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Testis-Specific Expression of mRNAs for a Unique Human Type 1 Hexokinase Lacking the Porin-Binding Domain

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ABSTRACT Several enzymes in the glycolytic pathway are reported to have spermatogenic cell-specific isozymes. We reported recently the cloning of cDNAs representing three unique type 1 hexokinase mRNAs (*mHk1-sa, mHk1-sb,* and *mHk1-sc*) present only in mouse spermatogenic cells and the patterns of expression of these mRNAs (Mori et al., 1993: Biol Reprod 49:191–203). The mRNAs contain a spermatogenic cell-specific sequence, but lack the sequence for the porin-binding domain that somatic cell hexokinases use to bind to a pore-forming protein in the outer mitochondrial membrane. We now report the cloning of cDNAs representing three unique human type 1 hexokinase mRNAs (*hHK1-ta, hHK1-tb,* and *hHK1-tc*) expressed in testis, but not detected by Northern analysis in other human tissues. These mRNAs also contain a testis-specific sequence not present in somatic cell type 1 hexokinase, but lack the sequence for the porin-binding domain. The *hHK1-tb* and *hHK1-tc* mRNAs each contain an additional unique sequence. The testis-specific sequence of the human mRNAs is similar to the spermatogenic cell-specific sequence of the mouse mRNAs. Furthermore, Northern analysis of RNA from mouse, hamster, guinea pig, rabbit, ram, human, and rat demonstrated expression of type 1 hexokinase mRNAs lacking the porin-binding domain in the testes of these mammals. These results suggest that hexokinase may have unique structural or functional features in spermatogenic cells and support a model proposed by others for hexokinase gene evolution in mammals.

Key Words: Hexokinase, Human, Testis, Gene expression, Spermatogenesis

INTRODUCTION

The developmental program of spermatogenesis is a multistep process which includes mitotic, meiotic, and postmeiotic phases characterized by dramatic shifts in the patterns of gene expression (for review, see Eddy et al., 1993). Cognate genes expressed uniquely in spermatogenic cells have been identified for three glycolytic enzymes: phosphoglycerate kinase (*Pgk-2*) (VandeBerg et al., 1976; McCarrey and Thomas, 1987; Boer et al., 1987), lactate dehydrogenase (*Ldh-3*) (Goldberg, 1963; Sakai et al., 1987; Millan et al., 1987), and glyceraldehyde 3-phosphate dehydrogenase (*Gapd-s*) (Welch et al., 1992a, 1995; Mori et al., 1992). In addition, we recently cloned cDNAs representing three hexokinase mRNAs (*mHk1-sa, mHk1-sb, mHk1-sc*) unique to mouse spermatogenic cells (Mori et al., 1993). Although these three cDNAs showed 99% identity to the somatic cell *mHk1* cDNA sequence throughout most of their coding regions, they differed from it at the 5′ end. They shared a spermatogenic cell-specific sequence, and each had a unique sequence immediately 5′ to this common sequence. They also lacked the sequence for the porin-binding domain (PBD) present in this region of the somatic cell type 1 hexokinase.

Hexokinase (HK, E.C.2.7.1.1) catalyzes the first step in glucose metabolism, utilizing ATP to phosphorylate glucose and produce glucose-6-phosphate. In mammals there are four hexokinase isozymes which vary in their tissue distribution and kinetic properties (Katzen et al., 1968). The type 1–3 hexokinase isozymes each consist of a single polypeptide chain of approximately 100 kDa which is inhibited by the glucose-6-phosphate product. The type 4 hexokinase (glucokinase) is similar to yeast hexokinase, being a single polypeptide of approximately 50 kDa and insensitive to inhibition by glucose-6-phosphate (for review, see Wilson, 1984). Expression of glucokinase is limited to hepatocytes and pancreatic β cells, and is regulated differently in these two cell types (Magnuson and Shelton, 1989; Newgard et al., 1990). These observations led several investigators (Holroyde and Trayer, 1976; Vowles and Easterby, 1979; Ureta, 1982) to postulate that mammalian hexokinases may have evolved from an ancestral hexokinase similar to present-day yeast hexokinase and mammalian liver glucokinase. This may have occurred by tandem gene duplication, with retention of catalytic function by the C-terminal half of the new gene and evolution of the N-terminal half to acquire regulatory function. The recent cloning

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of genes for mouse (Arora et al., 1990), rat (Schwab and Wilson, 1989), bovine (Griffin et al., 1991), and human (Nishi et al., 1988) type 1 hexokinase, as well as rat type 2 (Thelen and Wilson, 1991) and type 3 hexokinase (Schwab and Wilson, 1991), has provided evidence in support of the gene duplication hypothesis.

