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Several Novel Transcripts of Glyceraldehyde-3-Phosphate Dehydrogenase Expressed in Adult Chicken Testis

J. Mezquita, M. Pau, and C. Mezquita*

Laboratori de Genètica Molecular, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Facultat de Medicina, Universitat de Barcelona, 08036 Barcelona, Spain

Abstract Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in addition to being a classic glycolytic enzyme, is a multifunctional protein involved in relevant cell functions such as DNA replication, DNA repair, translational control of gene expression, and apoptosis. Although the multifunctional nature of GAPDH suggests versatility in the mechanisms regulating its expression, no major qualitative changes and few quantitative changes in the GAPDH transcripts have been reported. While studying the expression of GAPDH during spermatogenesis, we detected alternative initiations to TATA box and alternative splicings in the 5′ region of the pre-mRNA, resulting in at least six different types of mRNAs. The amount and the polyadenylation of the GAPDH transcripts increased in mature testis in relation to immature testis and further increased when cell suspensions from mature testis were exposed to heat shock. These results suggest that alternative initiation, alternative splicing, and polyadenylation could provide the necessary versatility to the regulation of the expression of this multifunctional protein during spermatogenesis. J. Cell. Biochem. 71:127–139, 1998.

Key words: GAPDH gene expression; spermatogenesis; meiotic and postmeiotic cells; heat shock; polyadenylation

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH) is a tetrameric nicotinamide-adenine dinucleotide (NAD)-binding glycolytic enzyme distributed in multiple cellular compartments. GAPDH is present in both prokaryotes and eukaryotes and is conserved across great evolutionary distances [Stone et al., 1985]. The number of genes and pseudogenes of GAPDH is different in different organisms [Piechaczyk et al., 1984]. Only one gene, an incomplete processed pseudogene, and a single mRNA species have been detected in chicken [Stone et al., 1985; Lum and Linial, 1997]. Other organisms possess a low number of gene copies (10–30 copies in man, hare, guinea pig, and hamster) or show high reiteration (>200 copies in mouse and rat) [Piechaczyk et al., 1984]. Despite the high number of GAPDH gene copies in mammals, only one somatic isoform and one isoform expressed in spermatids have been reported [Mori et al., 1992; Welch et al., 1995].

During spermatogenesis, GAPDH is essential for regulation of glucose metabolism in both primary spermatocytes and spermatids [Nakamura et al., 1984]. The enzyme is present in the principal piece of mammalian spermatozoa [Westhoff and Kamp, 1997]. In addition to being a glycolytic enzyme, GAPDH is a multifunctional protein involved in a variety of cell functions [Sirover, 1997] that may be relevant for spermatogenesis. GAPDH participates in the mechanisms of DNA replication [Baxi and Vishwanatha, 1995], DNA repair [Meyer-Siegler et al., 1991], nuclear tRNA export [Singh and Green, 1993], and translational control of gene expression [Nagy and Rigby, 1995; Sugahara et al., 1995; De et al., 1996; McGowan and Pekala, 1996; Schultz et al., 1996]. Roles as a microtubule binding protein and as a protein involved in endocytosis have also been reported [Kumagai and Sakai, 1983; Durrieu et al., 1987;

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*Correspondence to: C. Mezquita, Fisiologia, Facultat de Medicina, Universitat de Barcelona, Casanova 143, 08036 Barcelona, Spain. E-mail: mezquita@medicina.ub.es

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Muronetz et al., 1994; Federici et al., 1996; Robbins et al., 1995].

Recently, it has been proposed that GAPDH overexpression is implicated in the apoptotic death of cultured cerebellar granule cells and cerebral cortical neurons [Sunaga et al., 1995; Chuang and Ishitani, 1996]. Antisense oligonucleotides to GAPDH prevent such cell death [Ishitani and Chuang, 1996]. In addition to GAPDH overexpression, nuclear translocation of the protein participates in neuronal and nonneuronal apoptosis [Sawa et al., 1997].

The expression of GAPDH changes with hypoxia [Graven et al., 1994], calcium influx [Chao et al., 1990], during the cell cycle, and in proliferating and tumor cells [Persons et al., 1989; Tokunaga et al., 1987; Mansur et al., 1993; Epner and Coffey, 1996; Gong et al., 1996]. Despite the multifunctional nature of GAPDH, which would require versatility in the mechanism involved in the regulation of its expression, the GAPDH transcript is often regarded as a static marker and is commonly used as a reporter molecule to estimate the amount of mRNAs present in Northern blots. In this paper we show for the first time that, in addition to quantitative changes in expression, several qualitative changes in the GAPDH transcripts occur during avian spermatogenesis. The new GAPDH transcripts expressed during chicken spermatogenesis are the result of alternative initiation of transcription, alternative splicing, and polyadenylation. We are interested in the expression of GAPDH during spermatogenesis both as a model of transcriptional and posttranscriptional regulation and because of the potential importance of this multifunctional protein in the differentiation of the male gamete.

MATERIALS AND METHODS

cDNA Synthesis

Whole-size cDNA from mature and immature testis and from kidney was obtained by using the CapFinder protocol from Clontech (Palo Alto, CA). Briefly, first-strand cDNA was obtained with a modified oligo(dT) primer [CDS/3′ polymerase chain reaction (PCR) primer, 2 μM], 2 μg of total RNA, and a CapSwitch oligonucleotide (2 μM) added as an extended template at the 5′ end of the mRNA. After denaturation at 72°C for 2 min and cooling on ice, the reaction was incubated with 100 units of MMLV reverse transcriptase and dNTP (1 mM) at 42°C for 1 h. Two microliters of this first-strand reaction were amplified by PCR using KlenTaq polymerase, dNTPs (0.2 mM), and the 5′PCR and CDS/3′PCR primers (0.2 μM each) from Clontech for 1 min at 95°C, followed by 20 cycles at 95°C for 15 sec and at 68°C for 5 min.

Rapid Amplification of cDNA Ends (RACE)

The primer ATGTTGCTGGGGTCACGCTCCTG spanning intron 4 and the 5′PCR primer from Clontech were used for 5′RACE; the primer CATGGCATCCAAGGAGTGAGC at the 3′ untranslated region (UTR) and the CDS/3′PCR primer (Clontech) were used for 3′RACE. The 5′PCR primer by itself amplified the whole GAPDH cDNA, except for 100 nucleotides at the 3′UTR. RACE-PCR conditions were as follows: 20 pmol of each primer, 10 nmol of dNTPs, and 2.0 units of a high fidelity thermostable DNA polymerase (Expand High Fidelity, Boehringer Mannheim, Indianapolis, IN). Incubations were performed in a final volume of 50 μl; 3 min at 94°C; 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min; final extension was at 72°C for 7 min.

