Genomics **74,** 385–395 (2001)
doi:10.1006/geno.2001.6556, available online at http://www.idealibrary.com on IDEAL®

Molecular Cloning of a Novel Human Acid Phosphatase Gene (ACPT) That Is Highly Expressed in the Testis

George M. Yousef,*,† Maria Diamandis,* Klaus Jung,‡ and Eleftherios P. Diamandis*,†,[1]

*Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5 Canada; †Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, M5G 1L5 Canada; and ‡Department of Urology, University Hospital Charite, Humboldt University, Berlin, 10098 Germany

Received January 9, 2001; accepted April 3, 2001

Acid phosphatases are enzymes capable of hydrolyzing orthophosphoric acid esters in an acid medium. Prostatic acid phosphatase has served as a tumor marker for metastatic prostate cancer for many years. We have cloned a new human acid phosphatase gene (named testicular acid phosphatase, ACPT), which is expressed mainly in testis and to a lower extent in the prostate, trachea, and other tissues. This gene maps to chromosome 19q13.4, in an area that harbors many cancer–related genes. The testicular acid phosphatase gene is composed of 11 exons, and the protein is predicted to have a luminal domain, a transmembrane domain, and a cytoplasmic domain. The N-terminal end of the protein encodes a signal peptide. The protein has approximately 50% homology with both the prostatic and the lysosomal acid phosphatases, and the position of the cysteine residues, the N-glycosylation sites, and the histidine catalytic site are conserved among the three proteins. The testicular acid phosphatase gene is up-regulated by androgens and is down-regulated by estrogens in the prostate cancer cell line LNCaP. Our preliminary results indicate that this gene exhibits a lower level of expression in testicular cancer tissues than in their normal counterparts.

INTRODUCTION

Acid phosphatases (ACPs; orthophosphoric monoester phosphohydrolases) are a group of enzymes capable of hydrolyzing esters of orthophosphoric acid in an acid medium (Romas *et al.*, 1979). Four true isoenzymes (that is, forms with differences originating at the structural level of the gene) have been identified: erythrocytic, lysosomal, prostatic, and macrophagic acid phosphatases (Moss *et al.*, 1995). The different forms are often associated with particular characteristics that may not necessarily be absolute or specific. Erythroid acid phosphatase (ACP1)[2] is a small molecule that belongs to the group of acid phosphatases that are not inhibited by tartrate. The gene is 18 kb in length, consists of 7 exons (Bryson *et al.*, 1995), and has been mapped to the short arm of chromosome 2 (Ferguson-Smith *et al.*, 1973). The human lysosomal acid phosphatase gene (ACP2) was cloned by Pohlmann *et al.* (1988) and was assigned to chromosome 11. ACP2 is about 9 kb in size and is composed of 11 exons. The luminal domain is encoded by exons 2 to 10; exon 1 encodes the signal sequence and the first 8 amino acids, while exon 11 encodes the transmembrane and cytoplasmic domains (Geier *et al.*, 1989; Pohlmann *et al.*, 1988). Human prostatic acid phosphatase was the first urological tumor marker. It has been used for over 50 years for the diagnosis and prognosis of prostate cancer (Sharief and Li, 1994; Vihko *et al.*, 1988). This marker has now been largely replaced by prostate-specific antigen (PSA), which is a more sensitive and specific biomarker for the diagnosis and monitoring of prostatic carcinoma (Diamandis, 1998). Human prostatic acid phosphatase (ACPP or ACP3) is formed of 10 exons, spanning an area of approximately 40 kb on chromosome 3 (Sharief *et al.*, 1989; Sharief and Li, 1992, 1994; Vihko *et al.*, 1988; Yeh *et al.*, 1987). The gene encoding human macrophage acid phosphatase has been cloned and mapped to chromosome 19p13.2–p13.3 (Cassady *et al.*, 1993; Ketcham *et al.*, 1989; Lord *et al.*, 1990), has an open reading frame of 975 bp, and is resistant to inhibition by tartarate.

The chromosomal band 19q13.3–q13.4 is nonrandomly rearranged in a variety of solid tumors and harbors malignancy-related genes, including the RRAS oncogene and the apoptosis regulator gene BAX (Apte

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AF321918.

[1] To whom correspondence and reprint requests should be addressed at Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada. Telephone: (416) 586-8443. Fax: (416) 586-8628. E-mail: ediamandis@mtsinai.on.ca.

[2] Abbreviations used: ACP, acid phosphatase; ACPP, prostatic acid phosphatase; PSA, prostate-specific antigen; ACP2, lysosomal acid phosphatase; ACP1, erythroid acid phosphatase; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends; ACPT, testicular acid phosphatase.

0888-7543/01 $35.00

TABLE 1

EST Clones with >97% Identity to Exons of the Testicular Acid Phosphatase Gene

GenBank Accession No.	Tissue of origin	IMAGE ID	Homologous exons
AA861338	Testis	1407392	6–10
AA868001	Pooled testis, fetal lung, and B-cells	1461124	6–10
AA861459	Testis	1406569	6–10
AA983914	Pooled testis, fetal lung, and B-cells	1583318	6–10
AA005037	Pooled fetal liver and spleen	428530	6–10

et al., 1995). In our previous work, we cloned and mapped a number of kallikrein genes located in this region, including PSA and other genes that were found to be differentially expressed in a variety of tumors, including prostate cancer (Diamandis *et al.*, 2000; Yousef *et al.*, 1999, 2000a; Yousef and Diamandis, 1999). Here, we describe the cloning of a new gene, the testicular acid phosphatase gene, which is highly homologous to prostatic and lysosomal acid phosphatases. Our preliminary data indicate that this gene is regulated by steroid hormones and that its expression is significantly lower in testicular cancer tissues than in normal testicular tissues.

MATERIALS AND METHODS

Cloning of the testicular acid phosphatase gene. Sequencing information around the chromosomal region 19q13.4 and bacterial artificial chromosome (BAC) localization maps were obtained from the Lawrence Livermore National Laboratory (LLNL). Using different gene prediction programs, a putative new gene was identified in this region. The BAC clone that contains this gene (BC 781134) was obtained from the LLNL. This clone was digested, blotted on a membrane, and hybridized with gene-specific probes for the putative testicular acid phosphatase gene. Probes were synthesized according to the predicted sequence of the new gene, and positive fragments were subcloned and sequenced to verify the structure of the putative gene.

Expressed sequence tag (EST) searching. The predicted exons of the putative new gene were subjected to homology search using the BLASTN algorithm (Altschul *et al.*, 1997) on the National Center for Biotechnology Information Web server (http://www ncbi.nlm.nih.gov/BLAST/) against the human EST database (dbEST). Five clones with >97% identity were obtained from the IMAGE Consortium (Lennon *et al.*, 1996) through Research Genetics Inc. (Huntsville, AL) (Table 1). The clones were propagated, purified, and sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

Rapid amplification of cDNA ends (5′ RACE). According to the EST sequence data and the predicted structure of the gene, two gene-specific primers were designed (RN, 5′ CTC CAG GGC CTC CTG GTA CT 3′; and RN2, 5′ GGA ATA CCT GGG GAC AGG AC 3′). Two rounds of RACE reactions (nested polymerase chain reaction (PCR)) were performed with 5 μl Marathon Ready cDNA from human testis (Clontech, Palo Alto, CA) as a template. The reaction mix and PCR conditions were in accordance with the manufacturer's recommendations.

