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Expression of the Spermatogenic Cell-Specific Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDS) in Rat Testis

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ABSTRACT The spermatogenic cell-specific variant of glyceraldehyde 3-phosphate dehydrogenase (GAPDS) has been cloned from a rat testis cDNA library and its pattern of expression determined. A 1,417 nucleotide cDNA has been found to encode an enzyme with substantial homology to mouse GAPDS (94% identity) and human GAPD2 (83% identity) isozymes. Northern blotting of rat tissue RNAs detected the 1.5 kb *Gapds* transcript in the testis and not in RNA from liver, spleen, epididymis, heart, skeletal muscle, brain, seminal vesicle, and kidney. The rat *Gapds* mRNA was first detected at day 29 of postnatal testis development, an age which coincides with the initial post-meiotic differentiation of round spermatids. When isolated rat spermatogenic cell RNA was probed for *Gapds* expression, transcripts were detected only in round spermatids and condensing spermatids, but not in pachytene spermatocytes, demonstrating haploid expression of the *Gapds* gene. However, immunohistochemical staining of rat testis sections with anti-GAPDS antisera did not detect GAPDS in round spermatids, but localized the protein only to stage XIII and later condensing spermatids as well as testicular spermatozoa, indicating that *Gapds* expression is translationally regulated. The current results are similar to those previously obtained for mouse GAPDS and human GAPD2, suggesting that reliable comparisons can be made between these species in toxicant screening and contraceptive development. *Mol. Reprod. Dev. 73: 1052–1060, 2006.* Published 2006 Wiley-Liss, Inc.†

Key Words: glycolysis; metabolism; motility; gene expression

INTRODUCTION

The spermatogenic process involves multiple stages with the immature germ cells passing through mitotic, meiotic, and postmeiotic phases of differentiation before developing into spermatozoa. During maturation and subsequent acquisition of motility, male germ cells make a switch in energy metabolism, shifting from oxidative phosphorylation to glycolysis (Williams and Ford, 2001). The glycolytic pathway involves the sequential action of ten enzymes, which metabolize glucose into pyruvate and produce four molecules of ATP. Pyruvate is then channeled to the mitochondria for further ATP production via the Kreb's cycle. Interestingly, evidence exists that all the ten glycolytic enzymes are uniquely regulated in the testis and are either products of spermatogenic cell-specific genes, unique splicing events, or spermatogenic cell-specific expression patterns (hexokinase, Mori et al., 1993; phosphoglucose isomerase, Buehr and McLaren, 1981; phosphofructokinase, Manzano et al., 1999; aldolase, Gillis and Tamblyn, 1984; triose phosphate isomerase, Russell and Kim, 1996; glyceraldehyde 3-phosphate dehydrogenase, Welch et al., 1992; phosphogycerate kinase, Vandeberg et al., 1973; phosphoglycerate mutase, Broceno et al., 1995; enolase, Edwards and Grootegoed, 1983; pyruvate dehydegenase Dahl et al., 1990). Because of this observation, it has been proposed that the primary energy source supporting sperm function is supplied by glycolysis and that the germ cell specific isozymes have evolved to accommodate the unique milieu of the reproductive tract. Glycolytic metabolism has been shown to be necessary for sperm motility and in vitro fertilization (Fraser and Quinn, 1981; Cooper, 1984; Hoshi et al., 1991; Urner and Sakkas, 1996; Williams and Ford, 2001). In contrast, inhibition of oxidative

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phosphorylation (Ford and Harrison, 1981) or a targeted disruption of the haploid expressed testis-specific cytochrome c gene (Narisawa et al., 2002) do not block fertilization. The glyceraldehyde 3-phosphate dehydrogenase enzyme converts glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate and occupies a pivotal location in the glycolysis, separating the metabolic steps utilizing ATP from the ATP generating reactions. We previously identified a spermatogenic cell-specific variant of glyceraldehyde 3-phosphate dehydrogenase (mouse GAPDS, Welch et al., 1992; human GAPD2, Welch et al., 2000) which is expressed in both mouse and human spermatozoa. The GAPDS protein ultimately localizes to the fibrous sheath of the sperm flagellum (Bunch et al., 1998) where the resulting ATP output would be available for immediate use by flagellar dynein. Glycolysis in spermatozoa has been suggested as potential target for contraceptive action and can be affected by exposure to several halogenated compounds which block GAPD activity, subsequently altering sperm motility (Jones and Cooper, 1999; Bone and Cooper, 2000; Bone et al., 2001). There also exists the possibility that some cases of infertility may result from a toxicant mediated disruption of the sperm glycolytic pathway. Since the rat is a widely used animal model in toxicology, it was necessary to first characterize the rat GAPDS enzyme in order to examine a possible link between sperm glycolysis and toxicant or contraceptive induced male infertility.