Purification of PCR Products, Cloning, and Sequencing

PCR products were purified free of oligonucleotides with Millipore Ultrafree-MC 30,000 filters. Direct cloning was done by using the pGEM-T vector from Promega (Madison, WI). When possible, PCR products were blunt ended with Pfu DNA polymerase and ligated to pCR-Script SK+ (Stratagene, La Jolla, CA). Sequencing was done with the Licor MWG Biotech GmbH automatic sequencer by using the Thermo Sequenase cocktail from Amersham (Arlington Heights, IL). The forward and reverse primers of the vector were labeled with IRD41, and the following cycle sequencing program was used: 95°C for 3 min and then 30 cycles at 95°C for 15 sec, 64°C for 15 sec, and 70°C for 30 sec.

Preparation of RNA, Electrophoresis, Northern Hybridization, and Immunological Detection

Total RNA was prepared with the TriPure Isolation Reagent from Boehringer Mannheim according to the specifications of the manufacturer. Samples of total RNA (20–40 μg) obtained from chicken testis (6 and 25 weeks old), kidney, heart, liver, brain, and 6-day-old embryos were electrophoresed through 2 M formaldehyde, 1.2–1.6% agarose gels in 0.02 M MOPS,

5 mM sodium acetate (pH 7), and 1 mM EDTA. The gel was transferred to nylon membranes positively charged (Nytran Plus, Schleicher and Schuell, Keene, NH) in 10× standard saline citrate and fixed by ultraviolet cross linking. Blots were hybridized using ExpressHyb hybridization solution (Clontech) labeled with nonradioactive digoxigenin-specific probes as described below. Hybridization conditions and the immunological chemiluminisce detection procedure were as described in Engler-Blum et al. [1993].

DNA Probes

Probes were obtained by PCR from recombinant clones by using the forward and reverse primers of the vector, labeled by using Taq DNA polymerase and a dNTP mix containing DIG-11-dUTP (Boehringer Mannheim), and then purified free of nucleotides with Millipore Ultrafree MC 30,000 filters. Five to 20 ng/ml of probe were used in each hybridization. Recombinants having most of the whole transcript were used to obtain the GAPDH probe. A recombinant containing intron-1-less 5′ variant was used to obtain a specific probe of this transcript. The probe was obtained with the reverse primer from the vector and the specific primer AGTGTTCCTGCGGGGAGAGACC. To detect the TATA-plus transcripts specifically, the oligonucleotide GGCGCAGTGCCGCGCCGCCGCCCTTTATAGA CACGGAGGC, labeled at the 5′ end with digoxigenin (MWG-Biotech Synthesis Laboratories, Ebersberg, Germany; 70 pmol/ml), was used.

RNAase H Treatment

The method described by Kleene et al. [1984] was used: 20–30 µg of RNAs from heart and from immature and mature testis were separately mixed with 2 µg of oligo d(T) (Pharmacia Biotech, Uppsala, Sweden), denatured at 65°C for 2 min, and let to anneal for 15 min on ice. Then, 2 units of RNase H (Pharmacia Biotech) were added and incubated at 37°C for 30 min. Samples were extracted with phenol-chloroform, ethanol precipitated, and analyzed in agarose-formaldehyde 1.6% gels (as previously described) in parallel with control samples from the same tissues.

PCR-MIMIC

To determine the relative levels of the different GAPDH mRNA, PCR-MIMIC (competitive DNA fragments for use in quantitative PCR) was used [Siebert and Kellogg, 1995]. Mimics were generated by two successive PCR amplifications of heterologous DNA: Blue-Script SK+ was used as heterologous DNA for analysis of intron-1-less and TATA-plus GAPDH cDNAs, and pUC19 was used as heterologous DNA for somatic GAPDH cDNA. For the first PCR, two composite primers were used. One of the primers contained the upstream target primer sequence linked to 21 nucleotides that anneal to one strand of the heterologous DNA fragment. The other primer contained the downstream target primer sequence linked to 20 nucleotides that anneal to the opposite strand of the heterologous DNA. During amplification, the target primer sequences were incorporated into the PCR product. A dilution (1:1,000) of this PCR product was used to perform a second PCR with the shorter target primers. PCR was purified through Millipore 30,000 Ultra-free filters. Mimic products were then quantified in agarose gels by running an aliquot along DNA quantitative markers (Bio-Rad, Richmond, CA). PCR-MIMICs were diluted from 10 to 10^{-6} amol/µl. For competition experiments, 2 µl of mimic dilutions and 1 µl of a 1:200 dilution of cDNA (Cap-Finder protocol) were incubated with 20 pmol of each target primer and 25 µl of master mix (Boehringer Mannheim) in a final volume of 50 µl. Samples were analyzed (5 µl) in 3% agarose gels (1.5% SeaKem, 1.5% NuSieve). PCR was at 94°C for 3 min, 30 cycles at 94°C for 45 sec, 65°C for 45 sec, and 72°C for 90 sec, and a final extension at 72°C for 7 min.

Upstream composite primers (target upstream primers from intron-1-less, somatic, and TATA-plus transcripts, respectively, are underlined):

ACTGGGCCGGCGGTCTGCGAGGGTTTTCC-

CAGTCACGACG;

ACCTTCTCACTGCGCGCTGGAGGGTTTTC-

CCAGTCACGACG;

GTGTCTATAAAGGGCGGCGAGGGTTTTCC-

CAGTCACGACG.

Downstream composite primer (target downstream primer is underlined):

ATGTTGCTGGGGTCACGCTCCTGAGGAAA-

CAGCTATGACCATG.

Primer Extension Analyses of GAPDH mRNA Start Sites

A new nonradioactive primer extension method has been developed. This method uses primers labeled with IRD-800 whose extension products can be analyzed in a LICOR automatic sequencer. Total RNA from kidney and immature testis (340 µg) and 250 µg from mature testis and yeast (control RNA) were hybridized with 200 fmol of a primer (CAGATCGATGAAGGGATCATTGATGGCC AC) from the second exon of the GAPDH transcript labeled at the 5′ end with IRD800 (MWG). Hybridization conditions and processing of samples were as described by Schmidt et al. [1997], with minor modifications. Samples were hybridized overnight in 10 µl buffer [10 mM Pipes, pH 7.0; 400 mM NaCI; 1 mM EDTA; 0.05% sodium dodecylsulfate (SDS)] at 45°C and then diluted with 100 µl of dilution buffer (10 mM TrisCIH, pH 7.5; 300 mM NaCI; 0.5 mM EDTA), phenol-chloroform extracted, precipitated, and washed with ethanol. Pellets were resuspended in 100 µl of a reaction mixture containing reverse transcriptase buffer (BRL Superscript II buffer), DTT 5 mM, 0.3 mM of each dNTP, 2 units of RNasin, and 200 units of Superscript II reverse transcriptase (BRL, Gaitherburg, MD). After 1 h incubation at 50°C, samples were treated with RNases (1 µg RNase A/20 units RNase T1; Ambion, Austin, TX) for 10 min at room temperature and with Proteinase K (7 µg) for 10 min at 37°C. Samples were then mixed with 200 µl of dilution buffer containing 0.1% SDS, phenol-chloroform extracted, precipitated, and washed with ethanol. Pellets were redisolved in water and one-third of the solution was mixed with two volumes of sequencing dye, denatured for 1 min at 75°C, and analyzed in a 6% sequencing gel with a LICOR automatic sequencer, along with sequences obtained from GAPDH TATA-plus and intron-less recombinant clones sequenced with the same primer.

