Journal of Andrology, Vol. 21, No. 2, March/April 2000

Human Glyceraldehyde 3-Phosphate Dehydrogenase-2 Gene Is Expressed Specifically in Spermatogenic Cells

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ABSTRACT: Although the process of glycolysis is highly conserved in eukaryotes, several glycolytic enzymes have unique structural or functional features in spermatogenic cells. We previously identified and characterized the mouse complementary DNA (cDNA) and a gene for 1 of these enzymes, glyceraldehyde 3-phosphate dehydrogenase-s *(Gapds)*. This gene is expressed only in spermatids. The enzyme appears to have an essential role in energy production required for fertilization, and it is reported to be susceptible to inhibition by certain environmental chemicals. We have now cloned and sequenced the cDNA for the human homologue of glyceraldehyde 3-phosphate dehydrogenase *(GAPD2)* and determined the structure of the gene. The messenger RNA (mRNA) was detected in testis, but not in 15 other human tissues analyzed by Northern blot technique. The deduced GAPD2 protein contains 408 amino acids and is 68% identical with somatic cell GAPD. GAPD2 has a 72-amino acid segment at the amino terminal end that is not present in somatic cell GAPD. This segment is proline-rich but contains smaller stretches of polyproline and is 30 amino acids shorter than the comparable segment of mouse GAPDS. The structure of the human *GAPD2* gene was determined by polymerase chain reaction (PCR) to identify exon-intron junctions in a genomic clone and in total genomic DNA. The locations of these junctions in the *GAPD2* gene corresponded precisely to those of the 11 exon-intron junctions in the mouse *Gapds* gene. Immunohistochemical studies found that GAPD2 is located in the principal piece of the flagellum of human spermatozoa, as are GAPDS in mouse and rat spermatozoa. GAPD2 extracted from human spermatozoa and analyzed by Western blot technique migrated with an apparent molecular weight of ~56 000, although the calculated molecular weight is 44 501. The conserved nature of the mouse, rat, and human enzymes suggests that they serve similar roles in these and other mammalian species.

Key words: Glycolysis, testis, spermatogenesis, flagellum.
J Androl 2000;21:328–338

The major features of human spermatozoa are well described, but relatively little is known about the proteins responsible for their unique structures and functions. Many genes that are transcribed only in spermatogenic cells have been identified in other mammals, are regulated developmentally, and encode proteins essential for the production or function of the spermatozoon (Eddy et al, 1991, 1993). The high degree of conservation of genes during evolution allows much of the knowledge gained from molecular genetic studies on other mammals to be extrapolated to human beings. However, the process of spermatogenesis varies between species, and there are structural diversities between spermatozoa from different mammals. One approach to understanding this variability is to define the similarities and differences between genes and proteins involved in the development and function of spermatozoa of human beings and other mammals.

Glycolysis is a fundamental and highly conserved component of the energy production process in eukaryotes. Nevertheless, as many as 7 of the 10 enzymes in the glycolytic pathway may have spermatogenic cell–specific isozymes (Eddy et al, 1994). Two of these, phosphoglycerate kinase-2 (PGK2; Boer et al, 1987; McCarrey and Thomas, 1987) and glyceraldehyde 3-phosphate dehydrogenase-s (GAPDS; Welch et al, 1992), are products of genes expressed only in spermatogenic cells, but they represent highly conserved homologues of constitutively expressed genes. Unique transcripts for 3 other glycolytic enzymes also have been identified in spermatogenic cells.

Supported in part by the Andrew W. Mellon Foundation and NIH Grants U54HD35041 (UNC Laboratories for Reproductive Biology) and CA16086 (UNC Lineberger Comprehensive Cancer Center).

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Received for publication August 25, 1999; accepted for publication September 21, 1999.

The type 1 hexokinase (HK1) enzyme of mouse and human spermatogenic cells differs from the somatic cell enzyme in the amino terminal–coding region as a result of alternative exon usage (Mori et al, 1993, 1996, 1998). In addition, the messenger RNA (mRNA) for triosephosphate isomerase (TPI) are larger in germ cells than in somatic cells because of the utilization of an alternative polyadenylation signal (Russell and Kim, 1996), and mRNA are larger for phosphoglycerate mutase-2 (PGAM-2) because of an extended poly(A) tail (Broceno et al, 1995). Furthermore, other genes expressed only in spermatogenic cells are homologues of constitutively expressed genes encoding enzymes involved in energy production. These include genes for lactate dehydrogenase-C (LDHC; Millan et al, 1987, Sakai et al, 1987), cytochrome c_T (CYT; Virbasius and Scarpulla, 1988), the pyruvate dehydrogenase E1α subunit (PDHA2; Dahl et al, 1990), and glucose 6-phosphate dehydrogenase (G6PD; Hendricksen et al, 1997). Most of these studies have been done in rodents, but the human *PGK2* (McCarrey and Thomas, 1987), *LDH3* (Cooker et al, 1993), and *PDHA2* (Dahl et al, 1990) genes have been cloned.

Glyceraldehyde 3-phosphate dehydrogenase (GAPD, EC1.2.1.12) is an NAD-dependent enzyme that removes hydrogen and adds phosphate to glyceraldehyde 3-phosphate to form 1,3-diphosphoglycerate. The enzyme has been suggested to be a key regulator of glycolysis in spermatogenic cells and the target of environmental compounds that disrupt male fertility (Mohri et al, 1975; Jones, 1978; Stevenson et al, 1985). The mouse *Gapds* gene is expressed only in spermatids, with transcripts first appearing in cap-phase round spermatids (steps 4–7) and reaching maximum levels in condensing spermatids (steps 9-11; Mori et al, 1992; Welch et al, 1992). The mouse GAPDS protein is not detected in spermatids by immunohistochemistry until later (steps 12–13), suggesting that regulation of translation occurs (Bunch et al, 1998). Although the predicted molecular weight of GAPDS protein is 47 445, it was found by Western blot analysis to migrate at ~69 200. The difference between the predicted and observed molecular weights is likely caused by the 105–amino acid proline-rich domain at the N-terminus (Bunch et al, 1998). The protein is restricted to the principal piece of the flagellum and is tightly associated with the fibrous sheath (Fenderson et al, 1988; Bunch et al, 1998). The fibrous sheath is an unusual cytoskeletal structure that is believed to serve an essential role in stiffening the flagellum and modulating its bending (Eddy and O'Brien, 1994). The association of GAPDS with the fibrous sheath is consistent with it having a role in regulating sperm motility.

