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Identification of Three New Alternate Human Kallikrein 2 Transcripts: Evidence of Long Transcript and Alternative Splicing

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In a search for prostate-specific genes in the human expressed sequence tag (EST) database, we identified a seemingly unique EST cluster C81. Experimental data linked C81 to the human hKLK2 gene that encodes a prostate specific serine protease-human glandular kallikrein (hK2). We uncovered a full-length hKLK2 cDNA corresponding to a 3.0 kb hKLK2 mRNA by PCR and sequence analysis. The 3.0 kb transcript accounts for about 25% of the hKLK2 transcripts as compared to the previously known 1.5 kb transcript. We also identified a third spliced form of the hKLK2 gene produced by alternative splicing between intron III and exon 4. This spliced form was detected in normal prostate, prostate cancer and the prostate adenocarcinoma cell line LNCaP. The identification of long hKLK2 transcript and an alternative spliced form of the hKLK2 gene indicates that regulation of the gene is complex.

Key Words: **EST; kallikrein; alternative splicing; hKLK2 gene.**

Expressed sequence tags (ESTs) are fragments of cDNA sequences generated from different cDNA libraries which are derived from one particular tissue, cell type or tumor (1). Therefore, the pattern of library containing EST sequences corresponding to a gene provides an estimate of expression pattern to this gene in different tissues. By using a combination of EST database analysis and RNA expression analysis, we have identified several genes that are preferentially expressed in normal prostate, prostate cancer and other tissues non-essential for life (2–4). These genes are now being characterized and they may provide potential targets for cancer diagnosis or cancer therapy.

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The human kallikrein gene family (hKLK) is composed of four genes, hKLK1, hKLK2, hKLK3 (5, 6), hKLK4 and two putative hKLK-like gene fragment (7). The hKLK1 gene encodes pancreatic/renal glandular kallikrein called hK1, which is mainly expressed in pancreas, renal and salivary gland. The hKLK2 and hKLK3 genes encode hK2 and hK3 (also known as prostate specific antigen or PSA), which are synthesized primarily in the prostate epithelium (6, 8, 9). The newly discovered hKLK4 gene has also been reported to be preferentially expressed in human prostate (10). These hKLK genes are organized as a cluster in chromosome 19. hKLK1 is located 30 kb upstream of hKLK3 in a tail-to-tail fashion, and hKLK2 is located 12 kb downstream of hKLK3 in a head-to-tail fashion (11). PSA has been extensively studied as a marker for monitoring disease progression and treatment response among patients with prostate cancer (12, 13). Although PSA is a well-characterized and important marker for prostate cancer, there are also reports on prostate cancer patients who are PSA negative in the serum (14, 15). Recent reports have demonstrated that hK2 is expressed in every case of prostate cancer, and that the expression is incrementally increased from benign epithelium to primary cancer and lymph node metastases (15–17). Measurement of hK2 level in the serum has the potential to become another important diagnostic test for prostate cancer.

The hKLK2 gene is composed of 5 exons and 4 introns and encodes a transcript of 1.5 kb. Two isoforms of hKLK2 cDNA have been identified in the literature (5, 9, 18). The major isoform contains an open reading frame of 783 bp (5), The minor one contains an open reading frame of 669 bp due to an alternative splice acceptor site between exon 4 and 5 (18). In this report, by searching ESTs that are specifically expressed in normal prostate and prostate cancer, we have identified three novel transcripts. Two are RNAs that corre-

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TABLE I

Primers Used in This Study

Name	Position	Sequence
C81-1	2778–2758	5′-TGCTGCCCCAGTATCTGTAACCA-3′
C81-3	2398–2421	5′-CCACACATAGCACCGGAGATATG-3′
C81-7	1596–1571	5′-GCCAAGTTGCCAGTCTTCGTAACTGC-3′
K-6	1–23	5′-GGACACCTGTGTCAGCATGTGG-3′
K-5	487–508	5′-GGCAGCATCGAACCAGAGGAG-3′
K-17	550–541	5′-CATTGGACAGGAGATGGAGG-3′

sponds to 3.0 kb hKLK-2 cDNAs with long 3′ UTR. The third is a novel alternative spliced form of hKLK-2 which presumably encodes a truncated form of hK-2 protein.