Cell Suspensions and Heat-Shock Conditions

Hubbard White Mountain roosters (6–12 months) were used in the experiments. Mature testes (5–10 g) were decapsulated and minced finely with forceps. The minced tissue was gently suspended in 10 vol of minimum essential medium (S-MEM, Life Technologies, Inchinnan, Scotland) containing 0.1% trypsin. The suspension was incubated at 31°C for 30 min in an orbital air incubator and filtered through four layers of surgical gauze. Fetal calf serum was then added to 10%. Aliquots of 1–6 $\times$ 10^9 cells (10 ml) were used as control and heat-shock samples. Heat shock was conducted at 46°C for 2 h in a water bath orbital incubator. Controls were incubated at 40°C for 2 h. Cells were centrifuged at 1,000*g* for 5 min. The pellet was extracted with 1 ml of TriPure (Boehringer Mannheim).

Fractionation of Chicken Testis Cells

Cells from adult chicken testis were fractionated by centrifugal elutriation as previously described [Roca and Mezquita, 1989]. The following cell types were obtained: testicular spermatozoa and residual bodies (3 ml/min flow rate), elongated spermatids devoid of residual bodies (11 ml/min flow rate), early spermatids containing 70% round spermatids and 30% elongating spermatids (20 ml/min flow rate), and meiotic and premeiotic cells (37 ml/min flow rate). The purity of the fractions was determined by phase-contrast microscopy.

RESULTS

Novel GAPDH Transcripts in Adult Chicken Testis Resulting From Alternative Initiation of Transcription and Differential Splicing

Figure 1 shows diagrammatically the somatic GAPDH transcript and several novel transcripts observed in adult chicken testis. From 29 clones randomly sequenced from adult chicken testis, 10 were identical to the somatic type GAPDH, 11 initiates were upstream from the TATA box (TATA-plus transcripts), four initiates were downstream from the TATA box within the first intron (intron-1-less transcripts), and five showed alternative initiations and alternative splicings either of intron 1 or intron 2. All the GAPDH clones sequenced from kidney, 15 clones, were of the somatic type. The somatic type of GAPDH transcript shows essentially the same sequence previously reported [Panabieres et al., 1984], with few changes (Fig. 2). One of the nucleotide changes occurs in the coding region, giving rise to an amino acid substitution at position 197: glycine, in our sequence, instead of aspartic, the residue previously reported. Glycine, however, is the amino acid found at position 197 in GAPDH from quail [Weiskirchen et al., 1993], in agreement with our sequence.

The alternative sequences of the 5′UTR found in mature testis are shown in Figure 3. In several clones, transcription starts upstream from the TATA box, incorporating the TATA

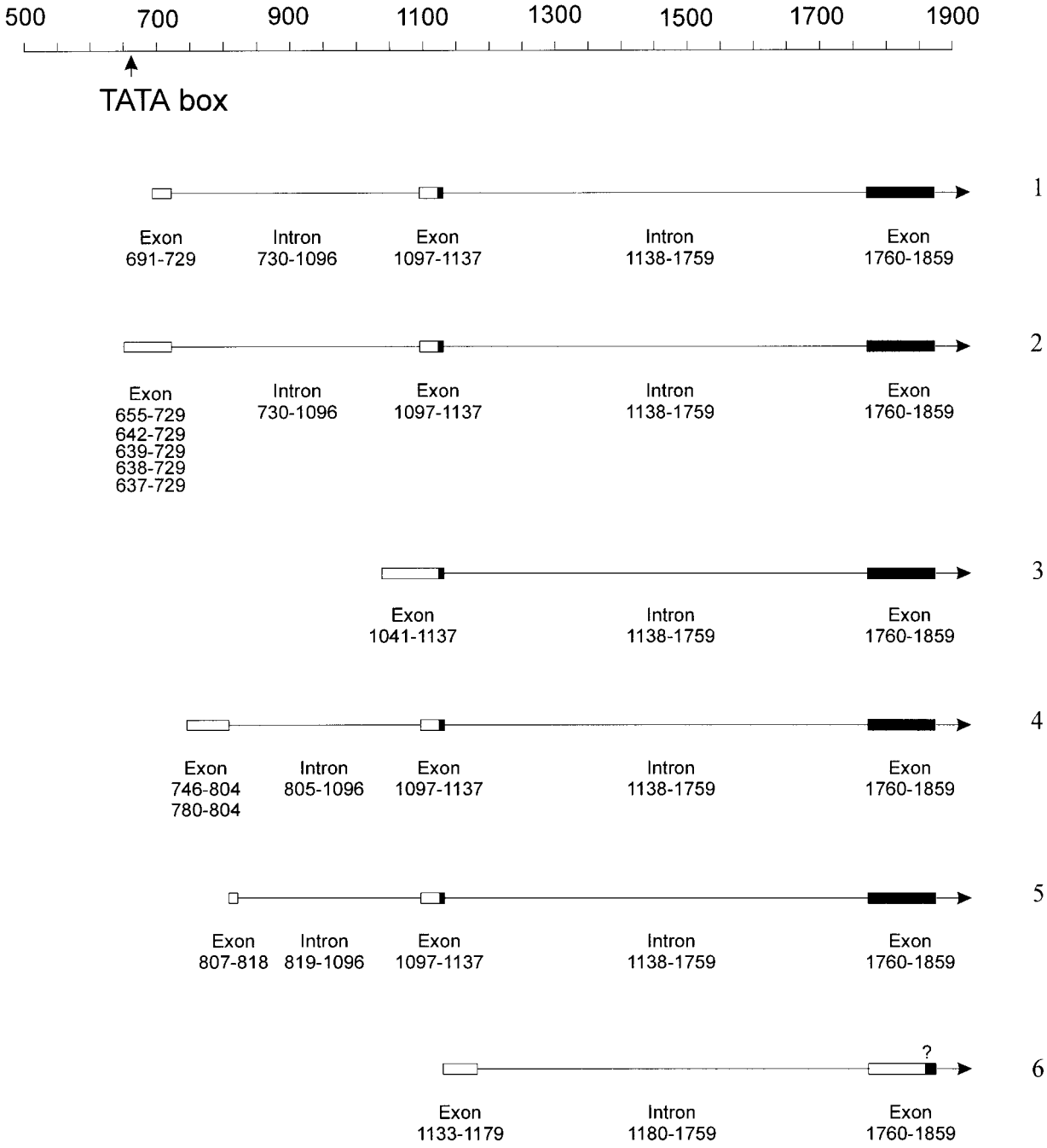

Fig. 1. Comparison of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) initiation sites and splicings of mRNAs from adult chicken testis. Six different forms of transcripts are shown, from top to bottom. **1**: Somatic form. **2**: Alternative initiation sites placed upstream from the TATA box, giving rise to TATA-plus transcripts. **3**: Alternative initiation site placed downstream from the TATA box, giving rise to intron-1-less transcripts. **4**, **5**: Alternative initiation sites and alternative splicing of intron 1. **6**: Alternative initiation site placed within exon 2 and alternative splicing of intron 2, giving rise to a transcript lacking the initiation codon. The nucleotide numbers correspond to those in the chicken GAPDH gene (GeneBank accesion number M11213) [Stone et al., 1985]. All the transcripts use the same reading frame. The question mark in transcript 6 indicates the position of the first ATG in frame at nucleotide 1857.

sequence in the 5′UTR. Whereas the 5′UTR of the somatic type of GAPDH consists of 57 nucleotides, the TATA-plus transcripts have a 5′UTR of 107 nucleotides. As in TATA-less genes, we observed some heterogeneity in the site of transcription initiation (transcript 2 in Figs. 1, 3). Upstream of the new initiation site, the sequence of the gene is extraordinarily G+C rich and contains several sp1 binding sequences [Stone et al., 1985].