This study reports the isolation and characterization of the human *GAPD2* cDNA gene. The name of this gene was chosen according to the 1997 Guidelines for Human Gene Nomenclature (www.gene.ucl.ac.uk/nomenclature/guidelines). The deduced GAPD2 protein has many features in common with mouse GAPDS, including testis-specific expression, localization to the principal piece of the flagellum, and a proline-rich domain at the N-terminus that is not present in the GAPD of somatic cells.

Materials and Methods

Isolation and Characterization of Human GAPD2 cDNA

A λgt11 human testis cDNA library (Clontech, Palo Alto, Calif) was screened by hybridization (Benton and Davis, 1977) with a probe consisting of the full-length mouse *Gapds* cDNA (Welch et al, 1992) to obtain human *GAPD2* cDNA clones. Nitrocellulose filters were processed at high stringency with wash conditions of 65°C in 0.1× sodium chloride/sodium citrate (SSC) buffer containing 0.1% sodium dodecyl sulfate (SDS). Positive plaques were isolated by dilution cloning, and λgt11 DNA containing *GAPD2* cDNA inserts was purified by the method of Kaslow (1986). Human *GAPD2* cDNA was excised by *Eco*RI digestion, and the insert cDNA subcloned into M13 bacteriophage for dideoxynucleotide sequencing (Sanger et al, 1977). Sequences were assembled and analyzed by GCG (Devereux et al, 1984) and Microgenie (Beckman, Palo Alto, Calif) programs.

Northern Blot Analysis

Human tissue Northern blots (Clontech) were hybridized as described (Wahl et al, 1979) with a full-length human *GAPD2* cDNA probe. The blots were washed at high stringency (65°C in 0.1× SSC and 0.1% SDS), and bands were imaged by autoradiography at −70°C with XAR-5 x-ray film (Eastman Kodak, Rochester, NY) and Cronex Lightning-Plus screens (NEN Life Science Products, Boston, Mass). A mouse somatic *Gapd* cDNA clone (Sabath et al, 1990) was used as a control probe.

Isolation and Exon Mapping of the Human GAPD2 Gene

A human *GAPD2* genomic clone was isolated from a human male genomic DNA library (Strategene, La Jolla, Calif) by hybridization screening with the human *GAPD2* cDNA (Welch et al, 1995). The exon structure of the human *GAPD2* gene was predicted from splice junctions previously identified from the human somatic *GAPD* (Ercolani et al, 1988), chicken somatic *Gapd* (Stone et al, 1985), and mouse *Gapds* (Welch et al, 1995) gene sequences. Primers were designed to flank the predicted exon-intron splice sites (Table). Polymerase chain reaction (PCR) conditions were optimized by the PCR Optimizer Kit (Invitrogen, Carlsbad, Calif). After PCR amplifications with the genomic clone as template, products were separated by agarose gel electrophoresis, and excised bands were purified with a gel extraction kit according to the supplier's recommendations (Qiagen, Valencia, Calif). The amplified products were then sequenced from both directions to identify the splice junctions and intron sequences. Direct PCR amplification of human genomic DNA was used to verify the integrity of the human *GAPD2*

Oligonucleotide primer sequences (5′ to 3′ orientation)

Intron A (upper)	AATGTCACCGTTGTCCAGTT
Intron A (lower)	GCCACAGACACCATCTTAGG
Intron B (upper)	TGCCTCCTCACCCCGCTACT
Intron B (lower)	ATCCAGGCGCGCAGGACCAG
Intron C (upper)	CTGCATGGAGAAGGGTGTTA
Intron C (lower)	ATCGGCCGTGGGTGGAGTCA
Intron D (upper)	GTCGTGGACAACCATGAGAT
Intron D (lower)	GAAGCTGCCTGTATGGAGAG
Intron E (upper)	CAGCTTCGGACCACATCTCT
Intron E (lower)	GCCAGGGTTATAGTCATTTT
Intron F (upper)	ATGGGTGTCAATGAAAATGA
Intron F (lower)	CGAGGGGAGCCAAACAGTTG
Intron G (upper)	CGTCCTGCACCACCAACTGT
Intron G (lower)	GGCCCGTCCACTGTCTTCTG
Intron H (upper)	GTGCCCACCAGAACATCATC
Intron H (lower)	CCTTGGCTGCTGCTTTTACA
Intron I (upper)	CATCAAGGAGGCTGTAAAAG
Intron I (lower)	TGGGTATCACCGAGGAAGTC
Intron J (upper)	ATACCCACTCGTCCATCTTC
Intron J (lower)	CTTCCCGTTTCACTTGTCTC

genomic clone and to confirm the exon-intron locations (GenBank accession numbers AF216631–AF216641).

Chromosomal Localization of Human GAPD2 *Gene*

A probe consisting of the entire human *GAPD2* genomic clone, including the bacteriophage lambda vector, was labeled with biotin-uridine 5′-triphosphate (UTP) by nick translation and used for fluorescence in situ hybridization (FISH) on metaphase chromosome spreads of human peripheral blood lymphocytes (BIOS, New Haven, Conn). The signal was localized by fluorescence microscopic examination after incubation with fluorescein-conjugated avidin. An E2A control probe (Mellentin et al, 1989) was used to confirm the human chromosome 19 localization.

Immunolocalization of GAPD2 Protein

The preparation and characterization of rabbit antisera used in this study were described previously (Bunch et al, 1998). Antiserum A1 was raised against a 37-amino acid synthetic peptide corresponding to a region of the deduced sequence of mouse GAPDS (Welch et al, 1992), and it binds specifically to GAPDS in mouse spermatids and spermatozoa. Antiserum C1 was raised against a synthetic peptide corresponding to the same region of the deduced sequence of rat GAPD (Forte et al, 1985), and it binds to mouse somatic GAPD, but not to mouse GAPDS. GAPD2 was localized in human spermatozoa by indirect immunofluorescence. Samples of human spermatozoa were provided by the Andrology Laboratory, Department of Obstetrics and Gynecology, School of Medicine, University of North Carolina at Chapel Hill. These samples were obtained from normal, healthy donors and were cryopreserved with 10% glycerol. They were thawed and washed in Dulbecco phosphate-buffered saline solution (PBSS), spread onto slides (Superfrost Plus, Fisher Scientific, Pittsburgh, Pa), air-dried, and fixed for 15 minutes with 3% paraformaldehyde in PBSS. The spermatozoa were permeabilized with 0.5% Triton X-100 and blocked with 2% goat serum (15 minutes) and 5% nonfat dry milk in PBSS (15 minutes) prior to incubation with primary antibody for 2 hours. Following additional washes with PBSS, the cells were incubated for 20 minutes with fluorescein isothiocyanate–labeled, affinity-purified goat anti-rabbit IgG (Cappel/Organon Teknika Corp, West Chester, Pa) diluted 1:100. Paired epifluorescence and phase-contrast photomicrographs were taken with Ektachrome P1600 color reversal film (Eastman Kodak).