Tissue expression. Total RNA isolated from 25 different human tissues was purchased from Clontech. We prepared cDNA as described below and used it for PCRs. Tissue cDNAs were amplified at various dilutions. To exclude nonspecific amplification, the PCR products were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The inserts were then sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from normal and malignant tissues using Trizol reagent (Gibco BRL, Gaithersburg, MD) following the manufacturer's instructions. The RNA concentration was determined spectrophotometrically. Two micrograms of total RNA was reverse-transcribed into first-strand cDNA using the Superscript preamplification system (Gibco BRL). The final volume was 20 μl. Two gene-specific primers were designed (F1MD, 5′ ATGGCCGGCCTGGGGTTTTG 3′; and R6PD, 5′ ACATGTTGGGGTCCAGTGTCA 3′), and 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 $MgCl_2$, 200 μM dNTPs (deoxynucleoside triphosphates), 150 ng of primers, and 2.5 units of HotStar DNA polymerase (Qiagen Inc., Valencia, CA) on a Perkin–Elmer 9600 thermal cycler. The cycling conditions were 94°C for 15 min to activate the polymerase, followed by 45 cycles of 94°C for 30 s, 64°C for 30 s, and 72° for 1 min, and a final extension step at 72°C for 10 min. Another pair of primers (F1PD, 5′ CAGTCTGACCAACACGCAAG 3′, and R6PD, 5′ ACATGTTGGGGTCCAGTGTCA 3′) was used for tissue expression and tumor screening. Equal amounts of PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least two exons to avoid contamination by genomic DNA.

Prostate cancer cell line and hormonal stimulation experiments. The LNCaP prostate cancer cell line was purchased from the American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in RPMI medium (Gibco BRL) 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 to 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 mRNA extraction.

Testicular cancer tissue specimens. Normal and cancerous testicular tissue samples were obtained from 14 patients who had undergone radical orchiectomy for testicular tumors at the Charite University Hospital, Berlin, Germany. Six tumors were seminomas, and 8 tumors were nonseminomas. The patients did not receive any hormonal therapy before surgery. The use of these tissues for research purposes was approved by the Ethics Committee of the Charite Hospital. Fresh testicular tissue samples were obtained from the cancerous and noncancerous parts of the same testis that had been removed. Small pieces of tissue were dissected immediately after removal of the testis and stored in liquid nitrogen until analysis. Histological analysis from all the tissue pieces was performed as previously described (Meyer *et al.*, 1997), to ensure that the tissue was either malignant or benign. The tissues were pulverized with a hammer under liquid nitrogen, and RNA was extracted using RNeasy minispin columns (Qiagen) following the manufacturer's instructions.

RESULTS

Cloning and Characterization of the Testicular Acid Phosphatase Gene

The BAC clone (BC 781134) was obtained from the LLNL, purified, digested, and probed with a testicular

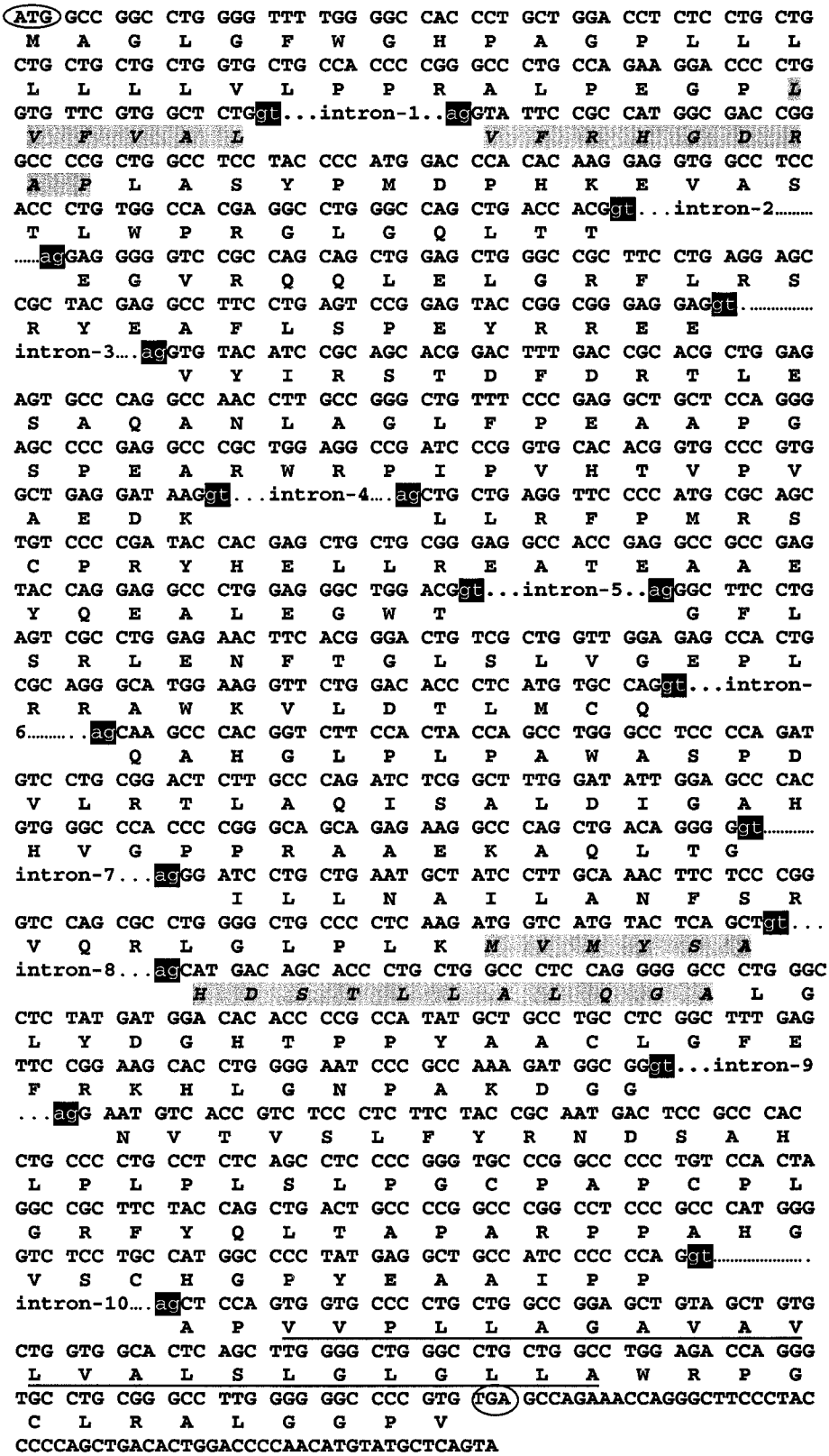

FIG. 1. Genomic organization and partial genomic sequence of the testicular acid phosphatse gene. Intronic sequences are not shown except for the splice junction areas. The start and stop codons are encircled, and the exon–intron junctions are boxed. The translated amino acids of the coding region are shown underneath in single-letter code. Amino acids of the histidine phosphatases phosphohistidine signature motif and the histidine acid phosphatases active site signature motif are highlighted in gray. Residues of the transmembrane domain are underlined. Intron boundaries are shown in lowercase letters. The full sequence of the gene and its splice variants can be found in GenBank (GenBank Accession No. AF321918).