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                                                  accttctcactg
  13 cgcgctggggccgttgacgtgcagcaggaacactataaaggcgag
  58 atggtgaaagtcggagtcaacggatttggccgtattggccgcctg
     M  V  K  V  G  V  N  G  F  G  R  I  G  R  L
 103 gtcaccagggctgccgtcctctctggcaaagtccaagtggtggcc
     V  T  R  A  A  V  L  S  G  K  V  Q  V  V  A
 148 atcaatgatcccttcatcgatctgaactacatggtttacatgttc
     I  N  D  P  F  I  D  L  N  Y  M  V  Y  M  F
 193 aaatatgattctacacacggacacttcaagggcactgtcaaggct
     K  Y  D  S  T  H  G  H  F  K  G  T  V  K  A
 238 gagaacgggaaacttgtgatcaatgggcacgccatcactatcttc
     E  N  G  K  L  V  I  N  G  H  A  I  T  I  F
 283 caggagcgtgaccccagcaacatcaaatgggcagatgcaggtgct
     Q  E  R  D  P  S  N  I  K  W  A  D  A  G  A
 328 gagtatgttgtggagtccactggtgtcttcaccaccatggagaag
     E  Y  V  V  E  S  T  G  V  F  T  T  M  E  K
 373 gctggggctcatctgaagggtggtgctaagcgtgttatcatctca
     A  G  A  H  L  K  G  G  A  K  R  V  I  I  S
 418 gctccctcagctgatgcccccatgtttgtgatgggtgtcaaccat
     A  P  S  A  D  A  P  M  F  V  M  G  V  N  H
 463 gagaaatatgacaagtccctgaaaattgtcagcaatgcatcgtgc
     E  K  Y  D  K  S  L  K  I  V  S  N  A  S  C
 508 accaccaactgcctggcacccttggccaaggtcatccatgacaac
     T  T  N  C  L  A  P  L  A  K  V  I  H  D  N
 553 tttggcattgtggagggtcttatgaccactgtccatgccatcaca
     F  G  I  V  E  G  L  M  T  T  V  H  A  I  T
 598 gccacacagaagacggtggatggcccctctgggaagctgtggaga
     A  T  Q  K  T  V  D  G  P  S  G  K  L  W  R
 643 gatggcagaggtgctgcccagaacatcatcccagcgtccactggg
     D  G  R  G  A  A  Q  N  I  I  P  A  S  T  G
 688 gctgctaaggctgtggggaaagtcatccctgagctgaatgggaag
     A  A  K  A  V  G  K  V  I  P  E  L  N  G  K
 733 cttactggaatggctttccgtgtgccaacccccaatgtctctgtt
     L  T  G  M  A  F  R  V  P  T  P  N  V  S  V
 778 gttgacctgacctgccgtctggagaaaccagccaagtatgatgat
     V  D  L  T  C  R  L  E  K  P  A  K  Y  D  D
 823 atcaagagggtagtgaaggctgctgctgatgggcccctgaagggc
     I  K  R  V  V  K  A  A  A  D  G  P  L  K  G
 868 atcctaggatacacagaggaccaggttgtctcctgtgacttcaat
     I  L  G  Y  T  E  D  Q  V  V  S  C  D  F  N
 913 ggtgacagccattcctccacctttgatgcgggtgctggcattgca
     G  D  S  H  S  S  T  F  D  A  G  A  G  I  A
 958 ctgaatgaccatttcgtcaagcttgtttcctggtatgacaatgag
     L  N  D  H  F  V  K  L  V  S  W  Y  D  N  E
1003 tttggatacagcaaccgtgttgtggacttgatggtccacatggca
     F  G  Y  S  N  R  V  V  D  L  M  V  H  M  A
1048 tccaaggagtgagccaggcacacagccccccctgctgcctagggaa
     S  K  E  *
1093 gcaggacccttgttggagcccctgctcttcaccaccgctcagtt
1138 ctgcatcctgcagtgagaggccagttctgttcccttctgtctccc
1183 ccactcctccaatttcttcctccacctgggggaggtgggagaggc
1228 tgatagaaactgatctgtttgtgtaccaccttacatcaataaaag
1273 tgttcaccatctgaag(a)n
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Fig. 2. Nucleotide and amino acid sequence of chicken glyceraldehyde-3-phosphate dehydrogenase. The predicted amino acid sequence is shown below the nucleotide sequence. This sequence has been deposited in the GeneBank database under the accession number AF047874. The polyadenylation signal is underlined.

GAPDH of somatic tissues possess an intron (intron 1) in the 5′ noncoding region of the gene. A number of clones isolated from adult chicken testis initiate downstream from the somatic initiation site, within intron 1, giving rise to intron-1-less transcripts. The length of the new 5′UTR is 76 nucleotides (transcript 3 in Figs. 1, 3). Upstream from the new initiation site, the gene sequence contains several sp1 binding sites.