Sequence comparisons between bovine, rat, human, and mouse type 1 hexokinase indicated that the N-terminal 15 amino acids are 100% conserved (Griffin et al., 1991). This hydrophobic domain is the putative porin-binding domain (PBD) (Arora et al., 1990; Griffin et al., 1991) that is necessary and sufficient for type 1 hexokinase binding to porin (Adams et al., 1991; Smith and Wilson, 1991). Porin or the voltage-dependent anion channel (VDAC) is the pore-forming protein involved in the movement of adenine nucleotides across the outer mitochondrial membrane. The PBD of hexokinase interacts with porin on the outer mitochondrial membrane and provides hexokinase with preferred access to the ATP generated in the mitochondrion (for review, see Adams et al., 1991). An evolutionary model for eukaryotic hexokinase genes, proposed by Griffin et al. (1991), suggests that recruitment of an exon may have added a PBD to the N-terminal end of the ancestral hexokinase gene after the tandem duplication event, followed by evolution of the N-terminal half into a regulatory domain. In place of the PBD of mouse somatic cell type 1 hexokinases, the mouse spermatogenic cell-specific type 1 hexokinase mRNAs (*mHk1-sa*, *mHk1-sb*, and *mHk1-sc*) contain a spermatogenic cell-specific sequence (Mori et al., 1993). The junction between the spermatogenic cell-specific sequence and the remainder of the coding region of these mRNAs coincided precisely with the junction between the PBD sequence and the remainder of the coding region of the mouse somatic *Hk1* mRNA. The finding that unique type 1 hexokinase mRNAs lacking the PBD are expressed in mouse spermatogenic cells appeared to support prior models for the evolution of hexokinase genes. Therefore, we hypothesized that unique type 1 hexokinase mRNAs lacking the PBD are also expressed in the spermatogenic cells of other mammalian species. To test our hypothesis, we used the 5′ rapid amplification of cDNA ends (RACE) method and identified three cDNAs representing unique human type 1 hexokinase mRNAs (*hHK1-ta, hHK1-tb*, and *hHK1-tc*) expressed in the testis, but not detected by Northern analysis in other human tissues. We report here the characterization of these unique human testis-specific type 1 hexokinase mRNAs and the expression of type 1 hexokinase mRNAs lacking the PBD in testes of seven mammalian species.

The nucleotide sequence data reported here are deposited in GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers U38226, U38227, and U38228.

MATERIALS AND METHODS

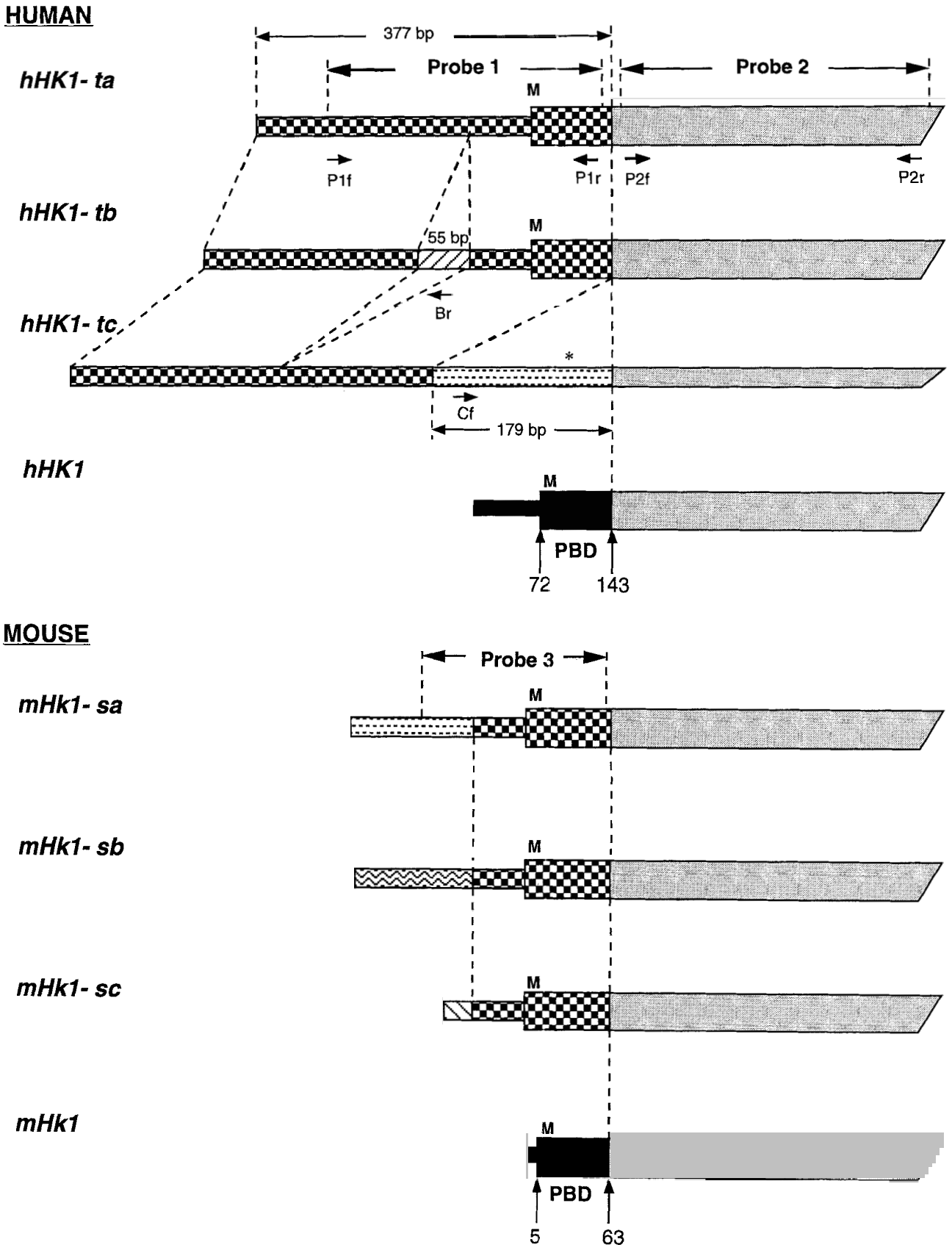
CD-1 mice and Sprague–Dawley rats were obtained from Charles River (Raleigh, NC) or the National Institute of Environmental Health Sciences (NIEHS) breeding colony. Testes from guinea pig, hamster, ram, and New Zealand rabbit were obtained through the Comparative Medicine Branch at NIEHS. All procedures involving animals were performed in accordance with USPHS guidelines and approved previously by the NIEHS Animal Care and Use Committee.

