting the direction of the coding sequence. Gene lengths and distances between genes are rounded to the nearest 0.5 kb. The site of the gap is marked with an asterisk. Telomeric to TLSP, there are likely another three kallikrein-like genes (Yousef *et al.*, 1999a; and GenBank Accession No. AF161221) but these are not as yet well characterized. For gene names and more details, see text. Figure is not drawn to scale.

AF164623), KLK-L1 (Yousef *et al.*, 1999c; Nelson *et al.*, 1999) (GenBank Accession No. AF135023), and KLK-L2 (Yousef and Diamandis, 1999) (GenBank Accession No. AF135028). The gaps in the 300-kb genomic sequence were partially filled as follows:

(a) The margins of the first gap were found to contain the 5' and 3' ends of the KLK2 gene; this enabled us to fill this gap with the published genomic structure of the KLK2 gene (GenBank Accession No. M18157).

(b) The margins of the third gap (gaps are numbered from centromere to telomere) were found to have the 3' and 5' ends of the zyme gene mRNA sequence; thus, a radiolabeled probe specific for the zyme gene was used to screen a human BAC library, and two positive clones were obtained. Restriction analysis was performed, followed by Southern blotting, and a fragment containing the zyme gene was obtained and sequenced, thus filling this gap (Yousef *et al.*, 1999b).

(c) The second gap (between KLK-L1 and KLK-L2 genes) still exists, and we used the *Eco*RI restriction map of this area to define its length approximately (Fig. 1).

Further support for the relative locations of these genes was obtained by performing PCRs with genespecific primers to screen genomic DNA clones. The most centromeric group of genes (PSA, KLK2, KLK-L1, KLK-L2, and zyme) were found to be clustered in one genomic BAC clone, and the next group (HSCCE, neuropsin, KLK-L3, and NES1) were found to be clustered together in another clone, as expected from the data of Fig. 1.

Cloning of the KLK-L3 Gene

A putative new gene, formed by three exons, was predicted by computer analysis of the genomic sequence. The predicted exons were subjected to sequence homology search against the human EST database (dbEST) and revealed an EST clone (GenBank Accession No. AA583908) that exhibited 99% identity with our putative gene. This EST was obtained, purified, and sequenced, and the sequence was aligned by BLAST software (Altschul *et al.*, 1997) against the genomic area that contains the putative gene. An additional exon, downstream of our predicted structure, was identified. The 3' end of the gene was verified by (a) the presence of the serine residue (S) of the catalytic triad in a well-conserved region; this highly conserved motif (GDSGGP) always occurs at the beginning of the last exon in all known kallikreins; (b) the presence of a stop codon that is in-frame with the predicted amino acid sequence; and (c) the presence of a 19-poly(A) stretch at the end of the EST that was not found in the genomic sequence.

To verify the accuracy of the cDNA sequence of the gene, PCRs were performed using gene-specific primers for the first and last exons of the predicted structure of the gene (L3-F2 and L3-R1) with cDNA isolated from different human tissues as putative templates. A positive band of the expected size was isolated from testis cDNA and fully sequenced. Its sequence was aligned by BLAST against the genomic sequence to define the exon/intron boundaries unequivocally. For further characterization of the 5' end of the gene, 5'RACE reaction was performed using Marathon Ready cDNA from testis as a template. This allowed us to identify an additional exon that contains the start codon and 5' untranslated region. The full sequence of the gene has now been deposited with GenBank (Accession No. AF135026).

Structural Characterization of the KLK-L3 Gene

As shown in Fig. 2, the KLK-L3 gene is formed of five coding exons and four intervening introns, although, as with other kallikreins, the presence of a further upstream untranslated exon(s) could not be ruled out (Luo *et al.*, 1998; Yoshida *et al.*, 1998; Yousef *et al.*, 1999b). All of the exon/intron splice sites conform to the consensus sequence for eukaryotic splice sites (Iida, 1990). The gene further follows strictly the common structural features of the human kallikrein multigene family, as described below.

The predicted protein-coding region of the gene is formed of 753 bp, encoding a deduced polypeptide with a predicted molecular mass of 27.5 kDa. A potential translation initiation codon is found at position 28 of the predicted first exon (numbers of nucleotides refer to GenBank Accession No. AF135026). This codon does not match well with the consensus Kozak (1987) sequence; however, it has a purine at position (-3), which occurs in 97% of vertebrate mRNAs (Kozak, 1987), and it is almost identical to the sequence of the zyme gene flanking the start codon (Yousef *et al.*,

CACTGGACGGGTGCACGTTCAGGATCCAGGTGCCCAGGGGTC(ATG)AAG CTG GGA CTC к L. G $\begin{array}{c} \textbf{CTC TGT GCT CTG CTC TCT CTG CTG GCA G g i g a ... intron l ... ccag GG CAT GGC L C A L L S L L A \\ \end{array}$ LLSLL TGG GCA GAC ACC CGT GCC ATC GGG GCC GAG GAA TGT CGC CCC AAC TCC CAG E E C R С т R Α I G Α $\begin{array}{c} \text{CCT TGG CAG GCC GGC CTC TTC CAC CTT ACT CGG CTC TTC TGT GGG GCG ACC} \\ \text{P } W & \text{Q} & \text{A} & \text{G} & \text{L} & \text{F} & \text{H} & \text{L} & \text{T} & \text{R} & \text{L} & \text{F} & \text{C} & \text{G} & \text{A} & \text{T} \end{array}$ $\begin{array}{c} \text{CTC ATC AGT GAC CGC TGG CTG CTC ACA GCT GCC CAC TGC CGC AAG CCEtera.....}\\ L & I & S & D & R & W & L & L & T & A & H & C & R & K & P \\ \end{array}$ intron 2gcagG TAT CTG TGG GTC CGC CTT GGA GAG CAC CAC CTC TGG AAA Y L W V R L G E H H L W K TGG GAG GGT CCG GAG CAG CTG TTC CGG GTT ACG GAC TTC TTC CCC CAC CCT W E РE 0 LF R V Т DF Р Н G GGC TTC AAC AAG GAC CTC AGC GCC AAT GAC CAC AAT GAT GAC ATC ATG CTG G F N K D L S A N D H N D \overline{D} I M L $\begin{array}{cccc} \text{ATC CGC CTG CCC AGG CAG GCA CGT CTG AGT CCT GCT GTG CAG CCC CTC AAC \\ \textbf{1} & \textbf{R} & \textbf{L} & \textbf{P} & \textbf{R} & \textbf{Q} & \textbf{A} & \textbf{R} & \textbf{L} & \textbf{S} & \textbf{P} & \textbf{A} & \textbf{V} & \textbf{Q} & \textbf{P} & \textbf{L} & \textbf{N} \end{array}$ CTC AGC CAG ACC TGT GTC TCC CCA GGC ATG CAG TGT CTC ATC TCA GGC TGG V S P G M Q C L I S G acag CG CTG TTT CCA GTC A L F P V GGG GCC GTG TCC AGC CCC AAG Ggtat..... intron S Р S ACA CTG CAG TGT GCC AAC ATC AGC ATC CTG GAG AAC AAA CTC TGT CAC TGG Q C A N I S I LEN KL С Н L GCA TAC CCT GGA CAC ATC TCG GAC AGC ATG CTC TGT GCG GGC CTG TGG GAG I S D S M L С G L W н A GGG GGC CGA GGT TCC TGC CAG gtg a intron ...acag GGT GAC TCT GGG GGC G D GG G G RGSCQ CCC CTG GTT TGC AAT GGA ACC TTG GCA GGC GTG GTG TCT GGG GGT GCT GAG S GTLAGV v G G A E $\begin{array}{cccc} ccc \ TGC \ TGC \ AGA \ CCC \ CGG \ CGC \ CCC \ GCA \ GTC \ TAC \ AGC \ GTA \ TGC \ CAC \ TAC \\ P \ C \ S \ R \ P \ R \ R \ P \ A \ V \ Y \ T \ S \ V \ C \ H \ Y \end{array}$ CTT GAC TGG ATC CAA GAA ATC ATG GAG AAC(TGA) L D W I Q E I M E N GCCCGCGCGCCACGGGGGCACCTTGGAAGACCAAGAGAGGCCCGAAGGGCACGGGGTAGGG