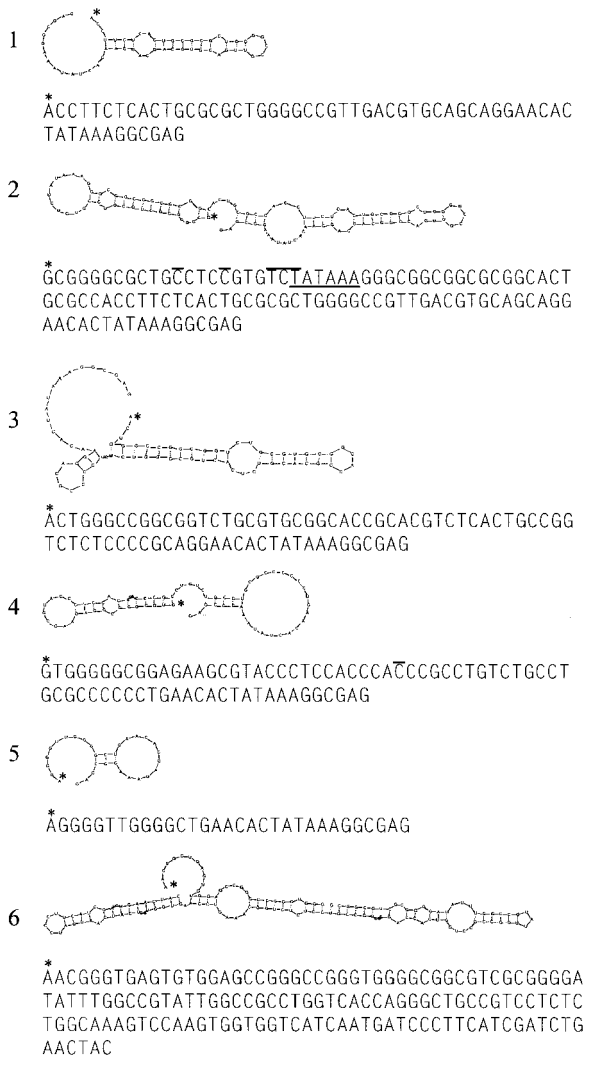

Fig. 3. The 5′ untranslated regions (UTRs) of several glyceraldehyde-3-phosphate dehydrogenase transcripts expressed in adult chicken testis. **1**: Somatic form. **2**: TATA-plus transcripts. **3**: Intron-1-less transcript. **4, 5:** Transcripts resulting from alternative initiation sites and alternative splicing of intron 1. **6**: Transcript resulting from an alternative initiation site placed within exon 2 and alternative splicing of intron 2. Predicted secondary structures of the 5′UTRs were obtained by using the program RNAdraw designed by Ole Matzura (Department of Medical Biophysiscs, Karolinska Institute, Sweden). Calculated free energy values for the structures at 37°C were −38.4 kJ/mol (1), −119 kJ/mol (2), −114 kJ/mol (3), −60 kJ/mol (4), −1.0 kJ/mol (6), and −178.5 kJ/mol (6). These sequences have been deposited in the GeneBank database under the accession numbers AF047874, AF047875, AF047876, AF047877, AF047878, and AF047879.

In addition to the GAPDH transcript variants previously described, we sequenced two clones from mature chicken testis, with alternative initiations and alternative splicings of intron 1 (transcripts 4 and 5 in Figs. 1, 3). Another clone shows alternative initiation and

alternative splicing of intron 2 (transcript 6 in in Figs. 1, 3). Transcript 6 initiates at the end of the second exon, downstream from the initiation codon ATG. This form, if translated from the first ATG in frame, placed in the third exon at nucleotide 1857 (Fig. 1), would result in a protein lacking the NAD binding domain. Computer analysis indicates that the lengthened 5′-untranslated sequences of transcripts 2, 3 and 6 allow the formation of hairpin structures (Fig. 3). These structures could increase the stability of the transcripts.

Figure 4 shows the different initiation sites of mature testis GAPDH identified by primer extension analysis. In contrast with mature testis, other tissues such as kidney or immature testis show no heterogeneity in the start of transcription. The relative amounts of the different GAPDH transcripts were quantified by PCR-MIMIC in mature testis, immature testis, and kidney (Fig. 5). The somatic transcript is the predominant form in all tissues studied. Only mature testis possesses a significant amount of intron-1-less and TATA-plus transcripts, in agreement with the results obtained with Northern, primer extension, and sequence analyses.

TATA-Plus 5′ Leader Sequence and the Intron-1-Less Transcript of GAPDH, Specifically Expressed in Adult Testis, Are Not Present in Immature Testis and Somatic Tissues

With nonradioactive probes, one specific for the TATA-plus transcript and one specific for the intron-1-less GAPDH transcript, we analyzed Northern blots from brain, heart, and immature and mature chicken testis. The TATA-plus transcripts and the intron-1-less GAPDH transcripts were detectable only in adult chicken testis (Fig. 6), indicating that these transcripts are expressed specifically in meiotic and/or postmeiotic stages of spermatogenesis.

GAPDH Is Abundantly Expressed and Polyadenylated in Adult Testis in Relation to Immature Testis and Somatic Tissues

RNA was isolated from three sources: (1) immature testis enriched with spermatogonia,

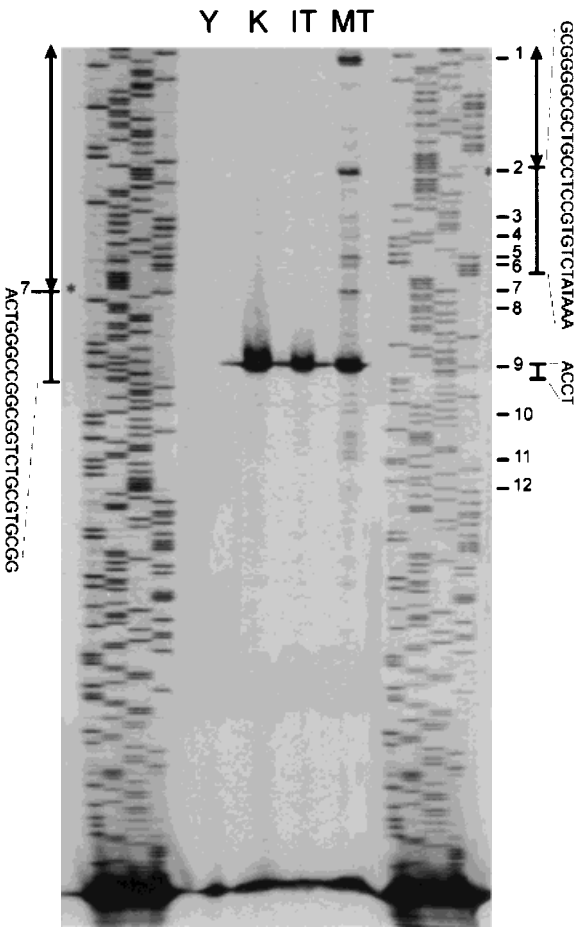

Fig. 4. Primer extension analyses of glyceraldehyde-3-phosphate dehydrogenase mRNA start sites. Primer extension assays were performed by using a primer specific to a sequence in the second exon of the somatic transcript. This primer hybridizes with the sequence between nucleotides 142 and 171, downstream of the canonical site described for the somatic transcript. Total RNA from kidney (K), immature testis (IT), and mature testis (MT) was used. A control sample (Y) of torulla yeast RNA was also used. The amount of product loaded in the gel corresponds to 80 µg primed-extended RNA (mature testis and control yeast) or to 100 µg (kidney and immature testis). Kidney and immature testes show only the canonical initiation site, a cluster of bands around A in the sequence ACCTT. No other bands were detected in kidney or immature testis even when loading 100 µg of primer-extended RNA. In contrast, the mature testis lane shows several bands that correspond to the size of the different initiation sites characterized by sequencing. Additional bands are also detected only in mature testis. These could be either truncated transcripts or uncharacterized initiation sites. The most marked bands other than the canonical one correspond to the TATA-plus and intron-1-less transcripts. Numbers indicate the products that correspond in size to the alternative initiation sites identified by sequencing. **1**: The band located 247–248 nucleotides upstream of the primer has not been identified by sequencing. It may correspond to a longer TATA-plus form. **2–6:** *TATA-plus transcripts.* **7**: Intron-1-less transcript. **8**: Alternative initiation and alternative splicing of intron 1. **9**: Somatic-type transcript. **10**: Alternative initiation and alternative splicing of intron 1. **11**: Alternative initiation and alternative splicing of intron 1. **12**: Alternative initiation site, placed within exon 2, and alternative splicing of intron 2. Left and right of the primer extension products, the sequences of the intron-1-less and TATA-plus recombinant clones were run as markers. These sequences were obtained with the same primer used for primer extension. The loading order is ACGT.