MATERIALS AND METHODS

Tissue and Cell Preparations

Protocols for animal experimentation were reviewed and approved by the US EPA Animal Care and Use Committee. Sprague–Dawley rats were obtained from Charles River (Raleigh, NC). Animals were euthanized by carbon dioxide inhalation followed by cervical dislocation. Tissues for RNA isolation were immediately frozen in liquid nitrogen, and stored at −75°C until used. Mixed spermatogenic cells, round spermatids, and condensing spermatids plus residual cytoplasts were obtained from Sprague–Dawley rats by sequential enzymatic dissociation and unit gravity sedimentation using methods described previously [Romrell et al., 1976; Bellvé et al., 1977, O'Brien, 1993]. Pachytene spermatocytes were similarly isolated by unit gravity sedimentation using testes from 22-day-old rats.

Isolation and Characterization of Rat *Gapds* cDNA

E. coli strains and DNA vectors were maintained by standard procedures (Sambrook et al., 1989). Rat *Gapds* cDNA clones were isolated from a Lambda Zap rat testis cDNA library (Stratagene, La Jolla, CA) by hybridization screening (Benton and Davis, 1977) with a full length mouse *Gapds* probe (Welch et al., 1992). Nitrocellulose filters were washed at high stringency in a 65°C solution of 0.1× SSC containing 0.1% SDS. Positive bacteriophage plaques were isolated by dilution cloning. The pBluescript phagemids containing the rat *Gapds* sequences were excised in vivo using the Rapid Excision system (Stratagene). Phagemid DNA for sequencing was isolated using the Wizard DNA plasmid purification system (Promega, Madison, WI). Insert sequences were determined using a PCR-based dideoxynucleotide cycle sequencing method (Sanger et al., 1977) and deoxyadenosine 5′-[α-^{35}S] thio-triphosphate label incorporation (SequiTherm Excel II, Epicentre Technologies, Madison, WI). The sequence information was assembled and analyzed using the Omiga nucleic acid analysis program (Accelrys, San Diego, CA).

Northern Blotting

For RNA isolation, tissues were first pulverized to a fine powder under liquid nitrogen using a mortar and pestle. Excess liquid nitrogen was allowed to evaporate and the powdered tissue was homogenized in Trizol reagent (Life Technologies, Grand Island, NY) and total RNA isolated according to the manufacturer's instructions. RNA concentration and purity were assessed by monitoring the A260/A280 ratio for a value of 1.5 or greater and RNA integrity was verified by observation of the 18S and 28S ribosomal bands after electrophoresis through nondenaturing agarose gels followed by ethidium bromide staining. Northern blotting of RNA has been described previously (Welch et al., 1992). Briefly, RNA was separated on 1.25% agarose gels using 1% formaldehyde as a denaturant and transferred by capillary action to Magnagraph nylon membrane (DuPont, Wilmington, DE). Loading equivalence was verified by UV shadowing of lanes to visualize the RNA samples. RNA integrity was assessed by visualization of the 18S (1.6 kb) and 28S (4.7 kb) bands and by hybridization with a β-actin (1.4 kb) control probe. The 18S ribosomal, 28S ribosomal, and β-actin mRNA bands were also used as molecular weight markers. DNA probes were labeled with [α-^{32}P]-deoxycytidine triphosphate by random priming using the Prime-It II system (Stratagene), and separated from unincorporated nucleotides on prepacked Sephadex G-50 columns (Amersham, Arlington Heights, IL). After hybridization, blots were rinsed, and subjected to stringent washing at 65°C in a solution of 0.1× SSC, 0.1% SDS. XAR-5 X-ray film (Eastman Kodak, Rochester, NY) and Cronex Lightning-plus screens (DuPont) were used for autoradiography at −70°C.

Immunocytochemistry

The preparation of GAPDS antisera in rabbits has been described previously (Bunch et al., 1998). Antiserum B1 was developed using a 14 amino acid synthetic peptide, which corresponds to a region of the mouse GAPDS protein that is perfectly conserved in the rat protein. Testes were collected from adult rats, fixed in Bouin's solution, dehydrated in ethanol, embedded in paraffin, and sectioned at 8 μm. Sections were incubated with GAPDS antiserum A1 or GAPD antiserum B1 (either blocked or unblocked with antigenic peptide (1 μg/ml)), followed by biotinylated goat anti-rabbit IgG and horseradish peroxidase conjugated to avidin

according to manufacturer's instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Immunocomplexes were detected using 3,3′-diaminobenzidine tetrahydrochloride (Aldrich, Milwaukee, WI) as a chromogen. Slides were counter stained with Gill's hematoxylin (Fisher Scientific, Atlanta, GA). Photographs were taken using Ektochrome 160T film (Eastman Kodak).