FIG. 2. Genomic organization and partial genomic sequence of the KLK-L3 gene. Intronic sequences are not shown except for the splice junctions. Introns are shown with lowercase letters and exons with uppercase letters. For the full sequence, see GenBank Accession No. AF135026. The start and stop codons are encircled, and the exon–intron junctions are boxed. The translated amino acids of the coding region are shown underneath in single-letter code. The catalytic residues are inside triangles. A putative polyadenylation signal is underlined.

1999b). It should also be noted that most kallikreins do not have the consensus G nucleotide at position (+4).

Nucleotides 6803–6808 (AGTAAA) closely resemble a consensus polyadenylation signal (Proudfood and Brownlee, 1976) and are followed by a stretch of 19 poly(A) nucleotides not found in genomic DNA, after a space of 14 nucleotides. No other potential polyadenylation signals were discernible in the 3' untranslated region, suggesting that the above motif is indeed the polyadenylation signal. The same polyadenylation signal motif was predicted for the KLK1 and KLK2 genes (Evans *et al.*, 1988; Schedlich *et al.*, 1987).

Although the KLK-L3 protein sequence is unique,

comparative analysis revealed that it is homologous to other members of the kallikrein multigene family. KLK-L3 shows 40% protein identity with the TLSP gene product and 38 and 33% identity with the KLK-L2 and KLK1 proteins, respectively. Hydrophobicity analysis revealed that the amino-terminal region is quite hydrophobic (Fig. 3), consistent with the possibility that this region may harbor a signal sequence, analogous to other serine proteases. Computer analysis of the amino acid sequence of KLK-L3 predicted a cleavage site between amino acids 19 and 20 (GWA-DT). Sequence alignment (Fig. 4) also revealed a potential cleavage site (Arg²²), at a site homologous to other

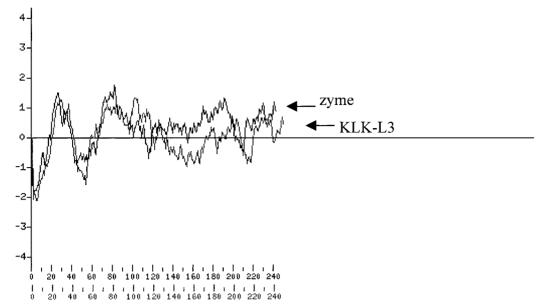


FIG. 3. Plot of hydrophobicity and hydrophilicity, comparing the pattern of the KLK-L3 with that of the zyme gene. Note the hydrophobic region around the first 20 amino acids, likely representing the signal peptide.

serine proteases (lysine (K) or arginine (R) is present in most cases) (Keil, 1971). Several evenly distributed hydrophobic regions throughout the KLK-L3 polypeptide are consistent with a globular protein, similar to other kallikreins and serine proteases. The dotted region in Fig. 4 indicates an 11-amino-acid loop characteristic of the classical kallikreins (PSA, KLK1, and KLK2) but not found in KLK-L3 or other members of the kallikrein multigene family (Yousef *et al.*, 1999b,c; Little *et al.*, 1997; Nelson *et al.*, 1999; Yousef and Diamandis, 1999).

Twenty-nine "invariant" amino acids surrounding the active site of serine proteases have been described (Dayhoff, 1978). Of these, 26 are conserved in KLK-L3. One of the unconserved amino acids (Ser¹⁶⁸ instead of Pro) is also found in prostase, KLK-L2, and enamel matrix serine proteinase (EMSP1) genes. The second (Leu⁵⁸ instead of Val) is also found in TLSP and KLK-L2 genes, and the third is Ala²⁶ instead of Gly. According to protein evolution studies, each of these changed amino acids represents a conserved evolutionary change to a protein of the same group (Miyata et *al.*, 1979). Twelve cysteine residues are present in the putative mature KLK-L3 protein; 10 of them are conserved in all the serine proteases that are aligned in Fig. 4 and would be expected to form disulfide bridges. The other two (C^{136} and C^{238}) are not found in PSA, KLK1, KLK2, or trypsinogen; however, they are found at similar positions in prostase, HSCCE, zyme neuropsin, and TLSP genes and are expected to form an additional disulfide bond.

To predict the phylogenetic relatedness of the KLK-L3 gene with other serine proteases, the amino acid sequences of the kallikrein genes were aligned together using the Clustal X multiple alignment program, and a distance matrix tree was predicted using the neighbor-joining/UPGMA method (Fig. 5). Phylo-

genetic analysis separated the classical kallikreins (KLK1, KLK2, and PSA) and grouped KLK-L3 with TLSP, neuropsin, zyme, HSCCE, and prostase/KLK-L1, consistent with previously published studies (Little *et al.*, 1997; Nelson *et al.*, 1999).

Tissue Expression and Hormonal Regulation of the KLK-L3 Gene

As shown in Fig. 6, the KLK-L3 gene is primarily expressed in thymus, testis, spinal cord, cerebellum, trachea, mammary gland, prostate, brain, salivary gland, ovary, and skin (the last two tissues are not shown in the figure). Lower levels of expression are seen in fetal brain, stomach, lung, thyroid, placenta, liver, small intestine, and bone marrow. No expression was seen in uterus, heart, fetal liver, adrenal gland, colon, spleen, skeletal muscle, pancreas, or kidney. To verify the RT-PCR specificity, representative PCR products were cloned and sequenced. Figure 7 shows that KLK-L3 gene is regulated by steroid hormones in the human breast cancer cell line BT-474.