To isolate the 5′ end of type 1 hexokinase cDNAs representing mRNAs expressed in human testis, 5′-RACE-Ready human testis cDNA (Clonetech, Palo Alto, CA) was employed in the amplification reaction with the Amplitaq kit (Perkin-Elmer Cetus, Norwalk, CT) and DNA thermal cycler (Perkin-Elmer Cetus) according to the suppliers' instructions. Reverse primer 1 (5′-ACATCTGCTCCTTCCACTCCGCTCG – 3′; nt 611–635) and reverse primer 2 (5′ – GCTTCCACTGCCGTG-CACGATGTTC – 3′; nt 429–453) were designed from the sequence of the human somatic cell type 1 hexokinase cDNA (Nishi et al., 1988).

The amplification products were separated on a 1.5% low melting temperature grade (LTG) agarose gel (Sigma, St. Louis, MO) and stained with ethidium bromide. The DNA products were isolated from the gel and directly ligated into the TA cloning vector (Invitrogen, San Diego, CA). The DNA inserts were analyzed by double-strand dideoxy nucleotide sequencing using the Sequenase (US Biochemical, Cleveland, OH) or Auto-Sequencer Core kit (Toyobo Co., Ltd., Osaka, Japan). Analyses of the nucleotide and deduced amino acid sequences were performed with GCG (Genetics Computer Group, Inc., Madison, WI), Microgenie (Beckman Instr., Palo Alto, CA), and PC/GENE (IntelliGenetics, Inc., Mountain View, CA) computer software.

Probes for the testis-specific sequence shared by *hHK1-ta, hHK1-tb*, and *hHK1-tc* (probe 1; *hHK1-ta*, nt 80–343 in Fig. 1; forward primer 1 = P1f: 5′-AATAGG-CAAGACCAGCAACC-3′; reverse primer 1 = P1r: 5′-AGCTGCTGTAGCCGATTCTC-3′), and the sequence common to these transcripts and to the somatic *hHK1* (probe 2; *hHK1*, nt 148–409 in Fig. 1; forward primer 2 = P2f: 5′-GACAAGTATCTGTATGCCATGC-3′; reverse primer 2 = P2r: 5′-CGGACTCCATGTGAA-CATTC-3′) were used for Northern analysis of RNA from human tissues (see Fig. 4) or testes of different mammalian species (see Fig. 6). Probe 3 (*mHk1-sa*, nt 49–263 in Fig. 1 forward primer 3 = P3f: 5′-AGCACCACTT-CTCTCTAGGA-3′; reverse primer 3 = P3r: 5′-CCTC-TGTGAGAGGGGGTCGT-3′) contains the mouse spermatogenic cell-specific sequence shared by *mHk1-sa, mHk1-sb*, and *mHk1-sc*, and part of the region unique to *mHk1-sa*. Probe 3 was used for Northern analysis of RNA from testes of different mammalian species (see Fig. 6), or juvenile rats of various ages (see Fig. 7). These probes were prepared using a modified PCR labeling technique (Welch et al., 1992b) with ^{32}P-dCTP (Amersham, Arlington Heights, IL) or digoxigenin-dUTP (Boehringer Mannheim, Mannheim, Germany). A human testis cDNA library (Clontech) was screened as described by Benton and Davis (1977) using probe 1.

Isolations of poly $(A)^+$ RNA and of DNA were carried out as described previously (Mori et al., 1993). The separation of RNA and DNA by agarose gel electrophoresis for Northern and Southern blotting analysis was accom-


HUMAN
hHK1- ta
377 bp
Probe 1
Probe 2
M
P1f
P1r
P2f
P2r
hHK1- tb
55 bp
M
Br
hHK1- tc
*
Cf
179 bp
hHK1
M
PBD
72
143
MOUSE
Probe 3
mHk1- sa
M
mHk1- sb
M
mHk1- sc
M
mHk1
M
PBD
5
63

plished as previously described (Welch et al., 1992b). Human testis RNA and multiple tissue Northern blots were purchased from Clontech.

To confirm the expression of *hHK1-tb* and *hHK1-tc* mRNA in human testis, reverse transcription followed by the polymerase chain reaction (rt-PCR) was performed using a GeneAmp RNA PCR kit (Perkin-Elmer) according to the supplier's instructions. Combinations of primers used in rt-PCR were as follows and their locations are shown in Figure 1: P1f with P1r; P1f with Br (5′-TTTGCTTGTTCCTTCATTTGG-3′; *hHK1-tb*, nt 265–285 in Fig. 2); and Cf (5′-CAGACCGAGGCTTG-CAGTGG-3′; *hHK1-tc*, nt 443–462 in Fig. 2) with P2r. After human testis RNA (Clontech) was reverse transcribed into single-strand cDNA, the PCR reaction was repeated for 35 cycles (1 min at 94°C, 1 min at 58°C, 30 sec at 72°C).