Immunofluorescence

Antiserum B1 raised against mouse GAPDS was used to localize the GAPDS protein in rat spermatozoa. Washed cauda epidiymal sperm were fixed for 10–15 min by dilution into an equal volume of PBS containing 3.7% formaldehyde (Mallinckrodt Chemical, Paris, KY). Free aldehyde groups were then blocked with 50 mM glycine in PBS. Sperm were permeablized with methanol for 1 min at −20°C. After nonspecific reactivity was blocked by incubation in PBS containing 10% goat serum (Life Technologies) for 15 min at room temperature, sperm were incubated with primary antibody for 2 hr at 37°C. Primary antibody was diluted 1:8,000 in PBS and was blocked by incubation with the antigenic peptide (100 μg/ml) for 1–16 hr at 4°C as a negative control. After washing in PBS, sperm were incubated for 1 hr at 37°C with fluorescein isothiocyanate-labeled, affinity purified goat anti-rabbit IgG (Cappel/Organon Teknika Corporation, West Chester, PA) diluted 1:100. Paired phase-contrast and fluorescence photomicrographs were taken with Ektachrome P800/1600 color reversal film (Eastman Kodak) at ASA 800.

RESULTS

Nucleotide and Predicted Amino Acid Sequences of the Rat *Gapds* cDNA

The rat *Gapds* cDNA sequence was determined to consist of 1,417 nucleotides and encompassed the entire *Gapds* coding region (Fig. 1). The coding sequence contained an ATG initiation codon and TAA stop codon at locations consistent with the mouse *Gapds* transcript, with a canonical poly-adenylation signal (AATAAA) being found in the 3′-UTR. Mouse GAPDS (Welch et al., 1992) and human GAPD2 (Welch et al., 2000) enzymes both possess a unique extension of the amino terminus, a characteristic feature of this isozyme that is not contained in somatic GAPD. The predicted amino acid sequence in the rat also possesses this amino terminal extension and the three conserved regions characteristic of this sequence (Fig. 2). The initial 19 amino acids of all three enzymes are highly conserved with a 89% identity between rat and mouse GAPDS, and a 74% identity between the rat and human enzymes. This region is followed immediately by an alternating cysteine and proline region consisting of 15 residues in the rat (aa 20–34). Mouse GAPDS contains a region of similar size (12 residues) while in the human GAPDH2, the sequence is limited to three amino acids. A second proline-rich region is present in all three species and spans 59 residues in the rat (aa 48–96; 73% proline), 67 residues in the mouse (69% proline), and 42 residues in the human (50% proline). A comparison of the catalytic region of the three GAPDS sequences and the rat somatic GAPDH (Fort et al., 1985) shows 94% (rat/mouse) and 83% (rat/human) identities between GAPDS and GAPDH2. By contrast, a comparison of the catalytic region of rat GAPDS with rat somatic GAPDH revealed only a 68% identity. Despite this divergence, the residues comprising the NAD+ and substrate binding sites are highly conserved with substitution of a tyrosine for phenylalanine at residue 197 of rat GAPDS. This replacement also occurs in both the mouse and human enzymes.

The *Gapds* Gene Is Expressed Only in Haploid Spermatogenic Cells of the Rat Testis

When a probe derived from the rat *Gapds* cDNA was hybridized to Northern blots containing total RNA from eight different rat tissues, an abundant 1.5-kb mRNA was found only in the testis (Fig. 3). *Gapds* mRNA could not be detected in rat somatic tissues or in the rat ovary despite extended exposure times. Faint bands were occasionally seen in heart and skeletal muscle at 1.3-kb, due to minor cross-hybridization with the abundant 1.3-kb somatic *Gapdh* mRNA present in these tissues (Welch et al., 1992). During postnatal testis development 1.5-kb *Gapds* mRNA was first detected in day 29 rat testis, an age coinciding with the initial appearance of round spermatids during the first wave of spermatogenesis (Fig. 4). The amount of *Gapds* mRNA increased until day 50 and then remained constant into maturity. Hybridization of isolated spermatogenic cell RNA with a rat *Gapds* probe detected *Gapds* mRNA in mixed spermatogenic cells, round spermatids, and condensing spermatids, but not pachytene spermatocytes (Fig. 5). These results were consistent with the initial appearance of the *Gapds* transcript in day 29 postnatal testis and demonstrate expression of *Gapds* mRNA only in haploid germ cells.

GAPDS Protein Is First Expressed in Condensing Spermatids

Immunochemical staining of rat testis sections with an antiserum directed against a mouse GAPDS peptide localized the enzyme to the germ cells of the adluminal compartment of the seminiferous tubules (Fig. 6A). The staining was specific and could be completely abolished by pre-incubation of the antiserum with the peptide immunogen (Fig. 6B). Higher resolution imaging showed that while staining was absent from stage IX round spermatids (Fig. 6C), a faint signal appeared in stage XIII condensing spermatids (Fig. 6D). The staining intensity increased through stage V (Fig. 6E) and stage VI tubules (Fig. 6F), but was always localized over the developing flagella and cytoplasm of the condensing spermatids. The staining began to decrease in stage VII (Fig. 6G), coincident with the decrease in cytoplasmic volume of the condensing spermatids. This decrease continued into stage VIII (Fig. 6H), although intense staining of the flagella was still observed in spermatids just prior to spermiation. The earlier appearance of the