Western Blot Analysis of GAPD2 Protein

Protein separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were carried out as described previously (Bunch et al, 1998). Human and mouse sperm samples were lysed with SDS sample buffer (2% SDS, 40 mmol/L dithiothreitol [DTT], 0.0625 mol/L Tris-HCl [pH 6.8], 10% glycerol) and heated to 85°C for 6 minutes. Protein concentrations were determined with the bicinchoninic acid (BCA) microassay (Pierce, Rockford, Ill). Sperm proteins were separated on 10 or 12% polyacrylamide gels by the Laemmli (1970) method for SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, Mass) according to standard methods (Towbin et al, 1979). Molecular weight standards (BenchMark Protein Ladder, Life Technologies, Grand Island, NY or Rainbow Molecular Weight Markers, Amersham Pharmacia Biotech, Piscataway, NJ) were included on each gel. Duplicate blots were blocked with 5% nonfat dry milk and incubated for 1 hour with antiserum A1 (diluted 1:3000) or antiserum A1 (1:3000) preincubated with 100 μg/mL antigenic peptide for 60 minutes at room temperature. The blots were washed and incubated for 30 minutes with horseradish peroxidase-labeled goat anti-rabbit IgG (diluted 1:30 000, Kirkegaard and Perry, Gaithersburg, Md). Immunoreactivity was detected by enhanced chemiluminescence (Renaissance Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products).

Measurement of GAPD2 Enzymatic Activity

Aliquots of spermatozoa were thawed on ice, washed with PBSS containing protease inhibitors (10 μg/mL aprotinin and 10 μg/mL leupeptin), and recovered by centrifugation at 300 × *g* for 10 minutes. Spermatozoa were permeabilized with 0.3% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS) detergent for 30 minutes on ice and processed by centrifuge at 10 000 × *g* for 5 minutes. Pellets and supernatants were assayed for enzymatic activity, and boiled sperm were included in assays as a negative control. GAPD2 activity was determined in a standard enzyme assay (Worthington Manual, 1993) by measuring the change in absorbance at 340 nm that occured on reduction of NAD^+ to NADH. The reaction mixture contained 0.25 mmol/L NAD^+, 3.3 μmol/L DTT, 0.3 mmol/L glyceraldehyde-3-phosphate, 5 mmol/L potassium fluoride (enolase inhibitor), 0.5 mmol/L oxalate (pyruvate kinase inhibitor), 0.1 mmol/L oxamate (lactate dehydrogenase inhibitor), 1×10^6 spermatozoa in 15 mmol/L sodium pyrophosphate, and 30 mmol/L sodium arsenate. Values for enzyme activity represent the average of 2 to 3 separate experiments, each done in quadruplicate and presented in IU + SEM.