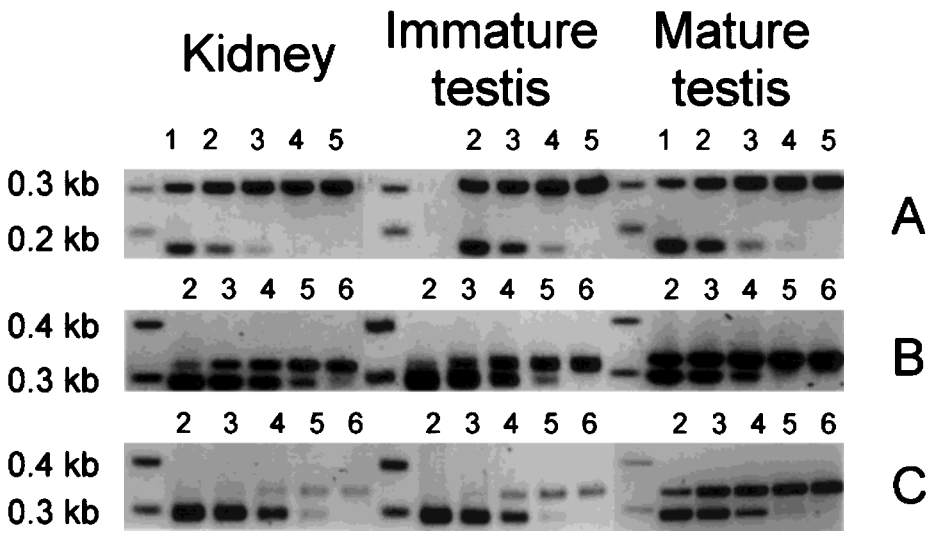

Fig. 5. Competitive polymerase chain reaction (PCR) analysis using a constant amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and 10-fold serial dilutions of a GAPDH MIMIC. One-tenth of PCR products was resolved on a 3% EtBr-agarose-gel (see Materials and Methods). **Lanes 1–6:** 2×10^{1}, 2×10^{0}, 2×10^{-1}, 2×10^{-2}, 2×10^{-3}, and 2×10^{-4} amol GAPDH-MIMIC, respectively. A ladder 100 (Pharmacia) was used as size marker. **A:** GAPDH somatic transcript. **B:** Intron-1-less GAPDH transcript. **C:** TATA-plus GAPDH transcript.

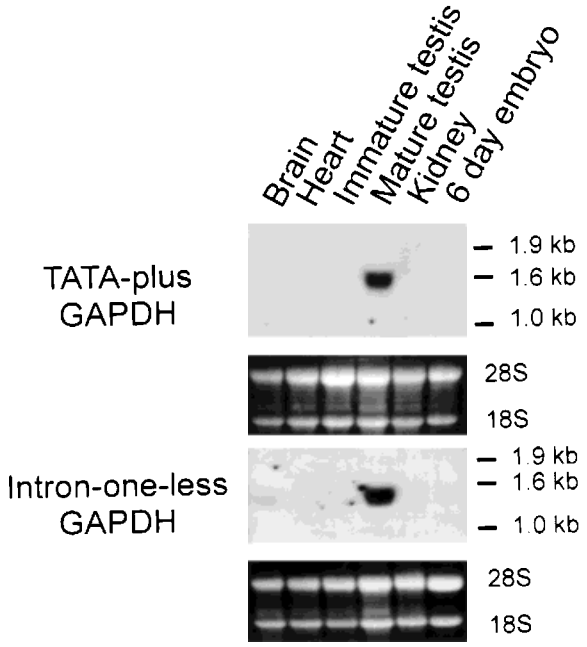

Fig. 6. Northern blotting analysis of the TATA-plus and intron-1-less glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts in brain, heart, immature testis, mature testis, kidney, and 6-day-old embryo. Samples were electrophoresed for 2.5 h. The probes are described in Materials and Methods. Panels with 28S and 18S ribosomal RNAs show the equalization of RNA samples used for analysis.

(2) adult chicken testis enriched with meiotic and postmeiotic cells, and (3) different somatic tissues: brain, heart, and 6-day-old embryos. The RNAs were analyzed by Northern blot hybridization with a probe from the coding region. The probe detected an abundant GADPDH transcript in adult testis and less amounts in prepuberal testis, brain, heart, and 6-day-old embryos (Fig. 7). In addition to differences in the amount of transcripts, there are marked differences in heterogeneity. The heterogeneity is due in part to different degrees of polyadenylation (Fig. 8). The major part of the transcript expressed in the heart has a short polyadenylation tail. Mature testis, however, display the highest level of heterogeneity, with very long polyadenylation tails. The heterogeneity of the GAPDH transcripts in mature testis does not disappear completely after RNase H treatment because of the existence of alternative transcripts differing in the 5′UTR.

Cellular Origin of GAPDH Transcripts Expressed in Adult Testis

To determine more precisely which spermatogenic cells express the specific GAPDH transcripts found in adult testis, we separated chicken male germ cells by centrifugal elutriation. The following fractions were used: (1) meiotic and premeiotic cells, (2) early spermatids, and (3) late spermatids. Both the TATA-plus transcript and the intron-1-less transcript are present in fractions containing meiotic cells, early spermatids, and late spermatids (Fig. 9). The transcripts are abundant in early spermatids and decrease markedly in late spermatids. In these cells, the electrophoretic mobility of the GAPDH transcripts increases. The size of