MATERIALS AND METHODS

RT-PCR were performed on cDNA from 24 different human tissues by using the human rapid–scan gene expression panels (Origene). The thermocycling protocol was: initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and elongation at 72°C for 3 minutes. The PCR reactions were analyzed on agarose gel and specific products were cloned into TA vectors (Invitrogen) and sequenced on an automated capillary sequencer, using Perkin–Elmer's dRhodamine terminator cycle sequencing kit (Perkin–Elmer Applied System).

Northern blot (MTN, Clontech) and multiple tissue mRNA dot blot (Clontech) hybridization are essentially as described before (4). Briefly, the RNA membranes were blocked for 3 hours at 45°C in hybridization solution. Probes labeled with ^{32}P either by random primer extension or by end labeling (Lofstrand Labs Limited) were added to the membrane and hybridize for 15 hour at 45°C. The membrane were washed twice with 2× SSC/0.1% SDS at room temperature and twice with 0.1× SSC/0.1% SDS at 55°C. The membranes were exposed to X-ray film for 1-2 days before development.

The primers used are listed in Table I. The oligo nucleotide probe A used for Northern blot (shown in Fig. 2B) is an antisense primer at position 838–886. 5′-GGTGTCCAGCATCCAGAAAGGCCAAGTGATGCCAGAACGTGAGGTGGCA-3′. Oligo nucleotides were synthesized by Sigma-Genosys.

Total RNA was isolated from human prostate cancer tissues by using TRIzol Reagent (Gibco BRL) as instructed by the company. Poly(A) RNA was isolated from LNCaP cell line by using the FastTrack kit (InVitrogen). Either 1.5 μg of total RNA from prostate cancer tissue or 500 ng of mRNA from LnCAP were reverse transcribed to single strand cDNA by oligo dT primer using SMART RACE kit from Clontech. These two sources of cDNA together with normal prostate cDNA purchased from Clontech were used to identify the alternate splicing form of hKLK2 gene as illustrated in Fig. 4B.

RESULTS

Prostate-Specific Expression of C81 Cluster

As part of a search for cDNAs that are preferentially present in prostate and prostate cancers, we found a cluster of ESTs containing 7 members from 5 different prostate or prostate cancer cDNA libraries and no EST in other tissues or cancers. This consensus cDNA sequence contains 538 bases with a poly A tail preceded by the AATAAA polyadenylation signal (data not shown). The EST cluster was designated as C81.

To verify the specificity of C81, we conducted a RT-PCR analysis by using the human rapid-scan panel with primers C81-1 and C81-3 as described in Materials and Methods. We detected an expected 380 bp fragment only in prostate among 24 different tissues examined as shown in Fig. 1A. This PCR product, C81-1-3, was sequenced and confirmed to be the expected fragment of the C81 cDNA. The specific expression of C81 was further confirmed by RNA dot blot analysis with the same DNA fragment C81-1-3 as hybridization probe (Fig. 1B). These experimental results verified that the EST database provided excellent guidance in finding a specifically expressed gene.

Identification of a Long Human Glandular Kallikrein 2 Transcript

Since C81 represents a highly specific cDNA in prostate and prostate tumor, we next analyzed the transcript it encodes. Northern blot analysis was conducted by using the same probe as that used for the RNA dot blot. As shown in Fig. 2A, a major band of 3.0 kb mRNA was revealed in the prostate sample.

To obtain the full-length cDNA of C81, we obtained and sequenced an EST clone nj70b10 (Research Genetics) from the cluster. The total sequence of this EST clone is 1450 bp long. BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) revealed an overlap between the 5′ of nj70b10 sequence and hKLK-2 cDNA 3′ UTR sequence directly upstream of the poly(A) stretch. The reported size of the hKLK-2 transcript is 1.5 kb, but a 3.0 kb band was also identified by Northern blot in the same report (18). We reasoned that the transcript discovered by us might correspond to the same 3.0 kb transcript, and the 1.5 kb hKLK2 transcript together with the 1.45 kb C81 sequence would approximately account for the 3.0 kb transcript. To test this hypothesis, we designed a primer k-6 located at the beginning of 5′ end of hKLK-2 cDNA and a primer C81-7 in the C81 sequence, down stream of the previously described hKLK-2 cDNA sequence. PCR analysis detected a DNA fragment of 1.6 kb by using prostate cDNA from Clontech (data not shown). This fragment was sequenced and the connection between the hKLK2 and C81 was confirmed (shown in Fig. 3). This result indicates that RNA polymerase can transcribe through the first polyadenylation signal of the hKLK2 gene and continue to the second signal before it stops.