DISCUSSION

Kallikreins are a subgroup of serine proteases, and these enzymes play important roles in diverse physiological processes (Clements, 1997). Recent evidence suggests that at least some members of the kallikrein family are implicated in breast, prostate, and other human cancers (Diamandis, 1998; Anisowicz *et al.*, 1996; Liu *et al.*, 1996; Diamandis and Yu, 1995; Goyal *et al.*, 1998; Stenman, 1999). Since in rodents there are many kallikrein genes, the restriction of this family to only three members in humans was somewhat surprising. Determination of the true size and cloning of all members of the human kallikrein gene family are im-

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PSA GGVLVHPONVITA/H ERNKSVILLGRHSLFHPEDT-GQVFQVSHSFPHPLYDMSLLKNR KLK2 GGVLVHPONVITA/H ERNKSVILLGRHSLFHPEDT-GQVFQVSHSFPHPLYDMSLLKNR KLK1 GGTLISDR/LLTA/H EGNVPONVITA/H trypsinogen GGSLINEGVVSAGH KKSIQVRLGENNLFDEDNT-ACFYNVSESPFHECRMSLENH trypsinogen GGSLINEGVVSAGH KKSIQVRLGENNLFDDNT-ACFYNVSESPFHECRMSLENK rutropsin GGVLVGGNVITA/H KKPYTVRLGOHLGCHNLQKEEGC-EQIFRVTDFFHPEGRNSLPRK- reuropsin GGVLVGGNVITA/H KKPYTVRLGDISLQNKOGE-EQIFRVTDFFHPEGRNSLPRK- ryme GGVLVNERVLTA/H KKPYTVRLGDISLQNKOGE-EQIFRVTDFFHPERSPHPLYDBAS ryme GGVLVNERVLTA/H KKNOLOVELGLADOFGGSUNVEASLSVRHPEYNRPLAN- PSA FLRFGDDSSHDIMLIRLSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSSHDIMLIRLSEPAE-LTDAVKVMLGLPTQEPALGTTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSSHDIMLIRLSEPAE-LTDAVKVVUGLPTGEVALGTTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSHDIMLIRLSEPAE-LTDAVKVVUGLPTGEVALGTTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSHDIMLIRLSEPAE-LTDAVKVVUGLPTQEVALGTTCYASGWGSIEPEEFLTP KLK1 TRQADEDYSHDIMLIRLSEPAE-LTDAVKVVUGLPTQEVALGTTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSHDIMLIRLSEPAE-LTDAVKVVUGLPTQENALGTTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSHDIMLIRLSEPAE-LTDAVKVVUGLPTQEN	PSA KLK2 KLK1 trypsinogen KLK-L3 TLSP neuropsin zyme HSCCE prostase	MWVPVVFLTLSVTWIGAAPLI-LSRIVGGWECEKHSQPWQVLVASRGRAVC MWDLVLSIALSVGCTGAVPLI-QSRIVGGWECEKHSQPWQVAVYSHGWAHC MWFLVLCLALSLGGTGAAPPI-QSRIVGGWECEQHSQPWQAALYHFSTFQC MKLGLLCALSLLAGHGWADTRAIGAEECRPNSQPWQAGLFHLTRLFC MRI-LQLILLALATGLVGGETRIIKGFECKPHSQPWQAALFEKTRLLC -MGRPRPRAAKTWMFLLLLGGAWAGHSRAQ-EDKVLGGHECQPHSQPWQAALFQGQQLLC MKKLMVVLSLIAAAWAEE-QNKLVHGGPCDKTSHPYQAALYTSGHLLC MARSLLLPLQILLSLALETAGEEAQGDKIIDGAPCARGSHPWQVALLSGNQLHC MA-TAGNPWGWFLGYLILGVAGSLVSGSCSQIINGEDCSPHSQPWQAALVMENELFC
KLK2GGVLVHPQSVLTAAFLKKNSQWUGRINLEPDEDT-QQEVPSHSPFPLZNNSLLENHKLK1GGILVHRQSVLTAAFISDNYQLWLGRINLEDDENT-AQFVHVSESFPHPGFNNSLLENHtrypsinogenGGSLINEQUVSACHYKSRIQVRLGENNIEDLENT-AQFVHVSESFPHPGFNNSLLENHRLK-13GATLISDRNLTAAFRKYPLWKIGEHNIEVLEGN-EQFINAAKIIRHPQVDRKTLNN-RLSPGATLISDRNLTAAFRKYPLWKIGEHNIEVLEGN-EQFINAAKIIRHPQVDRKTLNN-RLSCGGVLVGGNWLTAAFRKKYPLWKIGEHNIEVLGE-EQTRTATESFPHPGFNNSLPNK-prostaseGGVLVHPLWVLTAAFKKNNLQVFLGKINLKQESS-QEQSSWARVIHPDVDAASprostaseSGVLVHPQWVLSAFFPQNSTIGLGLHSLEADQEPGSQMVEASLSVRAVIHPDVDAASProstaseSGVLVHPQWVLSAFFPQNSTIGLGLHSLEADQEPGSQMVEASLSVRAPPEYNRPLLAN-PSAFLRPGDDSSHDUMLIRJSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEFELTPKLK2SLRPDEDSSHDUMLIRJSEPAF-ITDVVKVGLPTQEPALGTCYASGWGSIEPEFEFFFFKLK1TRQADEDYSHDUMLIRJSEPAF-ITDAVKAVDPLTDESRCVTAGTSCLISGWGAVSSRALFPNLSPDINNDIMLIRJSEPA-LSPAVQPLNLSQTCVSFGWGCTSSGAGASSFALFPPLSPDINNDIMLIRJSEPS-ITMAVRPITLSSRCVTAGTSCLISGWGAVSSFALFPprostaseDINNDIMLIRJARAK-LSELIQPLPLERDCSANTSCLISGWGCTTSPRENFPprostaseDINNDIMLIRJARAK-LSELIQPLPLERDCSANTSCLILGWGCTAGG-DFPProstase		CONTRACTOR DEPENDENT CONCLEMENT CONCOUCHEEDING VOMELLEND
KLK1 GGLUHRQNUTAAH trypsinogen GGSLINEQWVSAGH YKSRIQVRLGEHNIEDDENT-AQFVNSESFFHEGENSLLENH KLK-L3 GATLISDRULTAAH RAFULST GATLISDRULTAAH RUPSIN GATLIAPRMLLTAAH RUPSIN GGVLVGGNWUTAAH RUPSIN GGVLVGGNWUTAAH RUPSIN GGVLVGGNWUTAAH SKEXCE GGVLVGGNWUTAAH KKNESCE GGVLVMERWUTAAH KKNESCE GGVLVMERWUTAAH KKNESCE GGVLVMERWUTAAH KKNESCE GGVLVMERWUTAAH KKNESSEDININ KUSEPAE-LTDAVKVMDLPTQEPALGTCYASGWGSIEPEEFLTP Prostase SGVLVHPQWISSEDININ KUSEPAE-LTDAVKVMDLPTQEPALGTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSSEDININ KUSEPAE-LTDAVKVMDLPTQEPALGTCYASGWGSIEPEEFLTP KLK1 TRQADEDYSH DIMLI RLSEPAE-LTDAVKVMDLPTQEPALGTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSSEDININ KUSEPAE-ITMAVRETTSESCTTAGTSCLISGWGSTSSPQENEP TLSP DIMLINENGAELSPRAPSTCAUSTCVSPEMOCTICSGWGSTSSPQEKLFP TLSP DIMNDININ KUSSRAV-INARVSTUSCTOVSPEMOCTISGWGSTSSPQEKLFP Pyme DIMLINENGAE-LSSLIQPLELERDCSANTTSCLISGWGSTSSPQEKLFP Pyme DIMLININ KUNSQAE-LSSLVKVRISLADCTOVSGWGTVSTSPRENPP Symme DIMLIN		GGVLVHPOWVLTAAHOLKKNSOVWLCRHNLFFPEDT-GORVPVSHSFPHPLYNMSLLKHO