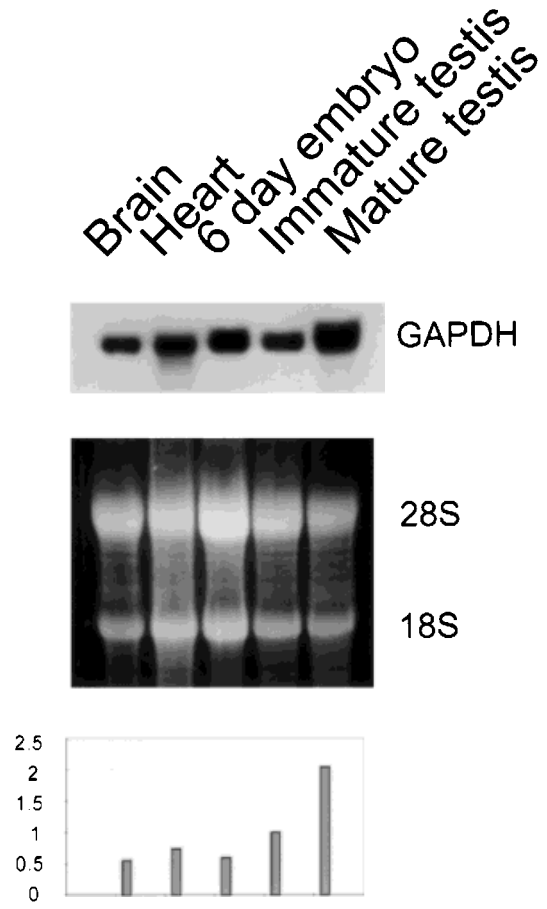

Fig. 7. Northern blotting analysis of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in different chicken tissues (brain, heart, 6-day-old embryos, immature testis, and mature testis). Total RNA (20 µg) was applied to each lane. Total RNA was prepared, electrophoresed for 2.5 h, and hybridized with the GAPDH probe described in Materials and Methods. The middle panel shows the equalization of RNA samples used for analysis. The lower panel represents the relative abundance of the GAPDH transcript. The values were determined by quantitative scanning densitometry of the Northern blots. Data were normalized by the relative intensity of the 28S ribosomal band.

the GAPDH transcripts in late spermatids is shorter than the polyadenylated forms and longer than the deadenylated forms obtained after RNase H treatment, indicating that shortening of the poly(A) tails is taking place during spermiogenesis. In early spermatids, certain mRNAs with long poly(A) tails are translationally repressed and become translationally active when the poly(A) tracts shorten in late spermatids [Kleene, 1996].

Heat Shock Increases Transcription of GAPDH and Polyadenylation of GAPDH Transcripts in Adult Chicken Testis

The amount of GAPDH transcript, already abundant in mature chicken testis, further in-

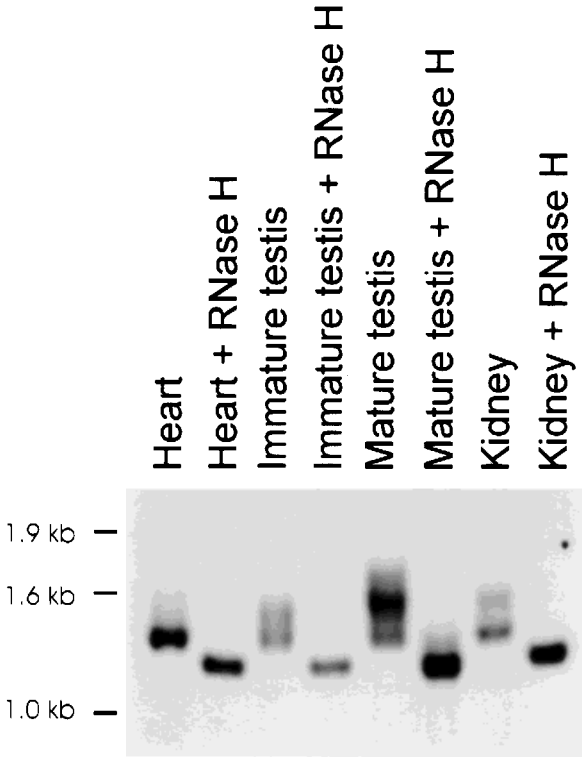

Fig. 8. Northern blotting analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA obtained from heart, immature testis, mature testis, and kidney before and after treatment with RNase H to digest the poly(A) tails. The GAPDH probe used is described in Materials and Methods. Samples were electrophoresed for 4 h.

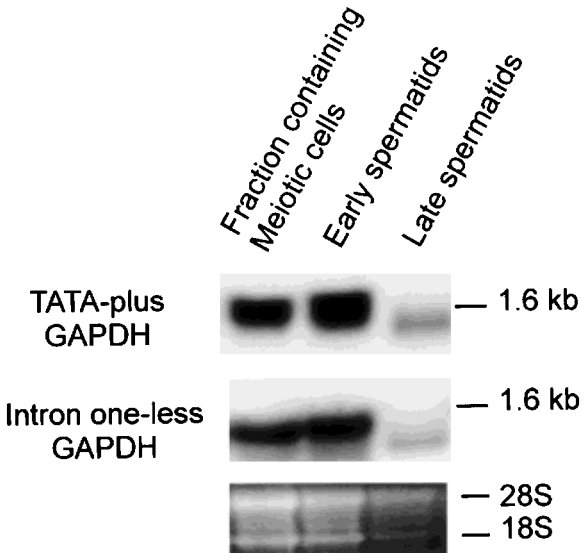

Fig. 9. Northern blotting analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA obtained from a fraction containing meiotic cells, early spermatids, and late spermatids. Cell fractions were obtained by centrifugal elutriation, as previously described [Roca and Mezquita, 1989]. Probes are described in Methods. The lower panel shows the equalization of RNA samples used for analysis.

creased when a testicular cell suspension was exposed to heat shock. Cells from adult chicken testis were exposed to 46°C for 2 h. Control cells were incubated at the internal body temperature of 40°C for the same length of time. Total RNA was prepared from heat-shocked and control cells and analyzed by Northern blot hybridization with a probe from the GAPDH coding sequence. The GAPDH transcript is more abundant in heat-shocked cells than in control cells (Fig. 10). The amount of GAPDH mRNA induced by heat shock is similar to the amount of the heat-shock inducible polyubiquitin gene UbI (results not shown) and much less than the amount of HSP70 induced under the same circumstances. In addition, heat shock increases the length of the GAPDH transcripts, giving rise to broader bands in Northern blots. Treatment with RNase H demonstrates that the larger sizes of GAPDH transcripts induced by heat shock are due to polyadenylation (results not shown). In immature testis, the amount of GAPDH transcript decreases with heat shock, and no polyadenylation was observed (Fig. 10).