In order to estimate the relative abundance of the two different sized transcripts, we conducted a Northern blot analysis by using probe A (see Materials and Methods) in the hKLK-2 cDNA sequence which is specific for hKLK-2 (less than 10% homology with PSA). The two major transcripts of the hKLK2 gene are 1.5

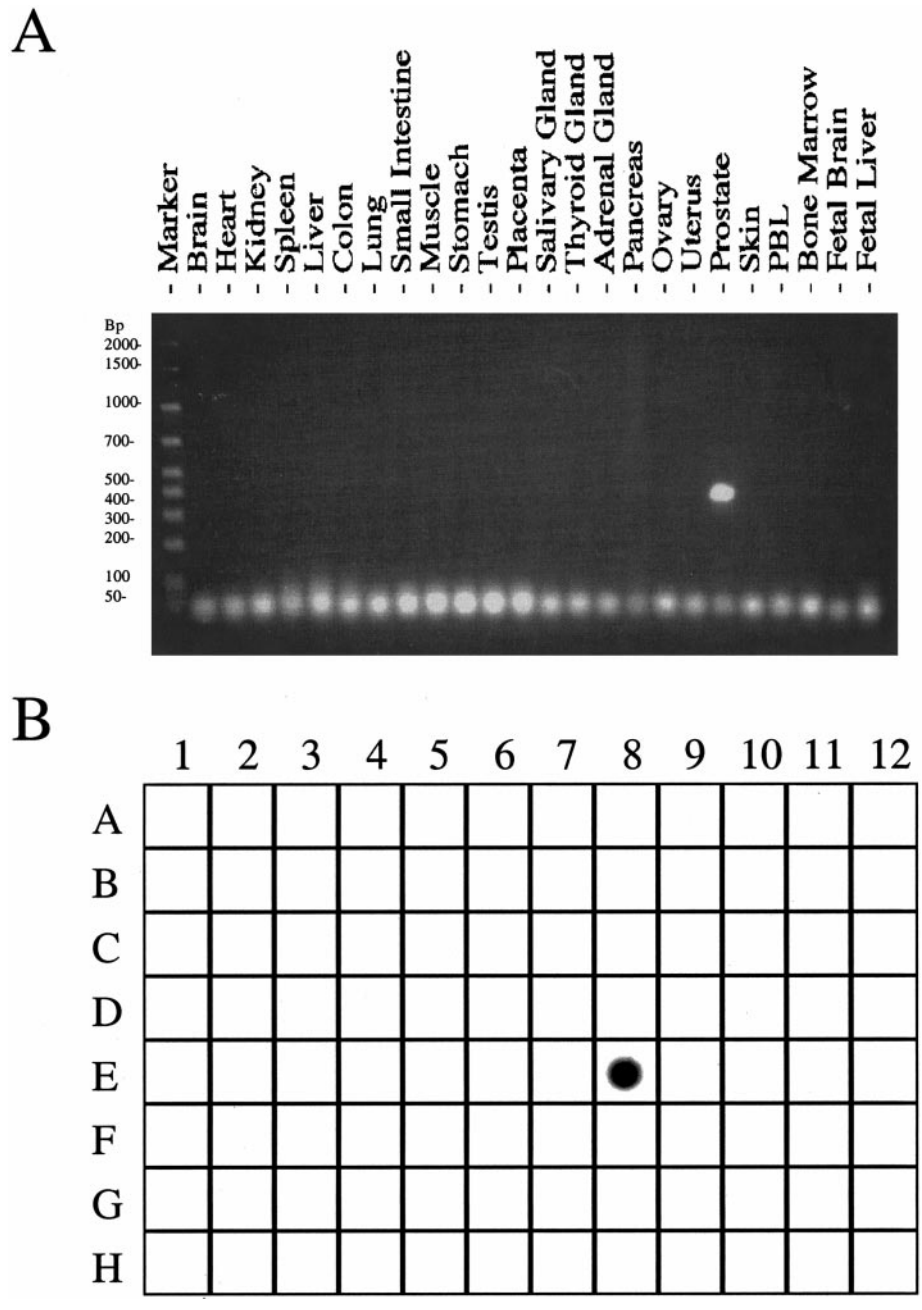

FIG. 1. Specific expression of C81. (A) Human rapid scan panel: 24 different sources of cDNA were amplified by using primers C81-1 and C81-3 under conditions described under Materials and Methods. After the PCR cycles, 5 μl of the high concentration (1000×) reactions were analyzed in the agarose gel and visualized by staining with EtBr. (B) Multiple tissue dot blot were hybridized with the C81-1-3 probe randomly labeled with ^{32}P. The film was exposed for overnight. The positive signal E8 is from prostate tissue.

kb and 3.0 kb in size as expected (shown in Fig. 2B). The expression level of the 3.0 kb transcript is approximately 25% that of the 1.5 kb transcript. The 4.7 kb band that is also seen can be the result of precursors of mature mRNA or another alternative mRNA of hKLK-2 gene. These results are consistent with other reports on the