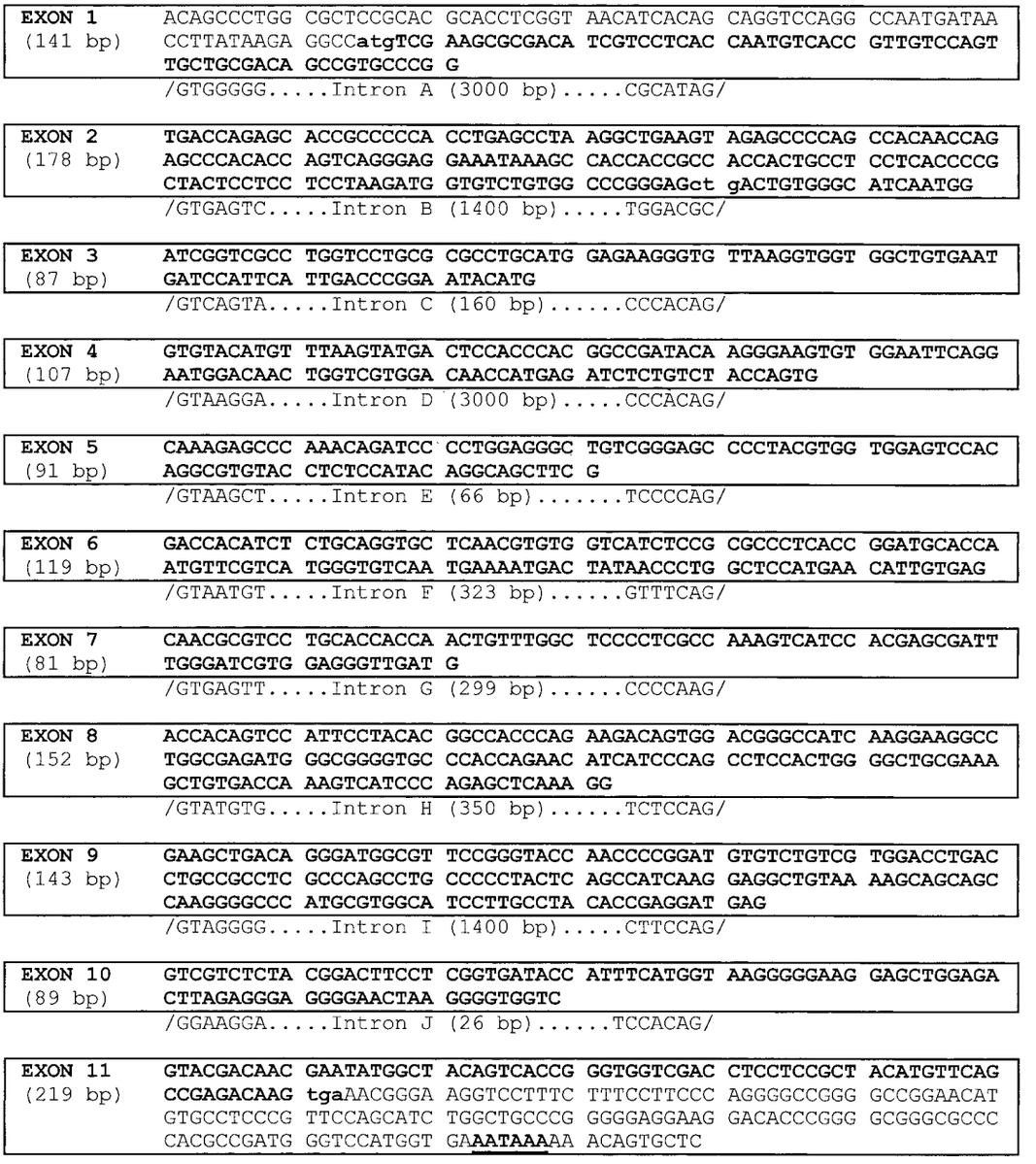

Figure 1. Human *GAPD2* genomic sequence. The human *GAPD2* gene consists of 11 exons and includes approximately 12 000 nucleotide base pairs. The coding region is indicated in bold, and the poly(A) addition signal is underlined in bold. The start and stop codons are indicated by lower case letters as is the CTG (Leu) codon in exon 2, which begins the region conserved with the somatic *GAPD* sequence. The exon-intron splice junctions were found to correspond exactly with those of the mouse *Gapds* gene although variations were seen in the size of the intervening sequences.

Results

Isolation and Characterization of Human GAPD2 cDNA

The cDNA clones for *GAPD2* were obtained from a human testis cDNA library, subcloned into M13 phage, and sequenced manually with standard dideoxynucleotide methods. The sequence of the longest cDNA insert was determined in both directions and confirmed by comparison with the sequences of 2 additional independently isolated cDNA clones. A total of 1447 nucleotides of the human *GAPD2* sequence was determined and consisted of 74 bp of 5′ untranslated region (UTR), 1227 bp of coding region, and 146 bp of 3′ UTR (Figure 1).

The human *GAPD2* cDNA was used as a probe on Northern blot analysis of human tissue polyA$^+$ mRNA samples. The cDNA hybridized specifically with a 1.5 kb mRNA, which is present only in the testis (Figure 2A). Some cross-hybridization was observed with the abundant 1.3-kb *GAPD* mRNA present in skeletal muscle, and no hybridization was observed with mRNA from heart,

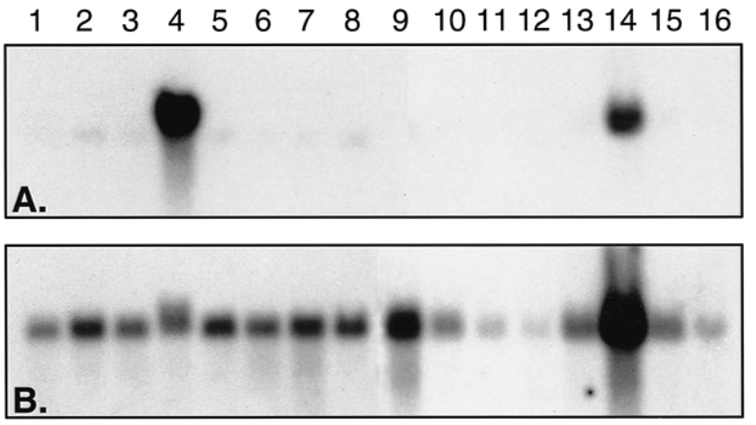

Figure 2. Northern blot analysis of *GAPD2* expression. Northern blot analysis of 16 human tissues **(A)** detected expression of the 1.5-kb *GAPD2* mRNA only in the testis, even after prolonged exposure times. The band seen in skeletal muscle is caused by cross-hybridization of the human *GAPD2* probe with the abundant 1.3-kb somatic *GAPD* mRNA present in this tissue. Northern blot analysis of *GAPD* expression **(B)** detected a 1.3-kb transcript in all tissues. Lane 1 are values for spleen; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, ovary; lane 6, small intestine; lane 7, colon; lane 8, leucocytes; lane 9, heart; lane 10, brain; lane 11, placenta; lane 12, lung; lane 13, liver; lane 14, skeletal muscle; lane 15, kidney; and lane 16, pancreas. The blot was exposed for 9 days, and 2 μg of polyA$^+$ RNA was loaded per lane.

brain, placenta, lung, liver, kidney, pancreas, spleen, thymus, prostate, ovary, small intestine, colon, or peripheral blood lymphocytes (Figure 2A). The same blots were hybridized with a mouse somatic *Gapd* probe, and the 1.3-kb *GAPD* mRNA was seen in all tissues (Figure 2B). It was most abundant in muscle tissue and least abundant in lung tissue. The probe also crosshybridized with the 1.5-kb *GAPD2* transcript in testis.

Comparison of Human and Mouse Sequences

Comparison of the deduced human GAPD2 and mouse GAPDS proteins by sequence alignment (Figure 3) indicated that they have 83% amino acid identity. All residues involved in forming the substrate-binding site and NAD$^+$ cofactor-binding pocket were conserved (Rossman et al, 1975; Wierenga et al, 1985). Human GAPD2 is 30 amino acids shorter than mouse GAPDS; the difference is caused by the presence of more prolines in the amino terminal segment of GAPDS. This suggests that the prolines are not critical for enzyme function and serve species-specific structural roles. However, the first 19 amino acids of GAPD2 and mouse GAPDS are highly conserved and may play an important role in the function or localization of these proteins. The deduced human GAPD2 and GAPD proteins have 68% identity. Mouse GAPDS and GAPD have 71% identity (Welch et al, 1992), and human and mouse GAPD have 94% amino acid identity. This indicates that a higher degree of identity exists between the GAPD2 and GAPDS proteins in human and mouse than exists between the somatic cell enzyme and the spermatogenic cell enzyme within either species.

Characterization of the Human GAPD2 *Gene*

The structure of the gene was determined from a human genomic DNA clone by PCR amplification across putative exon-intron junctions predicted by comparison with human *GAPD,* chicken *gapd,* and mouse *Gapds* gene sequences. PCR cycle-sequencing with the primers indicated in the Table verified that all splice junctions corresponded precisely to the positions predicted from the mouse *Gapds* gene (Figure 4). This demonstrates that the human *GAPD2* gene consists of 11 exons, with the same 2 exons that are separate in the chicken (exons 9 and 10) forming 1 exon (exon 9) in the mouse and human genes. This is in contrast to the human *GAPD* gene where 4 exons present in the chicken (exons 8–11) are represented by 1 exon (exon 8). Direct PCR amplification of human genomic DNA yielded the same results and verified the results obtained from the human *GAPD2* genomic clone. The sequences for introns A, B, D, H, and I were not determined in their entirety.