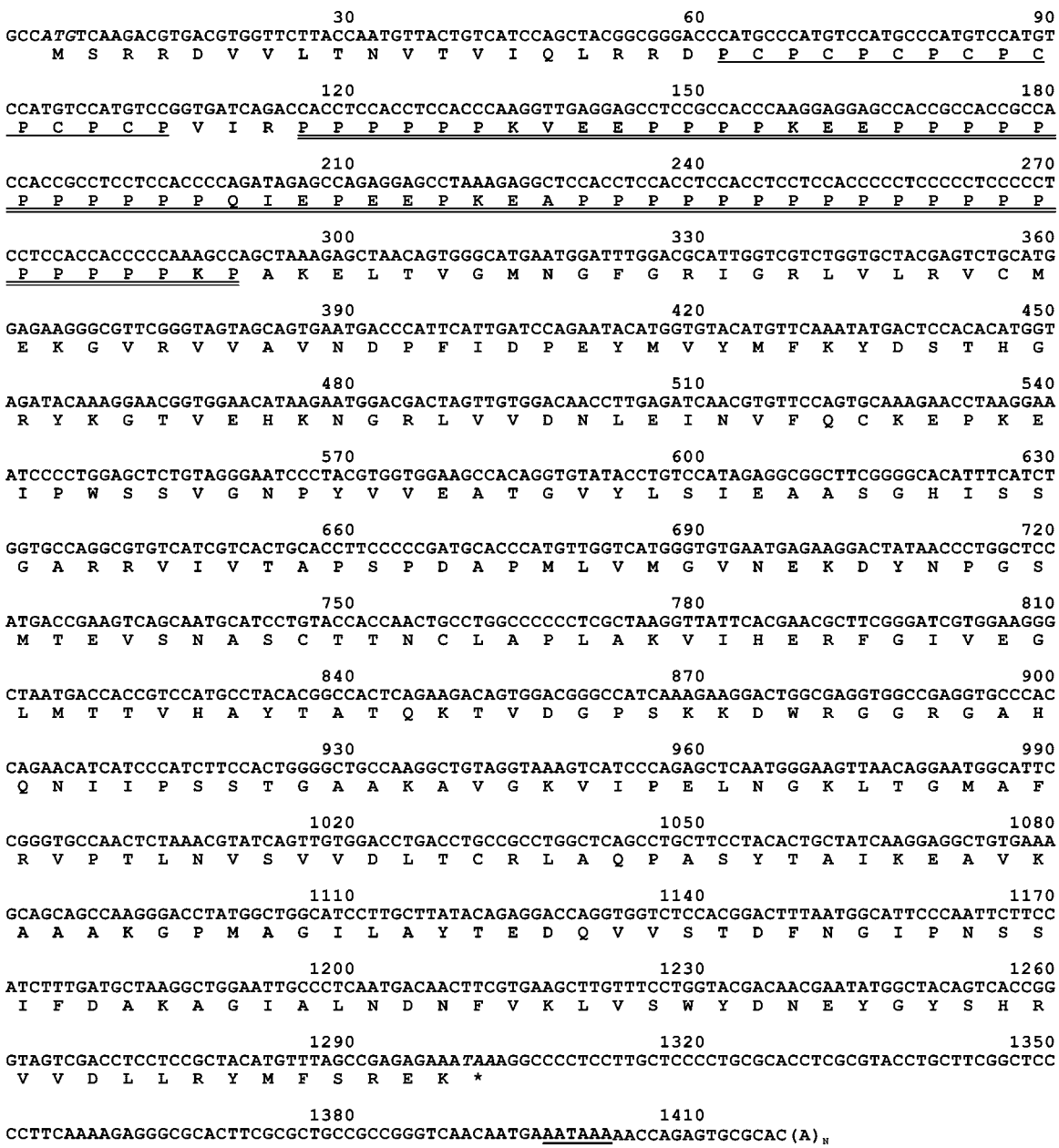

Fig. 1. Nucleotide and predicted amino acid sequence of rat *Gapds*. The rat *Gapds* cDNA consists of 1,417 bp including the poly-adenylation signal (nt 1,397–1,402) and contains the complete coding sequence (nt 4–1,299). Two cytidine-rich areas are present and encode alternating cysteine-proline (underlined, nt 60–104) and proline-rich (double underlined, nt 115–290) sequences. The open reading frame was terminated by a TAA stop codon (asterisk) and encodes a 432 amino acid GAPDS protein.

Gapds transcript in round spermatids, when compared to the synthesis of GAPDS protein in condensing spermatids, demonstrates translational regulation of the rat *Gapds* transcript similar to that seen with the mouse GAPDS enzyme (Bunch et al., 1998).

GAPDS Is Localized to the Principal Piece of the Spermatozoan Tail

Staining of fixed, permeablized rat spermatozoa localized GAPDS to the principal piece of the flagellum (Fig. 7A,B). Only faint autofluorescence was seen over the midpiece and mitochondria in controls where the primary antisera was preincubated with the immunogenic peptide (Fig. 7C,D). The staining pattern is consistent with the association between rat GAPDS and the fibrous sheath of the sperm tail, as has been previously demonstrated in mouse spermatozoa (Bunch et al., 1998) and similar to glycolytic enzymes enolase (Gitlits et al., 2000) and hexokinase (Mori et al., 1998) which have also been localized to the sperm tail.