hKLK2 transcripts (18). The entire cDNA from 5′ hKLK2 cDNA to C81 was completely sequenced and designated as hKLK-3.0 (shown in Fig. 3). This hKLK2-3.0 cDNA encodes the same amino acid sequence as reported in the literature (5, 18). The sequence of hKLK2-3.0 is consistent with the known sequence in the first 1477 bp with only 3 nucleotide differences: 973 (G → T), 1085 (C → G), and 1441 (T → A) and an insertion of C at position 1245, that does not affect the amino acid sequence. These results indicate

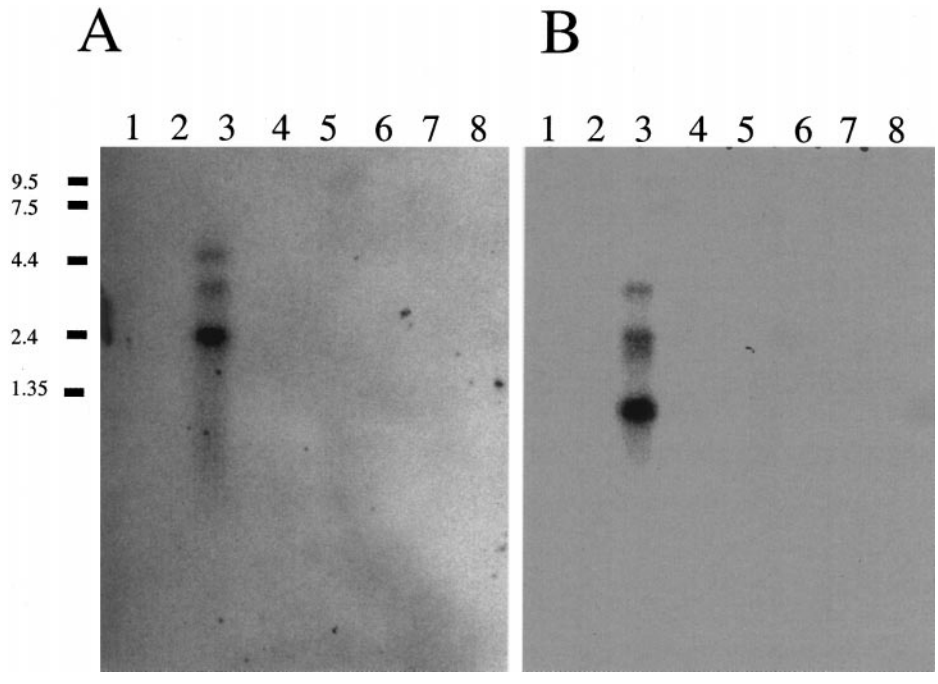

FIG. 2. Northern blot analysis of hKLK2 gene. (A) Northern blot (Clontech) containing 8 different tissues were probed with C81-1-3 which were random labeled with ^{32}P and hybridized for overnight. (B) The same Northern blots were probed with probe A which were end-labeled by protein kinase and hybridized for overnight. 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, peripheral leukocyte.

that, in addition to the 1.5 kb transcript, the glandular kallikrein 2 gene can also be transcribed into a 3.0 kb transcript with a long 3′UTR. The sequence of hK-3.0 has been deposited in the GenBank database (Accession Nos. AF188745, AF188746, and AF188747).