TABLE 2

Comparison of Structural Features of the Three Acid Phosphatase Genes

	Exon length			Intron phase[a]			Splice sites		
	ACP2[b]	ACPT[c]	ACPP[d]	ACP2	ACPT	ACPP	ACP2	ACPT	ACPP
Exon 1[e]	106	111	120	0	0	0	GT/AG	GT/AG	GT/AG
Exon 2	96	105	96	0	0	0	GT/AG	GT/AG	GT/AG
Exon 3	87	87	90	0	0	0	GT/AG	GT/AG	GC/AG
Exon 4	153	147	153	0	0	0	GT/AG	GT/AG	GT/AG
Exon 5	99	99	99	0	0	0	GT/AG	GT/AG	GT/AG
Exon 6	90	96	93	0	0	0	GT/AG	GT/AG	GT/AG
Exon 7	133	133	132	I	I	0	GT/AG	GT/AG	GT/AG
Exon 8	83	83	96	0	0	0	GT/AG	GT/AG	GT/AG
Exon 9	107	125	104	II	II	II	GT/AG	GT/AG	GT/AG
Exon 10	176	179	[f]	I	I	—	GT/AG	GT/AG	GT/AG
Exon 11[a]	116	116	—	—	—	—	—	—	—

[a] Intron phases are indicated by roman numerals. 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.
[b] ACP2, lysosomal acid phosphatase.
[c] ACPT, testicular acid phosphatase.
[d] ACPP, prostatic acid phosphatase.
[e] Includes only the coding region.
[f] Variable in different isoforms.

acid phosphatase-specific oligonucleotide probe. Positive fragments were sequenced, to obtain the genomic structure of the gene. EST database search, PCR using exon-specific primers derived from the predicted exons of the gene, 5′ RACE, and protein and domain homology search were used to identify the full structure of the testicular acid phosphatase gene.

The testicular acid phosphatase gene (official gene symbol ACPT, approved by the Human Gene Nomenclature Committee) is composed of 11 coding exons and 10 intervening introns and spans 5 kb of genomic nucleotide sequence (Fig. 1). All exon–intron boundaries comply with the GT/AG rule and are in good agreement with the consensus sequence of splice junctions (Breathnach and Chambon, 1981). The cDNA of the gene consists of 1281 bp, encoding a polypeptide chain of 426 amino acids with a predicted molecular mass of 46.1 kDa. The methionine translation initiation codon is located in the first exon, and, as is the case with most vertebrate mRNAs, the start codon is surrounded by a purine in position −3 and a (G) in position +4 (Kozak, 1991). No more sequence was obtained upstream of the start codon by 5′ RACE. Thus, the transcription initiation site is expected to be close to the translation start codon. The two most homologous genes, ACPP and ACP2, have short 5′ untranslated regions of 23 (Vihko *et al.*, 1988) and 9 nucleotides (Geier *et al.*, 1989), respectively. The coding region is followed by 61 bases of 3′ untranslated sequence before the poly(A) tail. All exons end with an intron phase of 0 except exons 7 and 10 which have an intron phase of I, and exon 9, which has an intron phase of II. A comparison of intron phases and other characteristics of ACPT, ACP2, and ACPP is shown in Table 2. At the protein level, testicular acid phosphatase shows 48% identity and 63% similarity with the human lysosomal acid phosphatase and 44% identity and 60% similarity with the prostatic acid phosphatase. No significant homology was found with erythroid or type 5 acid phosphatases. The amino acid sequence of the gene starts with a putative signal peptide of 28 amino acids (Fig. 2). This signal peptide has the structural features characteristic of a signal peptide (Nielsen *et al.*, 1997; Nielsen and Krogh, 1998): (1) a basic N-terminal region, a central hydrophobic region, and a more polar C-terminal region; (2) alanine is present at position −3; and (3) leucine is present at positions −7 to −13 with the exception of position −8 (Fig. 3).

The Prediction of Transmembrane Helices in Proteins program (Sonnhammer *et al.*, 1998) predicted that amino acids 1–390 represent a luminal (extracellular) domain, amino acids 391–413 represent a transmembrane domain, and amino acids 414–425 represent a cytoplasmic domain (Fig. 4). The same results were obtained by the TMpred program (Hofmann and Stoffel, 1993) (data not shown).

Scanning of the testicular acid phosphatase protein against the PROSITE database of protein families and domains (Hofmann *et al.*, 1999) revealed the presence of four potential N-glycosylation sites, a phosphohistidine signature domain, and a histidine acid phosphatase active site signature (Table 3). Screening of the CD (conserved domain) database at the National Center for Biotechnology Information (NCBI) revealed the presence of the same domains.

To study the phylogenetic relatedness of the new testicular acid phosphatase, we aligned its amino acid sequence with all known acid phosphatases, human alkaline phosphatase, and some known lysosomal enzymes using the Clustal X multiple alignment pro-