RESULTS

Characterization of Three Unique Human Hexokinase Type 1 mRNAs Lacking the Porin-Binding Domain (PBD)

The 5′ rapid amplification of cDNA ends (RACE) method was used to isolate the 5′ ends of three unique cDNAs representing type 1 hexokinase mRNAs (*hHK1-ta*, *hHK1-tb*, and *hHK1-tc*) expressed in the human testis. All contained a testis-specific sequence, while *hHK1-tb* and *hHK1-tc* each contained an additional unique sequence. None contained the PBD present at the 5′ end of the coding region of type 1 hexokinase in human somatic cells (*hHK1*) (Figs. 1, 2, 3). The deduced amino acid sequences of *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* immediately 3′ to this region were identical to that of *hHK1*. The human testis-specific sequence common to the *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* cDNAs was similar to the mouse spermatogenic cell-specific sequence of *mHk1-sa*, *mHk1-sb*, and *mHk1-sc*, but not to other sequences in GenBank. Comparison of the deduced amino acid sequence of the testis-specific region in human *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* with that of the spermatogenic cell-specific region in mouse *mHk1-sa*, *mHk1-sb*, and *mHk1-sc* indicated 44% similarity and 40% identity (Fig. 3). The location of the predicted translation initiation codon (ATG) of the human testis-specific hexokinase mRNAs coincides with that of the mouse spermatogenic cell-specific hexokinase mRNAs. Moreover, the junction between the human testis-specific sequence and the remainder of the predicted coding region of the hexokinase cDNAs coincides with the junction between the mouse spermatogenic cell-specific sequence and the predicted coding region of the mouse hexokinase cDNAs, and also with the junction between the PBD sequence and the remainder of the coding region of the human somatic *hHK1* cDNA. The role of these unique sequences is unknown, but the deduced amino acid sequence encoded by the testis-specific region of the human hexokinase mRNAs predicts an alpha helical conformation.

The expression of the *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* mRNAs was determined by Northern blot analysis using probe 1 and probe 2, and RNA isolated from various human tissues (Fig. 4). Probe 1, from the testis-specific region common to *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* (Fig. 1), recognized a ~4.3 kb mRNA in testis, but not in other human tissues (Fig. 4A), even when the blot was overexposed (data not shown). These data indicate that the testis-specific region of *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* is present in ~4.3 kb transcripts expressed abundantly in human testis, but not in the somatic tissues surveyed. In contrast, probe 2, from the region of *hHK1* with high identity to the testis-specific cDNAs (Fig. 1), hybridized weakly with a ~4.1 kb mRNA present in several human somatic tissues and strongly with a ~4.3 kb mRNA in human testis (Fig. 4B). When a human actin probe (Clontech) was used on the same blot (Fig. 4B), it detected a 2.2 kb transcript that was present in approximately equal amounts in all of the human tissues.

We used probe 1 to clone an *hHK1-ta* cDNA from a human testis cDNA library to confirm by an independent approach that *hHK1-ta* mRNA is expressed in human testis (data not shown). In addition, rt-PCR with primers specific to the unique sequences of *hHK1-tb* or *hHK1-tc* (P1f and P1r, P1f and Br, or Cf and P2r; see Fig. 1) was used to confirm that these transcripts are expressed in human testis (Fig. 5). However, the *hHK1-tc* transcript has stop codons in all three reading frames and probably is not translated into a functional protein.

Fig. 1. Comparison of the structural features of the 5′ end of the *hHK1-ta*, *hHK1-tb*, *hHK1-tc*, *hHK1*, *mHk1-sa*, *mHk1-sb*, *mHk1-sc*, and *mHk1* mRNAs. Sequence analysis of the *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* cDNAs indicated that they represented mRNAs that contain a testis-specific sequence at the 5′ end (checkered boxes; 377 bp). The *hHK1-tb* and *hHK1-tc* mRNAs each have an additional sequence [boxes containing cross-hatched lines (55 bp) and dashed lines (179 bp), respectively] not present in *hHK1* or *hHK1-ta* mRNAs. The junction between the testis-specific region and the remainder of the predicted coding sequences of the *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* mRNAs was the same as that between the PBD and the remainder of the somatic cell *mHk1* mRNA sequence, and also the junction between the spermatogenic cell-specific sequence in the *mHk1-sa*, *mHk1-sb*, and *mHk1-sc* mRNAs and the remainder of their sequences. The unique 5′ *mHk1-sa*, *mHk1-sb*, and *mHk1-sc* regions are represented by boxes containing dashed lines, wavy lines, and hatched lines, respectively. M indicates the predicted initiation codon (ATG). * indicates the stop codon in the *hHK1-tc* mRNA.

The predicted coding regions of the *hHK1-ta*, *hHK1-tb*, *hHK1*, *mHk1-sa*, *mHk1-sb*, *mHk1-sc*, and *mHk1* mRNAs are indicated by the wide boxes, while the 5′ untranslated regions are indicated by the narrow boxes. The regions encoding the porin-binding domain (PBD; Griffin et al., 1991) in the *hHK1* and *mHk1* mRNAs are indicated by solid boxes.