Alternative Splicing Forms of the hKLK-2 Gene

While sequencing of PCR fragments obtained with primers k-6 and C81-7 as described above, we identified another spliced form of the hKLK2 gene in addition to the two known isoforms. This third form, designated as pk-3, has a deletion of 13 nucleotides between exon 3 and 4 of hKLK2. This sequence is located at the position where during processing of the hKLK2 transcript, intron III is spliced out (see Fig. 4A). Comparison with the genomic sequence (5) revealed that in processing of the hKLK2 transcript, the predicted splicing acceptor site of intron III is CATAG, whereas in the case of pk-3 an alternate site from exon 4 CCCAG, which is 13 bp downstream, is used instead. This change in RNA gives rise to a frame shift and an immediate translational stop, that would result a truncated hK-2 protein missing 97 amino acids at the C-terminal.

To investigate the frequency of the pk-3 alternative spliced form, the hKLK2 cDNA fragments were amplified by PCR using primers k-5 and k-17 derived from exon 3 and exon 4. The expected sizes of the amplified fragment hKLK2 and pk-3 are 72 bp and 59 bp respectively. As shown in Fig. 4B, the two different bands were found after amplification of cDNA from prostate tumor, normal prostate and a prostate adenocarcinoma cell line, LNCaP. Both of the two fragments were sequenced and confirmed to be the right sequence from the hKLK2 gene (data not shown). The 72 bp fragment is more abundant than the 59 bp, indicating that a relatively small percentage of hKLK2 mRNA undergoes from this alternate splicing.

DISCUSSION

In this study, we identified three 3.0 kb hKLK2 transcripts, each with an extra 3′ UTR sequence and an alternatively spliced form of the hKLK2 gene by analysis of the EST database and experimental verification. The transcripts of hKLK2 gene are summarized in Fig. 5. The previously known alternate spliced forms of the hKLK2 cDNA is caused by an addition of 37 nucleotides as a result of a different splice donor site in intron IV: it occurs in about 20% of hKLK2 transcripts (18). The alternate spliced form we have identified was caused by alternate splicing in intron III which accounts for a minor percentage of the hKLK2 cDNA. The alternative splice acceptor site perfectly matches the splice accept consensus sequence PyN(C/A)AG (19). It

