

Protease/*KLK-L1* Is a New Member of the Human Kallikrein Gene Family, Is Expressed in Prostate and Breast Tissues, and Is Hormonally Regulated

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Abstract

By using the positional candidate gene approach, we were able to identify a novel serine protease gene that maps to chromosome 19q13.3–q13.4. Screening of expressed sequence tags allowed us to establish the expression of the gene and delineate its genomic organization (GenBank accession no. AF135023). We named this gene *KLK-L1*. Another group, by using a subtraction hybridization method, cloned the same gene and named it *protease* (GenBank accession nos. AF113140 and AF113141). Here, we describe the precise mapping and localization of the *protease/KLK-L1* gene between the known genes *KLK2* (human glandular kallikrein) and *zyme* (also known as *protease M/neurosin*). The direction of transcription of *protease/KLK-L1* is the same as that of *zyme* but opposite to that of *KLK2* and prostate-specific antigen genes. Contrary to the initial impression, *protease/KLK-L1* is expressed at high levels not only in prostate tissue but also in testis, mammary gland, adrenals, uterus, thyroid, and salivary glands. We have further demonstrated with *in vitro* experiments with the breast carcinoma cell line BT-474 that this gene is expressed and that its expression is up-regulated by androgens and progestins. On the basis of information on other genes that are localized in the same region (prostate-specific antigen, *KLK2*, *zyme*, and *normal epithelial cell specific-1* gene), we speculate that *protease/KLK-L1* may be involved in the pathogenesis and/or progression of prostate, breast, and possibly other malignancies.

Introduction

Four major strategies can be used for new gene identification, depending on the type and amount of information available for the target gene as well as the available genomic resources: functional cloning, the candidate gene approach, positional cloning, and the positional candidate approach (1). These different techniques do not necessarily proceed in isolation; they can often be interlinked and complementary to each other. In positional cloning (2), originally referred to as reverse genetics (3), the identification of the gene is made without any prior knowledge of the function of its protein product. Positional candidate cloning is the identification of a gene based on its position on the genetic map and on the availability of candidate genes mapped to the same region (4). This method is rapidly becoming predominant because of the increasing number of cloned and mapped genes and the availability of numerous ESTs² (5).

KLKs and KLK-L proteins are a subgroup of the serine protease enzyme family. These proteases exhibit a high degree of substrate specificity (6). In mouse and rat, large multigene families encode for KLKs (7). In the mouse genome, at least 24 genes have been identi-

fied (8), and a similar family of 15–20 KLKs has been found in the rat genome (9). Three human *KLK* genes have been described: PSA (or *KLK3*; Ref. 10), human glandular *KLK* (*KLK2*; Ref. 11), and tissue (pancreatic-renal) *KLK* (*KLK1*; Ref. 12). The mouse *KLK* genes are clustered in groups of up to 11 genes on chromosome 7, and the distance between the genes in the various clusters can be as small as 3–7 kb (8). All three human *KLK* genes have been assigned to chromosome 19q13.2–19q13.4 (13). New members of the human *KLK* gene family include *protease M* [Ref. 14; also named *zyme*; (15) or *neurosin* (16)] and *NES1* (17). Both genes have been assigned to chromosome 19q13.3.

PSA is considered the best diagnostic and prognostic marker for prostate cancer (18, 19), and *KLK2*, *zyme*, and *NES1* are now being evaluated as useful diagnostic and/or prognostic markers for prostate, breast, and ovarian cancer (19–21).

Recently, Nelson *et al.* (22) cloned a new gene, *protease*, by using a subtraction approach and demonstrated by Northern blot analysis that it is expressed primarily, if not exclusively, in prostatic tissue. They mapped *protease* to chromosome 19q13.3–q13.4, close to the human *KLK* gene locus (Ref. 22; see GenBank accession nos. AF 113140 and AF 113141).

In our efforts to identify additional genes that could be useful markers for cancer, we studied an area spanning ~300 kb of contiguous DNA sequence on human chromosome 19 (19q13.3–q13.4). Our approach independently allowed us to clone a gene, named *KLK-L1* (GenBank accession no. AF135023). Upon homology analysis, *protease* and *KLK-L1* are identical and represent the same gene. Here, we describe fine mapping of the *protease/KLK-L1* gene and its chromosomal localization in relation to a number of other homologous genes also mapping to the same region. In addition, we present extensive tissue expression studies and demonstrate that, in addition to prostate (which shows the highest expression), *protease/KLK-L1* is also expressed in female breasts, testis, adrenals, uterus, colon, thyroid, brain, spinal cord, and salivary glands. Furthermore, we demonstrate that this gene is up-regulated by androgens and progestins in the breast carcinoma cell line BT-474.

