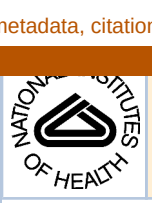

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Spermatogenic Cell-specific Type 1 Hexokinase is the Predominant Hexokinase in Sperm

Noriko Nakamura[1], **Haruna Shibata**[2], **Deborah A. O'Brien**[3], **Chisato Mori**[2], and **Edward M. Eddy**[1]

1 *Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA*

2 *Department of Bioenvironmental Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, JAPAN*

3 *Department of Cell and Developmental Biology, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA*

Abstract

Hexokinase is the first enzyme in the glycolytic pathway and utilizes ATP to convert glucose to glucose-6-phosphate (G6P). We previously identified three variant transcripts of *Hk1* that are expressed specifically in spermatogenic cells, have different 5′ untranslated regions, and encode a protein (HK1S, spermatogenic cell-specific type 1 hexokinase) in which the porin-binding domain (PBD) of HK1 is replaced by a novel N-terminal spermatogenic cell-specific region (SSR). However, the level of expression of the individual variant transcripts or of the other members of the hexokinase gene family (*Hk2*, *Hk3*, and *Gck*) in spermatogenic cells remains uncertain. We show that *Hk1*, *Hk2*, and *Hk3* transcripts levels are quite low in spermatocytes and spermatids and *Gck* transcripts are relatively abundant in spermatids, but that GCK is not detected in spermatozoa. Using real time RT-PCR (qPCR) with primers specific for each of the three variant forms and RNA from whole testis and isolated germ cells, we found that transcripts for *Hk1_v2* and *Hk1_v3*, but not for *Hk1_v1*, are relatively high in spermatids. Similar results were seen using spermatogenic cells isolated by laser-capture microdissection (LCM). Immunoblotting studies found that HK1S is abundant in sperm, and immunostaining confirmed that HK1S is located mainly in the principal piece of the sperm flagellum, where other spermatogenic cell-specific glycolytic enzymes have been found. These results strongly suggest that HK1, HK2, HK3 and GCK are unlikely to have a role in glycolysis in sperm and that HK1S encoded by *Hk1_v2* and *Hk1_v3* serves this role.

Keywords

spermatogenesis; isozyme; gene expression; glycolysis; testis

INTRODUCTION

Hexokinase (HK, E.C.2.7.1.1) is the initial enzyme in the glycolytic pathway and utilizes ATP to phosphorylate glucose and produce glucose-6-phosphate (G6P). There are four genes in the hexokinase family in mammals (reviewed by Wilson, 1995). Three encode isozymes (HK1, HK2 and HK3) of ~100 kDa that contain two homologous sequences (HK domain) in tandem and are inhibited by G6P. The fourth member of this family is glucokinase (GCK), a ~50 kDa

Correspond to: E. M. Eddy, LRDT, C4-01, NIH, NIEHS, Research Triangle Park, NC 27709, USA, Tel: (919)-541-3015, FAX: (919)-541-3800, E-mail: eddy@niehs.nih.gov.

protein that contains one HK domain and is not inhibited by G6P (Wilson, 1984). HK1 is present in most cell types, but is seen at highest levels in brain and erythrocytes, and HK2 is found predominately in insulin-sensitive tissue (skeletal muscle, heart and adipose tissue). HK3 is present at low levels in liver, lung and spleen (Katzen et al., 1968; Ureta, 1978; Preller and Wilson, 1992), while GCK is present mainly in hepatocytes and pancreatic cells (Magnuson and Shelton, 1989; Newgard et al., 1990).

We identified previously three mouse spermatogenic cell-specific variant transcripts from the *Hk1* gene. They were originally named *Hk1-sa*, *Hk1-sb*, and *Hk1-sc* (Mori et al., 1993), but are renamed *Hk1_v1*, *Hk1_v2*, and *Hk1_v3* to comply with guidelines of the Mouse Genome Nomenclature Committee (http://www.informatics.jax.org/mgihome/nomen/gene.shtml). The structural organization of the three *Hk1* variants is shown in Figure 1. The three variant transcripts encode spermatogenic cell-specific type 1 hexokinase (HK1S). Their sequences differ in their 5′ untranslated regions, but the open reading frames are alike except for a 69 nucleotide insert in *Hk1_v2* that we refer to as the *Hk1-sb* insert (SBI) (Mori et al., 1993). A novel feature common to all three variants is the encoding of a 24 amino-acid sequence at the N-terminus that we refer as the spermatogenic cell-specific region (SSR) (Mori et al., 1993). The N-terminal 20 amino acids of the ubiquitously expressed form of HK1 define the porin binding domain (PBD) (Arora et al., 1990; Griffin et al., 1991) that binds HK1 to porin (also known as voltage-dependent anion channels; VDACs) on the outer mitochondrial membrane; presumably giving HK1 preferential access to ATP produced by oxidative phosphorylation, (see Adams et al., 1991, Ceser and Wilson, 1998).

Previous reports indicated that a monoclonal antibody to rat brain HK1 bound to the proximal and middle portion of the mouse sperm flagellum (Visconti et al., 1996), while two antisera to the SSR region localized HK1S to the principal piece region in the mouse sperm flagellum (Mori et al., 1998; Travis et al., 1998). One SSR antiserum also bound to the surface of the head and the midpiece region of the flagellum (Travis et al., 1998).

In this study, we used real time RT-PCR (qPCR) to examine mRNA from testes of juvenile mice during the relatively synchronous first wave of spermatogenesis (days 10–30) to compare the steady-state transcript levels of the members of the hexokinase gene family (*Hk1*, *Hk2*, *Hk3*, and *Gck*). The same approach was used to determine at what stages of spermatogenesis the *Hk1* variants are first expressed and to compare their levels during this period. In addition, the relative steady-state levels for the variant transcripts and for the other hexokinase gene-family members in individual spermatogenic cell types were determined by qPCR with RNA from isolated mouse pachytene spermatocytes, round spermatids, and elongating spermatids, and with RNA from spermatogonia, pachytene spermatocytes, early spermatids and late spermatids collected by laser-capture microdissection (LCM). Western blotting and immunohistochemistry were used to determine when HK1 and GCK are expressed in testis and if they are present in sperm. Most of the ATP required for mouse sperm motility is produced by glycolysis (Miki et al., 2004). The present study confirms and extends previous suggestions that *Hk1* variant transcripts encode the hexokinase isozyme that participates in glycolysis in mouse spermatozoa.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich (Saint Louis, MO) unless indicated otherwise. The CD-1 mice used for isolation of RNA, immunohistochemistry and germ cell isolation were obtained from Charles River Laboratories (Raleigh, NC). the C57BL6/J mice used for laser capture studies were from Japan SMC (Hamamatsu, Japan). The care and use of animals were carried out according to U.S. Public Health Service (USPHS) guidelines and the studies were approved in advance by the Institutional Animal Care and Use Committee of

NIEHS or the University of North Carolina, or were performed in accordance with Chiba University animal experimentation guidelines.