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1    GGACACCTGTGTCAGCATGTGGGACCTGGTTCTCTCCATCGCCTTGTCTG TGGGGTGCACTGGTGCCGTGCCCCTCATCCAGTCTCGGATTGTGGGAGGC 100
101  TGGGAGTGTGAGAAGCATTCCCAACCCTGGCAGGTGGCTGTGTACAGTCA TGGATGGGCACACTGTGGGGGTGTCCTGGTGCACCCCCAGTGGGTGCTCA 200
201  CAGCTGCCCATTGCCTAAAGAAGAATAGCCAGGTCTGGCTGGGTCGGCAC AACCTGTTTGAGCCTGAAGACACAGGCCAGAGGGTCCCTGTCAGCCACAG 300
301  CTTCCCACACCCGCTCTACAATATGAGCCTTCTGAAGCATCAAAGCCTTA GACCAGATGAAGACTCCAGCCATGACCTCATGCTGCTCCGCCTGTCAGAG 400
401  CCTGCCAAGATCACAGATGTTGTGAAGGTCCTGGGCCTGCCCACCCAGGA GCCAGCACTGGGGACCACCTGCTACGCCTCAGGCTGGGGCAGCATCGAAC 500
501  CAGAGGAGT          GAGTCTTCAGTGTGTGAGCCTCCATCTC CTGTCCAATGACATGTGTGCTAGAGCTTACTCTGAGAAGGTGACAGAGTT 600
           TCTTGCGCCCCAG
                                    GTGAGTCATCCCTACTCCCAACATCTGGAGGGGAAAG
601  CATGTTGTGTGCTGGGCTCTGGACAGGTGGTAAAGACACTTGTGGGGGTG ATTCTGGGGGTCCACTTGTCTGTAATGGTGTGCTTCAAGGTATCACATCA 700
701  TGGGGCCCTGAGCCATGTGCCCTGCCTGAAAAGCCTGCTGTGTACACCAA GGTGGTGCATTACCGGAAGTGGATCAAGGACACCATCGCAGCCAACCCCT 800
801  GAGTGCCCCTGTCCCACCCCTACCTCTAGTAAATTTAAGTCCACCTCACG TTCTGGCATCACTTGGCCTTTCTGGATGCTGGACACCTGAAGCTTGGAAC 900
901  TCACCTGGCCGAAGCTCGAGCCTCCTGAGTCCTACTGACCTGTGCTTTCT GGTGTGGAGTCCAGGGCTGCTATGAAAAGGAATGGGCAGACACAGGTGTA 1000
1001 TGCCAATGTTTCTGAAATGGGTATAATTTCGTCCTCTCCTTCGGAACACT GGCTGTCTCTGAAGACTTCTCGCTCAGTTTCAGTGAGGACACACACAAAG 1100
1101 ACGTGGGTGACCATGTTGTTTGTGGGGTGCAGAGATGGGAGGGGTGGGGC CCACCCTGGAAGAGTGGACAGTGACACAAGGTGGACACTCTCTACAGATC 1200
1201 ACTGAGGATAAGCTGGAGCCACAATGCATGAGGCACACACACAGCAAGGA TGACGCTGTAAACATAGCCCACGCTGTCCTGGGGGCACTGGGAAGCCTAG 1300
1301 ATAAGGCCGTGAGCAGAAAGAAGGGGAGGATCCTCCTATGTTGTTGAAGG AGGGACTAGGGGGAGAAACTGAAAGCTGATTAATTACAGGAGGTTTGTTC 1400
1401 AGGTCCCCCAAACCACCGTCAGATTTGATGATTTCCTAGCAGGACTTACA GAAATAAAGAGCTATCATGCTGTGGTTTATTATGGTTTGTTACATTGATG 1500
1501 GGATACATACTGAAATCAGCAAACAAAACAGATGTATAGATTAGAGTGTG GAGAAAACAGAGGAAAACTTGCAGTTACGAAGACTGGCAACTTGGCTTTA 1600
1601 CTAAGTTTTCAGACTGGCAGGAAGTCAAACCTATTAGGCTGAGGACCTTG TGGAGTGTAGCTGATCCAGCTGATAGAGGAACTAGCCAGGTGGGGGCCTT 1700
1701 TCCCTTTGGATGGGGGGCATATCTGACAGTTATTCTCTCCAAGTGGAGAC TTACGGACAGCATATAATTCTCCCTGCAAGGATGTATGATAATATGTACA 1800
1801 AAGTAATTCCAACTGAGGAAGCTCACCTGATCCTTAGTGTCCAAGGTTTT TACTGGGGGTCTGTAGGACGAGTATGGAGTACTTGAATAATTGACCTGAA 1900
1901 GTCCTCAGACCTGAGGTTCCCTAGAGTTCAAACAGATACAGCATGGTCCA GAGTCCCAGATGTACAAAAACAGGGATTCATCACAAATCCCATCTTTAGC 2000
2001 ATGAAGGGTCTGGCATGGCCCAAGGCCCCAAGTATATCAAGGCACTTGGG CAGAACATGCCAAGGAATCAAATGTCATCTCCCAGGAGTTATTCAAGGGT 2100
2101 GAGCCCTTTACTTGGGATGTACAGGCTTTGAGCAGTGCAGGGCTGCTGAG TCAACCTTTTATTGTACAGGGGATGAGGGAAAGGGAGAGGATGAGGAAGC 2200
2201 CCCCCTGGGGATTTGGTTTGGTCTTGTGATCAGGTGGTCTATGGGGCTAT CCCTACAAAGAAGAATCCAGAAATAGGGGCACATTGAGGAATGATACTGA 2300
2301 GCCCAAAGAGCATTCAATCATTGTTTTATTTGCCTTCTTTTCACACCATT GGTGAGGGAGGGATTACCACCCTGGGGTTATGAAGATGGTTGAACACCCC 2400
2401 ACACATAGCACCGGAGATATGAGATCAACAGTTTCTTAGCCATAGAGATT CACAGCCCAGAGCAGGAGGACGCTGCACACCATGCAGGATGACATGGGGG 2500
2501 ATGCGCTCGGGATTGGTGTGAAGAAGCAAGGACTGTTAGAGGCAGGCTTT ATAGTAACAAGACGGTGGGGCAAACTCTGATTTCCGTGGGGGAATGTCAT 2600
2601 GGTCTTGCTTTACTAAGTTTTGAGACTGGCAGGTAGTGAAACTCATTAGG CTGAGAACCTTGTGGAATGCAGCTGACCCAGCTGATAGAGGAAGTAGCCA 2700
2701 GGTGGGAGCCTTTCCCAGTGGGTGTGGGACATATCTGGCAAGATTTTGTG GCACTCCTGGTTACAGATACTGGGGCAGCAAATAAAACTGAATCTTGTTT 2800
2801 TCAGACCTTAAAAAAAAAAAAAAAAAAAA
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FIG. 3. Nucleotide sequence of hKLK-3.0 and the other two alternate splicing forms. The new extra 3′ UTR sequences are dot dashed. The positions of polyadenylation signals are bold and underlined. The translation stop codons are indicated by bold. The position and the sequence of new splicing form with 13 base pairs deletion are indicated by dot lines, and the previously reported alternate splicing form (18) are indicated by italics.