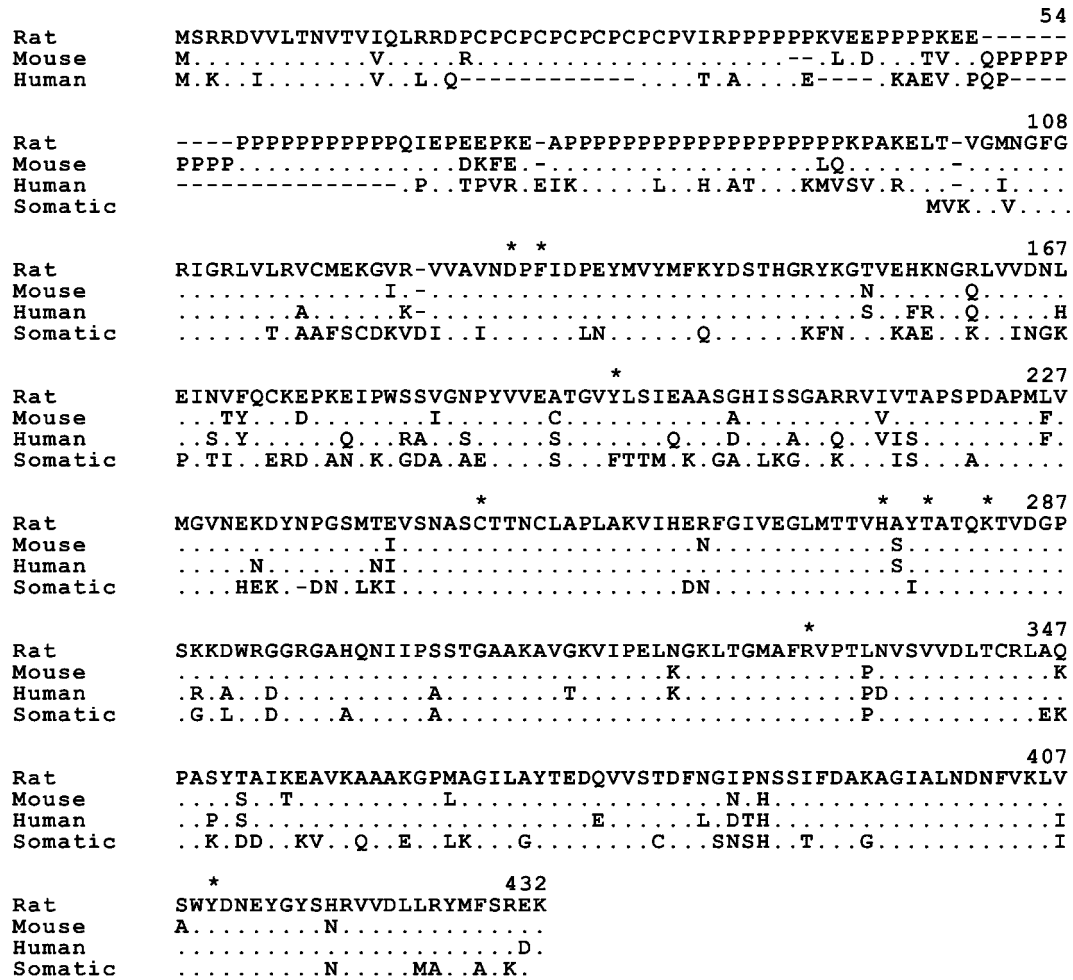

Fig. 2. Rat GAPDS homology with mouse GAPDS, human GAPDH2, and rat somatic GAPDH enzymes. Alignments of the rat sequence indicated 94% (mouse GAPDS), 83% (human GAPDH2), and 68% (rat somatic GAPDH) identities across the catalytic region (aa 100–432) with eight of nine catalytic residues (asterisks) being completely conserved (Asp 130, Phe 132, Tyr 197, Cys 248, His 275, Thr 278, Lys 282, Arg 330, Tyr 410). The single exception was the substitution of Phe for Try 197 in the somatic enzyme. While the amino terminal leader sequences are of variable length, substantial homology is also present in this region. Sequence identities are indicated by dots and while sequence gaps are designated by dashes.

DISCUSSION

The rat enzyme is the third spermatogenic cell-specific member of the *GAPDH* gene family to be characterized and these results suggest that extrapolations of GAPDS function in the rat can be made to other species, including human. Rat GAPDS has been shown to be very similar to the mouse (94% identity) and human (83% identity) amino acid sequences, with a high degree of identity seen in the proline-rich amino terminal sequence unique to this enzyme. It has also been demonstrated that expression of the rat enzyme is under a translational regulation similar to the mouse GAPDS, since the rat transcript is first found in round spermatids, but translation is detected only in condensing spermatids. The high degree of conservation among these spermatogenic cell-specific glycolytic enzymes suggests again that they possess unique properties related to their involvement in germ cell metabolism. The enzymatic properties of GAPDS are currently being investigated, but differences in substrate inhibition and activity are expected similar to adaptations reported for other spermatogenic cell-specific enzymes (lactate dehydrogenase C4; Blanco, 1980). GAPDS has already been shown to have a specific localization within the spermatozoon that contrasts sharply with the cytoplasmic location of the somatic GAPD protein and has been shown to be tightly bound to the fibrous sheath component of the principal piece of the sperm tail (Westhoff and Kamp, 1997; Bunch et al., 1998). The localization of additional metabolic enzymes such as hexokinase (Mori et al., 1998) and enolase (Gitlits et al., 2000) to the sperm tail, suggests that these energy producing components may be arranged in this fashion to directly supply ATP to the flagellar apparatus and support sperm motility.