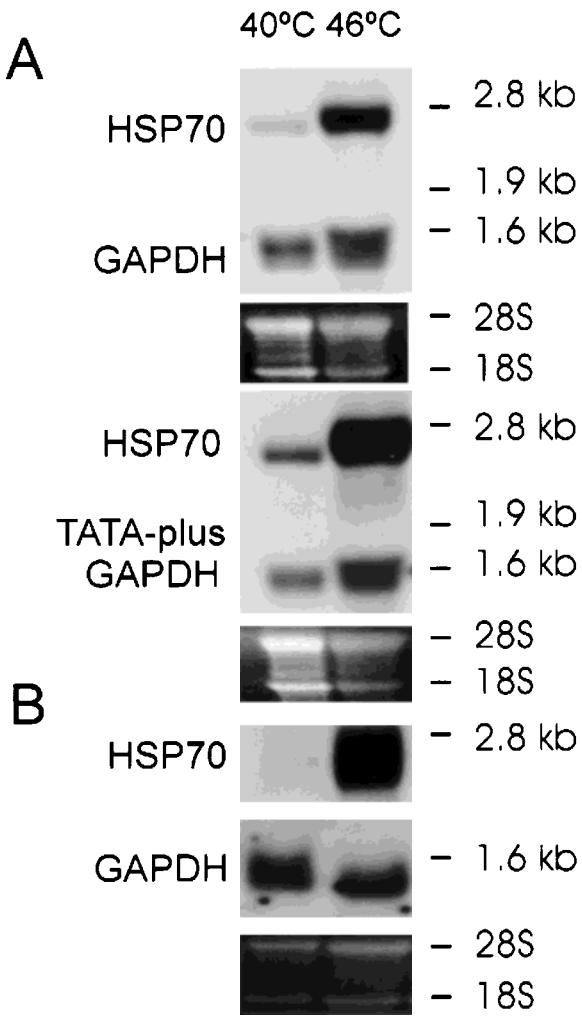

Fig. 10. Northern blotting analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA obtained from heat shocked adult testis cell suspensions (**A**) and prepuberal testis cell suspensions (**B**) in relation to control cells. Cell suspensions prepared from adult chicken testis and immature testis were exposed to 46°C for 2 h or were incubated for the same length of time at the chicken body internal temperature of 40°C. The expression of the protein HSP70 is used as a control of heat shock. Panels with 28S and 18S ribosomal RNAs show the equalization of RNA samples used for analysis.

DISCUSSION

The present study identifies for the first time several novel transcripts of the multifunctional protein GAPDH. The new transcripts are expressed in adult testis and differ from the canonical somatic form in the transcription start site and/or in the splicing of the first and second introns. The heterogeneity of the GAPDH transcripts in adult testis has been demonstrated by sequencing and by primer extension analysis.

One type of transcript found in adult testis results from the use of an initiation site placed upstream from the TATA box used in somatic tissues. The transcript incorporates the TATA sequence in the 5′UTR. The transcription start site is closer to the positive regulatory elements reported in the human GAPDH gene, placed from −181 to −144 relative to the somatic transcriptional initiation site [Aki et al., 1997]. We previously reported a similar observation in several genes expressed in meiotic and postmeiotic stages of chicken spermatogenesis: polyubiquitin UbI [Mezquita et al., 1997], polyubiquitin UbII [Mezquita et al., 1993], and carbonic anhydrase II [Mezquita et al., 1994]. The use of alternative promoters placed upstream from the TATA box, in addition to opening the possibility for new potential mechanisms of transcriptional control, produces extended 5′UTRs that could stabilize the message and ensure delayed translation at the end of spermiogenesis, when transcription is not longer active. The potential secondary structures of the TATA-plus GAPDH mRNA placed near the initiation site (Fig. 3) may block translation [Kozak, 1991; Sagliocco et al., 1993], in accordance with this interpretation. We do not know the reason for the functional TATA-less expression of GAPDH and other genes in chicken spermatogenesis. In mammals, an isoform of the GAPDH is expressed only in postmeiotic stages of spermato-

genesis [Welch et al., 1995]. The promoter region of this isoform contains no TATA box and, like other TATA-less genes, shows heterogeneity in the site of transcription initiation.

Another type of GAPDH transcript expressed only in meiotic and postmeiotic cells uses an initiation site placed downstream from the TATA box, within the 5′ intron 1, resulting in an intron-1-less transcript. We previously reported the same phenomenon, 5′ intron-less transcription, in the polyubiquitin gene UbII expressed in meiotic and postmeiotic stages of chicken spermatogenesis [Mezquita et al., 1993]. As we have suggested above for the TATA-plus GAPDH transcript, the alternative initiation site of the 5′ intron-1-less transcript would also offer new possibilities of regulation of gene expression at the level of transcription and translation. The lengthened 5′UTR of the 5′ intron-1-less transcript possesses secondary structures near the initiation site that can delay translation. Although the function of the first GAPDH intron is not known at present, it has been suggested that the first intron of GAPDH in maize cells would stimulate anaerobic expression and splicing efficiency [Kohler et al., 1996].

In addition to the 5′ intron-1-less transcripts and the TATA-plus transcripts, we have characterized two alternative forms of splicing of intron 1 transcript, placed in the 5′ region of the gene, and one alternative form of splicing of intron 2. Alternative splicing of the 5′ intron also occurs in the heat-inducible polyubiquitin gene UbI in meiotic and postmeiotic stages of chicken spermatogenesis [Mezquita et al., 1997]. Additional heterogeneity observed in the GAPDH transcripts is caused by polyadenylation. GAPDH transcripts in adult chicken testis are highly polyadenylated in relation to immature testis and somatic tissues. Polyadenylation appears to function in cytoplasmic control of mRNA translation and stability. Regulation of mRNA translation and stability is unusually important during meiotic and haploid phases of spermatogenesis [Kleene, 1996]. Numerous mRNAs in meiotic and postmeiotic spermatogenic cells are translationally repressed. These mRNAs become translationally active in late stages of spermiogenesis, when the poly(A) tails are shorter.

The main finding of this study, the heterogeneity of the 5′UTR of GAPDH expressed in meiotic and postmeiotic stages of spermatogenesis due to alternative initiation and alternative splicing, could provide the necessary versatility to the regulation of expression of this multifunctional protein during spermatogenesis. GAPDH has been proposed to be a key enzyme regulating glycolysis in spermatogenic cells [Nakamura et al., 1984]. It is also possible that GAPDH, abundantly expressed in meiotic and postmeiotic chicken cells, could play essential roles in nonglycolytic functions during spermatogenesis. Particularly interesting among these nonglycolytic functions is the ability of GAPDH to bind specifically to mRNAs and regulate translation [Sugahara et al., 1995; De et al., 1996; McGowan and Pekala, 1996]. Specific interaction of GAPDH with the 5′UTR RNA of hepatitis A virus may be an important determinant of viral translation activity [Schultz et al., 1996]. GAPDH also binds selectively AU-rich RNA and may be important in the regulation of turnover and translation of these mRNAs [Nagy and Rigby, 1995]. Further studies will show whether a similar function of GAPDH is responsible for the delayed translation of certain messages during spermiogenesis.

The present work shows that GAPDH mRNAs, already abundant in adult testis, increase when chicken testis cells are exposed to heat shock. GAPDH is a stress protein induced by heat shock and hypoxia [Yang et al., 1993; Graven et al., 1994; Laxalt et al., 1996]. In the yeast Saccharomyces cerevisiae, one GAPDH gene is induced by heat shock [Lindquist and Craig, 1988; Mager and Ferreira, 1993; Boucherie et al., 1995]. The increased GAPDH synthesis with heat shock may be beneficial because it enables the cell to increase the rate of glycolysis, thereby restoring the intracellullar level of ATP. It is also possible that the GAPDH induced by heat shock could play a cellular role distinct from glycolysis that is required by stressed cells. Further studies will show whether the increase in the amount of GAPDH mRNA and the increased polyadenylation we observed in adult chicken testis with heat shock may contribute to the development of thermotolerance during avian spermatogenesis, as has been reported in Xenopus laevis embryos [Nickells and Browder, 1988].

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