seems like that the sequence CCCAG is less preferred than CATAG with only a small fraction converted to this spliced form. A protein derived from the pk-3, caused by a frame shift of 13 nucleotides will encode a truncated hK-2 protein containing only 194 amino acids instead of 261.

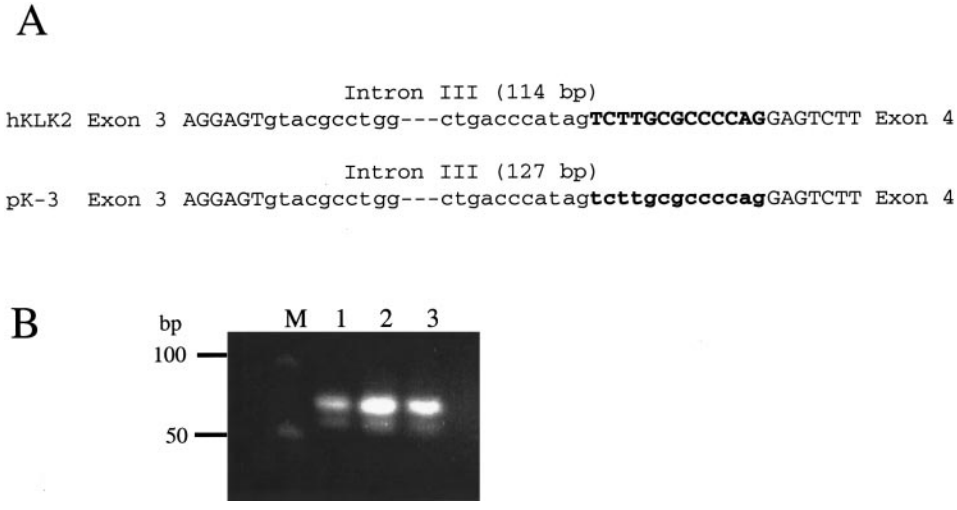

FIG. 4. The structure and the relative abundance of alternate splicing form of hKLK2 gene. (A) The illustration of the formation of the two hKLK2 mRNA PCR products caused by alternative splicing between intron III and exon 4. The exons are indicated by uppercase and the intron is in lowercase letters. The differences between the two alternative splicing forms are indicated in bold. (B) Agarose gel electrophoresis of the hKLK2 PCR product by primers k-5 and k-17 with different sources of cDNA. Lane 1, molecular weight marker; lane 2, prostate tumor; 3, normal prostate; 4, LnCAP.