Localization of Human GAPD2 *Gene to Chromosome 19q13.1*

The full-length human *GAPD2* genomic clone was used as a probe for FISH on metaphase chromosomes of human peripheral blood lymphocytes. Initial experiments indicated that the *GAPD2* probe hybridized to a group F chromosome, possibly chromosome 19. Cohybridization of metaphase spreads with the *GAPD2* probe and a second probe to *E2A*, previously localized to 19p and proximal 19q (Mellentin et al, 1989), confirmed the chromosome localization. It was found that 70% of the cells examined (56 of 80 total cells) exhibited specific labeling. In addition, measurements of 10 labeled chromosomes placed the *GAPD2* gene at 28% of the centromere to telomere distance on chromosome arm 19q, a position equivalent to 19q13.1.

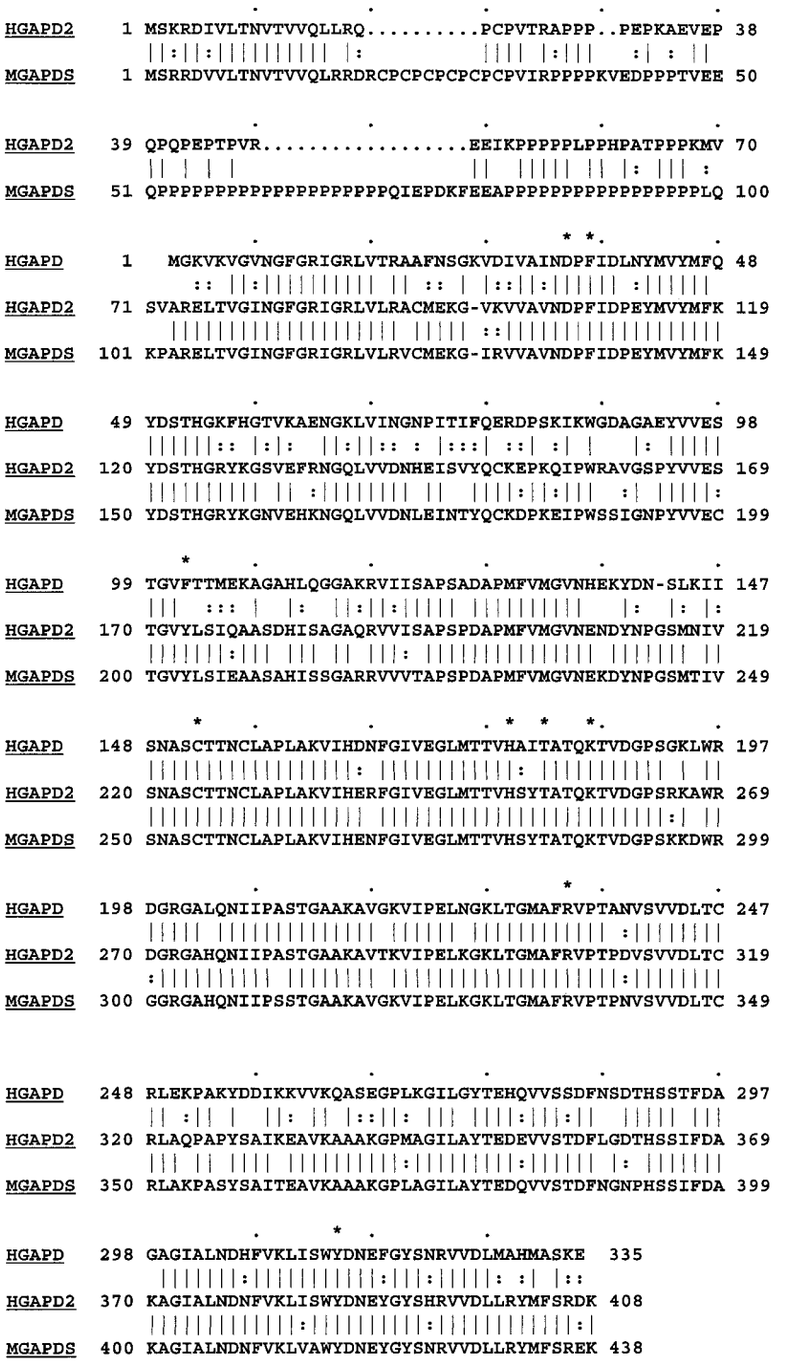

Figure 3. Comparison of human GAPD2, mouse Gapds, and human somatic GAPD deduced protein sequences. Alignment of these 3 sequences demonstrates extensive homology between their amino acid sequences. Some gaps are seen in the amino terminal region unique to human GAPD2 and mouse Gapds, but these gaps appear primarily in areas of repetitive cysteine-proline and polyproline sequences. The human GAPD2 sequence shows 83% identity with mouse Gapds, but only 68% identity with the human somatic GAPD sequence. Asterisks indicate the residues involved in forming the NAD^+-binding pocket and the substrate-binding site.

Immunolocalization of GAPD2 Protein in Human Spermatozoa

Antisera specific for either GAPDS (A1) or somatic GAPD (C1) were used to localize these proteins in human spermatozoa and somatic cells by indirect immunofluorescence. Immunoreactivity in fixed and permeabilized cells was recorded in paired epifluorescence and phase-contrast photomicrographs (Figure 5). GAPD2 was re-