Materials and Methods

DNA Sequences on Chromosome 19. Large amounts of DNA sequencing data for chromosome 19 are available from the Lawrence Livermore National Laboratory.³ We have obtained ~300 kb of genomic sequence from their web site, encompassing a region on chromosome 19q13.3–q13.4, where the known *KLK* genes are localized. This sequence is represented by nine contigs of variable lengths. By using the published sequences of PSA, *KLK2*, *NES1*, and *protease M* and the alignment program BLAST 2 (23), we identified the relative positions of these genes on the contiguous map.

Gene Prediction Analysis. For exon prediction analysis of the whole genomic area, we used a number of different computer programs. We originally tested all these programs using the known genomic sequences of the PSA, *protease M*, and *NES1* genes. The most reliable computer programs

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² The abbreviations used are: EST, expressed sequence tag; KLK, kallikrein; KLK-L, KLK-like; PSA, prostate-specific antigen; NES1, normal epithelial cell specific-1; RT-PCR, reverse transcription-PCR.

³ <http://www.bio.llnl.gov/genome/genome.html>.

Table 1 ESTs with >95% homology to exons of the prostate/CLK-L1 gene

GenBank accession no.	Source	Tissue	Homologous exons
AA551449	I.M.A.G.E.	Prostate	3, 4, 5
AA533140	I.M.A.G.E.	Prostate	4, 5
AA503963	I.M.A.G.E.	Prostate	5
AA569484	I.M.A.G.E.	Prostate	5
AA336074	TIGR ^a	Endometrium	2, 3

^a TIGR, The Institute for Genomic Research.

[GeneBuilder⁴ (gene prediction and exon prediction); Grail 2⁵; and GENEID-3⁶] were used for further use.

Protein Homology Searching. Putative exons of the newly identified gene were first translated to the corresponding amino acid sequences. BLAST homology searching for the proteins encoded by the exons were performed using the BLASTP program and the GenBank databases (23).

Searching ESTs. Sequence homology searching was performed using the BLASTN algorithm (23), obtained from the National Center for Biotechnology Information,⁷ against the human EST database (dbEST). Clones with >95% homology were obtained from the I.M.A.G.E. consortium (24) through Research Genetics Inc. (Huntsville, AL) and from The Institute for Genomic Research⁸ (Table 1). Clones were propagated, purified, and then sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

Breast Cancer Cell Line and Stimulation Experiments. The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (Manassas, VA). BT-474 cells were cultured in RPMI (Life Technologies, Inc., Gaithersburg, MD), supplemented with glutamine (200 mmol/liter), bovine insulin (10 mg/liter), 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 h before the experiments, the culture media were replaced with phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, 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 mRNA extraction.

RT-PCR. Total RNA was extracted from the breast cancer cells using Trizol reagent (Life Technologies, Inc.) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. Two µg of total RNA were reverse transcribed into first strand cDNA using the Superscript preamplification system (Life Technologies, Inc.). The final volume was 20 µl. On the basis of the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (Table 2), PCR was carried out in a reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTPs, 150 ng of primers, and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ) on a Perkin-Elmer (Foster City, CA) 9600 thermal cycler. The cycling conditions were: 94°C for 9 min, to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s and 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.

Tissue Expression of Prostase. Total RNA isolated from 26 different human tissues was purchased from Clontech (Palo Alto, CA). We prepared cDNA as described above for the tissue culture experiments and used it for PCRs with the primers described in Table 2. Tissue cDNAs were amplified at various dilutions.

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

⁴ <http://l25.itba.mi.cnr.it/~webgene/genebuilder.html>.

⁵ <http://compbio.ornl.gov>.

⁶ <http://apolo.imim.es/geneid.html>.

⁷ <http://www.ncbi.nlm.nih.gov/BLAST/>.

⁸ <http://www.tigr.org/tldb/db.html>.