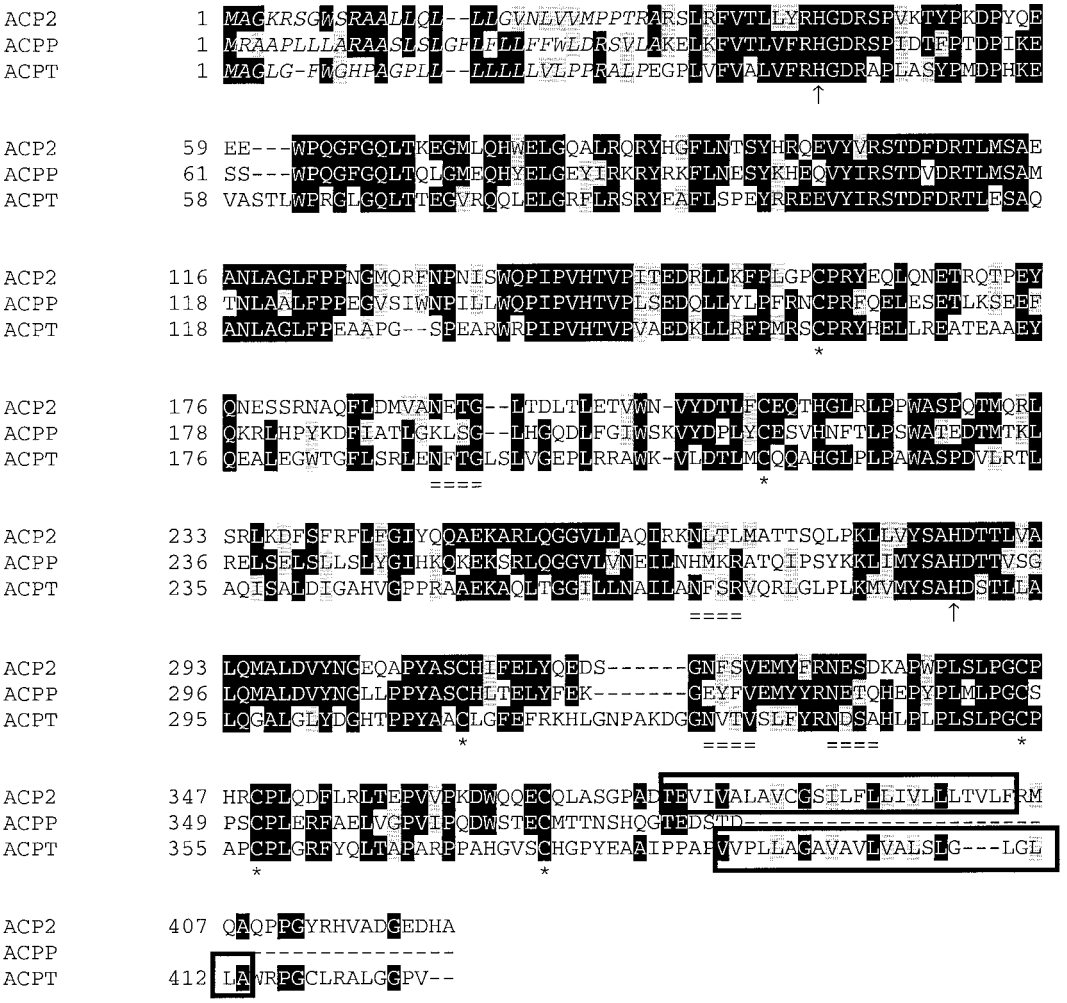

FIG. 2. Multiple alignment of the amino acid sequences of testicular acid phosphatase (ACPT), prostatic acid phosphatase (ACPP), and lysosomal acid phosphatase (ACP2) proteins. Numbers of the amino acid residues of each protein are shown on the left of each row. Identical residues are highlighted in black, and similar residues are highlighted in gray. The amino acid residues of the signal peptides are shown in italics. The positions of the conserved cysteine residues are marked by asterisks. N-glycosylation sites are double underlined, and transmembrane domains are boxed. The histidine residues important for catalytic activity are shown by an arrow.

gram. Distance matrix and parsimony trees were constructed using the neighbor-joining/UPGMA and Protpars parsimony methods. All possible trees grouped testicular acid phosphatase together with both ACPP and ACP2 in one group (Fig. 5).

Tissue Expression and Hormonal Regulation of the Testicular Acid Phosphatase Gene

We screened cDNA from 25 normal human tissues by PCR using gene-specific primers. The gene is expressed at highest levels in the testis. Lower levels of expression are found in the trachea, prostate, bone marrow, spinal cord, colon, fetal brain, heart, thymus, fetal liver, spleen, pancreas, and skeletal muscle (Fig. 6). To verify the RT-PCR specificity, the PCR products were cloned and sequenced. The multiple bands in some tissues represent splice variants, as exemplified in detail below.

Figure 7 shows that the testicular acid phosphatase gene is under steroid hormone regulation in the prostatic carcinoma cell line LNCaP. Expression of the gene, at the mRNA level, is up-regulated by mibolerone (a synthetic androgen) and dihydrotestosterone (DHT) and is down-regulated by estrogen (estradiol) and progestin (norgestrel).

Testicular Acid Phosphatase Gene Expression in Testicular Tumors

To investigate the possible differential expression of the testicular acid phosphatase gene in testicular tumors, we studied 14 pairs of matched cancerous/normal tissues of testicular tumors, including 6 seminomas and 8 nonseminomas (teratoma, embryonal carcinoma, and choriocarcinoma). cDNAs from these tissues were examined by PCR using gene-specific primers. The testicular acid phosphatase gene was found to be abundantly expressed in all 14 normal testicular tissues. Expression was very low or undetectable in 10 of the 14 cancerous counterparts, lower than normal in 2 tumors, and comparable to normal in

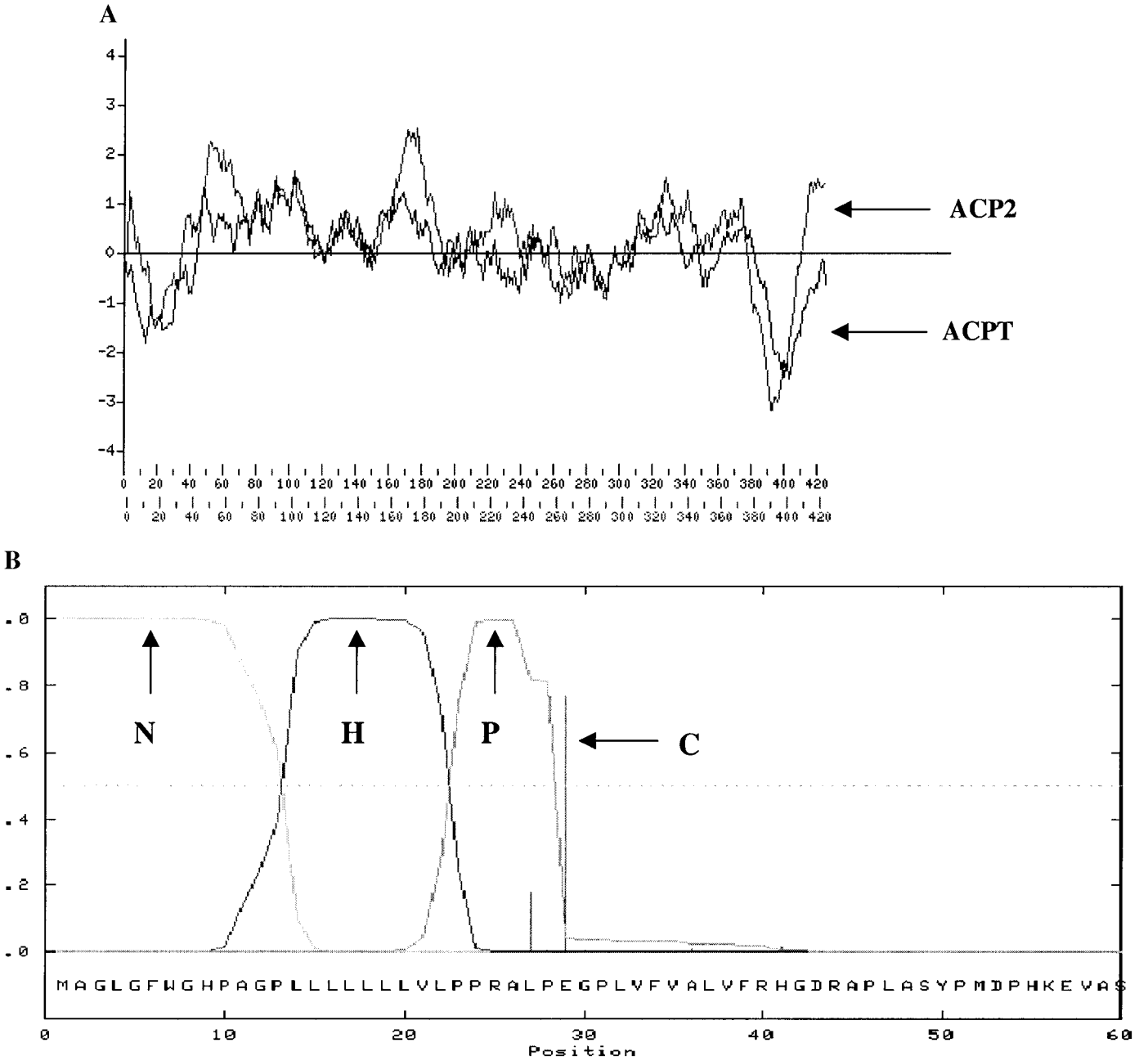

FIG. 3. (**A**) Plot of hydrophobicity and hydrophilicity of the testicular acid phosphatase (ACPT) and lysosomal acid phosphatase (ACP2). (**B**) Plot illustrating the different structural regions of the signal peptide of testicular acid phosphatase. N, basic N-terminal region; H, central hydrophobic region; P, polar C-terminal region; C, cleavage site (P ↓ E). Prediction of the signal peptide was performed using the SignalP server (Nielsen and Krogh, 1998).

another 2 tumors (Fig. 8). Actin, a housekeeping gene, was used as a control, and approximately the same level of expression for the latter was found in all normal and malignant tissues, indicating that the lower level of testicular acid phosphatase expression was not due to mRNA variation between samples.

