Expression of Lactate Dehydrogenases A and B During Chicken Spermatogenesis: Characterization of Testis Specific Transcripts

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Abstract   The substrates required for glycolysis change markedly at successive stages of spermatogenesis suggesting a considerable plasticity in the expression of glycolytic enzymes. Lactate dehydrogenase (LDH) isoenzymes, LDH-A and LDH-B, are expressed in premeiotic, meiotic cells, and early spermatids, both in avian and mammalian spermatogenesis. Highly polyadenylated forms, particularly of LDH-A, were detected in chicken testis. While mammals and columbid birds express the testis specific LDH-C gene in meiotic and postmeiotic cells, several LDH-B testis specific transcripts were detected in the corresponding cells during chicken spermatogenesis. These testis specific transcripts and the mRNA of mammalian LDH-C show several properties in common, such as temporal correlation of expression, mRNA stability, and repression of premature translation. These observations suggest that the testis specific transcripts could perform during chicken spermatogenesis the functions of the LDH-C mRNA in mammalian testis. J. Cell. Biochem. 79:15–27, 2000.   © 2000 Wiley-Liss, Inc.

Key words: 5'UTR; mRNA stability; polyadenylation; translation

Lactate dehydrogenases (LDH; EC 1.1.1.27) catalyze the reversible oxidation of lactate to pyruvate, using NAD^+ as a coenzyme. The LDH gene family is differentially expressed in tissue-specific patterns. In vertebrates there are three different subunits of LDH isozymes: LDH-A converts pyruvate to lactate under anaerobic conditions and predominates in skeletal muscle and liver; LDH-B kinetically favors the conversion of lactate to pyruvate and is found in aerobic tissues such as heart; and LDH-C, the third type of isoenzyme, with a broad substrate specificity, is expressed in mature testis in mammals and columbid birds [Goldberg, 1963, 1977; Zinkham et al., 1964; Markert et al., 1975; Holbrook et al., 1975; Li et al., 1983; Markert, 1984; Burgos et al., 1995]. The pigeon LDH-C isozyme is not present in other avian species and, despite the fact of being testis specific, it is not the orthologous of

the mammalian testicular LDH-C. The avian protein arose from a recent duplication of LDH-B, while the mammalian LDH-C originated from a duplication of LDH-A [Markert et al., 1975; Li et al., 1983; Mannen et al., 1997; Stock et al., 1997]. In addition to the testis specific isoenzyme, the somatic forms LDH-A and LDH-B are also expressed in mammalian testis [Thomas et al., 1990].

During spermatogenesis, spermatogonia utilize glucose as their major energy substrate [Nakamura et al., 1984]. However, spermatocytes and spermatids suffer a rapid decline in their ATP content in the presence of glucose and require lactate/pyruvate for the maintenance of their ATP concentration [Jutte et al., 1981; Mita and Hall, 1982]. Lactate is supplied to spermatocytes and spermatids by Sertoli cells, which convert glucose into lactate [Robinson and Fritz, 1981; Jutte et al., 1981; Grootegoed et al, 1986] and secret lactate towards the adluminal compartment, where it is the major energy substrate for meiotic cells and round spermatids. Lactate secretion by Sertoli cells is stimulated by the follicle-stimulating hormone [Mita et al., 1982; Jutte, et al., 1983].

At the end of spermatogenesis, spermatozoa, in contrast to round spermatids, possess a high glycolytic capacity [Brooks, 1976] and are able to use glucose/fructose as the major source of energy [Bajpai et al., 1998]. Sequestration of glycolytic enzymes to the motile apparatus of the sperm flagellum may be important for generation of the energy required for the hyper activated motility of fertilization [Fraser and Quinn, 1981; Bradley et al., 1996; Westhoff and Kamp, 1997; Mahadevan et al., 1997; Bunch et al., 1998].