Results

Identification of the Prostase/CLK-L1 Gene. Our exon prediction strategy of the 300-kb DNA sequences around chromosome 19q13.3–q13.4 identified a novel gene with a structure reminiscent of a serine protease. The major features of this gene were: its homology, at the amino acid and DNA levels, with other human *CLK* genes; the conservation of the catalytic triad (histidine, aspartic acid, and serine); the number of exons; and the complete conservation of the intron phases. Because the genomic and cDNA structure of our gene, named *CLK-L1*, was subsequently found to be identical to the structure reported independently by Nelson *et al.* (22), we conclude that the *CLK-L1* gene (GenBank accession no. AF135023) is the same gene as prostase (GenBank accession nos. AF113140 and AF 113141).

EST Sequence Homology Search. EST sequence homology search of the putative exons obtained from the gene prediction programs (as described above) against the human EST database (dbEST) revealed five ESTs with >95% identity to the putative exons of our gene (Table 1). Positive clones were obtained, and the inserts were sequenced from both directions. Alignment was used to compare between the EST sequences and the exons predicted by the computer programs, and final selection of the exon-intron splice sites was made according to the EST sequences. Furthermore, many of the ESTs were overlapping, further ensuring the accuracy of the data. The coding and genomic sequence of the gene, as submitted to GenBank (accession no. AF135023), are essentially the same as the data submitted by Nelson *et al.* (22).

Mapping and Chromosomal Localization of Prostase/CLK-L1 Gene. Alignment of the prostase/*CLK-L1* sequence and the sequences of other known *CLK* genes within the 300-kb area of the contigs constructed at the Lawrence Livermore National Laboratory enabled us to precisely localize all genes and to determine the direction of transcription, as shown in Fig. 1. The distance between PSA and *CLK2* genes was calculated to be 12,508 bp. The prostase/*CLK-L1* gene was 26,229 bp more telomeric to *CLK2* and was transcribed in the opposite direction. The *zyme* gene was ~51 kb more telomeric to the prostase gene and was transcribed in the same direction. The human stratum corneum chymotryptic enzyme gene, the neuropsin gene, and the *NES1* gene were all further telomeric to *zyme* and were all transcribed in the same direction as *zyme*. There was another putative novel *CLK-L* gene between prostase/*CLK-L1* and *zyme* that has not, as yet, been extensively characterized (GenBank accession no. AF135028).

Tissue Expression of the Prostase/CLK-L1 Gene. We have assessed by RT-PCR the tissues that express the prostase/*CLK-L1* gene. The experiments were performed at various dilutions of the cDNAs to obtain some information about the relative levels of expression. RT-PCR for actin was used as a positive control, and RT-PCR for the PSA cDNA was used as another positive control with tissue-restricted specificity. Positive ESTs for prostase/*CLK-L1* were used as controls for the PCR procedure. The PSA gene was found to be highly

Table 2 Primers used for RT-PCR analysis of various genes

Gene	Primer name	Sequence ^a	Product size (bp)
Prostase (<i>CLK-L1</i>)	RS	TGACCCGCTGTACCACCCCA	278
	RAS	GAATTCCTCCGCAGGATGT	
<i>pS2</i>	PS2S	GGTGATCTGCGCCCTGGTCCT	328
	PS2AS	AGGTGTCCGGTGGAGGTGGCA	
PSA	PSAS	TGCGCAAGTTCACCCCTCA	754
	PSAAS	CCCTCTCCTTACTTCATCC	
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT	372
	ACTINAS	TCTCCTTAATGTCACGCACGA	

^a All nucleotide sequences are given in the 5' → 3' orientation.

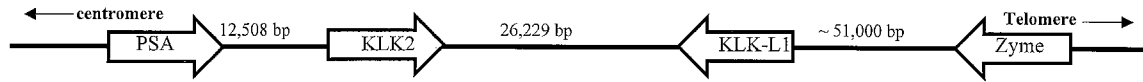


Fig. 1. A contiguous genomic sequence around chromosome 19q13.3–q13.4. Arrows, genes; the directions of the arrows represent the directions of the coding sequences. Distances between genes are in bp. Figure is not drawn to scale.