Splice Variants of the Testicular Acid Phosphatase Gene

PCR screening using gene-specific primers (F1MD and R6PD) revealed the presence of up to three closely spaced bands in some of the tissue cDNAs (Fig. 6). These bands were digested by two different restriction enzymes (to allow better separation), gel-purified, cloned, and sequenced. The middle band represents the regular form of the gene, the upper band represents splice variant 1, and the lower band represents splice variant 2 (Fig. 9). Another short splice variant was identified in the bone marrow (splice variant 3) (data not shown). Splice variant 1 is present in the testis, trachea, prostate, and bone marrow. In this variant, the first intron is not spliced out; thus, exons 1 and 2

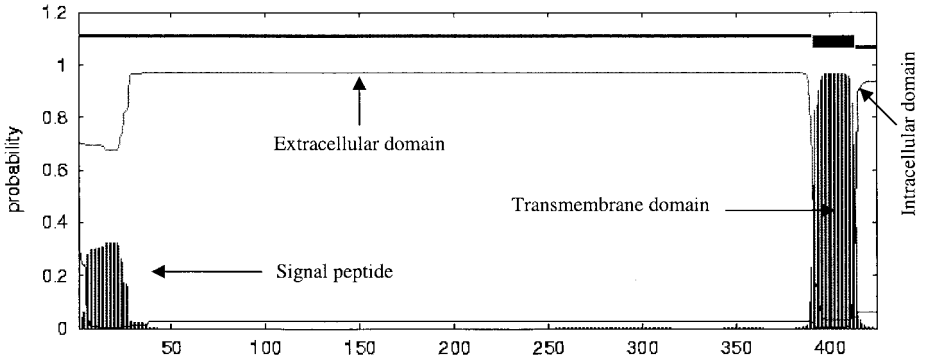

FIG. 4. A plot illustrating the predicted domains of testicular acid phosphatase using the TMHMM server. The signal peptide and extracellular, transmembrane, and intracellular domains are shown. For more details, see Results.

and the intervening intron are all parts of the first exon. This leads to frameshifting. The mRNA is predicted to encode a truncated protein of 92 amino acids. Splice variant 2 has the same structure as variant 1, in addition to a deleted exon 7. Splice variant 3 is similar to the regular form but is missing exons 4 and 5 and the last part of exon 3. This variant is predicted to encode a polypeptide chain of 333 amino acids.

Chromosomal Localization of the Testicular Acid Phosphatase Gene

PCR screening of the BAC clone (BC 781134) was performed using gene-specific primers for the testicular acid phosphatase gene and each of the 15 kallikrein genes that were recently cloned in this region (Diamandis *et al.*, 2000; Yousef *et al.*, 2000b). The clone was positive for KLK15, KLK1, and testicular acid phosphatase, but negative for KLK3 (PSA) and other kallikreins. We have recently constructed a detailed map of the human kallikrein gene locus (Yousef *et al.*, 2000b). We obtained contigs of different lengths from the BAC clone (BC781134), and by using long-PCR strategies, we were able to construct a larger contig that contains the testicular acid phosphatase gene, as well as the KLK1 and KLK15 genes. Sequence analysis indicated that the testicular acid phosphatase gene is the most centromeric and is transcribed from centromere to telomere. The KLK1 and the KLK15 genes are transcribed in the opposite direction. The KLK3 gene (coding for prostate-specific antigen) is the most telomeric and is transcribed in the same direction as the testicular acid phosphatase gene. A detailed map of the region, showing distances between these four genes and BAC clones, is shown in Fig. 10. No other genes were found to map between the ACPT and the KLK1 genes.

TABLE 3

Structural Domains and Postranslational Modification Sites of Testicular Acid Phosphatase

	Amino acid sequence	Position[a]
N-glycosylation sites	NFTG	191–194
	NFSR	269–272
	NVTV	330–333
	NDSA	339–342
Phosphohistidine signature of histidine phosphatases	LVFVALVFR**H**GDRAP	32–46
Active site signature of histidine acid phosphatases	MVMYSA**H**DSTLLALQGA	282–298
Transmembrane domain	VVPLLAGAVAVLVALSLGLGLLA	391–413

[a] Amino acid position according to GenBank Accession No. AF321918 and Fig. 1.

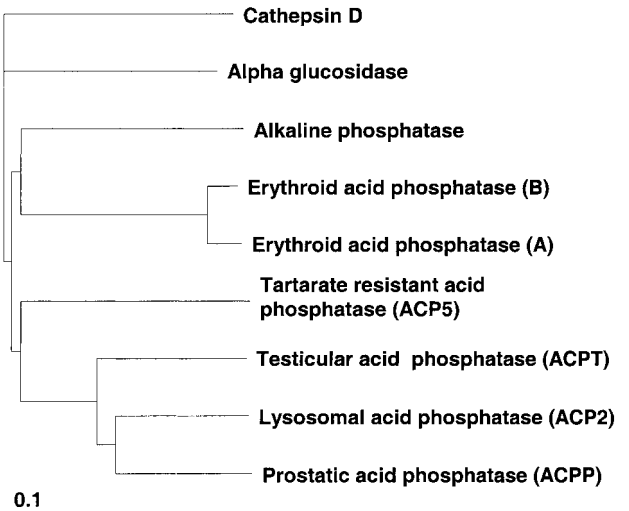

FIG. 5. Phylogenetic tree using the protein parsimony algorithm. All known human acid phosphatases were included in the analysis, in addition to human alkaline phosphatase and some lysosomal enzymes. The testicular acid phosphatase enzyme was aligned with both the ACPP and the ACP2 genes in one group, indicating that these genes arose from a common ancestral gene (see Discussion for details). Proteins included are as follows (the GenBank accession numbers are shown in parentheses): erythroid acid phosphatases A and B (NM_004300 and NM_007099), human lysosomal acid phosphatase (NP_001601), human prostatic acid phosphatase (NP_001090), ACP5 (tartrate-resistant acid phosphatase) (NP_001602), human alkaline phosphatase (NP_000469), alpha glucosidase (NP_004659), cathepsin D (NM_001909), and testicular acid phosphatase (AF321918).

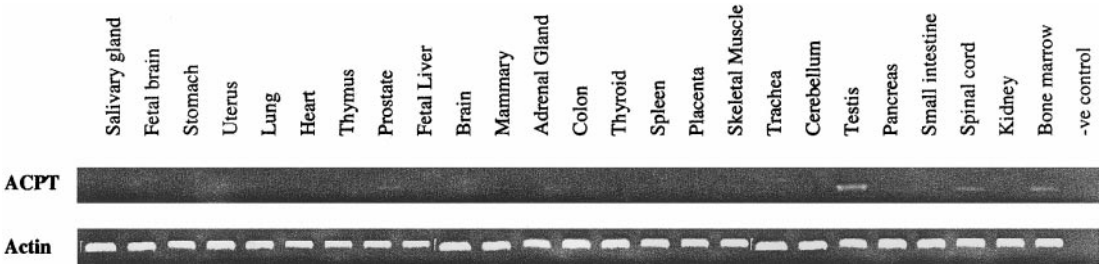

FIG. 6. Tissue expression of testicular acid phosphatase as determined by RT-PCR. The highest level of expression was found in the testis; however, lower levels were also found in many other tissues. More than one band was visible in some tissues. These bands represent the different splice variants of the gene. For splice variant discussion, see text and legend to Fig. 9.