Isolated spermatogenic cells

Spermatogenic cells were isolated as previously described (O'Brien, 1993). Purities of pachytene spermatocytes and round spermatids (steps 1–8) exceeded 90%. Elongating spermatids isolated by this method contained 30–40% nucleated spermatids (steps 9–16) and cytoplasts derived primarily from these same cells. Two independent preparations for each of the germ cell types were used.

Quantitative Real-Time RT-PCR (qPCR)

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) from testes of mice 10 to 30 days of age, brain of adults, and purified populations of spermatogenic cells. The cDNA templates for qPCR were synthesized from RNA samples using reverse-transcriptase (Applied Biosystems, Foster City, CA). Gene specific primer pairs for *Hk1-3*, *Gck*, and *Hk1* transcript variants, and for transcripts of ribosomal protein L7 (*Rpl7*) are shown in Table 1. The regions of *Hk1* and *Hk1* variants amplified by each primer pair are shown in Table 2. The qPCR analyses were performed using SYBR Green PCR Master Mix reagents (Applied Biosystems) and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's protocols. The cDNA served as a template in a 50 μl reaction mixture and was processed using an initial denaturation step at 95°C for 10 min, followed by 40–45 amplification cycles (95°C for 15 s, 50–60°C for 1 min) and one dissociation stage cycle (95°C for 15 s, 60°C for 15 s, 95°C for 15 s). The annealing temperature and amplification cycles for primers determined to produce a single amplicon by RT-PCR are shown in Table 1. The relative steady-state transcript levels were calculated using cycle time (Ct) values and the equation: Relative Quantity = $2^{-\Delta\Delta Ct}$. The expression levels were normalized using *Rpl7* as an internal control for each sample (Mayuehas and Klein, 1990). The relative ratios (folds) of transcript levels in each sample were calculated using the day 10 value as one. The qPCR reactions were performed in triplicate with each of the samples.

Laser-capture Microdissection (LCM)

Cryostat sections 7–8 μm in thickness were prepared from testes of adult C57BL/6 mice, fixed with ice-cold 5% acetic acid/95% ethanol, and stained with hematoxylin (Wako Pure Chemicals Industries, Osaka Japan). The PixCell II LCM system (Arcturus Engineering, Mountain View, CA) was used to microdissect individual spermatogenic cells from testis sections. The laser beam was adjusted to melt the thermoplastic film in a spot of the diameter visually corresponding to the diameter of the target cell and captures were performed using a 7.5 μm diameter beam. Power settings were 40–80 mW, and laser pulse durations were 0.5–1.0 ms. Spermatogonia, pachytene spermatocytes, early spermatids and late spermatids were collected and RNA was extracted using the Picopure RNA isolation kit (Arcturus). To determine if spermatogonia or pachytene spermatocytes samples were contaminated with other cell types, RT-PCR assays were performed using primers for *Kit* or *Hspa2* as markers of spermatogonia or pachytene spermatocytes, respectively The primer sequences for *Kit* were: Upper 5′-GCA TCA CCA TCA AAA ACG TG -3′; Lower 5′-GAT AGT CAG CGT CTC CTG GC - 3′; and for *Hspa2* were Upper 5′-CCG TGG AAG ACG AGA AAC TG -3′; Lower 5′-AGG TTT ACG CGG ACT CCA G -3′. After confirming that contamination was negligible, qPCR assays were carried out using the primers shown in Table 1 and SYBR Green PCR Master Mix reagents (Applied Biosystems) and the DNA Engine Opticon System (MJ Research Inc., Watertown, MA). The cDNAs generated by RT-PCR served as templates in 25 μl reaction mixtures. Duplicate aliquots of each sample were analyzed using an initial denaturation cycle (50°C for 2 min, 95°C for 10 min), followed by 40 to 48 amplification cycles (95°C for 15 s,

54°C or 57.2°C for 1 min). The annealing temperature used for *Hk1_v1* and *Hk1_v2* was 57.2° C, and for *Hk1_v3* and SSR was 54°C. The fold changes in gene expression were calculated using the equation: fold change = $2^{-\Delta\Delta Ct}$. The levels of *Hk1* variant transcripts were normalized using *Rpl7* as an internal control in each sample. The relative ratio (folds) for each sample was calculated using the level in spermatogonia as one.

Protein Extraction and Immunoblotting

Liver and testis were homogenized in RIPA buffer [20mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1% TritonX-100, 0.1% SDS, 0.5% NP-40, and Complete protease inhibitor cocktail (Roche Diagnostics GmbH, Indianapolis, IN)], incubated for 30 min on ice, centrifuged at 15,800 g for 30 min, and the soluble fraction collected. Sperm obtained from the cauda epididymis were incubated in M2 medium (Sigma) for 5 min at room temperature and pelleted in Eppendorf tubes by centrifugation at 7,500 g for 3 min. The sperm pellet was lysed in phosphate buffer saline (PBS) containing 0.1% TritonX-100, centrifuged at 4,528 g for 5 min at 4°C, and the soluble fraction collected. The samples were suspended in 2 × sample buffer [4% SDS, 100 mM Tris-HCl (pH 6.8), 20% glycerol, 2-mercaptoethanol, and 0.001% bromophenol blue].