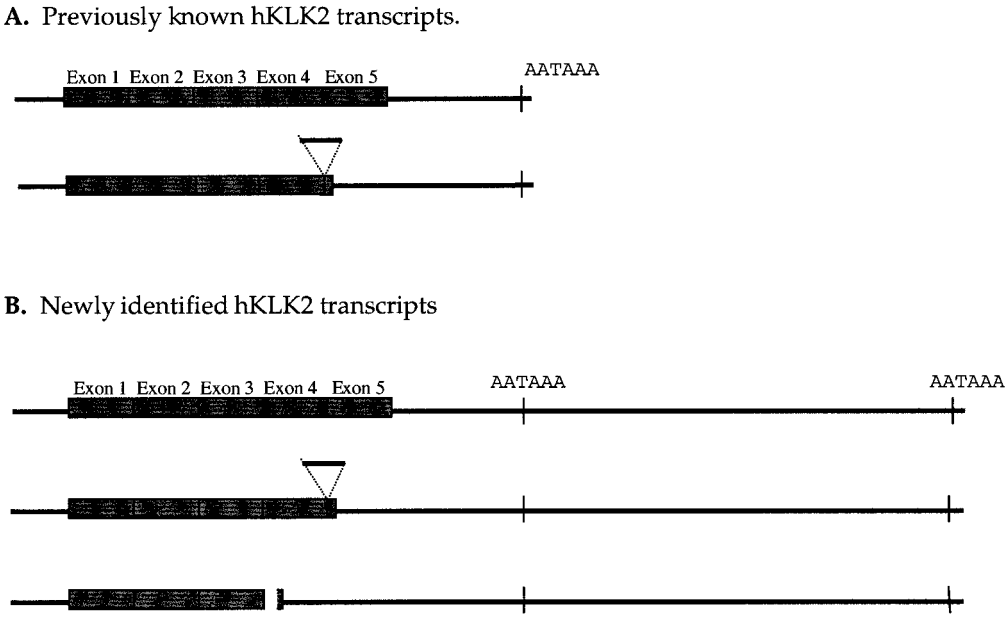

FIG. 5. Illustration of hKLK2 transcripts. The exons are indicated by filled boxes. The dark lines corresponding to the 5′ and 3′-UTR sequences. Polyadenylation signals are labeled as AATAAA. The picture is not drawn to scale.

We also investigated if the hKLK3 (the gene that encodes PSA) can be transcribed with an extra long 3′UTR. The sequence of human PSA gene has been deposited in GenBank (20). It extends from the known hKLK3 polyadenylation signal by about 650 bp and has 240 bp overlap with a sequence containing the enhancer region of the hKLK2 gene (21). Using these two sequences, we have constructed the full genomic sequence between the hKLK3 and hKLK2 genes. When that segment was analyzed using BLAST against the human EST database, three ESTs were pulled with high sequence identity (>97%). These ESTs presumably derived from hKLK3 cDNA with long 3′-UTR sequence. The full-length alternative hKLK3 cDNA would then contain a total of 2870 bp ending at the 3′ end of EST sequence with multiple polyadenylation signals ahead of poly A stretch. Consistent with the alternative hKLK3 transcripts, there was also a strong signal at 3.0 kb position in the Northern blot of hKLK3 transcripts published by Reigman (18). Therefore, it is possible that extra long 3′UTRs could be common to the genes of the kallikrein gene family.

At present, we do not know the function of extended 3′ UTR sequences. It is possible that these extra bases are needed for regulating mRNA stability or targeting of the mRNA in cells. Several *cis*-acting RNA elements in 3′-UTR have been identified in *Xenopus, Drosophila* and mouse oocytes and embryos (22, and references therein). Our identification of an additional 3′UTR may provide some information on the existence of other tissue specific UTR elements and the understanding of transcription and translation event of the hKLK2 gene. The identification of three new transcripts also raises the issue of the complexity of regulation for the hKLK2 gene.

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