The regions used to prepare probes 1, 2, and 3 for Northern blot analyses are shown in relation to the general structures of the *hHK1-ta* and *mHk1-sa* mRNAs, respectively. The locations of probe 1 for the human testis-specific sequence and probe 2 for the region conserved among the *hHK1-ta*, *hHK1-tb*, *hHK1-tc*, and *hHK1* mRNAs are shown by arrow bars. Probe 3 (215 bp) contains the mouse spermatogenic cell-specific sequence and part of the unique *mHk1-sa* region. The location and orientation of primers (P1f, P1r, P2f, P2r, Br, and Cf; see Materials and Methods) used to generate these probes and for PCR analyses are shown by arrows.

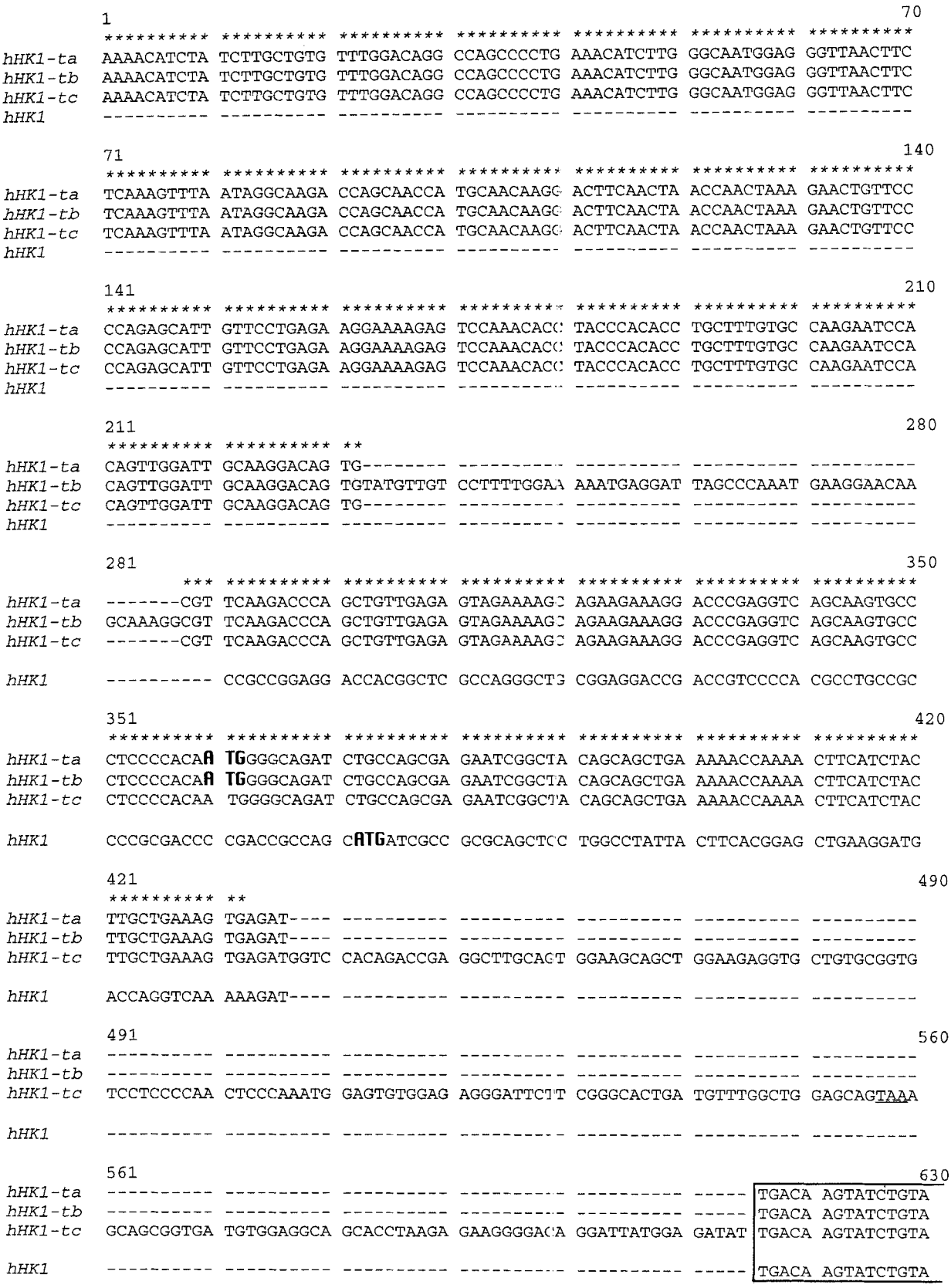


Fig. 2. Comparison of the nucleotide sequences at the 5′ end of the *hHK1-ta*, *hHK1-tb*, *hHK1-tc*, and *hHK1* cDNAs. The asterisks indicate the testis-specific sequence. The single-lined box indicates the initial part of the region conserved among the *hHK1-ta*, *hHK1-tb*, *hHK1-tc*, and *hHK1* mRNAs. The predicted initiation codons (ATG) are shown in bold. The predicted stop codon in the *hHK1-tc* mRNA is underlined.

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hHK1-t     MGQICQRESA TAAEKPKLHL LAESE IDKYLYA
mHk1-s     MGQNCQRGQA VDVEPKIRPP LTEEK IDKYLYA
hHK1             MIAAQL LAYYFTELKD DQVKK IDKYLYA
mHk1             MIAAQL LAYYFTELKD DQVKK IDKYLYA
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Fig. 3. Alignment of the deduced amino acid sequences encoded by the N-terminal regions of the *hHK1-t* (*hHK1-ta*, *-tb*, and *tc*), *mHk1-s*, (*mHK1-sa*, *-sb*, and *sc*), *hHK1* and *mHk1* mRNAs. Vertical lines indicate identity, and the vertical dots indicate conservative amino acid replacements. The underline indicates the porin-binding domain (PBD; Griffin et al., 1991).