Samples of sperm, liver and testis, prepared as indicated above, were separated on 10% gradient ready gels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred from the gel to Immobilon-P PVDF membrane (Millipore Corp., Bedford, MA) and individual proteins detected using anti-GCK (N-term) (ABGENT, San Diego, CA), anti-SSR (Mori et al., 1998) and anti-hexokinase type 1 polyclonal antibodies (Chemicon International, Inc., Temecula, CA). The primary antibody was detected using a peroxidase-labeled second antibody and the ECL procedure (Amersham Pharmacia Biotech, Piscataway, NJ) as recommended by the supplier.

Immunohistochemistry

Testes were fixed in Bouins fixative, dehydrated, and embedded in paraffin by standard procedures. Immunostaining with the anti-SSR antibody was carried out as described previously (Mori et al., 1998). The polyclonal antibody to hexokinase type 1 (Chemicon) was diluted 1:100 with Automation buffer (Biomeda Corp., Foster City CA) containing 1% bovine serum albumin (BSA), and the protein-antibody complex detected using an Elite ABC kit for rabbit IgG (Vector Laboratories, Burlingame CA).

RESULTS

Expression of Hexokinase Gene-Family Members in Testis

We used qPCR and RNA from testes of 10 to 30 day old juvenile mice to determine which members of the hexokinase gene family are expressed in the testis. This is the period when progression of the first wave of spermatogenesis is relatively synchronous throughout the testis. Transcript levels for total *Hk1* (ubiquitous and variant forms) and *Gck* increased between days 22–24 and became higher during days 24–28 (Fig. 2A). However, the *Hk2* and *Hk3* steady-state transcript levels quite low during this period. To determine if the increase in total *Hk1* transcript levels was due to increases in the ubiquitous form or the variant forms, we used qPCR with RNA from testis and primers specific for the ubiquitous form of *Hk1* (PBD-Hk1) (Figure 2A). Transcript levels of the ubiquitous *Hk1* were low through day 22 and became lower during days 22–30 (Fig. 2A), strongly suggesting that the *Hk1* variant transcripts are the major form of present in whole testis at this time (i.e., *Hk1*≫PDB-*Hk1*).

Expression of *Hk1* Variants in Testis

Subsequent studies used qPCR assays with the primers shown in Table 1 and RNA from whole testis of 10 to 30 day-old mice to determine when the *Hk1* variants are expressed during the first wave of spermatogenesis. The combined level of expression of *Hk1* variants was determined using primers specific for the SSR. The level was low during days 10 to 18 and increased substantially from days 20 to 30 (Fig. 2B), suggesting that expression of the *Hk1* variants occurs primarily during the post-meiotic phase of spermatogenesis. To determine when each *Hk1* variant is expressed, qPCR was carried out using primers specific for each of the variants (Table 1). These assays showed that *Hk1_v1* transcript levels remained low during days 10 to 30, *Hk1_v2* transcripts levels increase between days 22–24, and *Hk1_v3* transcripts levels increase between days 24–30 (Fig. 2B). The variant present at highest level between days 24 and 28 was *Hk1_v2*, and on day 30 was *Hk1_v3* (Fig. 2B). These results indicate that *HK1_v2* and *Hk1_v3* are responsible for most of the increase in SSR transcripts seen after day 22 in whole testis, corresponding to the period when spermatids develop during the first wave of spermatogenesis.

Expression of *Hk1* Variants in Spermatogenic Cells

Our next set of studies used qPCR assays with RNA from isolated populations of pachytene spermatocytes, round spermatids, and condensing spermatids to examine expression of the three *Hk1* variants in specific spermatogenic cell types. Transcript levels were low in pachytene spermatocytes for all three variants, increased somewhat in round spermatids, and were at highest levels in condensing spermatids (Fig. 3A). The level of ubiquitous *Hk1* transcripts (PBD) was quite low in all cell types, in contrast to the expression level of ubiquitous *Hk1* in brain (Fig. 3B). However, *Gck* transcript levels increased in round spermatids, and were the highest (in relative fold increases over the level in pachytene spermatocytes) in condensing spermatids (Fig. 3A).

In addition, qPCR assays were carried out with RNA from spermatogonia, pachytene spermatocytes, early spermatids, and late spermatids isolated using laser-capture microdissection (LCM). For all three of the *Hk1* variants, transcript levels were low in spermatogonia and pachytene spermatocytes and higher in spermatids. The *Hk1_v2* and *Hk1_v3* transcripts were present at their highest levels in late spermatids (Fig. 4).

Detection of Hexokinase Proteins in Sperm, Testis, and Liver

Immunoblotting with antibodies to the SSR (recognizing HK1S only), to HK1 (recognizing HK1 and HK1S), and to GCK was used to determine if these enzymes are detected in RIPA buffer extracts of sperm, testis and liver. The SSR antibody detected HK1S in sperm, but not in liver (Fig. 5A, middle panel), while the GCK antibody detected GCK in liver and testis, but not in sperm (Fig. 5A, left panel). However, a faint band in sperm extracts was seen with the GCK antibody after a long exposure (data not shown). In addition, the HK1 antibody stained protein bands strongly in sperm extracts and weakly in whole testis extracts (Fig. 5B, left panel) corresponding to the bands stained by the SSR antibody (Fig. 5B, middle panel). Antibodies specific for HK2 and HK3 were unavailable, but the low levels of *Hk2* and *Hk3* transcripts in testis suggests that these two enzymes are unlikely to be present in sperm in appreciable amounts. These results and the previous finding that *Hk1* transcripts are present in negligible amounts in isolated spermatogenic cells (Fig. 3A) strongly suggest that HK1S is by far the predominant hexokinase present in sperm.

Localization of HK1S in Testis and Spermatozoa

Immunohistochemistry was used on sections of adult mouse testis to determine when HK1S is synthesized during spermatogenesis. Strong staining was seen with the HK1 antibody in the

cytoplasm and flagella of step 15 and 16 spermatids (Figs. 6A, B). The SSR antibody also detected HK1S in these same locations in step 15 and 16 spermatids (Figs. 6C, D). These results are consistent with our previous findings (Mori et al., 1998). In addition, indirect immunofluorescence was used to localize HK1S in sperm from the mouse cauda epididymis. The principal piece was immunostained prominently by the HK1 antibody and the middle piece and head were immunostained at a lower level (Figs. 6 G, H). The principal piece also was immunostained prominently and the middle piece less prominently by the SSR antibody, but the head was immunostained infrequently (Figs. 6E, F).