Table 3 Tissue expression of prostate/KLK-L1 by RT-PCR analysis

High expression	Medium expression	Low expression	No expression
Prostate	Mammary gland	Salivary glands	Stomach
Testis	Colon	Lung	Heart
Adrenals	Spinal cord	Brain	Spleen
Uterus		Bone marrow	Placenta
Thyroid		Thymus	Liver
		Trachea	Pancreas
		Cerebellum	Kidney
			Fetal brain
			Fetal liver
			Skeletal muscle
			Small intestine

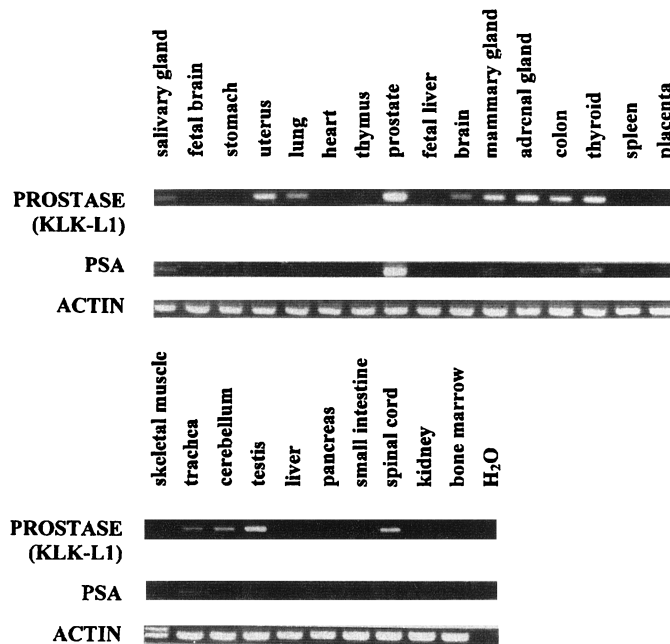


Fig. 2. Tissue expression of the prostate/KLK-L1 gene as determined by RT-PCR. Actin and PSA were used as control genes. Interpretations are presented in Table 3.

expressed in the prostate, as expected, and to a lower extent in mammary and salivary glands, as also expected from recent literature reports (19, 25). We further found very low expression of PSA in the thyroid gland, trachea, and testis, a finding that is in accordance with recent RT-PCR data reported by others (26).

The tissue expression of prostate/KLK-L1 is summarized in Table 3 and Fig. 2. This protease was primarily expressed in the prostate, testis, adrenals, uterus, thyroid, colon, central nervous system, and mammary tissues and, at much lower levels, in other tissues. Because Nelson *et al.* (22) reported almost exclusive expression of prostate/KLK-L1 to prostate, we verified the specificity of our RT-PCR procedure for prostate/KLK-L1 by cloning the PCR products from mammary, testicular, and prostate tissues and sequencing them. One example with mammary tissue is shown in Fig. 3. All cloned PCR products were identical in sequence to the cDNA sequence reported for the prostate/KLK-L1.

Hormonal Regulation of the Prostate/KLK-L1 Gene. We have used the steroid hormone receptor-positive breast carcinoma cell line BT-474 as a model system to evaluate whether prostate/KLK-L1

expression is under steroid hormone regulation. As shown in Fig. 4, the controls worked as expected, *i.e.*, actin positivity without hormonal regulation in all cDNAs, estrogen up-regulation only of the *pS2* gene, and up-regulation of the *PSA* gene by androgens and progestins. Prostate/KLK-L1 was up-regulated primarily by androgens and progestins, similar to *PSA*. This up-regulation was dose dependent, and it was evident at steroid hormone levels $\geq 10^{-10}$ M (data not shown).

Discussion

The *KLK3* gene encodes for *PSA*, a protein that currently represents the best tumor marker available (19). Because there are so many *KLK* genes in rodents, the restriction of this family to only three genes in humans was somewhat surprising (7). More recently, new candidate *KLK* genes in humans have been discovered, including *NESI* (17) and *zyme/protease M/neurosin* (14–16). The known *KLKs* and the newly discovered *KLK-L* genes share the following similarities: (a) they encode for serine proteases; (b) they have five coding exons; (c) they share significant DNA and protein homologies with each other; (d) they map in the same locus on chromosome 19q13.3–q13.4, a region that is structurally similar to an area on mouse chromosome 7, where all of the mouse *KLK* genes are localized; and (e) they appear to be regulated by steroid hormones. We propose that prostate/KLK-L1 is a member of the same family because these common characteristics are also shared by the newly discovered gene.