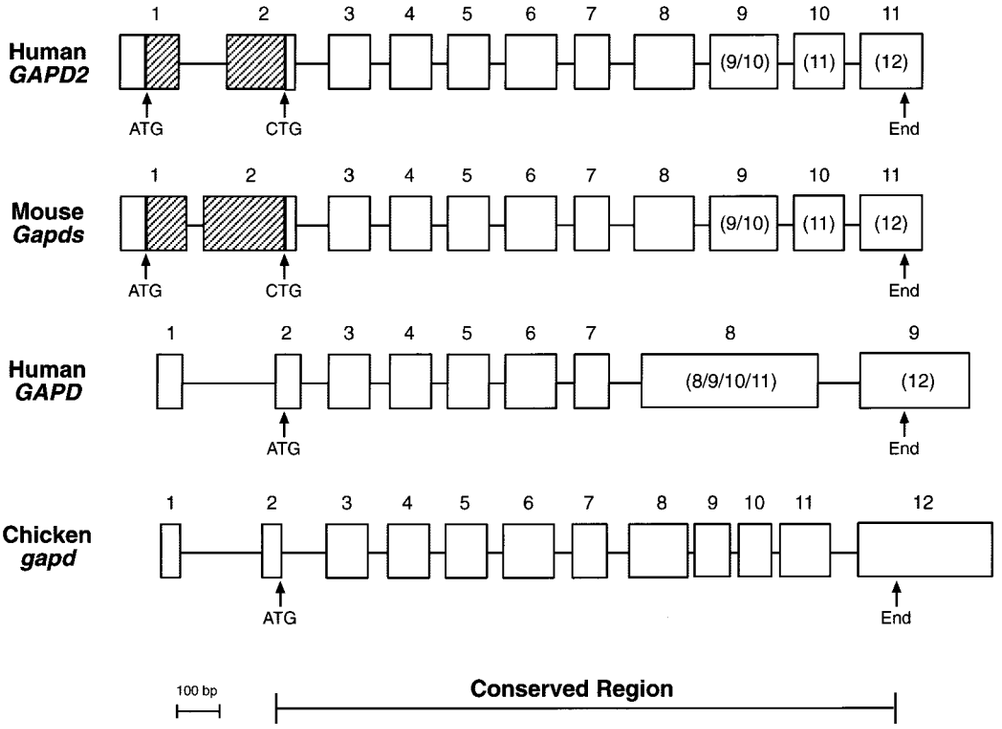

Figure 4. Exon-intron structure of the *GAPD2* gene. The exon-intron structure of human *GAPD2* and mouse *Gapds* genes are identical. The locations of introns in both *GAPD2* and mouse *Gapds* genes are perfectly conserved between the 2 species. Both genes are interrupted by 10 introns and exon 9, because both are equivalent to a fusion of exons 9 and 10 of chicken *gapd.* This is in contrast to the human *GAPD* gene in which exon 8 is equivalent to the fusion of chicken *GAPD* exons 8, 9, 10, and 11. ATG indicates the beginning of the coding sequence.

stricted to the principal piece of the flagellum in human spermatozoa (arrows, Figure 5A and C). Somatic cell GAPD was not detected in human spermatozoa, although somatic cells in the samples were intensely labeled with the C1 antiserum (Figure 5B and D). In previous studies, mouse spermatozoa had identical patterns of immunoreactivity with these antisera (Bunch et al, 1998).

Western Blot Analysis of GAPD2 Protein in Human Spermatozoa

Extracts of mouse and human spermatozoa were separated by SDS-PAGE and analyzed by Western blotting with antiserum A1. GAPDS from mouse spermatozoa migrated with an apparent molecular weight of ~69 200 (Figure 6), 45% greater than the calculated molecular weight of 47 445 (Bunch et al, 1998). GAPD2 extracted from human spermatozoa migrated with an apparent molecular weight of ~56 000 (average calculated from 5 separate blots), 24% greater than the calculated molecular weight of 44 501. Reactivity of mouse GAPDS and human GAPD2 was eliminated by preincubation of antiserum A1 with the antigenic peptide (A1 + peptide; Figure 6).

GAPD2 Activity Is Not Released by Permeabilization

When spermatozoa were treated with CHAPS detergent, GAPD2 enzyme activity (IU 1×10^{-6} sperm, mean ± SE) remained predominantly in the pellet (Figure 7). However, enzyme activity was destroyed when spermatozoa were boiled prior to permeabilization with CHAPS. These results demonstrate that GAPD2 is enzymatically active and suggest that it is tightly associated with relatively insoluble components of human spermatozoa, as in mouse spermatozoa (Bunch et al, 1998) and probably in boar (Westhoff and Kamp, 1997) and rabbit spermatozoa (Storey and Kayne, 1975).

Discussion

The human is the third species in which we have identified a cDNA for a *GAPD* gene family member encoding a protein localized to the principal piece of the flagellum in spermatozoa. Expression of the human *GAPD2* gene was detected by Northern blot analysis in testis tissues but not in 15 other tissues. The deduced protein contains 408 amino acids and is 83% identical to mouse GAPDS (Welch et al, 1992) and 94% identical to rat GAPDS (Welch et al, unpublished observations). The proteins in spermatozoa in all 3 species differ from their somatic cell counterparts by the presence of a proline-rich extension at the amino terminal end. Previous Northern blot studies demonstrated that rabbit, ram, and rat testes contain