GAPDH occupies a pivotal place in the glycolytic enzyme sequence. The enzymatic steps prior to GAPDH action utilize two molecules of ATP and is a net energy

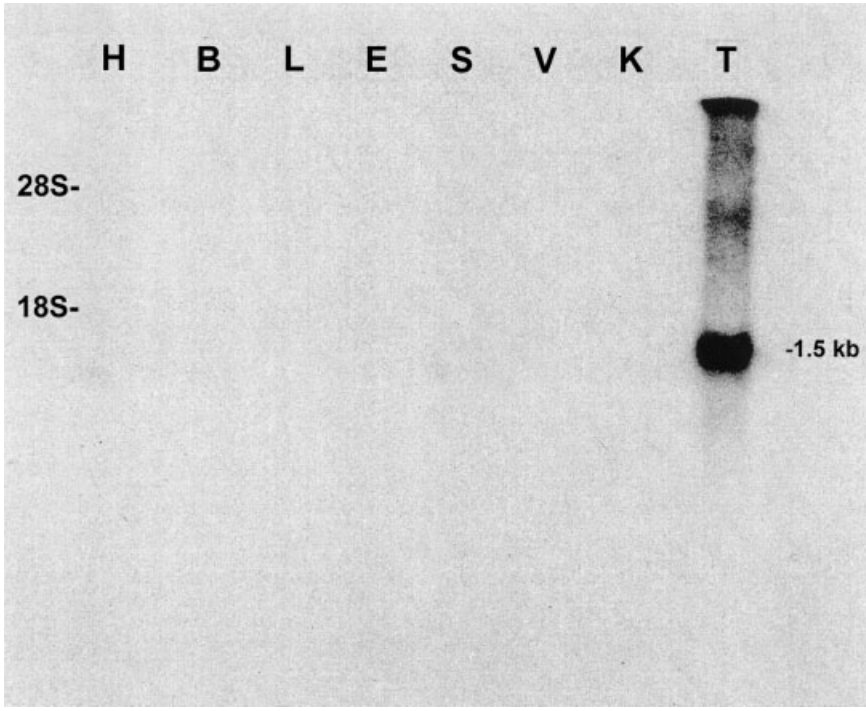

Fig. 3. The rat *Gapds* gene is expressed in the rat testis. Northern blot analysis of rat tissue RNAs detected the presence of a 1.5 kb *Gapds* mRNA only in the testis (T). No 1.5 kb transcript was seen in RNA from heart (H), brain (B), liver (L), epididymis (E), skeletal muscle (S) seminal vesicle (V), or kidney (K). Ten micrograms of total RNA was loaded per lane. The blot was washed in 0.1× SSC, 0.1% SDS at 65°C, and exposed for 48 hr. Equivalent loading and transfer was verified by UV shadowing of the membrane and RNA integrity was checked by hybridization with a β-actin control probe (not shown).

consuming process. Enzymatic actions subsequent to GAPDH action result in a net energy gain of two molecules of ATP. Therefore, inhibition of glycolysis at the level of GAPDH will not only block energy production, but actually converts glycolysis into an energy utilizing reaction and may impact ATP produced by other sources. Substantial effort has gone into identifying nonsteroidal male contraceptives. This research has identified a number of halogenated chemicals, which have been proposed to act by inhibiting sperm energy metabolism, especially glycolysis. One compound, S(+)alpha-chlorohydrin (ACH; Jones, 1978), an experimental contraceptive first described in 1970, has been extensively studied. ACH has been show to be converted to an active metabolite (S)-3-chlorolactaldehye (CLA) by the action of NADH+ dependant glycerol dehydrogenase (Jones and Stevenson, 1983; Stevenson and Jones, 1985) This metabolite is very similar structurally to glyceraldehyde 3-phosphate, the natural substrate of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and is believed to act as a competitive inhibitor of the GAPDH enzyme. The anti-contraceptive effect varies among species, possibly due to differences in the speed of ACH metabolism and excretion (Bone et al., 2001).