DISCUSSION

Acid phosphatase (EC 3.1.3.2) is represented by a number of enzymes that can be differentiated according to structural, catalytic, and immunological properties (Waheed *et al.*, 1985). The structural features of the newly identified testicular acid phosphatase gene are quite similar to those of the prostatic and lysosomal acid phosphatases at both the DNA and the protein levels. At the genomic level, the following structural features are conserved in all three genes: (a) the numbers of exons are similar (except for the lack of exon 11, which contains the transmembrane domain, in prostatic acid phosphatase); (b) the lengths of all exons are comparable or identical (except for exon 11 of ACP2); (c) the intron phases are conserved in all three genes (except for exon 7 in ACPP); and (d) all exon–intron boundaries follow the GT/AG rule (except for exon 3 in ACPP). Table 2 summarizes various structural features of the three genes.

At the protein level, multiple alignment of testicular acid phosphatase with the prostatic and lysosomal acid phosphatases (Fig. 2) indicates the presence of a high degree of homology (~50%) and the existence of many conserved domains. Furthermore, the positions of six cysteine residues are completely conserved in all three proteins (Fig. 2). Transmembrane prediction programs indicated that the testicular acid phosphatase protein contains a short cytoplasmic domain, a transmembrane domain, and a long luminal (extracellular) domain (Fig. 4). This pattern is quite similar to that of the human, rat, and mouse lysosomal acid phosphatases, and the positions of these three domains are nearly identical (Geier *et al.*, 1991). As is the case with ACPP, the testicular acid phosphatase gene was found to be up-regulated by androgens and down-regulated by estrogen (Fig. 7). In addition, phylogenetic analysis indicated that ACPT is more closely related to ACPP and ACP2 proteins and to a lesser degree to other acid phosphatases (Fig. 5). Although experimental evidence is lacking, the newly identified gene should be considered to encode an acid phosphatase, based on structural features and protein similarities. These data also suggest that these three enzymes (testicular, prostatic, and lysosomal acid phosphatases) belong to a multigene family that likely originated from an ancestral gene during the course of evolution. It has been suggested that the ancestral exon 11 was lost in the ACPP gene by deletion, or alternatively, this exon could have been added after gene duplication (Sharief *et al.*, 1989; Sharief and Li, 1992).

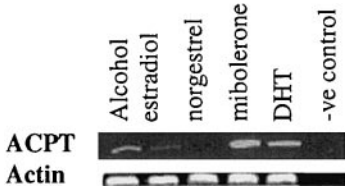

FIG. 7. Hormonal regulation of the testicular acid phosphatase gene in the LNCaP prostatic carcinoma cell line, as determined by RT-PCR. Steroids were added at 10^{-8} M final concentration. Actin (not regulated by steroid hormones) was used as a control gene. -ve, negative control.

A number of acid phosphatases, from both prokaryotes and eukaryotes, share two regions of sequence similarity, each centered around a conserved histidine residue. These two histidines seem to be involved in the enzymes' catalytic mechanism (Ostanin *et al.*, 1992; Van Etten *et al.*, 1991). The first histidine is located close to the N-terminal end, while the second is located in the C-terminal part of the enzyme (Table 3). Enzymes belonging to this family are called "histidine acid phosphatases" and include all mammalian lysosomal and prostatic acid phosphatases. These two histidines and their surrounding conserved sequences are present in the testicular acid phosphatase protein (Table 3 and Fig. 2). Furthermore, the predicted N-glycosylation sites match well with the corresponding sites for glycosylation of lysosomal and prostatic acid phosphatases (Table 3 and Fig. 2). These data provide further evidence that the new gene belongs to the acid phosphatase family.

The presence of alternatively spliced forms is not uncommon among acid phosphatases. Alternative splicing of exons 3 and 4 was shown to account for two different isoenzymes of ACP1 (Bryson *et al.*, 1995; Wo *et al.*, 1992), and a rare variant of ACP1, called ACP1*KUK, was identified in Czechoslovakia (Arnaud *et al.*, 1989, 1992). Also, Sharief *et al.* (1989) identified three mRNA clones for the ACPP gene. These clones have different lengths of the 3′ untranslated region.

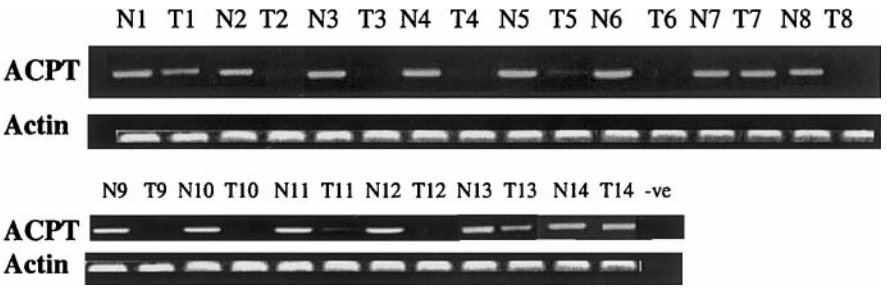

FIG. 8. Expression of the testicular acid phosphatase gene in 14 pairs of normal and cancerous testicular tissues, as determined by RT-PCR. The gene transcript was found to be abundant in all 14 normal testicular tissues, very low or undetectable in 10 the 14 cancerous counterparts, lower than normal in 2 tumors, and comparable to normal in another 2 tumors. Actin, a housekeeping gene, was used as a control to ensure quality and equal quantity of added cDNAs. -ve, negative control.

The 3′ untranslated region of the testicular acid phosphatase gene is relatively short in comparison to the human lysosomal and ACPP genes.

The length of the signal peptide (28 amino acids) is comparable to that of prostatic and lysosomal acid phosphatases (32 and 30 amino acids, respectively), and the predicted cleavage site occurs at a similar position in all three genes (Fig. 2).

The testicular acid phosphatase gene is up-regulated by androgens and is down-regulated by estrogens in the LNCaP prostate cancer cell line. The two available tumor markers for prostate cancer, PSA and ACPP, were also shown to be up-regulated by androgens in prostate cells (Diamandis, 1998; Moss *et al.*, 1995; Romas *et al.*, 1979; Yam *et al.*, 1980).

Testicular cancer is the most common malignancy in men ages 15–34 years, and its incidence has increased over the past 35 years (Hayes *et al.*, 1990; Parker, 1997). Testicular germ cell neoplasms originate from germ cells and proceed through a premalignant carcinoma *in situ* (CIS) stage. Time for progression from CIS to invasive tumor is roughly 5–7 years (Lutzker and Barnard, 1998). Testicular germ cell tumors exhibit nonrandom loss of DNA sequences scattered among several distinct chromosomal arms (Sandberg *et al.*, 1996), suggesting that the loss of tumor suppressor genes contributes to progression from CIS. In close proximity to ACPT, there are three genes that have been implicated in carcinogenesis, RRAS, BAX, and a newly identified homologue of Bcl-2 (Scorilas *et al.*, in press). However, we do not have any data showing either gain or loss of genetic material affecting these genes in cancer. A unique feature of testicular tumors is that they are "curable"; i.e., they are extremely sensitive to chemotherapy (Lutzker and Barnard, 1998), and early diagnosis of the disease by a reliable tumor marker is of extreme importance. The possible role of testicular acid phosphatase as a testicular biomarker or as a therapeutic target needs further experimentation.