Nelson *et al.* (22) used a subtraction approach, and we used the positional candidate approach (1, 4) to clone the same gene. These two gene identification methods are radically different. As more DNA sequence data become available, the method we used will become more popular. We verified that our gene is expressed by identifying

TGACCCGCTG TACCACCCCA GCATGTTCTG CGCCGGCGGA GGGCAAGACC AGAAGGACTC
CTGCAACGGT GACTCTGGGG GGCCCTGAT CTGCAACGGG TACTTCAGG GCCTTGTC
TTTCGGAAAA GCCCGGTGTG GCCAAGTTGG CGTGCCAGGT GCCTACACCA ACCTCTGCAA
ATTCACTGAG TGGATAGAGA AAACCGTCCA GGCCAGTTAA CTCTGGGGAC TGGGAACCCA
TGAAATTGAC CCCCAAATAC ATCCTGCGGA AGGAATTC

Fig. 3. Sequence of PCR product obtained with cDNA from female breast tissue using prostate/KLK-L1 primers. Primer sequences are underlined. The sequence is identical to the sequence obtained from prostatic tissue.

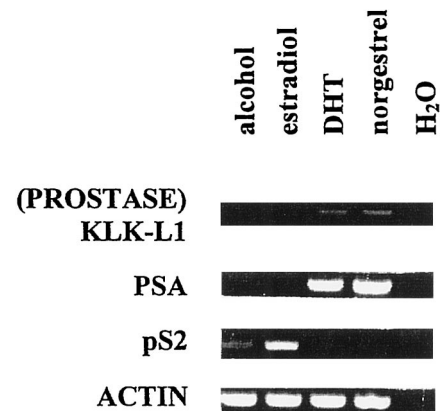


Fig. 4. Hormonal regulation of the prostate/KLK-L1 gene in the BT-474 breast carcinoma cell lines. DHT, dihydrotestosterone. Steroids were added at a final concentration of 10^{-8} M. Actin (not regulated by steroid hormones), *pS2* (up-regulated by estrogens), and *PSA* (up-regulated by androgens and progestins), were used as control genes. Prostate/KLK-L1 was up-regulated by androgens and progestins.

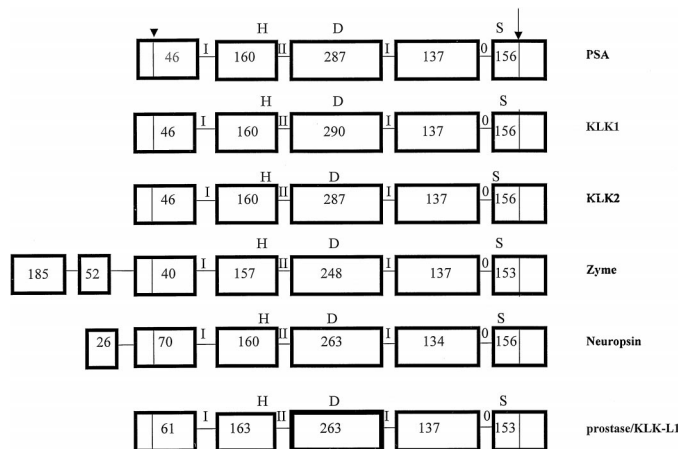


Fig. 5. Schematic diagram showing comparison of the genomic structure of PSA, *KLK1*, *KLK2*, *zyme*, *neuropilin*, and *prostate/KLK-L1* genes. Exons are represented by boxes, and introns are represented by the connecting lines. Arrowhead, start codons; vertical arrow, stop codons. Letters above boxes indicate relative positions of the catalytic triad: H, histidine; D, aspartic acid; and S, serine. Roman numerals, intron phases. The intron phase refers to the location of the intron within the codon: I, intron occurs after the first nucleotide of the codon; II, intron occurs after the second nucleotide of the codon; and 0, intron occurs between codons. Numbers inside boxes indicate exon lengths (in bp). Figure is not drawn to scale.

and sequencing five independent ESTs isolated from prostate as well as from other tissues (Table 1).

Many other *KLK-L* genes (including *NESI*, *zyme*, and *neuropilin*) contain one or more untranslated exons in the 5' end of mRNA (27, 28). Neither Nelson *et al.* (22) nor we were able to clarify this issue for *prostate/KLK-L1*. Although the coding sequence of the gene is now resolved, more work will be necessary to establish the exact transcription start site and the identification of any untranslated exons.

The *prostate* gene was mapped by Nelson *et al.* (22) to chromosome 19q13.3–q13.4. In our work, we extended this data by finding the exact localization of the gene and its position in relation to other genes in the area (Fig. 1). *Prostate/KLK-L1* lies between *KLK2* and *zyme*. Our recent work suggests that the *KLK* locus in humans may contain at least 13 *KLK-L* genes (29).