DISCUSSION

Using gene-specific primers and qPCR, we found that the steady-state *Hk1, Hk2* and *Hk3* transcript levels were quite low during the first wave of spermatogenesis in the postnatal testis, as well as in in isolated pachytene spermatocytes, round spermatids, and elongating spermatids. An earlier study using RT-PCR reported that *Hk1* is expressed in pachytene spermatocytes, round spermatids, and condensing spermatids in the mouse (Visconti et al., 1996), but the reasons for these differences are not obvious. We also found that *Gck* transcript levels were relatively high in whole testis and in condensing spermatids, but the GCK protein was not detected in appreciable amounts in sperm. While GCK was not detected in boar sperm, it was detected in dog sperm by western blotting and was localized to the flagellum and head by immunostaining (Fernández-Novell et al., 2004). Additional studies will be needed to determine if GCK has a significant role in mouse spermatids and if the presence of GCK in sperm is common or infrequent in other species. Since GCK is not inhibited by G6P like other members of the hexokinase family, there might be significant differences within species of glycolysis in spermatids and sperm and between species for glycolysis in sperm.

A goal of this study was to determine when and at what levels the *Hk1* variants are expressed during spermatogenesis. We found using both qPCR and LCM that *Hk1_v1* steady-state transcript levels are low in the mouse testis throughout the first wave of spermatogenesis, while *Hk1_2* and *Hk1_v3* levels are highest during the period corresponding to spermatid development. These results are consistent with earlier *in situ* hybridization and northern blotting studies that indicated *Hk1* variants are present at highest levels during the post-meiotic period of spermatogenesis (Mori et al., 1993). These results also are consistent with previous reports that *Hk1_v1* levels are relatively low in testis and isolated spermatogenic cells, while *Hk1_v2* levels are high in spermatids (Mori et al, 1993; Visconti et al., 1996). Thus, it is quite likely that *Hk1_v2* and *Hk1_v3* transcripts present in spermatids are the source of the HK1S protein and hexokinase activity in mouse sperm.

An unusual feature of glycolysis in mouse sperm is that some of the enzymes are products of genes or transcripts expressed specifically or predominantly in spermatogenic cells, often during the post-meiotic phase, which substitute for the cognate isozymes found in somatic cells. Genes for glyceraldehyde 3-phosphate dehydrogenase (*Gapdhs*) (Welch et al., 1992) and phosphoglycerate kinase-2 (*Pgk2*) (McCarrey and Thomas, 1987; Boer et al., 1987) are expressed specifically in spermatogenic cells. While the gene for lactate dehydrogenase C (*Ldhc*) (Millan et al., 1987; Sakai et al., 1987) is expressed predominantly in spermatogenic cells, LDHC also is detected in oocytes and early embryos (Coonrod et al., 2006).

Mouse *Gapdhs* transcripts are first seen in round spermatids (Welch et al., 1992; Mori et al., 1992), while GAPDHS is synthesized in condensing spermatids and becomes a component of the fibrous sheath in the sperm flagellum (Bunch et al., 1998). However, somatic cell GAPDH was not detected in mouse sperm by immuno-blotting or immuno-fluorescence (Bunch et al., 1998). Transcripts for mouse *Pgk1* are present at relatively low levels in spermatogonia and early spermatocytes and decline in amount throughout pachytene spermatocyte and spermatid

development (Kramer and Erickson, 1981; Goto et al., 1990; McCarrey et al., 1992), and PGK1 activity was not detected in sperm (McCarrey et al., 1992; 1996). Transcription of *Pgk2* begins early in pachytene spermatocyte development (Goto et al., 1990; McCarrey et al., 1992). However, PGK2 protein is first detected in step 12 spermatids (elongating spermatids) (Bluthmann et al., 1982; Kramer and Erickson, 1981), and is the only PGK present in sperm (McCarrey et al., 1996). Transcription of *Ldhc* begins in pachytene spermatocytes and continues in spermatids (Fujimoto et al., 1988; Thomas et al., 1990). The LDHC isozyme is detected from the development of preleptotene spermatocytes and throughout the remainder of spermatogenesis (Goldberg and Hawtry, 1967; Hawtry and Goldberg, 1968; Hintz and Goldberg, 1977; Wieben, 1981; Li et al. 1989) and is localized to the principal piece of mouse and human sperm (Beyler et al., 1985). Although the initiation of transcription of these three genes is quite similar, GAPDHS and LDHC protein synthesis begins in early pachytene spermatocytes, while PGK2 synthesis is delayed until after meiosis.

There are additional similarities between HK1S and the other glycolytic enzymes in sperm. HK1S co-localizes with GAPDHS and LDHC in the principal piece region of the flagellum. In addition, GAPDHS and HK1S have novel peptide sequences at their N-terminus, the mouse GAPDHS having a proline-rich105 amino acid N-terminal extension not present in GAPDH, while HK1S has the SSR peptide sequence which replaces the PBD sequence of HK1. A possible role of these novel sequences is to target the proteins to the principal piece or anchor them to the fibrous sheath. This has been tested for HK1S in non-homologous cell systems. In one study, a green fluorescent protein (GFP)-tagged SSR peptide expressed in HK1-deficient M+R42 cells targeted to the endoplasmic reticulum and plasma membrane (Travis et al., 1999). Another study reported that an epitope-tagged SSR peptide expressed in NIH3T3 cells was found in the cytosolic fraction of cell homogenates (Nakamura et al., 2003). While the reasons for the differences in these results are not obvious, it is questionable if the results can be extrapolated to spermatogenic cells and further studies are needed to determine if the SSR targets HK1S to specific sites in elongating spermatids and spermatozoa. It also remains to be determined if the novel 23 amino acid region encoded within *Hk1_v2* leads to differential targeting of this HK1S variant, serves a structural role, alters the enzymatic activity, or has some other role.