Testicular cancer is one of the hormonally related cancers (Henderson and Feigelson, 2000). The role of steroid hormones in the development of testicular cancer is not clear, but two recent reports suggest that one possible cause of testicular cancer may be suboptimal androgen levels (James, 2000) and environmental estrogen exposure (Parker, 1997). One possible mechanism of action of these hormones is through altering the expression of hormonally regulated genes. Testicular acid phosphatase is one potential candidate gene.

In conclusion, we cloned a new acid phosphatase gene that is expressed mainly in testis and has a high degree of homology and structural similarity to prostatic and lysosomal acid phosphatases. This new gene is under steroid hormonal regulation, and its expression is low in testicular cancer tissues. The clinical applicability of this gene for diagnosis, prognosis, monitoring and therapy of testicular cancer should be worth examining in future studies.

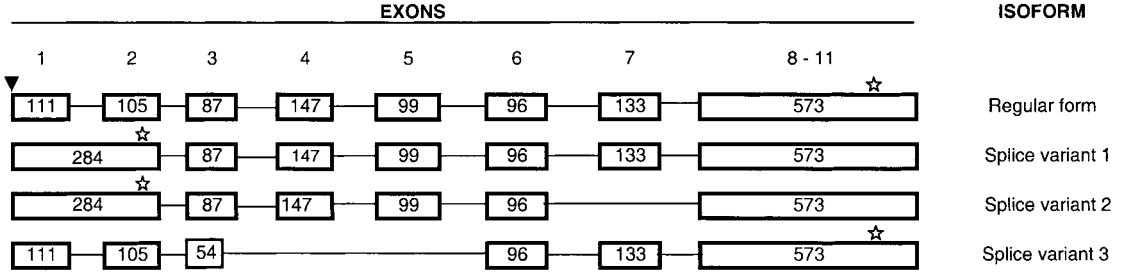

FIG. 9. Schematic presentation of the different splice variants of the testicular acid phosphatase gene. Exons are shown by boxes and introns by the connecting lines. Numbers inside boxes represent the exon lengths in basepairs. The arrowhead points to the common start codon, and stars point to the position of the stop codons. For full sequence information, see GenBank Accession No. AF321918. The exon–intron boundaries for exons 8–11 are not shown. The alternative splicing and/or exon skips create frameshifts, which lead to premature termination. Figure is not drawn to scale. For more details, see text.

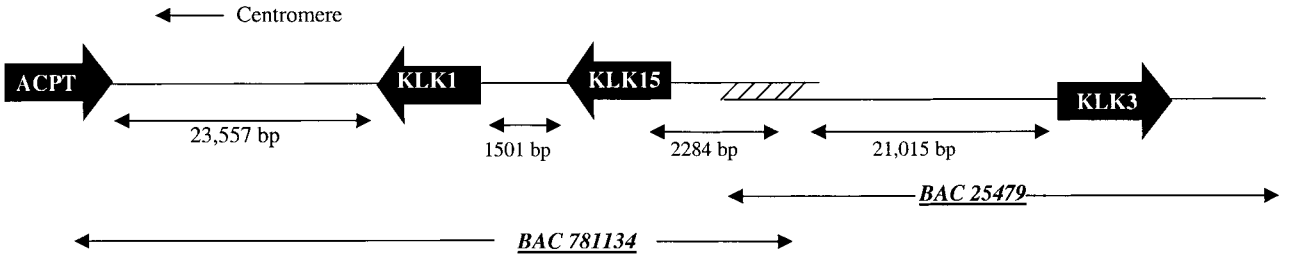

FIG. 10. The relative locations of testicular acid phosphatase (ACPT), pancreatic/renal kallikrein (KLK1), KLK15, and prostate-specific antigen (KLK3) genes on chromosome 19q13.4. Two overlapping BAC clones are identified, and the overlap region is hatched. Genes are represented by horizontal arrows indicating the direction of transcription. The distances between genes are shown in basepairs. Figure is not drawn to scale.

ACKNOWLEDGMENT

We thank Dr. L. Ashworth, Biology and Biotechnology Program, Lawrence Livermore National Laboratory, for valuable discussions and advice.

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25:** 3389–3402.

Apte, S. S., Mattei, M. G., and Olsen, B. R. (1995). Mapping of the human BAX gene to chromosome 19q13.3–q13.4 and isolation of a novel alternatively spliced transcript, BAX delta. *Genomics* **26:** 592–594.

Arnaud, J., Vavrusa, B., Sevin, J., and Constans, J. (1989). Human red-cell acid phosphatase (ACP1): A new mutant (ACP1*KUK) detected by isoelectric focusing, kinetics of thermostability and substrate activity. *Hum. Hered.* **39:** 288–293.

Arnaud, J., Vavrusa, B., Wiederanders, G., and Constans, J. (1992). Human red-cell acid phosphatase (ACP1): Kinetic and thermodynamic characterization of the KUK variant. *Hum. Hered.* **42:** 140–142.

Breathnach, R., and Chambon, P. (1981). Organization and expression of eucaryotic split genes coding for proteins. *Annu. Rev. Biochem.* **50:** 349–383.

Bryson, G. L., Massa, H., Trask, B. J., and Van Etten, R. L. (1995). Gene structure, sequence, and chromosomal localization of the human red cell-type low-molecular-weight acid phosphotyrosyl phosphatase gene, ACP1. *Genomics* **30:** 133–140.

Cassady, A. I., King, A. G., Cross, N. C., and Hume, D. A. (1993). Isolation and characterization of the genes encoding mouse and human type-5 acid phosphatase. *Gene* **130:** 201–207.

Diamandis, E. P. (1998). Prostate-specific antigen—Its usefulness in clinical medicine. *Trends Endocrinol. Metab.* **9:** 310–316.

Diamandis, E. P., Yousef, G. M., Luo, L. Y., Magklara, A., and Obiezu, C. V. (2000). The new human kallikrein gene family: Implications in carcinogenesis. *Trends Endocrinol. Metab.* **11:** 54–60.

Ferguson-Smith, M. A., Newman, B. F., Ellis, P. M., Thomson, D. M., and Riley, I. D. (1973). Assignment by deletion of human red cell acid phosphatase gene locus to the short arm of chromosome 2. *Nat. New Biol.* **243:** 271–274.

Geier, C., von Figura, K., and Pohlmann, R. (1989). Structure of the human lysosomal acid phosphatase gene. *Eur. J. Biochem.* **183:** 611–616.

Geier, C., von Figura, K., and Pohlmann, R. (1991). Molecular cloning of the mouse lysosomal acid phosphatase. *Biol. Chem. Hoppe-Seyler* **372:** 301–304.

Hayes, R. B., Brown, L. M., Pottern, L. M., Gomez, M., Kardaun, J. W., Hoover, R. N., O'Connell, K. J., Sutzman, R. E., and Javadpour, N. (1990). Occupation and risk for testicular cancer: A case-control study. *Int. J. Epidemiol.* **19:** 825–831.