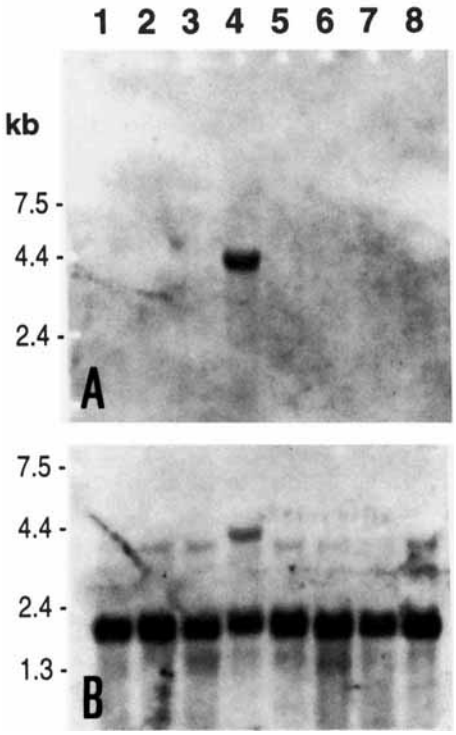

Fig. 4. Tissue-specific expression of human type 1 *hHK1-t* mRNAs. Northern analyses were carried out using RNA isolated from various human tissues hybridized with probe 1 **(A)** and probe 2 **(B)**. Probe 1 for the testis-specific region of the *hHK1-ta*, *hHK1-tb*, and *hHK1-tc* mRNAs (see Fig. 1) recognized a ~4.3 kb mRNA present in testis, but not in other human tissues (A). In contrast, probe 2 for the region conserved among *hHK1-t* and *hHK1* mRNAs (see Fig. 1) hybridized weakly with a ~4.1 kb mRNA in several human somatic tissues and strongly with a ~4.3 kb mRNA in human testis (B). When a human actin probe (Clontech) was used on the same blot (B), it detected a 2.2 kb transcript that was approximately equivalent in all tissues. RNA was isolated from **lane 1** spleen, **lane 2** thymus, **lane 3** prostate, **lane 4** testis, **lane 5** ovary, **lane 6** small intestine, **lane 7** colon, and **lane 8** peripheral blood leukocyte. Two micrograms of poly $(A)^+$ RNA were present in each lane.

Conserved Expression of mRNAs for a Unique Hexokinase Type 1 Lacking the PBD in Testes of Different Mammals

Northern analysis was performed on equivalent amounts of poly $(A)^+$ RNA isolated from testes of mouse, hamster, guinea pig, rabbit, and human. Hybridization with probe 1 (containing the human testis-specific *hHK1-t* region, see Fig. 1) demonstrated that ram and guinea pig testes contained ~4.3 kb mRNAs homologous to the human testis-specific type 1 hexokinase mRNAs (Fig. 6A). In contrast, probe 3 (from mouse, containing the *mHk1-s* spermatogenic cell-specific region and part of the *mHk1-sa* unique region, see Fig. 1) recognized ~4.3 kb and ~3.8 kb mRNAs in hamster testis (Fig. 6C), and a ~4.3 kb mRNA in rat testis (Fig. 7). Probe 3 also recognized faintly a ~4.3 kb mRNA in guinea pig, ram, and human testis (Fig. 6C). When the blot was heavily overexposed, probe 3 detected a ~4.3 kb mRNA in rabbit testis (data not shown). Although we reported previously that probe 3 recognized ~4.1 kb mRNA in mouse testis (Mori et al., 1993), Northern analysis in this study indicated that the *mHk1-s* transcripts are ~4.3 kb. The results with probe 2, from *hHK1-ta* and containing a sequence of high identity between somatic *HK1* and testis *HK1-t*, demonstrated that similar amounts of mRNA are present in each lane (Fig. 6B). Since probe 3 was shown previously to be specific for type 1 hexokinase mRNAs lacking the PBD sequence (Mori et al., 1993), these results strongly suggest that similar transcripts are expressed in the testes of these mammals.

Northern blot analysis of total RNA from testes of rats of various ages using probe 3 revealed that a ~4.3 kb mRNA was present in low abundance in juvenile rat testes at day 23 and at higher abundance at day 29 (Fig. 7). This suggests that rat type 1 hexokinase mRNAs lacking the PBD are expressed at lower abundance in pachytene spermatocytes and at higher abundance in spermatids, and that spermatogenic cell-specific type 1 hexokinase mRNAs lacking the PBD are expressed in the rat as they are in the mouse.