The mechanical force for flagellar motion is generated by ATP-dependent dynein arms on the axoneme outer microtubules (reviewed by Eddy, 2006). Although it was commonly assumed that sperm mitochondria produced the ATP required for motility, the principal piece region is 75–90% of the length of the flagellum and there was not a good explanation for how ATP moved from the middle piece to the distal part of the flagellum. The localization of HK1S, GAPDHS and LDHC in the principal piece strongly suggested that glycolysis occurs in this region of the flagellum.

This was confirmed using gene targeting to disrupt the *Gapdhs* gene (Miki et al., 2004). In sperm lacking GAPDHS, forward motility was eliminated, the males were infertile, and ATP levels were dramatically reduced. However, mitochondrial oxygen consumption was unchanged, indicating that glycolysis rather than oxidative phosphorylation is the major source of ATP for sperm motility in mice. This was confirmed by the findings that disruption of the *Pgk2* gene also results in greatly reduced ATP levels, severe impairment of flagellar activity, and male infertility (Danshina et al., 2006). The fibrous sheath and principal piece region containing HK1S and the other glycolytic enzymes is in close proximity to the axoneme throughout most of the flagellum. This arrangement serves to minimize the time and distance for ATP to diffuse from the site of generation to the dynein arms on the outer microtubules of the axoneme to power sperm motility.

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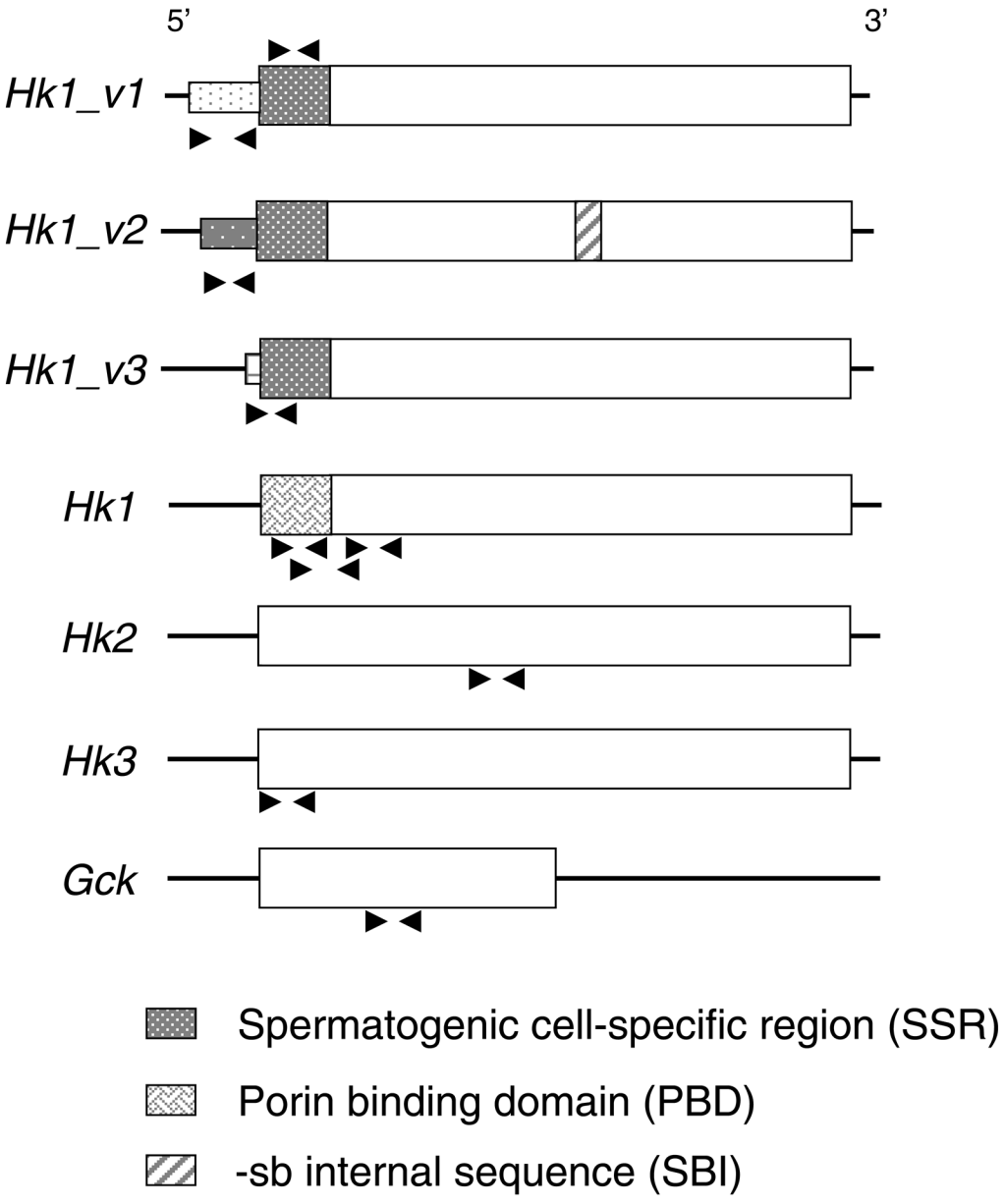

Fig. 1.

The structures of the cDNAs of hexokinase gene-family members. The coding regions of the *Hk1* variants and *Hk1 Hk2, and Hk3* are similar in length, while that for *Gck* is approximately half that of the other hexokinase family members. The 5′untranslated regions of *Hk1*, *Hk1_v1*, *Hk1_v2*, and *Hk1_3* differ in their lengths and sequences. Pair of facing arrows indicates the positions of the sequence-specific primers used in this study.