trypsinogenGGSLINEQGVVSACH_VKSRLQVRLGEHNLEVLEGN=DQFINAAKITRHPQVDRKTLNN- RLK-13RLK-13GATLIARAH-RKPYLWVRLGEHNLEVLEGN=DQFINAAKITRHPQVDRKTLNN- neuropsinGGVLVGENVLITAAH-KKPYLWVRLGOHSLQNKDGP=DQFPVDGSTPHPCFNKDLSAN- TKSCCrgwmGGVLVHERWVLTAAH-KKPYLVVRLGOHSLQNKDGP=DQEIPVVDSTPHPCVNSSD-VE- ymmProstaseSGVLVHPQWLTAAH-KKPNLQVFLGKHNLRQRESS-QBQSSVVRAVIHPDYDAAS prostasePSAFLRPGDDSSHDLMLRLSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTP KLK2KLK1TRQADEDYSHDLMLRLSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTP KLK1KLK2SLRPEDEDSSHDLMLRLSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTP KLK2KLK1TRQADEDYSHDLMLRLSEPAE-LTDAVKVVDLPTQEPALGTTCYASGWGSIEPEEFLTP KLK2KLK2SLRPEDEDSSHDLMLRLSEPAE-LTDAVKVVDLPTQEPALGTTCYASGWGSIEPEEFLTP KLK2KLK1TRQADEDYSHDLMLRLPRQAR-LSPAVQPLNLSCTCVSGWGCLISGWGAVSSFKALFP TLSPDHNDDIMLRLPRQAR-LSPAVQPLNLSCTCVSGWGCLISGWGAVSSFKALFP TLSPDHNDDIMLRLPRQAR-LSSAVQPLNLSCTCVSGWGTTSFPLNFP TypsinogenHVNDDIMLRLPRQAR-LSSLUPPLERDCSANTTSCHILGWGKTADGDFP ProstasePSAKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSDSGGLVCNGVLQGITS KLK1DDLQCUDAVLSQAKCEASYPGKITSMFCVGFLEGGKDSCQCDSGGLVCNGVLQGITS TypsinogenDELQCLDAVLSQAKCEASYPGKITSMFCVGFLEGGKDSCQCDSGGLVCNGVLQGITS NLK2KLK1DDLQCUDAVLSQAKCEASYPGKITSMFCVGFLEGGKDSCQCDSGGLVCNGVLQGITS SLKL-13VTLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGKSCCDSGGLVCNGVLQGITS prostaseTVLQCVNVSVSEEVCKKYPVNLVKKKNKLDTIAANP KLK2WGEPCALPERPSUYTKVVHYKKWIKDTIAANP KKLK2PSAWGEPCALPERPSUYTKVVHYKWIKMITTAANP KLK13GGPCATKLPSVYTKVCKVYNVLDSYKWIEDTIAENS t		
KLK-L3 GATLISDRHLTAAH KKYYLWYRLGEHLUKWEGP-DOLFWIDFFPHOFNKDLSAN- TLSP TLSP GATLIAPRWLTAAH KKYYLWLGQHNLQKEEGC-EQTRTATESFPHOFNKSLPNK- neuropsin GGULVGGWVLTAAH KKPKYTVRLGDBSLQNKDGP-EQEIPWQSITHPCYNSD-VE- zyme GGULVINERWLTAAH KKPKYTVRLGDBSLQNKDGP-EQEIPWQSITHPCYNSD-VE- prostase SGVLVHPERWLTAAH KKNEYTVRLGDBSLQNKDGP-EQEIPWQSITHPCYNSD-VE- gongusse PSA FLRPGDDSHDUMLRLSPAF-ITDVVKVLGPTQEPALGTTCYASGWGSIEPEEFLTP KLK2 SLRPDEDSSHDUMLRLSPAF-ITDVVKVLGPTQEPALGTTCYASGWGSIEPEEFLTR KLK1 TRQADDDYSHDUMLRLSPAF-ITDVVKVLGPTQEPALGTTCYASGWGSIEPEFFLRP KLK-13 Trypsinogen DIMLKLSSRAV-INAVSTISLFTAPPATGTKCLISGWGASSGADP KLK-L3 DHNDDIMLRLPDQAF-LSPAVQPLNLSGTCVSFGMQCLISGWGAVSSPKALFP TLSP DHNDDIMLRLPDQAF-LSPAVQPLNLSGTCVSGWGVTSFRENFP Prostase prostase DHNDIMLWKNSQAR-LSPAVQPLNLSGTCVSGWGVTSFRENFP prostase DIMLKLDESVS-ESDTIRSISIASQCPTAGNSCUSSWGLLANGRMP PSA KKLQCVDLHVISNDVCAQVHPKVTKFMLCAGRWTGGKDTCGDSGGLVCNGVLQGITS KLK1 DDLQCVDLKILPNDECKKAHVQKVTDFMLCVGHLEGGKDTCVDSGGLVCNGVLQGITS KLK2 RULCANTILENKLCHWAYPGHISDSMLCACLWEGGRSCCGDSGGLVCNGULQGITS SQUVQUKUSVVSEVCSKLYPPLYPRMCGASSKG-ADTCQDSGGLVCNGULQGITS KLK2 WLQCANTSLLENKLCHWAYPGHISDSMLCACLWEGGRSCCGDSGGLVCNGULQGITS SQUCDCLDAPVLSQACEASYPGKITSNMFCVGFLGGKDSCGDSGGLVCNGULQGIVG YTLCCANTSLLENKLCHWAYPGHISDSMLCACLWEGGR		
TLSPGATLIAPRNLLTAAHLUKPRYIVHLGQHLQKEEGG-EQUETATATESFPHFGFNNSLPNK- neuropsinGGVLUNGRNVLTAAHLUKKKKYYTVRLGDHSLQNKDGP-EQUETATATESFPHFGFNNSLPNK- neuropsinregveGGVLUNERVLTAAHLUKKKKYYTVRLGDHSLQNKDGP-EQUETATATESFPHFGFNNSLPNK- structurer prostaseGGVLUNERVLTAAHLUKKKKYYTVRLGDHSLQNKDGP-EQUETATOSFPHGFNNSLPNK- HSCCE GGVLUNERVLTAAHLUKKKYYTVRLGDHSLQNKDGP-EQUETATOSFNULTAAHLUKKKYYTVRLGDHSLQNKDGP-EQUETATOSFNULTAAHLUKKYYTVRLGDHSLQNKUGASSSTRANDAAHLUKKYYTVRLGDHSLANDAAHLUKKYYTVRLGDHSLANDAAHLUKKYYTVRLGDHSLANDAAHLUKKYYTVRLGDHSLANDAAHLUKKYYTVRLGDHSLANDAAHLUKKYYTVRLGDHSLANDAAHLUKKYYTVRLGDHSLANDAAHLUKKYYTVRLGDHSLANDAAHLUKYYTVRLGAGNAUSSYKALFP prostaseTILSPDINNDJINLIKLDSVYTVRTAAHLUKYTVRVLANDAAHLUSSACVTDFMLCAGHWGGKSCGSDSGGFLVCNGVLQGITS KLK1DDLQCLDALPUNGAAAUSEKVTEFMLCAGHWGGKSCGSDSGGFLVCNGVLQGITS KLK2KKLQCVVLHUNSNDVCAQVHQKVTVFMLCAGRWTGGKSCGSDSGGFLVCNGVLQGITS KLK2SSAKKLQCVDLHUNSNDVCAQVHQKVTFMLCAGRWTGGKSCGSDSGGFLVCNGVLQGITS RUKYCQTNKVTVSVSEUCSKLYDPLYNDSMCAGAGHDQKDSCNGSSGGFLVCNGLQGVVS YLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGRGSCGDSGGFLVCNGLQGVVS YLQCNNSVVSEUCSKLYDPLYNDSYNGLEGGRDDQKDSCNGSSGGFLVCNGLQGVS YLQCNVSVVSEUCSKLYDPLYNDSYNGLANDAAHLUKSTAAHLUKYTVYNKNINTAANS YLQCNNSVVSEUCSKLYDPLYNDSYNKWIKDTIAANP KLK2WGEPCCALPERPSLYTKVVHYKWHKNTUAANN MGEPCCALPERPSLYTKVVHYKWHKNTUA		
zymeGGVLIHPLAVLTAAH*KKPNLQVFLGKHNLRQRESS-QEQSSVVRAVIHEDPYDAAS HSCCEHSCCEGGVLVNERVUTAAFCKNPEYTVHLGSDTLGDRRAQRIKASKSFRHPGYSTQT prostaseSGVLVNERVUTAAFCKNPEYTVHLGSDTLGDRRAQRIKASKSFRHPGYSTQT prostaseSGVLVNERVUTAAFCKNPEYTVHLGSDTLGDRRAQRIKASKSFRHPGYSTQT prostasePSAFLRPGDDSSHD.MTLRLSPAFA-TDVXVKUDLPTQEPALGTCYASGWGSIEPEEFLFP KLK2SLRPEDEDSSHD.MTLRLSPAFA-TDVXVVULPTEEPEVGSTCLASGWGSIEPEEFLFP KLK1TRQADEDYSHDLMLIRLSPAFA-TDVXVVUELPTEEPEVGSTCLASGWGSIEPEFLFR KLK1KLK1TRQADEDYSHDLMLIRLSPAFA-TDVXVVUELPTEEPEVGSTCLASGWGSIEPENFSFP TrypsinogenDHNDD.MTLQLRQAS-LSPAVPLNLSQTCVSPGWCTLSGWGXSSPKALFP PoweropsinDHNDD.MTLQLRQAS-LGSKVKPISLADHCOPCQCKCTVSGWGTVTSPRENFP zymeDHNDD.MTLQLRQAS-LGSKVKPISLADHCOPCQCKCTVSGWGTVSPRENFP prostasePSAKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSDSGFLVCNGVLQGITS KLK2RL2QCVSLHLLSNDWCARAYSEKVTEFMLCAGLWTGGKDTCCDSSGELVCNGVLQGITS KLK2RL2QCVSLHLLSNDWCARAYSEKVTEFMLCAGLWTGGKDTCCDDSGELVCNGVLQGITS KLK2PSAKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGLWTGGKDTCCDDSGELVCNGVLQGITS KLK2RL2QCUDKVLISPQCCEASYPGKITSNMFCVGFLEGGKDSCCDDSGELVCNGULQGVS KLK-L3VLQCANISILENKLCHWAYPGITDCMNCASUPEGGKDSCCDDSGELVCNGLQGVS KLK-L3VLQCANISILENKLCHWAYPGITDCMNCAGESKG-ADTCCDDSGELVCNGLQGVS KLK-L3PSAWGSEPCALPERPSLYTKVVHYRKWIKDTIAANP KLK2WGSEPCALPERPSLYTKVVHYRKWIKDTIAANP KLK1WGSEPCALPERPSLYTKVVHYRKWIKDTIAANP KLK1SKLK-L3GGAEPCAQKKREVYTKVVHYRKWIKDTIAANP KLK1WGOPCAITRKPGVYTKVCKYVNUYKWIKDTIAANP KLK1WGSPCGRSDRFREVYTNVCCKYTWNIQKTIQAK- MGOPCAKSKREVYTNVCKYTWNIQKTIQAK-		GATLIAPR <mark>WLLTAAHC</mark> LKPRYIVHLGQHNLQKEEGC-EQTRTATESFPHPGFNNSLPNK-