DISCUSSION

We have cloned cDNAs representing three unique human type 1 hexokinase mRNAs (*hHK1-ta*, *hHK1-tb*, and *hHK1-tc*) which are expressed in the testis, but not in other human tissues surveyed by Northern analysis. All lack the sequence for the PBD present in somatic cell type 1 hexokinase, but contain a testis-specific sequence near the 5′ end. The junction between the testis-specific sequence and the remainder of the mRNA sequence coincides with that between the PBD and the remainder of the sequence of the somatic *hHK1* mRNA. The *hHK1-t* and *hHK1* mRNA sequences also appear to be identical 3′ to this junction. In addition, *hHK1-tb* has a unique sequence inserted within the noncoding region of the testis-specific sequence, while *hHK1-tc* has a unique segment inserted between the testis-specific region and remainder of the sequence. However, the *hHK1-tc* transcript has stop codons in all three reading frames and probably is not translated. Another mRNA in the testis that apparently is untranslated is a product of the *Xist* gene (McCarrey and Dilworth, 1992).

The deduced testis-specific amino acid sequence of the *hHK1-ta* and *hHK1-tb* mRNAs was 40% identical with that encoded by the spermatogenic cell-specific sequence of the mouse *mHk1-sa*, *mHk1-sb*, and *mHk1-sc* mRNAs

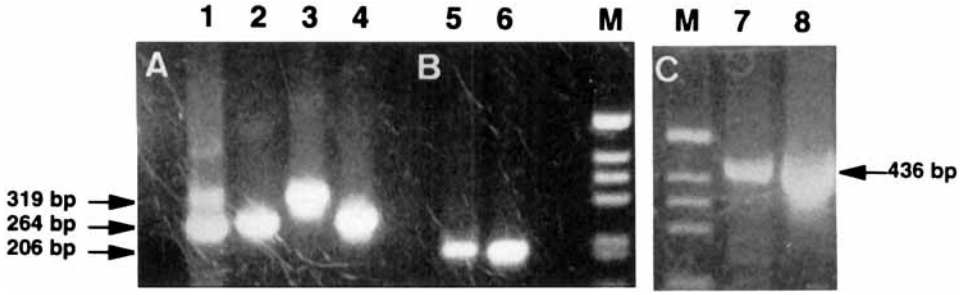

Fig. 5. Detection of multiple type 1 hexokinase transcripts in human testis. Reverse transcription and PCR (rt-PCR) was carried out on human testis poly $(A)^+$ RNA (**lanes 1, 5,** and **7**). PCR was performed using *hHK1-tc* cDNA (**lanes 2** and **8**), *hHK1-tb* cDNA (**lanes 3** and **6**), and *hHK1-ta* cDNA **(lane 4)** as template. **Lane M** was 1 kb DNA ladder. Primers used for rt-PCR were **(A)** P1f and P1r; **(B)** P1f and Br; and **(C)** Cf and P2r (see Fig. 1). Detected in human testis were *hHK1-ta* mRNA (264 bp band in A, lane 1), *hHK1-tb* mRNA (319 bp band in A, lane 1; 206 bp band in B, lane 5), and *hHK1-tc* mRNA (436 bp band in C, lane 7).

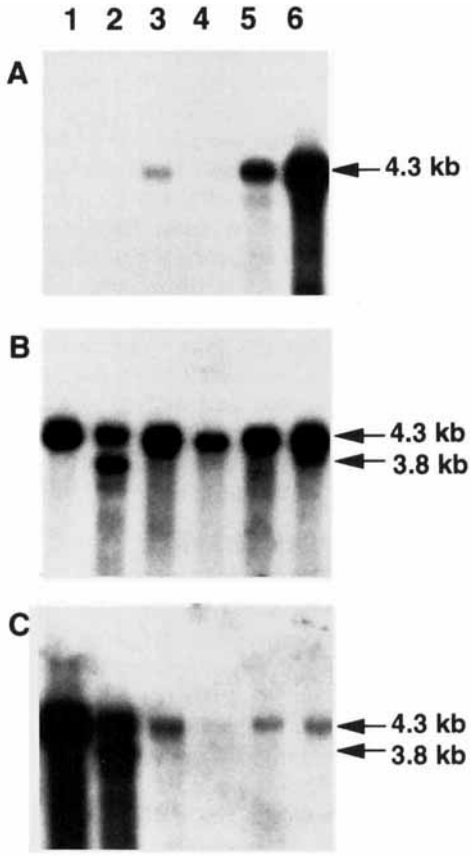

Fig. 6. Unique type 1 hexokinase gene expression in the testis of different mammalian species. Northern analyses were performed using probe 1 **(A)**, probe 2 **(B)**, and probe 3 **(C)**. RNA was isolated from **lane 1** mouse, **lane 2** hamster, **lane 3** guinea pig, **lane 4** rabbit, **lane 5** ram, and **lane 6** human testes. Two point five micrograms of poly$(A)^+$ RNA was loaded per lane. The human testis-specific *hHK1-t* probe (probe 1) hybridized with mRNA from human, ram, and guinea pig testis (A, lanes 6, 5, and 3, respectively), while the mouse spermatogenic cell-specific probe (probe 3) hybridized with mRNA from all six mammals (C).

(Mori et al., 1993). Furthermore, the junctional site between the testis-specific region in the human *hHK1-t* mRNAs and the region shared with the somatic *hHK1* mRNA is the same as that between the spermatogenic cell-specific sequence in the mouse *mHk1-s* mRNAs and the region they share with the somatic cell *mHk1* mRNA. These similarities, as well as the nucleic acid and deduced amino acid sequence homologies of the human testis-specific sequence and mouse spermatogenic cell-specific sequence, suggest that the human mRNAs are also expressed in spermatogenic cells. Moreover, our Northern blot analysis of RNA from testes of rats of various ages showed that expression of a unique type 1 hexokinase mRNA lacking the PBD is regulated developmentally, suggesting that its transcription also occurs in spermatogenic cells. Further studies, using in situ hybridization or Northern analysis of RNA from isolated germ cells, will be needed to confirm that the human *hHK1-t* mRNAs are expressed specifically in spermatogenic cells.