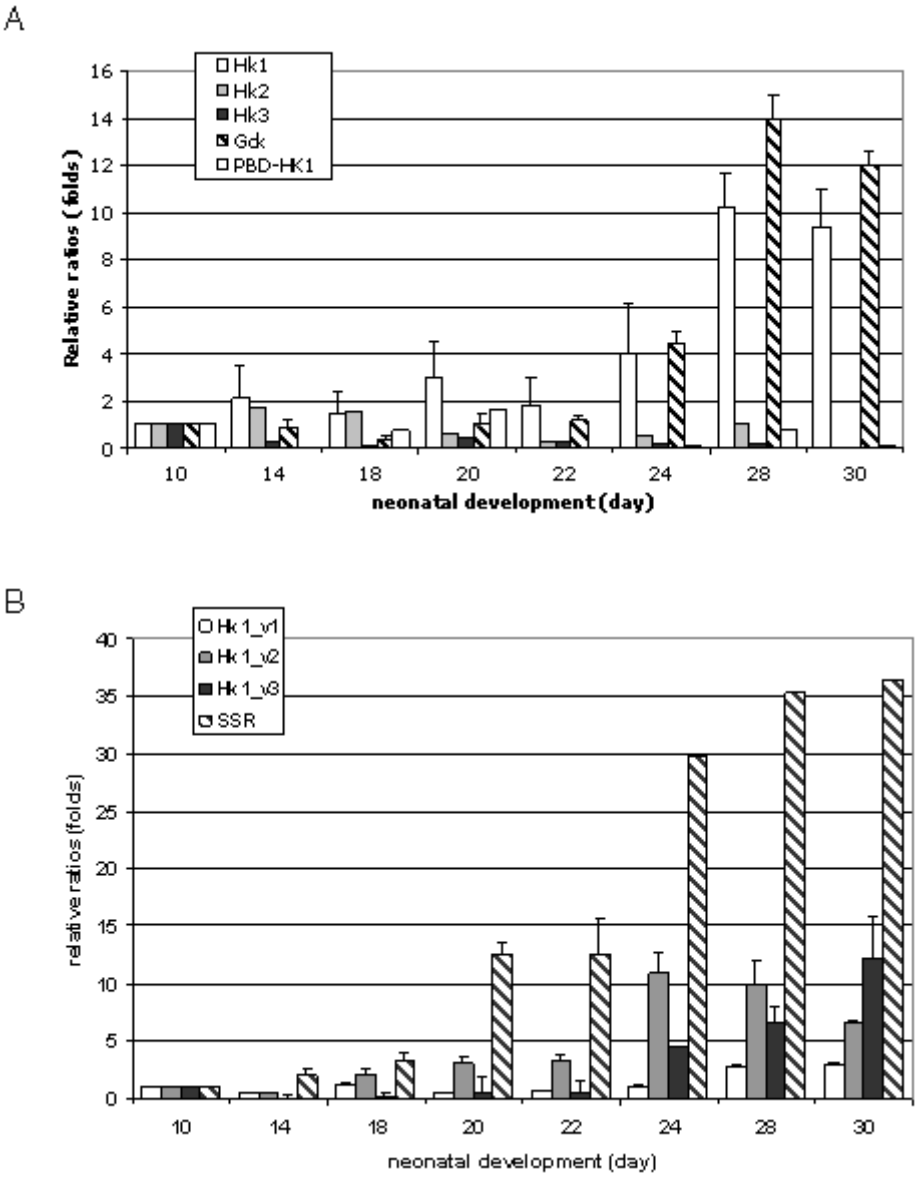

Fig. 2.

Expression of hexokinase gene-family members and of *Hk1* variants in testis. **A:** Primers specific for each of the hexokinase gene family members were used to assay by qPCR their transcription in testes of 10 to 30 day-old mice. (*Hk1*, white bars; *Hk2*, gray bars; *Hk3*, black bars; *Gck*, spotted bars; PBD-Hk1, grey-spotted bars) **B:** Primers specific for each of the *Hk1* variants and for the sequence encoding the SSR were used to assay by qPCR their expression in testes of 10 to 30 day old mice. (*Hk1_v1*, white bars; *Hk1_v2*, gray bars; *Hk1_v3*, black bars; SSR, diagonal line bars) Expression levels were determined as described in Materials and Methods and shown here as ratios (folds) relative to the level on day 10, with that level set at one. Data are expressed as means ± SEM.

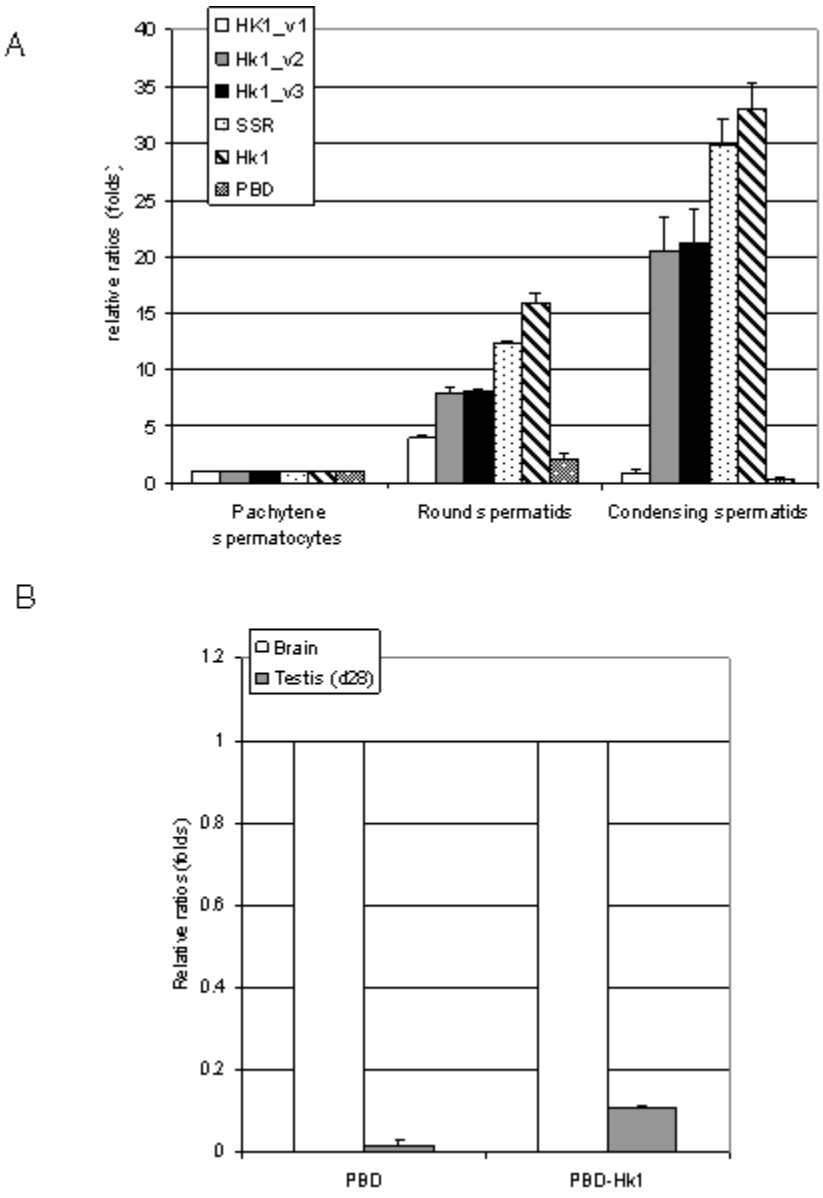

Fig. 3.