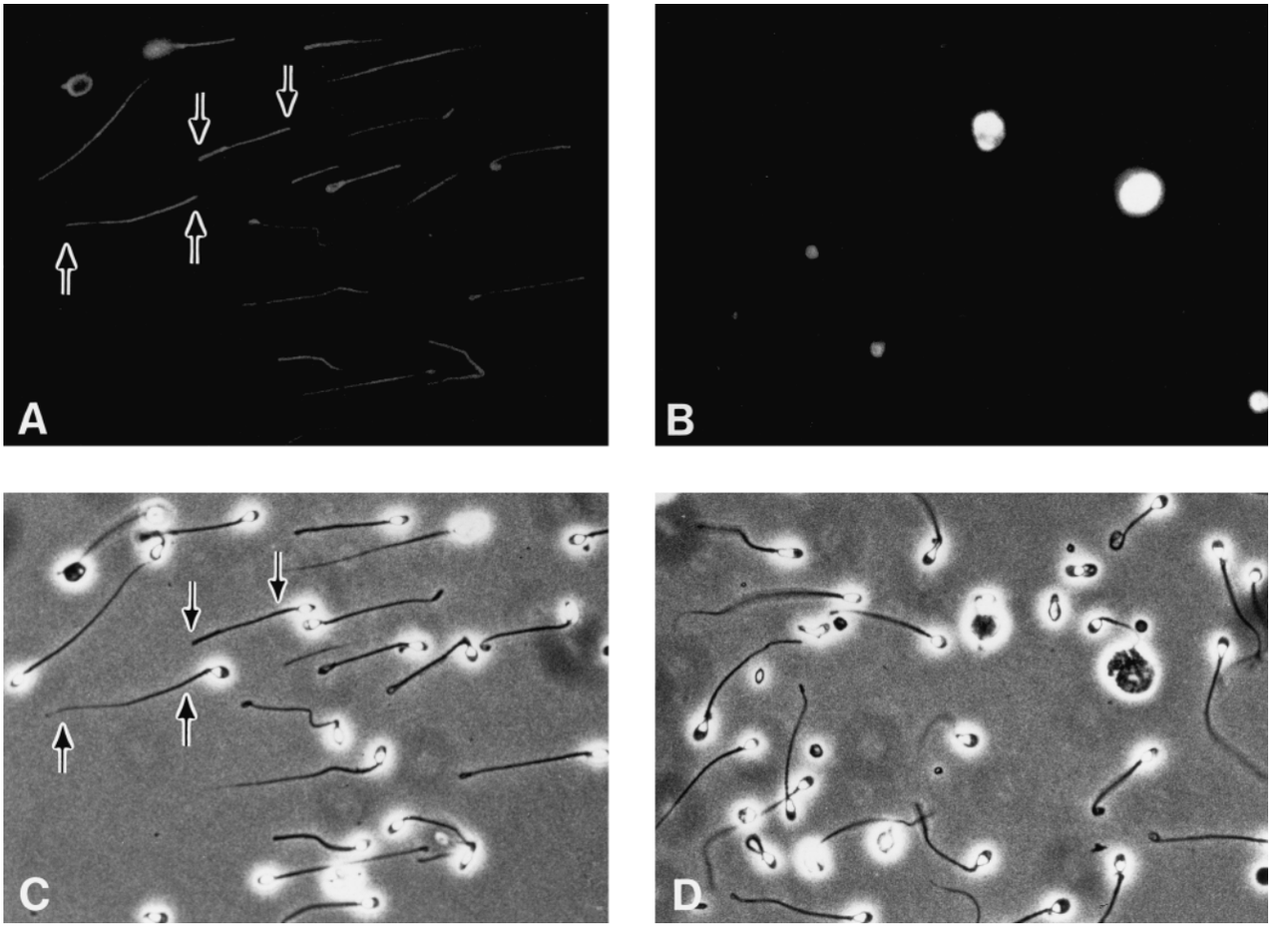

Figure 5. Immunolocalization of GAPD2 protein in human spermatozoa. Human spermatozoa were dried onto positively charged slides, fixed, and permeabilized prior to immunostaining. Epifluorescence **(A, B)** and phase-contrast **(C, D)** photomicrographs were taken of identical fields after staining with each antiserum. GAPD2 immunoreactivity detected with antiserum A1 was restricted to the principal piece of the sperm flagellum **(A, C)**. Antiserum to somatic GAPD (C1) reacted with contaminating somatic cells, but not with human spermatozoa **(B, D)**. Arrows indicate sperm flagellum.

mRNA that hybridized with a mouse *Gapds* probe (Welch et al, 1995). These findings and the report that a GAPD is present in the principal piece of boar spermatozoa (Westhoff and Kamp, 1997) strongly suggest that a homologous gene was expressed in spermatogenic cells of other mammals.

The *GAPD2* sequence is highly similar to 2 others recently submitted to GenBank. Large-scale sequencing of segments of human chromosome 19 yielded a 46 275-bp sequence (GenBank accession number AC002389) derived from chromosome 19q13.1 that contains a region homologous to the mouse *Gapds* gene. The chromosome location of this gene agrees with the location of *GAPD2* determined by FISH. The deduced protein sequence matches that of *GAPD2*, except that exon 7 was indicated to start 27 nt toward the 5′ end of the junction that we identified between intron F and exon 7, and it appears to incorrectly add 9 amino acids to the deduced protein. The chromosome segment sequenced also contains a gastric H,K-ATPase gene (Maeda et al, 1990) situated downstream of *GAPD2*. Another sequence reported recently (GenBank accession number AJ005371) was indicated to be a human testis-specific cDNA. It contains a deduced protein-coding region that matches GAPD2, but it lacks sequences present at the 5′ and 3′ UTRs of the *GAPD2* cDNA sequence reported here. In addition, expressed sequence tags (ESTs) from human testis (GenBank accession number AL048801) and human ocular ciliary body (GenBank accession number R88358) have been reported that have marked homology with a region of *GAPD2*. The significance of the ciliary body EST remains to be determined.

The GAPD gene family apparently has undergone extensive duplication by retroposon integration in mammals, with the human genome containing approximately 12 pseudogenes (Ercolani et al, 1988) and the mouse genome harboring several hundred GAPD-like sequences (Piechaczyk et al, 1984; Garcia-Meunier et al, 1993). The

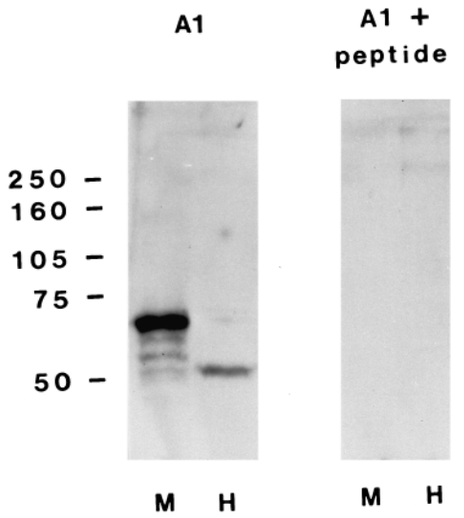

Figure 6. Western blot analysis of the GAPD2 protein. Proteins extracted from mouse spermatozoa (15 μg/lane) and human spermatozoa (30 μg/lane) were separated on a 12% polyacrylamide gel (450 volt-hours) and detected on Western blots with antiserum A1. Molecular weights ($\times 10^{-3}$) determined from standards are indicated on the left. GAPD2 migrated with an apparent molecular weight of ~56 000 in extracts of human spermatozoa. For comparison, GAPDS in extracts of mouse spermatozoa migrated with an apparent molecular weight of ~69 000. As a control, a duplicate blot was incubated with A1 antiserum preabsorbed with the antigenic peptide (A1 + peptide), and no immunoreactivity with GAPD2 or GAPDS was seen. M indicates mouse spermatozoa; H, human spermatozoa.