HSCCEGGVLVNERWVLTAAHCKMNEYTVHLGSDTLGDRRAQRIKASKSFRHEGYSTQT SGVLVHPOWVLSAAFCFONSYTTGLGLHSLEADQEPGSQMVEASLSVRHPEYNRPLLAN-PSAFLRPGDDSSHDLMLIRLSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTP KLK2KLK2SLRPDEDSSHDLMLIRLSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTP KLK1TRQADEDYSHDLMLIRLSEPAK-ITDVVKVLGLPTQEPALGTTCYASGWGSIEPEEFLTP KLK1KLK2SLRPDEDSSHDLMLIRLSEPAK-ITDVVKVLGLPTQEPALGTTCYASGWGSIEPEEFLTP KLK-13HSCEDINLDLKLSSRAV-INARVSTISLPTAPPATGTKCLISGWGNTSSPENFSP TLSPTLSPDINDDLMLIRLSPQAR-LSPAVQPLNLSQTCVSPGMQCLISGWGXSSPKALFP neuropsinPONNDLMUKMASPVS-ITWAVRPILSSRCVTAGTSCLISGWGSTSSPQIRLP prostaseDINNDLMLWLMSQAR-LSSMVKKVISLSABHCTQPGQKCTVSGWGTTSPDVTFP prostasePSAKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGLLVCNGVLQGITS KLK2RSLQCVSLHLLSNDMCARAYSEKVTEFMLCAGLWTGGKDTCQDSGELWCNGVLQGITS trypsinogenDELQCLDAPVLSQAKCEASYPGKITSDMTCVGFLEGGKDSCCDDSGELWCNGVLQGUGS tLVDGVLKILSNDMCARAYSEKVTEFMLCAGLWEGGRSCCDDSGELWCNGVLQGUSS tLWDSVSEEVCSKLYDPITDTMVCASVQEGKDSCCDDSGELWCNGULQGVS tLK-L3VILQCANISILENKLCHWAYPGNITDTMVCAGSKGGADSCCDDSGELWCNGQLQGVS BSCCESDLWCVDVKLSPQCTVYKKVLNYKKIKDTIAANP KLK4WGSEPCALPERFSLYTKVVHYRKWIKDTIVANP KLK4WGSEPCALPERFSLYTKVVHYRKWIKDTIVANP KLK2KLK-L3GGAEPCSRPRRAVTSVCKYLDWIQETMKNN- MGDPCCRASKKLSYKWIEDTIAENS trypsinogenKLK-L3GGAEPCSRPRRAVTSVCKYVNYKWIKNTIAANS KLK-L3GGAEPCSRPRRAVTSVCKYVNYKWIKNTIAANS KLK-L3GGAEPCSRPRRAVTSVCKYVNYKWIKNTIAANS MCK-L3SSAWGSEPCALPERFSLYTKVVHYRKWIKDTIAANP MCK1KLK1WGYPCGRSEKKFGVYTKVCKYVDWIQETMKNN- neuropsinPSAWGSPCCARPERSLYKVKVYNVYR	neuropsin	
prostaseSGVLVHPQNVLSAAFCFQNSYTIGLGLHSLEADQEPGSQMVEASLSVRHPEYNRPLLAN-PSAFLRPGDDSSHDLMLLRLSEPAE-LTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKLK2SLRPDEDSSHDLMLLRLSEPAK-ITDVVKVLGLPTQEPALGTTCYASGWGSIEPEEFLTPKLK1TRQADEDYSHDLMLLRLTEPADTITDAVKVVELPTEPEVGSTCLASGWGSIEPEEFLSPKLK1TRQADEDYSHDLMLLRLTEPADTITDAVKVVELPTEPEVGSTCLASGWGSIEPEEFSPtrypsinogenDIMLKLSSRAV-INARVSTISLPTAPPATGTKCLISGWGNTASSGADYPKLK-13DIMLKLSRAV-INARVSTISLPTAPATGTKCLISGWGNTSSPDIRLPneuropsinDIMLKLSPAC-LSSRVQFLNLSQTCVSGWGTUSGWGNTSSPDIRLPpeuropsinDIMLKLARPAK-LSELIQPLPLERDCSANTTSCHILGWGKTADGDFPHSCCEHNQDLMLLQLRDQAS-LGSKVKPISLADHCTQPGGKCTVSGWGTTSPDVTPPprostaseDIMLIKLDSVS-ESDTIRSISIASQCPTAGNSCLVSGWGLLANGRMPKLK2RSLQCVSLHLLSNDWCAQVHPQKVTKFMLCAGRWTGGKSTCSCDSGFLVCNGVLQGITSKLK1DDLQCVDLKILPNDECKKAHVQKVTDFMLCVGHLEGGKDTCVCDSGGELVCNGVLQGTSKLK1DDLQCVDLKILPNDECKKAHVQKVTDFMLCAGLWTGGKDTCCDDSGELVCNGVLQGVSKLK-13VTLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGRDSCQDSGGELVCNGLQGVSKLK-14DTLQCAVITHLVSREECEHAYPGGITDMVCASSVEGGNDSCQDSGELVCNGLQGUSrypsinogenDELQCLDAPVLSQAKCEDAYPGITDMVCASVEGGKDSCQDSGELVCNGLQGUSrypsinogenDTIQCAYITHLVSREECEHAYPGGITDMVCASSVEGGNDSCQDSGELVCNGLQGUSrypsinogenDTIQCAYITHLSSREECEHAYPGITDMVCASSDEGDSGELVCNGLAGIISneuropsinDTIQCAYITHUSKEECEHAYPGITDMVCASSDEGDSGELVCNGLAGGISrypsinogenWGSEPCALPERPSLYKVVHYKWIKDTIAANPKLK1WGPECGALPERPSLYTKVVHYKWIKDTIAANSKLK-13GGABECSRPRRAVITSVCHYLDSYVWIEDTIAENStrypsinogenWGCDCATTRKPGVYTKVC	-	GGVLIHPL <mark>WVLTAA<i>H</i>C</mark> KKPNLQVFLGKHNLRQRESS-QEQSSVVRAVIHPDYDAAS
PSAFLRPGDDSHPSAFLRPGDDSHKLK2SLRPDESSHOLMLIRLSDDSHSLRPDESSHOLMLIRLSDDSHOLMLIRLSEPAK-ITDVVKVLGLPTOEPALGTTCYASGWGSIEPEEFLRPKLK1TRQADEDYSHOLMLIRLSDSSHOLMLIRLSEPAK-ITDVVKVLGLPTOEPALGTTCYASGWGSIEPEEFLRPKLK-L3DIMLIRLSPDHNDIMLIRLSPDHNNDIMLIRLRCL3DHNNDIMLIRUROPSinDHNNDIMLIRUROPSinDHNNDIMLIPTSPDHNNDIMLIRUROPSinDHNNDIMLIProstase		
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KLK2RSLQCVSLHLLSNDMCARAYSEKVTEFMLCAGLWTGGKDTCGGDSGGPLVCNGVLQGITSKLK1DDLQCVDLKILPNDECKKAHVQKVTDFMLCVGHLEGGKDTCVGDSGGPLWCDGVLQGVTStrypsinogenDELQCLDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSCQGDSGGPLVCNGQLQGVVSKLK-L3VTLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGRGSCQGDSGGPLVCNGTLAGVVSTLSPHTLRCANITIIEHQKCENAYPGNITDTMVCASVQEGGKDSCQGDSGGPLVCNQSLQGIISneuropsinDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG-ADTCQGDSGGPLVCDGALQGITSzymeDTIQCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGPLVCBHLRGLVSHSCCESDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGPLVCRGTLQGLVSprostaseTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGPLICNGYLQGLVSKLK2WGSEPCALPERPSLYTKVVHYRKWIKDTIVANPKLK1WGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVCHYDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDFGVYTQVCKFTKWINDTMKKHR	prostase	
KLK1DDLQCVDLKILPNDECKKAHVQKVTDFMLCVGHLEGGKDTCVGDSGGPLMCDGVLQGVTStrypsinogenDELQCLDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSKLK-L3VTLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGRGSCQGDSGGPLVCNGTLAGVVSTLSPHTLRCANITIIEHQKCENAYPGNITDTMVCASVQEGGKDSCQGDSGGPLVCNQSLQGIISneuropsinDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG-ADTCQGDSGGPLVCDGALQGITSzymeDTIQCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGPLVCGDHLRGLVSHSCCESDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGPLVCRGTLQGLVSprostaseTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSCGPLICNGYLQGLVSKLK2WGSEPCALPERPSLYTKVVHYRKWIKDTIAANPKLK1WGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVVNYVKWIKNTIAANSKLK-L3GGAEPCSRPRRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR	PSA	KKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCS <mark>CD<i>S</i>GGP</mark> LVCNGVLQGITS
trypsinogenDELQCLDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSKLK-L3VTLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGRGSCQGDSGGPLVCNGTLAGVVSTLSPHTLRCANITIIEHQKCENAYPGNITDTMVCASVQEGGKDSCQGDSGGPLVCNQSLQGIISneuropsinDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG-ADTCQGDSGGPLVCDGALQGITSzymeDTIQCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGPLVCGDHLRGLVSHSCCESDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGPLVCRGTLQGLVSprostaseTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGPLICNGYLQGLVSKLK2WGSEPCALPERPSLYTKVVHYRKWIKDTIAANPKLK1WGYPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVVNYVKWIKNTIAANSKLK-L3GGAEPCSRPRRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR	KLK2	
KLK-L3VTLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGRGSCQGDSGGFLVCNGTLAGVVSTLSPHTLRCANITIIEHQKCENAYPGNITDTMVCASVQEGGKDSCQGDSGGFLVCNQSLQGIISneuropsinDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG-ADTCQGDSGGFLVCDGALQGITSzymeDTIQCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGFLVCCGDHLRGLVSHSCCESDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGFLVCRGTLQGLVSprostaseTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGFLICNGYLQGLVSPSAWGSEPCALPERPSLYTKVVHYRKWIKDTIAANPKLK2WGPEPCALPEKPAVYTKVVHYRKWIKDTIAANPKLK1WGYPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVVNYVKWIKNTIAANSKLK-L3GGAEPCSRPRRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR		
TLSPHTLRCANITIIEHQKCENAYPGNITDTMVCASVQEGGKDSCQGDSGGFLVCNQSLQGIISneuropsinDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG-ADTCQGDSGGFLVCDGALQGITSzymeDTIQCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGFLVCGDHLRGLVSHSCCESDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGFLVCRGTLQGLVSprostaseTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGFLICNGYLQGLVSPSAWGSEPCALPERPSLYTKVVHYRKWIKDTIAANPKLK2WGPEPCALPEKPAVYTKVVHYRKWIKDTIAANPKLK1WGYPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVYNYVKWIKNTIAANSKLK-L3GGAEPCSRPRRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR		
neuropsinDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG-ADTCQGDSGGFLVCDGALQGITSzymeDTIQCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGFLVCGDHLRGLVSHSCCESDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGFLVCRGTLQGLVSprostaseTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGFLICNGYLQGLVSPSAWGSEPCALPERPSLYTKVVHYRKWIKDTIVANPKLK2WGPEPCALPEKPAVYTKVVHYRKWIKDTIAANPKLK1WGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVYNYVKWIKNTIAANSKLK-L3GGAEPCSRPRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR		
zymeDTIQCAYIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSGGFLVCGDHLRGLVSHSCCESDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGFLVCRGTLQGLVSprostaseTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGFLICNGYLQGLVSPSAWGSEPCALPERPSLYTKVVHYRKWIKDTIVANPKLK2WGPEPCALPEKPAVYTKVVHYRKWIKDTIAANPKLK1WGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVVNYVKWIKNTIAANSKLK-L3GGAEPCSRPRRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR		
HSCCESDLMCVDVKLISPQDCTKVYKDLLENSMLCAGIPDSKKNACNGDSGGFLVCRGTLQGLVSprostaseTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDSCNGDSGGFLICNGYLQGLVSPSAWGSEPCALPERPSLYTKVVHYRKWIKDTIVANPKLK2WGPEPCALPEKPAVYTKVVHYRKWIKDTIAANPKLK1WGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVYNYVKWIKNTIAANSKLK-L3GGAEPCSRPRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR		
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KLK2WGPEPCALPEKPAVYTKVVHYRKWIKDTIAANPKLK1WGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENStrypsinogenWG-DGCAQKNKPGVYTKVYNYVKWIKNTIAANSKLK-L3GGAEPCSRPRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR	PSA	WGSEPCALPERPSLYTKVVHYRKWIKDTIVANP
trypsinogenWG-DGCAQKNKPGVYTKVYNYVKWIKNTIAANSKLK-L3GGAEPCSRPRPAVYTSVCHYLDWIQEIMENTLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR		
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TLSPWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN-neuropsinWGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKGzymeWGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK-HSCCEWGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR	11 5	
neuropsin WGSDPCGRSDKPGVYTNICRYLDWIKKIIGSKG zyme WGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK- HSCCE WGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR		
zyme WGNIPCGSKEKPGVYTNVCRYTNWIQKTIQAK- HSCCE WGTFPCGQPNDPGVYTQVCKFTKWINDTMKKHR		-
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FIG. 4. Alignment of the deduced amino acid sequence of KLK-L3 with members of the kallikrein multigene family. Genes are (from top to bottom; the GenBank Accession number is given in parentheses): PSA (P07288), KLK2 (P20151), KLK1 (NP_002248), trypsinogen (P07477), KLK-L3 (AF135026), trypsin-like serine protease (TLSP) (BAA33404), neuropsin (BAA28673), zyme (Q92876), human stratum corneum chymotryptic enzyme (HSCCE) (AAD49718), and/prostase/KLK-L1 (AAD21581). Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are in boldface italics, and the 29 invariant serine protease residues are denoted by (. Cysteine residues are marked by (). Conserved areas around the catalytic triad are highlighted in black. The arrowheads () represent the potential cleavage sites. The dotted area represents the kallikrein loop sequence.