Expression of *Hk1* variants and of *Hk1* in isolated spermatogenic cells and of *Hk1* transcripts in brain and testis. **A**: Primers specific for the *Hk1* variants (*Hk1_v1*, *Hk1_v2*, *Hk1_v3*) and for *Hk1* (primers within the sequence encoding the PBD) were used to assay by qPCR their transcript levels in isolated spermatogenic cells. Expression levels were determined as indicated for Figure 2. (*Hk1_v1*, white bars; *Hk1_v2*, gray bars; *Hk1_v3*, black bars; SSR, spotted bars; *Hk1*, diagonal line bars; *Gck*, horizontal line bars; PBD, grey-spotted bars) **B**: RNA from brain and testis (day 28) were assayed by qPCR using a primer pair within the sequence for the PBD (labeled PBD) and with one primer in the PBD and the other primer in the region common to all *Hk1* transcripts (labeled PBD-Hk1). (Brain; white bar; testis, gray bar) Expression levels are shown here as ratios (folds) relative to the level on brain, with that level set at one.

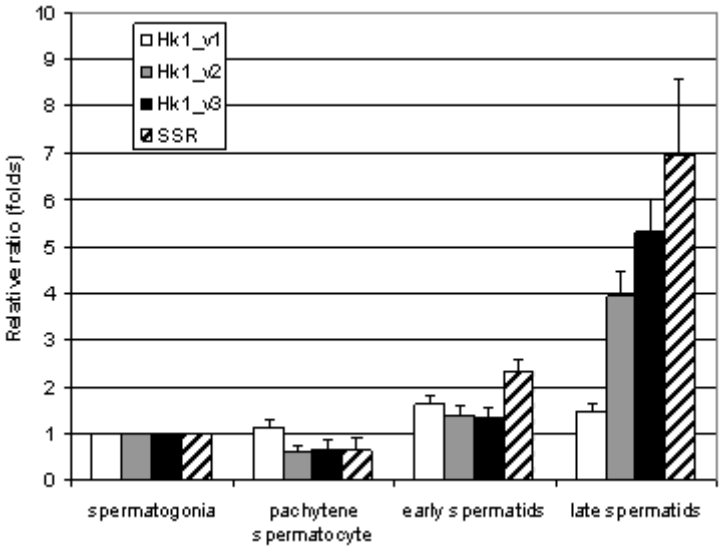

Fig. 4.

Expression of *Hk1* variants determined using LCM. RNA from spermatogenic cells isolated using LCM was assayed by qPCR as described in the Methods and Materials. Primers specific for *Hk1* variants (*Hk1_v1*, *Hk1_v2*, *Hk1_v3*), *Hk1* and SSR used were identical to those used for the studies in Figure 2. Expression levels were determined as indicated for Figure 2. (*Hk1_v1*, white bars; *Hk1_v2*, gray bars; *Hk1_v3*, black bars; SSR, diagonal line bars).