Irwin *et al.* (30) have proposed that the serine protease genes can be classified into five different groups, according to intron position. The established *KLKs* (*KLK1*, *KLK2*, and *PSA*), *trypsinogen*, and *chymotrypsinogen* belong to a group that has: (a) an intron just downstream from the codon for the active site histidine residue, (b) a second intron downstream from the exon containing the codon for the active site aspartic acid residue, and (c) a third intron just upstream from the exon containing the codon for the active site serine residue. As seen in Fig. 5, the genomic organization of *prostate/KLK-L1* gene is very similar to this group of genes. The lengths of the coding parts of exons 1–5 are 61, 163, 263, 137, and 153 bp, respectively, which are close or identical to the lengths of the exons of the *KLK* genes and also similar or identical to those of other newly discovered genes in the same chromosomal region, like the *NESI* (27), *zyme/protease M/neurosin* (14–16),⁹ and *neuropilin* (28) genes.

Nelson *et al.* (22) reported that *prostate* is almost exclusively expressed in prostatic tissue, as determined by Northern blot analysis. Our more sensitive RT-PCR protocol reveals that this enzyme is also expressed in significant amounts in other tissues, including testis, female mammary gland, adrenals, uterus, thyroid, colon, brain, lung, and salivary glands (Fig. 2 and Table 3). Except for testis, the other tissues mentioned here were not studied by Nelson *et al.* (22). The specificity of our RT-PCR primers was verified by sequencing the

obtained PCR products, with one example shown in Fig. 3. The finding of *prostate/KLK-L1* expression in mammary gland and other tissues should not be surprising. The classical *KLKs* *PSA* and *KLK2*, which are predominantly prostatic proteins, were shown to be expressed at significant amounts in female breast (25, 31, 32). Moreover, our tissue culture studies with the breast carcinoma cell line BT-474 further confirm not only the ability of these cells to produce *prostate/KLK-L1* but also hormonal regulation of *prostate/KLK-L1* in a manner similar to that reported by Nelson *et al.* (22) for the prostatic carcinoma cell line LNCaP (Ref. 33; Fig. 4). The functional characterization of the *prostate/KLK-L1* gene promoter has not, as yet, been reported.

The diagnostic/prognostic and other clinical applications of the *prostate/KLK-L1* gene are currently unknown. Nelson *et al.* (22) reported a number of possible functions for this protein. An interesting theme is now developing involving the group of homologous genes on chromosome 19q13.3 (*PSA*, *KLK2*, *prostate*, *zyme*, and *NESI*). The combined data to date suggest that all of them are expressed in prostate and breast tissues, and all of them are hormonally regulated. At least three genes (*PSA*, *zyme*, and *NESI*) are significantly down-regulated in breast cancer (14, 17, 34, 35), and one of them (*NESI*) appears to be a novel tumor suppressor (21). *PSA* appears to be an inducer of apoptosis and a negative regulator of cell growth (36, 37). *KLK2* may be part of a pathway that activates pro-*PSA* (38–40). It is conceivable that all these genes are part of a cascade pathway that plays a role in cell proliferation, differentiation, or apoptosis by regulating (positively or negatively) growth factors or their receptors or cytokines through proteolysis (41). Also interesting is the linkage of locus 19q13 to solid tumors and gliomas (42), which raises the possibility that some of the genes in the region may be disrupted by rearrangements. This possibility has not, as yet, been examined.

In conclusion, we cloned independently, by the positional candidate approach, a gene that was recently cloned by subtractive hybridization (22). This new gene encodes for a serine protease that shows homology with other members of the *KLK* gene family and maps to the same chromosomal location. Many structural features of the *KLKs* are conserved in *prostate/KLK-L1*. Here, we present the precise mapping of this gene between the two known genes, *KLK2* and *zyme*. We further demonstrate that *prostate/KLK-L1* is expressed in many tissues, in addition the prostate, including the female breast. We propose that this gene should not be called “*prostate*” because, as in the situation with *PSA*, it will represent a misnomer. We have further demonstrated, using breast carcinoma cell lines, that *prostate/KLK-L1* can be produced by these cells and that its expression is significantly up-regulated by androgens and progestins. On the basis of information for other homologous genes in the area (*PSA*, *zyme*, and *NESI*), we speculate that *prostate/KLK-L1* may be involved in the pathogenesis and/or progression of prostate, breast, and, possibly, other cancers. Recently, the similarities between prostate and breast cancers have been reviewed (43). The newly identified gene can be added to the list of genes that may play a role in prostate and breast cancer.

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⁹ Unpublished data.

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