portant to our understanding of the contribution of the kallikreins to human biology.

In this report, we have precisely defined the human kallikrein gene locus and constructed the first detailed map describing the relative positions of the kallikreins and other kallikrein-like genes (Fig. 1). This map is consistent with previous reports on the localization of the classical kallikreins and the approximate mapping

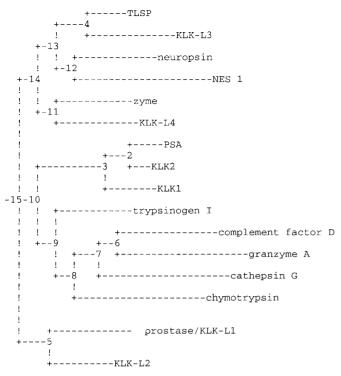


FIG. 5. Dendrogram of the predicted phylogenetic tree for some serine proteases and kallikrein genes. Neighbor-joining/UPGMA method was used to align KLK-L3 with other members of the kallikrein gene family. Gene names and accession numbers are listed in the legend to Fig. 4. The tree grouped the classical kallikreins (KLK1, KLK2, and PSA) together and aligned the KLK-L3 gene in one group with TLSP, neuropsin, and NES 1 genes. KLK-L4 (Gen-Bank Accession No. AF135024) lies further telomeric to TLSP (Yousef *et al.*, 1999a).

of some new kallikreins by radiation hybrid and FISH techniques (Riegman *et al.*, 1992; Luo *et al.*, 1998; Anisowicz *et al.*, 1996; McCormack *et al.*, 1995). It should be noted, however, that the lengths of certain segments of this map (as depicted in Fig. 1) are dependent on the EcoRI restriction map of the area and are measured in terms of approximate kilobase units. Also, the measure of intervals between genes may change slightly in the future, since some kallikreins may have an extra 5'exon(s) that has not as yet been identified. Kallikreins with verified 5' untranslated exons include NES1 (Luo *et al.*, 1998), zyme (Yousef *et al.*, 1999b), and neuropsin (Yoshida *et al.*, 1998). This map is also directional; it indicates that PSA and KLK2 genes are transcribed in the same direction (centromere to telo-

mere) and that the rest of the kallikrein-like genes are transcribed in the reverse direction (Fig. 1).

An early report indicated that KLK1 is located approximately 31 kb centromeric to PSA (Riegman *et al.*, 1992). Our map extends only 24 kb centromeric to PSA, and for this reason, KLK1 was not precisely localized. Also, a recent report failed to define precisely the location of KLK1 in relation to the other kallikreins (Stephenson *et al.*, 1999). Thus, the exact location of the KLK1 gene is still to be defined from linear chromosome 19 sequencing data. The possibility still exists that this locus is extended further and that other kallikrein-like genes may be located upstream of PSA or downstream from TLSP. Evidence for this has been described elsewhere (Yousef *et al.*, 1999a).