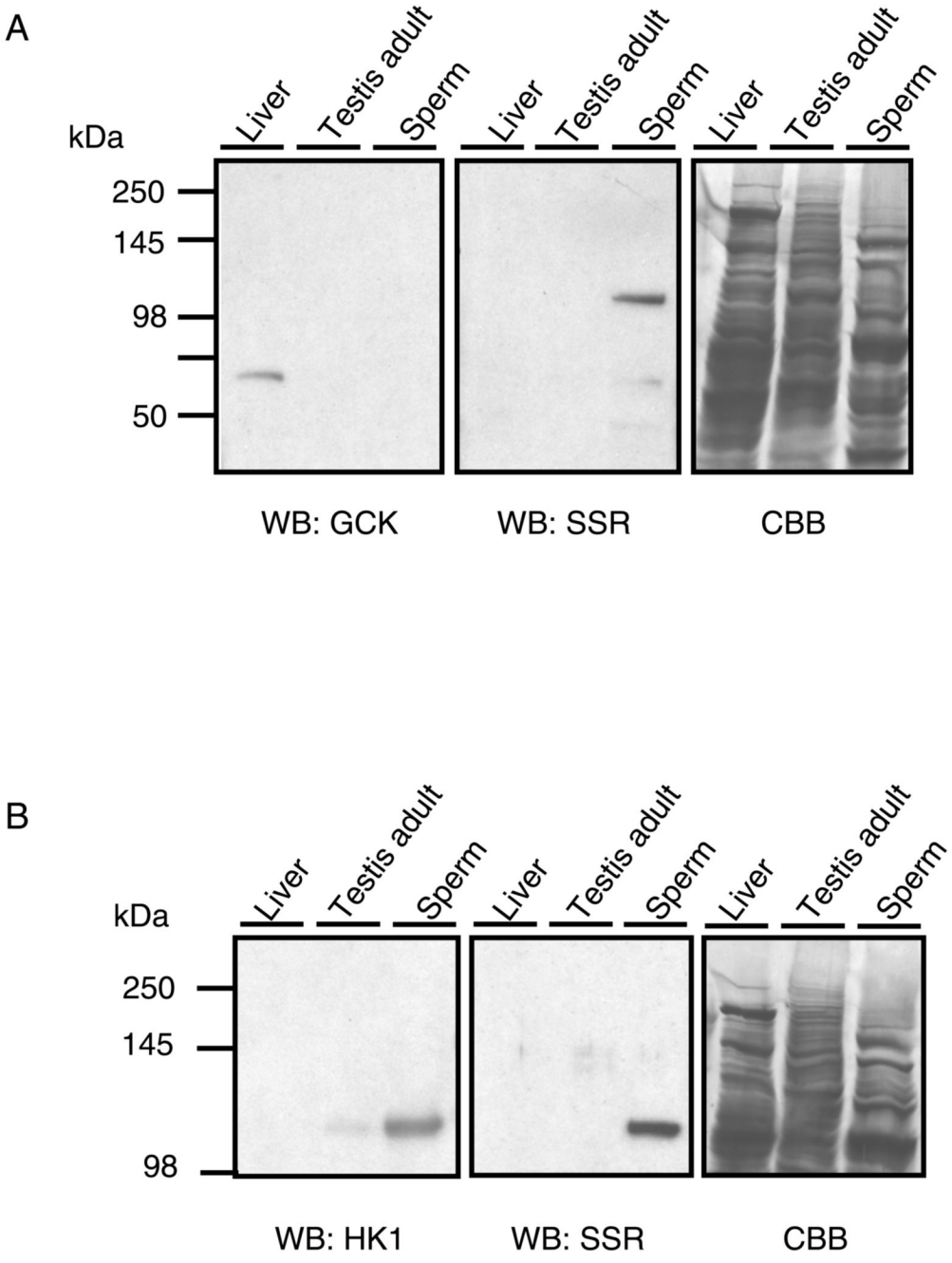

Fig. 5.

Expression of GCK and HK1 in liver, testis and sperm. Protein extracts from liver, testis and sperm were obtained as described in Materials and Methods. Protein (15μg) was loaded in each lane, separated by SDS-PAGE (A and B, right panels) and transferred to membranes. This was repeated at least three times and representative results are shown. The membranes first were probed with antibody to GCK (**A**, left panel) or to HK1, (**B**, left panel) and then were stripped and probed with antiserum to the SSR (**A** and **B**, middle panels). The antibody to GCK detected a protein only in liver (**A**, left panel), and the antibody to HK1 detected a protein in sperm (**A** and **B**, middle panels). The gels were stained with Coomassie blue to monitor protein loading (**A** and **B**, right panel).

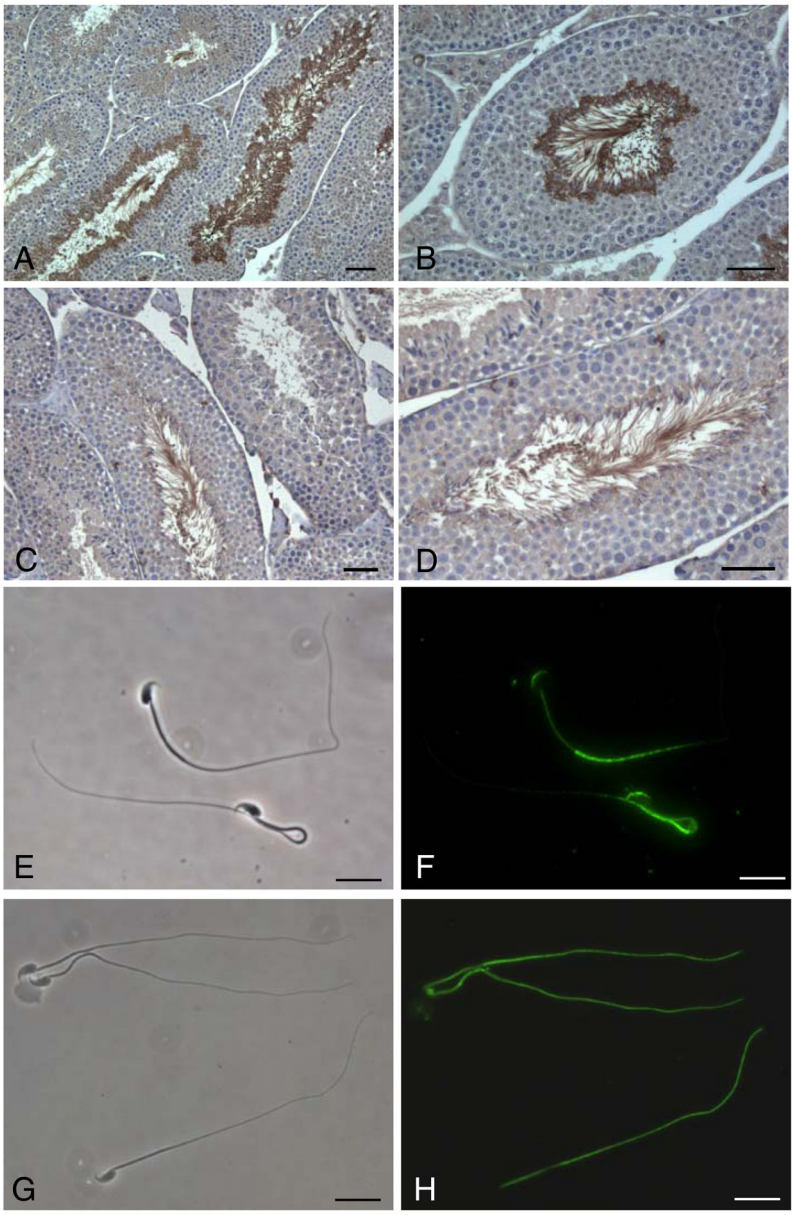

Fig. 6.

Immuno-localization of HK1S in mouse testis and sperm. Sections of adult mouse testis were immunostained with antibodies to HK1 (**A**, **B**) or to the SSR (**C**, **D**). The HK1 antibody reacted strongly with step15–16 spermatids (**A**, **B**) and the SSR antibody also reacted with condensing spermatids (**C**, **D**). (Bars: 50 μm) Epididymal sperm was stained with antibodies to SSR (**E**, **F**) or to HK1 (**G**, **H**). The antibody to SSR stained the principal piece region more intensely than the middle piece, but seldom stained head (**F**). The antibody to HK1 stained the principal piece region and to a lesser degree the middle piece and head (**H**). Phase micrographs (**E**, **G**) show the same sperm that are immuno-stained in the fluorescence micrographs (**F**, **H**). (Bars: 8 μm).