Northern blot analysis of RNA from testes of seven different mammals (mouse, hamster, guinea pig, rabbit, ram, human, and rat) suggested that mRNA containing a testis-specific type 1 hexokinase sequence and lacking the PBD sequence is conserved among these species. Probe 1 (from the *hHK1-t* testis-specific sequence) hybridized with mRNA from guinea pig and ram, but not mouse, hamster, or rabbit. However, probe 3 (from the *mHk1-s* spermatogenic cell-specific sequence) hybridized with mRNA from all species. Since we have shown previously that probe 3 hybridizes with spermatogenic cell *mHk1-s* mRNA lacking the PBD sequence, but not with somatic cell *mHk1* mRNA containing the PBD sequence (Mori et al., 1993), these results suggest that the mRNAs present in the testes of each of these mammals also lack the PBD sequence.

Probe 2 (from the sequence common to *hHK1* and *mHk1* in both testes and somatic cells) and probe 3 hybridized with ~4.3 kb and ~3.8 kb transcripts in the hamster testis. This suggests that at least two different type 1 hexokinase mRNAs are expressed in hamster testis. Since we have identified three unique hexokinase mRNAs lacking the PBD sequence in the testis of mouse and human, expression of multiple transcripts for type 1 hexokinase in spermatogenic cells may be a common feature in mammals. Though the mechanisms involved in the generation of multiple hexokinase transcripts in

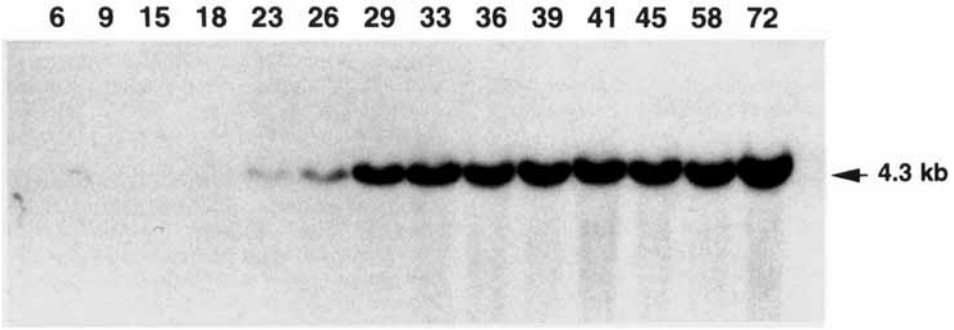

Fig. 7. Unique type 1 hexokinase mRNA expression during rat testis development. Probe 3 was used to detect mRNA in testes from rats from 6 to 72 days of age. Hybridization first occurred at day 23, when pachytene spermatocytes are the most advanced spermatogenic cells. Hybridization was more pronounced at day 29, when round spermatids are becoming abundant during the first wave of spermatogenesis. Five micrograms of total RNA was loader per lane.

spermatogenic cells have not yet been determined, the findings with glucokinase (Magnuson et al., 1989; Newgard et al., 1990) lead us to hypothesize that the use of different transcription start sites and alternate splicing of exons are responsible for producing multiple transcripts from a hexokinase gene expressed only in spermatogenic cells.

Previous studies have indicated that the hexokinase found in testis and sperm may be different from that in other tissues. Sosa et al. (1972) reported that a rat sperm type hexokinase unlike those in somatic tissues (Katzen, 1967; Katzen et al., 1968) could be detected by starch gel electrophoresis. Although testes from 10- and 15-day-old rats lacked the sperm type hexokinase activity, it was detected at 20 days and increased in amount between 25 and 30 days of age (Sosa et al., 1972). The sperm type hexokinase was the predominant type in testes from 50-day-old rats (Sosa et al., 1972). The developmental patterns of expression of the rat sperm type hexokinase generally correspond to that of the rat testis-specific hexokinase mRNA and the mouse spermatogenic cell-specific hexokinase mRNAs (Mori et al., 1993), suggesting that it is the translation product of one or more *Hk1-s* mRNAs. Recently, Kalab et al. (1994) reported the germ cells in the testis of mice possess a unique tyrosine-phosphorylated form of hexokinase lacking the PBD.

Hexokinase is one of the rate-limiting enzymes of glycolysis, catalyzing the phosphorylation of glucose to glucose-6-phosphate. In somatic cells, hexokinase is soluble and also associates with a voltage-dependent anion channel (porin) in the outer mitochondrial membrane via a PBD present in the N-terminal region of the enzyme (Wilson, 1984; Smith and Wilson, 1991). Our previous (Mori et al., 1993) and present studies identifying mRNAs which encode type 1 hexokinases lacking the PBD suggest that unique enzymes are translated from these mRNAs, which probably do not bind to the outer mitochondrial membrane and have structural and functional characteristics specific to spermatogenic cells or spermatozoa. Furthermore, these results appear to support and extend models for the evolution of hexokinase genes (Griffin et al., 1991) by suggesting that recruitment of an exon may have been responsible for the addition of a spermatogenic cell-specific domain to the N-terminal end of an *Hk1* precursor gene after the tandem duplication event.

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