Traditionally, kallikreins are characterized by their ability to liberate lysyl-bradykinin (kallidin) from kininogen (Clements, 1997). In humans, only KLK1 meets this "functional" definition. KLK2 and KLK3 are assigned to the same family based on the strong structural similarities of the genes and proteins and the close localization of these genes in the same chromosomal region (Rittenhouse et al., 1998; Richards et al., 1982). More recently, a new structural concept has emerged to describe kallikreins. Richards and co-workers introduced the concept of a "kallikrein multigene family" in mice, to refer to these genes (Richards et al., 1982; Van Leeuwen et al., 1986). This definition is not based much on the specific enzymatic function of the gene product, but more on its sequence homology and its close linkage on mouse chromosome 7.

Irwin *et al.* (1988) proposed that the serine protease genes can be classified into five different groups according to intron position. The established kallikreins (KLK1, KLK2, and PSA), trypsinogen, and chymotrypsinogen belong to a group that has (1) an intron just downstream from the codon for the active site histidine residue. (2) a second intron downstream from the exon containing the codon for the active site aspartic acid residue, and (3) a third intron just upstream from the exon containing the codon for the active site serine residue. Our results indicate the presence of some more common structural features that are found in all kallikreins (including the newly identified KLK-L3 gene): (1) All genes are formed of five coding exons and four intervening introns (with the possibility that some genes may have an extra 5' untranslated exon(s) (Luo

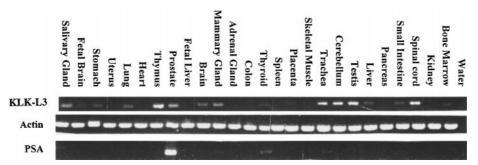


FIG. 6. Tissue expression of the KLK-L3 gene as determined by RT-PCR. Actin and PSA are control genes. For discussion, see text.

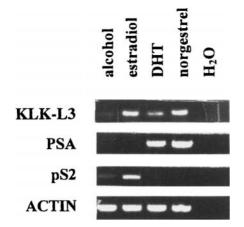


FIG. 7. Hormonal regulation of the KLK-L3 gene in the BT-474 breast carcinoma cell line. DHT, dihydrotestosterone. Steroids were at 10^{-8} M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens), and PSA (upregulated by androgens and progestins) are control genes. KLK-L3 is upregulated by progestins, estrogens, and androgens, in that order. For more details, see text.

et al., 1998; Yoshida *et al.*, 1998; Yousef *et al.*, 1999b) (Fig. 8). (2) The exon lengths are usually comparable. (3) The intron phases are always conserved (I-II-I-0) (see the legend to Fig. 8 for a description of intron phases). (4) These genes are clustered in the same chromosomal region, apparently without any intervening non-kallikrein-like genes (Fig. 1). Thus, we can conclude that all the recently identified serine proteases that are present in this region (zyme, HSCCE, neuropsin, NES1, prostase/KLK-L1, KLK-L2, and TLSP), together with the newly identified kallikrein-

like gene (KLK-L3), could be considered members of the expanded human kallikrein multigene family.

The chromosomal band 19q13 is nonrandomly rearranged in a variety of human solid tumors including ovarian cancers (Mitelman, 1994), and the currently available data indicate that the kallikrein gene locus is related to many malignancies. At least three kallikrein genes (PSA, zyme, and NES1) are down-regulated in breast cancer (Anisowicz et al., 1996; Liu et al., 1996; Yu et al., 1998; Sauter et al., 1996), and NES1 appears to be a novel tumor suppressor gene (Goyal et al., 1998). Furthermore, PSA exhibits potent antiangiogenic activity (Fortier et al., 1999). It is possible that some of these kallikreins are involved in a cascade pathway, similar to the coagulation or apoptotic process, whereby proforms of proteolytic enzymes are activated and then act upon downstream substrates. Such activity was found for the KLK2 gene product, which acts upon and activates pro-PSA (Kumar et al., 1997; Lovgren et al., 1997).

We have shown here that the expanded human kallikrein gene family has a similar number of members as the rodent family of genes. Although the precise localization of this family of genes has now been achieved, its functional role and connection to human disease are still incompletely understood. Some new compelling data have raised the possibility that at least some of these genes behave as tumor suppressors (Goyal *et al.*, 1998), as negative regulators of cell growth (Lai *et al.*, 1996), as antiangiogenic molecules (Fortier *et al.*, 1999), and as apoptotic molecules (Balbay *et al.*, 1999). The paramount diagnostic value of some members is also well established (Rittenhouse *et*

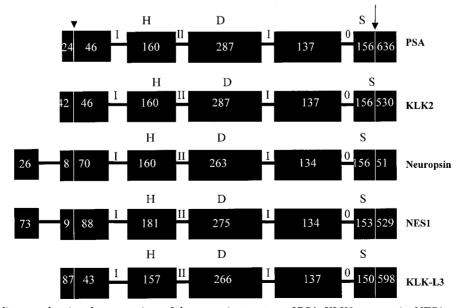


FIG. 8. Schematic diagram showing the comparison of the genomic structure of PSA, KLK2, neuropsin, NES1, and KLK-L3 genes. Exons are shown by black boxes and introns by the connecting lines. Arrowheads show the start codon, and arrows show the stop codon. Letters above boxes indicate relative positions of the catalytic triad; H denotes histidine, D denotes aspartic acid, and S denotes serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I indicates that the intron occurs after the first nucleotide of the codon, II indicates that the intron occurs after the second nucleotide, and 0 indicates that the intron occurs between codons. Numbers inside boxes indicate exon lengths in basepairs. Figure is not drawn to scale.

al., 1998; Diamandis, 1998; Stenman, 1999). For these reasons, it is important to check all members of this family of genes as potential diagnostic or prognostic markers or as candidate therapeutic targets.

The newly identified KLK-L3 gene is expressed in many tissues, including skin, thymus, central nervous system, breast, prostate, and testis. The wide range of tissue expression of KLK-L3 should not be surprising since, by using the more sensitive RT-PCR technique, many kallikrein genes were found to be expressed in a wide variety of tissues. For example, PSA, KLK2, prostase/KLK-L1, and KLK-L2 are now known to be expressed in breast and many other tissues (Rittenhouse *et al.*, 1998; Yousef *et al.*, 1999b,c; Diamandis and Yu, 1995).

Like many other kallikreins, KLK-L3 is regulated by steroid hormones but in a more complex fashion than PSA and KLK2, which are up-regulated by androgens and progestins (Zarghami *et al.*, 1997). In the breast carcinoma cell line studied, KLK-L3 appears to be upregulated by progestins > estrogens > androgens (Fig. 7). Some other kallikrein-like genes behave similarly (Yousef *et al.*, 1999b). The functional characterization of the KLK-L3 promoter will further clarify the precise mechanism of KLK-L3 regulation by steroid hormones. The possible biological function of KLK-L3 is under investigation.

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