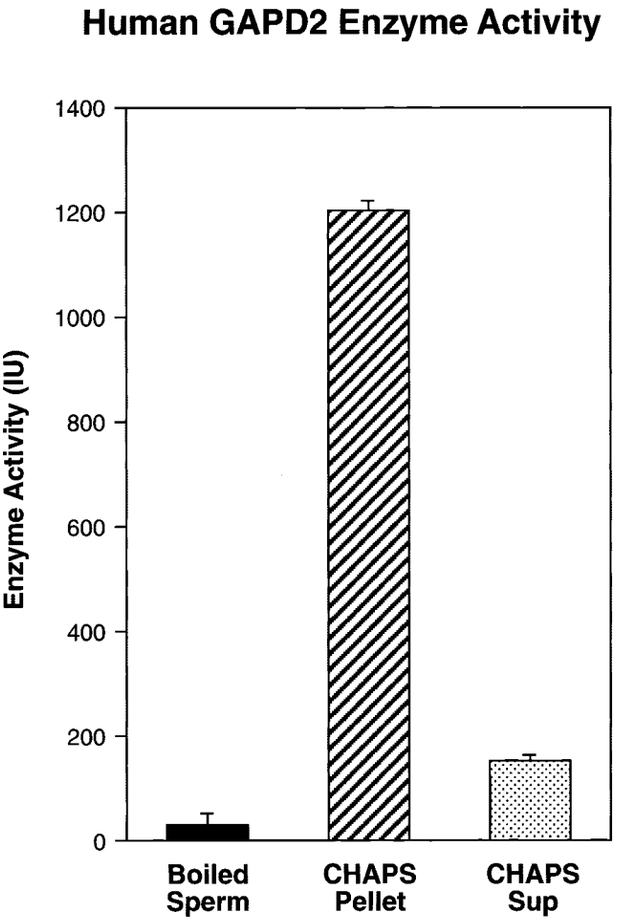

Figure 7. GAPD2 enzymatic activity in human spermatozoa. Enzyme activity (IU/1 $\times$ 10^6 spermatozoa, mean ± SE) was measured in the pellet and supernatant from human spermatozoa permeabilized with 0.3% CHAPS. Boiled sperm served as a negative control. Considerable GAPD2 enzyme activity was associated with the sperm pellet, whereas little enzyme activity was released into the supernatant with CHAPS treatment. Boiling the spermatozoa destroyed enzyme activity.

genes for 3 other enzymes expressed exclusively in spermatogenic cells and involved in energy production, *PGK2* (McCarrey and Thomas, 1987), *PDHA2* (Dahl et al, 1990), and *G6PD* (Hendricksen et al, 1997), appear to have originated as retroposons. It is nevertheless unlikely that the *GAPD2* gene arose by this process. It contains introns, which are lacking in retroposons, and its intron-exon organization is identical to the mouse *Gapds* gene and different from the human *GAPD* gene. Instead, the exon-intron organization of *GAPD2* and *Gapds* are similar to the more primitive chicken *gapd* gene. Although unique *gapd* mRNAs are present in chicken testis, they are transcribed from the same gene as mRNAs in somatic tissues and result from the use of alternative transcription initiation sites and splicing processes (Mezquita et al, 1998). Although these results do not rule out the possibility that another *gapd* gene is expressed in chicken testis, we suggest that the genes expressed in somatic and spermatogenic cells in mammals arose from a common ancestral gene after the evolutionary divergence of birds and mammals.

The GAPD2 protein sequence contains the residues required to constitute the NAD^+ cofactor-binding pocket and the glyceraldehyde 3-phosphate substrate-binding site that are necessary for enzyme activity (Rossman et al, 1975; Wierenga et al, 1985). Proline-rich domains at the amino terminal ends of human GAPD2 and mouse GAPDS are absent from human and mouse somatic cell GAPD. This domain is shorter in human GAPD2 than in mouse GAPDS because there are fewer polyproline and proline-rich sequences. We provided evidence (Bunch et al, 1998) that the abundant proline residues (51) in the initial portion of the mouse GAPDS were responsible for the anomalous migration (45% greater molecular weight than calculated) of the protein separated by SDS-PAGE. GAPD2 has fewer proline residues (23) and migrated at 24% greater molecular weight than calculated, lending further support to the suggestion that the anomalous migration is caused by the abundance of proline residues in the amino terminal domain.

These studies demonstrated that GAPD2 is present in the principal piece of human spermatozoa, the same location as GAPDS in the mouse (Bunch et al, 1998), and

that most of the GAPD2 activity was present in the pellet after spermatozoa were extracted with detergent. This strongly suggests that GAPD2, like GAPDS, is associated with the fibrous sheath. The evidence that enzymes involved in glycolysis are associated with insoluble sperm components (Storey and Kayne, 1975) led to the suggestion that they are anchored to the sperm cytoskeleton at sites of high energy utilization (Eddy et al, 1994). Glycolysis is required for in vitro fertilization and vigorous motility of spermatozoa in mice (Fraser and Quinn, 1981) and human beings (Mahadevan et al, 1997). Furthermore, inhibition of oxidative phosphorylation with oligomycin failed to inhibit fertilization (Fraser and Quinn, 1981), suggesting that ATP production by mitochondria is not required for this process. The functional significance of the subcellular localization of GAPD2 in spermatozoa remains to be determined. However, anchoring the enzyme to the fibrous sheath would minimize the path of diffusion for ATP generated during glycolysis to reach motor proteins of the flagellum.

In summary, we have identified and characterized the cDNA sequence and the intron-exon structure of the human *GAPD2* gene and have shown that it is a homologue of the mouse *Gapds* gene. This new member of the *GAPD* gene family in human beings is expressed specifically in the testis. The *GAPD2* gene encodes a protein that localizes to the principal piece of the flagellum of human spermatozoa, the same location where GAPDS is found in mouse spermatozoa. These data strongly suggest that the *GAPD2* gene was expressed specifically in spermatogenic cells. The GAPDS protein has a calculated molecular weight of 44 501 but migrates at ~56 000 molecular weight by SDS-PAGE. The anomalous migration is probably caused by the abundance of proline residues in the 72-amino acid segment at the amino terminal end. GAPD2 is 68% identical to somatic cell GAPD with the proline-rich amino terminal segment, which is a major difference between these 2 proteins. The exon-intron organization of the human *GAPD2* and mouse *Gapds* genes is more similar to the *gapd* gene of chicken than the human *GAPD* or mouse *Gapd* genes. This suggests that the genes expressed in somatic cells and spermatogenic cells of mammals arose from a common ancestor after the evolutionary divergence of birds and mammals.

Acknowledgments

The authors thank Teddy Devereux and Dr. Rachel Bienstock for the constructive comments on the manuscript. This document has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the view of the Agency; likewise, mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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