Other chlorinated compounds have been shown to affect fertility and may also act by blocking glycolysis. An ACH analog, the industrial solvent, epichlorohydrin (ECH), is also metabolized to CLA and has been shown to reduce male fertility by decreasing sperm motility (Toth et al., 1989; Slott et al., 1990, 1997). A number of additional reproductive toxicants have been proposed to act by inhibiting glycolytic activity in spermatozoa. Ornidazole, an anti-fungal compound (Bone et al., 2000), 1-chloro-3-hydroxypropanone (CHOP), 1,6-dichloro-1, 6-dideoxy-D-fructose (DCDF), 6-chloro-6-deoxyglucose (Bone et al., 2001) have all been proposed to act through the 3-chlorolactaldehyde metabolite and presumably by blocking glycolytic energy production (Bone and Cooper, 2000). This is further supported by the lack of any reproductive effect from metranidazole, an ornidazol analog, which is not metabolized to CLA (Bone et al., 1997; Cooper et al., 1997).

These compounds have been shown to inhibit sperm motility and have been tested earlier as potential male contraceptive agents acting by inhibition of the glycolytic pathway, although these studies were largely discontinued after adverse neurological effects were noted in primates (Jones and Cooper, 1999). Adverse epididymal effects have also been noted and Jelks and Miller (2001) demonstrated that ACH inhibits GAPDH activity in organs other than the testis. The contraceptive potential of these compounds has been re-examined in recent years, in an attempt to identify analogs that can circumvent the adverse effects noted earlier. To date these studies have not been able to substantially improve the contraceptive potential of the test compounds, although these agents still rely upon conversion to the active CLA

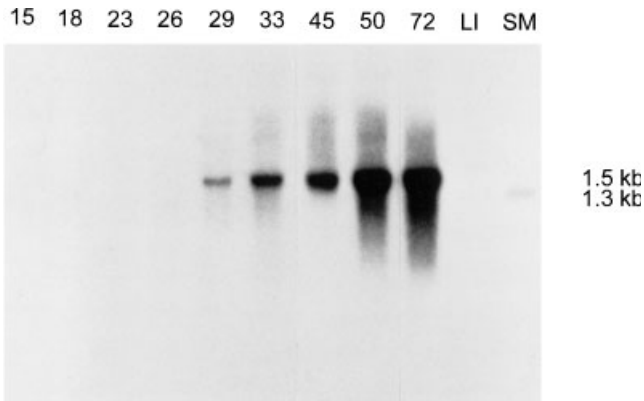

Fig. 4. Developmental expression of *Gapds* in rat testis. The 1.5 kb *Gapds* mRNA was first detected in postnatal day 29 rat testis, coinciding with the initial differentiation of round spermatids. The amount of signal increased until day 50 and subsequently remained stable. No 1.5 kb signal was seen in rat liver or skeletal muscle controls. Days of age are given over each testis lane with liver (LI) and skeletal muscle (SM) on the right. Ten micrograms of total RNA was loaded per lane. The blot was washed in 0.1× SSC, 0.1% SDS at 65°C, and exposed for 48 hr.

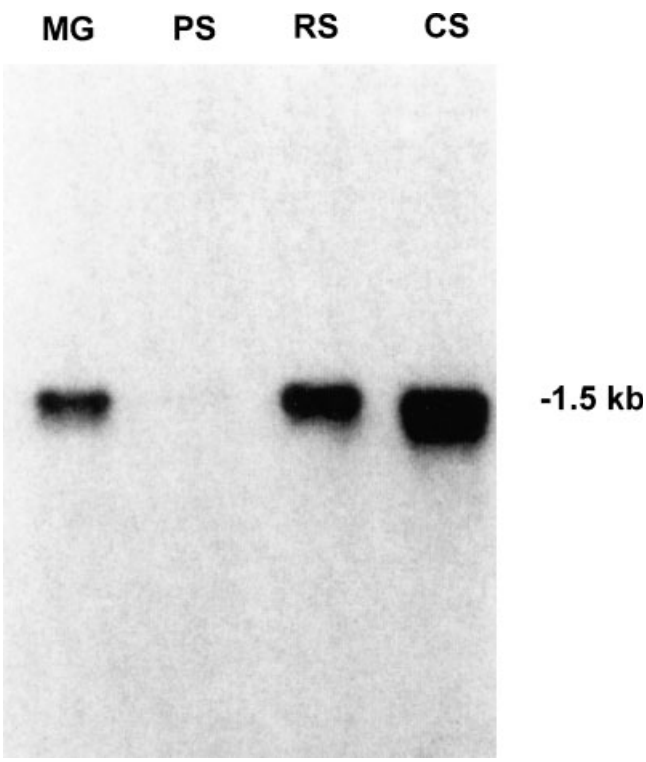

Fig. 5. The rat *Gapds* gene is expressed in haploid spermatogenic cells. Hybridization of spermatogenic cell RNA with the rat *Gapds* probe detected *Gapds* mRNA in mixed spermatogenic cells (MG), round spermatids (RS), and condensing spermatids (CS), but not in pachytene spermatocytes (PS). The results demonstrate that *Gapds* transcripts are expressed only in haploid germ cells. Ten micrograms of total RNA was loaded per lane. The blot was washed in 0.1× SSC, 0.1% SDS at 65°C, and exposed for 48 hr.