Henderson, B. E., and Feigelson, H. S.(2000). Hormonal carcinogenesis. *Carcinogenesis* **21:** 427–433.

Hofmann, K., Bucher, P., Falquet, L., and Bairoch, A. (1999). The PROSITE database, its status in 1999. *Nucleic Acids Res.* **27:** 215–219.

Hofmann, K., and Stoffel, W. (1993). TMbase—A database of membrane spanning proteins segments. *Biol. Chem. Hoppe-Seyler* **347:** 166.

James, W. H. (2000). A possible cause of testicular cancer. *Br. J. Cancer* **82:** 2023.

Ketcham, C. M., Roberts, R. M., Simmen, R. C., and Nick, H. S. (1989). Molecular cloning of the type 5, iron-containing, tartrate-resistant acid phosphatase from human placenta. *J. Biol. Chem.* **264:** 557–563.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: Intimations of translational control. *J. Cell Biol.* **115:** 887–903.

Lennon, G., Auffray, C., Polymeropoulos, M., and Soares, M. B. (1996). The I.M.A.G.E. Consortium: An integrated molecular analysis of genomes and their expression. *Genomics* **33:** 151–152.

Lord, D. K., Cross, N. C., Bevilacqua, M. A., Rider, S. H., Gorman, P. A., Groves, A. V., Moss, D. W., Sheer, D., and Cox, T. M. (1990). Type 5 acid phosphatase. Sequence, expression and chromosomal localization of a differentiation-associated protein of the human macrophage [Published erratum appears in *Eur. J. Biochem.* 1990 **191**(3):775]. *Eur. J. Biochem.* **189:** 287–293.

Lutzker, S. G., and Barnard, N. J. (1998). Testicular germ cell tumors: Molecular understanding and clinical implications. *Mol. Med. Today* **4:** 404–411.

Meyer, A., Jung, K., Lein, M., Rudolph, B., Schnorr, D., and Loening, S. A. (1997). Factors influencing the ratio of free to total prostate-specific antigen in serum. *Int. J. Cancer* **74:** 630–636.

Moss, D. W., Raymond, F. D., and Wile, D. B. (1995). Clinical and biological aspects of acid phosphatase. *Crit. Rev. Clin. Lab. Sci.* **32:** 431–467.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10:** 1–6.

Nielsen, H., and Krogh, A. (1998). Prediction of signal peptides and signal anchors by a hidden Markov model. *Ismb* **6:** 122–130.

Ostanin, K., Harms, E. H., Stevis, P. E., Kuciel, R., Zhou, M. M., and Van Etten, R. L. (1992). Overexpression, site-directed mutagenesis, and mechanism of *Escherichia coli* acid phosphatase. *J. Biol. Chem.* **267:** 22830–22836.

Parker, L. (1997). Causes of testicular cancer. *Lancet* **350:** 827–828.

Pohlmann, R., Krentler, C., Schmidt, B., Schroder, W., Lorkowski, G., Culley, J., Mersmann, G., Geier, C., Waheed, A., Gottschalk, S.,

et al. (1988). Human lysosomal acid phosphatase: Cloning, expression and chromosomal assignment. *EMBO J.* **7:** 2343–2350.

Romas, N. A., Rose, N. R., and Tannenbaum, M. (1979). Acid phosphatase: New developments. *Hum. Pathol.* **10:** 501–512.

Sandberg, A. A., Meloni, A. M., and Suijkerbuijk, R. F. (1996). Reviews of chromosome studies in urological tumors. III. Cytogenetics and genes in testicular tumors. *J. Urol.* **155:** 1531–1556.

Scorilas, A., Kyriakopoulou, L., Yousef, G. M., Ashworth, L. K., Kwamie, A., Diamandis, E. P. (2001). Molecular cloning, physical mapping, and expression analysis of a novel gene, BCL2L12, encoding a proline-rich protein with a highly conserved BH2 domain of the Bcl-2 family. *Genomics* **72:** 217–221.

Sharief, F. S., Lee, H., Leuderman, M. M., Lundwall, A., Deaven, L. L., Lee, C. L., and Li, S. S. (1989). Human prostatic acid phosphatase: cDNA cloning, gene mapping and protein sequence homology with lysosomal acid phosphatase. *Biochem. Biophys. Res. Commun.* **160:** 79–86.

Sharief, F. S., and Li, S. S. (1992). Structure of human prostatic acid phosphatase gene. *Biochem. Biophys. Res. Commun.* **184:** 1468–1476.

Sharief, F. S., and Li, S. S. (1994). Nucleotide sequence of human prostatic acid phosphatase ACPP gene, including seven Alu repeats. *Biochem. Mol. Biol. Int.* **33:** 561–565.

Sonnhammer, E., Heijne, G., and Krogh, A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. *In* "Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology," pp. 175–182, AAAI Press, California.

Van Etten, R. L., Davidson, R., Stevis, P. E., MacArthur, H., and Moore, D. L. (1991). Covalent structure, disulfide bonding, and identification of reactive surface and active site residues of human prostatic acid phosphatase. *J. Biol. Chem.* **266:** 2313–2319.

Vihko, P., Virkkunen, P., Henttu, P., Roiko, K., Solin, T., and Huhtala, M. L. (1988). Molecular cloning and sequence analysis of cDNA encoding human prostatic acid phosphatase. *FEBS Lett.* **236:** 275–281.

Waheed, A., Van Etten, R. L., Gieselmann, V., and von Figura, K. (1985). Immunological characterization of human acid phosphatase gene products. *Biochem. Genet.* **23:** 309–319.

Wo, Y. Y., McCormack, A. L., Shabanowitz, J., Hunt, D. F., Davis, J. P., Mitchell, G. L., and Van Etten, R. L. (1992). Sequencing, cloning, and expression of human red cell-type acid phosphatase, a cytoplasmic phosphotyrosyl protein phosphatase. *J. Biol. Chem.* **267:** 10856–10865.

Yam, L. T., Li, C.-Y., and Lam, K. W. (1980). The non-prostatic acid phosphatases. *In* "Male Accessory Sex Glands" (E. Spring-Mills and E. S. E. Hafez, Eds.), pp. 183–196, Elsevier/North-Holland, Amesterdam.

Yeh, L. C., Lee, A. J., Lee, N. E., Lam, K. W., and Lee, J. C. (1987). Molecular cloning of cDNA for human prostatic acid phosphatase. *Gene* **60:** 191–196.

Yousef, G. M., Chang, A., and Diamandis, E. P. (2000a). Identification and characterization of KLK-L4, a new kallikrein-like gene that appears to be down regulated in breast cancer tissues. *J. Biol. Chem.* **275:** 11891–11898.

Yousef, G. M., Chang, A., Scorilas, A., and Diamandis, E. P. (2000b). Genomic organization of the human kallikrein gene family on chromosome 19q13.3–q13.4. *Biochem. Biophys. Res. Commun.* **276:** 125–133.

Yousef, G. M., and Diamandis, E. P. (1999). The new kallikrein-like gene, KLK-L2. Molecular characterization, mapping, tissue expression, and hormonal regulation. *J. Biol. Chem.* **274:** 37511–37516.

Yousef, G. M., Obiezu, C. V., Luo, L. Y., Black, M. H., and Diamandis, E. P. (1999). Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated. *Cancer Res.* **59:** 4252–4256.