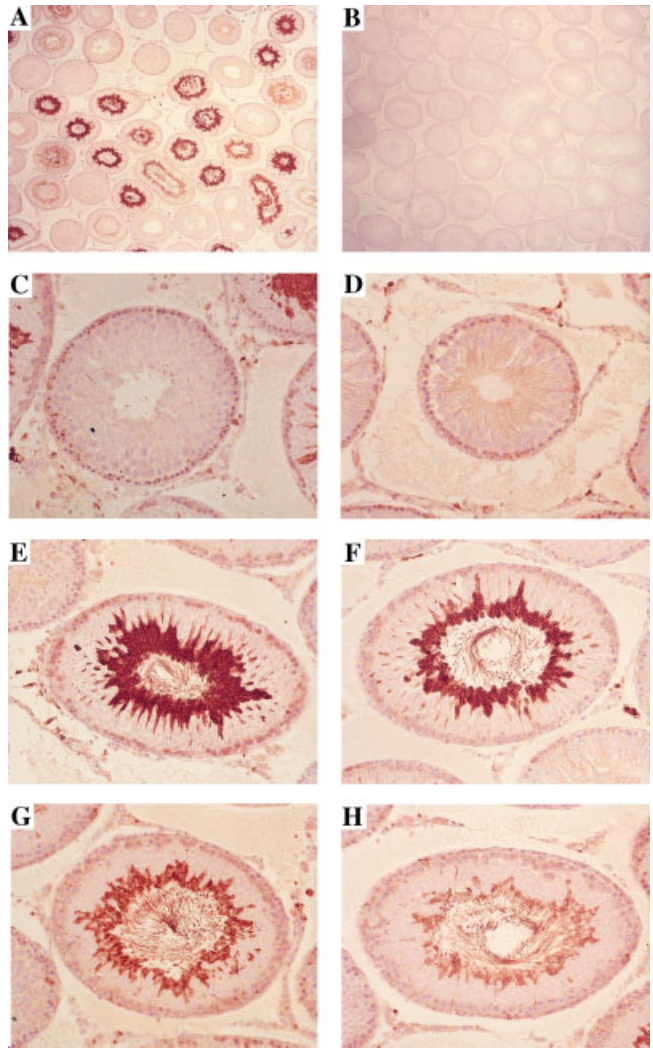

Fig. 6. Rat GAPDS protein is first expressed in condensing spermatids. Rat testis sections were stained with an antiserum (1:8,000 dilution) directed against a mouse GAPDS peptide localized the enzyme to germ cells in the adluminal compartment of seminiferous tubules (**A**). The staining was specific and could be completely abolished by preincubation of the antiserum with the peptide immunogen (1 μg/ml, **B**) although nonspecific staining of spermatocyte nuclei was still present. Increased magnification showed that while staining was absent from round spermatids (stage IX, **C**), a faint signal appeared in stage XIII condensing spermatids (**D**). The staining intensity increased through stage V (**E**) and stage VI (**F**) tubules, but was always localized over the developing flagella and cytoplasm of the condensing spermatids. The staining began to decrease in stage VII (**G**) coincident with the decrease in cytoplasmic volume of the condensing spermatids. The staining continued to diminish into stage VIII (**H**), although an intense staining of the flagella was still evident in sperm just prior to spermiation.

metabolite by glycerol kinase and do not address the presence of the sperm-specific GAPDS enzyme. It seems likely that successful development of this contraceptive approach will involve the synthesis of CLA analogs whose activity can be specifically targeted to unique catalytic properties of sperm-specific glycolytic enzymes

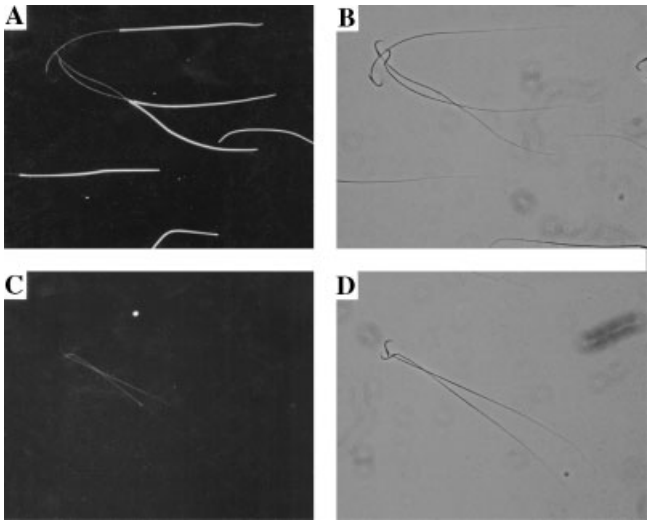

Fig. 7. GAPDS is located in the principal piece of the sperm tail. Staining of fixed permeablized rat spermatozoa localized GAPDS to the principal piece of the flagellum (**A**, **B**). Only a faint autofluorescence was seen in controls in which the primary antisera was blocked with the immunogenic peptide (**C**, **D**).

(Jones and Cooper, 1999) including GAPDS. However, for a single compound to be effective across multiple species, the differences in metabolic inactivation and clearance seen with ACH (Bone et al., 2002) will also need to be overcome.

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