Although much is known on the function of glycolytic enzymes and on the biochemical mechanisms involved in their catalytic action [Martínez Arias and Pettersson 1996; Martínez Arias et al., 1998], transcriptional and post-transcriptional regulation of their expression is less understood. At present, a great deal of interest in LDH enzymes is related with their expression in cancer cells [reviewed in Dang et al., 1997]. The dramatic changes in the substrates required for energy at successive stages of spermatogenesis suggest a considerable plasticity in the expression of glycolytic enzymes during this process. Spermatogenesis is an excellent model to study the expression of several glycolytic enzymes that are codified by genes specifically expressed in the testis or by testis specific transcripts [Eddy, 1995; Cooker et al., 1993; Mezquita et al., 1998]. Previously we have characterized several chicken testis specific transcripts for the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) [Mezquita et al., 1998]. Our results indicated that alternative initiation, alternative splicing, and polyadenylation could provide the necessary versatility to the regulation of the expression of this glycolytic enzyme during the differentiation of the male gamete. The purpose of the present work was to determine whether the expression of other glycolytic enzymes, such as lactate dehydrogenases, shows similar plasticity in their transcripts. We report here marked quantitative and qualitative differences in the expression of LDH-A and LDH-B during chicken spermatogenesis. Several novel LDH-B transcripts, resulting from alternative initiation of transcription and possible alternative splicing, have been characterized. LDH-B and particularly LDH-A are highly polyadenylated in chicken testis in relation to somatic tissues. In addition, we report that chicken LDH-B mRNA expressed during spermatogenesis is stable

and most of it is not polysome-associated like mammalian LDH-C and other testis specific transcripts.

MATERIALS AND METHODS
cDNA Synthesis

Whole-size cDNA from adult testis was obtained by using the Amplifinder RACE protocol from Clontech (Palo Alto, CA). Briefly, first-strand cDNA was prepared using an oligo d(T) primer (10 pmols) or N6 random hexanucleotides (100 ng), 2 µg of poly A^+ RNA, an ultrapure dNTP mix (40 nmols each) and AMV reverse transcriptase (12 units) in reverse transcriptase buffer containing 60 units of RNase inhibitor. First RNA plus primer was denatured at 65°C for 5 min. Then a reverse transcriptase master mix was added and incubated at 52°C for 30 min. RNA was hydrolyzed adding NaOH 6N and incubating at 65°C, 30 min. cDNA was purified with GENO-BIND. An Anchor primer (Clontech) was ligated at the 5′ end for RACE analysis of the cDNA. AmpliFINDER Anchor (8 pmols) and T4 RNA ligase (10 units) were used in ligase buffer. Ligation was carried at room temperature for 18 h. One µl of 1:10 dilution of the ligation mixture was used for RACE experiments.

Rapid Amplification of cDNA Ends (RACE)

The primers GTCTGAAGGAACAAGCTGC-CATGC and CTTGCCGAGGATGCTGATGGC in conjunction with the anchor primer AGGT-TCCAGAATCGATAGTG were used in two consecutive PCR reactions for 5′ RACE. The primers GGTTGTTGAAAGTGCCTATGAGG and CGAACTGGGCCATTGGTCTTAGC and the oligo d(T) primer T_{30} (AGC)(AGCT) were use in similar PCR reactions for 3′RACE. RACE-PCR conditions were as follow: 20 pmols of each primer, 40 nmol of dNTPs, 2 mM Mg^{++} and 1 unit of DNA Polymerase (Taq and High Fidelity Pyrococus GB-D Polymerase, Life Technologies, Paisley, Scotland). Incubations were performed, in a final volume of 50 µl, for 30 s at 94°C and 30 cycles of 30 s at 94°C, 30 s at 60°C; and 2 min at 68°C.

Purification of PCR Products, Cloning, and Sequencing

PCR products were purified free of oligonucleotides with Millipore Ultrafree-MC 30,000 filters. Then, blunt ended with Pfu DNA poly-

merase and ligated to the vector pCR-Script SK+ (Stratagene, La Jolla, CA). JM109 cells were made electrocompetent after gently washing them with ddH$_2$O, then electroporated with 1:5 dilution of the ligated insert in pCR-Script. White colonies were grown in 5 ml of LB-Ampicillin media and recombinant DNA minipreps were obtained using the *Promega Wizard Plus SV Minipreps DNA Purification System A1470* (Madison, WI).

Sequencing was done with the Licor MWG Biotech GmbH automatic sequencer by using the SequiTherm EXCEL ll Long-Read (Epicentre Technologies, Madison, WI) protocol with the forward or reverse primers labeled with IRD700 (MWG-Biotech GmbH, Ebersberg, Germany) and a cycle-sequencing program of 3 min at 95°C, 30 cycles of 15 s at 95°C, 15 s at 64°C, and 30 s at 70°C.

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

Total RNA was prepared with TRIZOL Isolation Reagent from Life Technologies, according to the specifications of the manufacturer. RNA was obtained from prepuberal testis, adult testis, and several somatic tissues: heart, kidney, skeletal muscle, and liver. RNA concentrations and purity were determined by absorbance at 260 and 280 nm. The samples (15–30 μg) were electrophoresed in 1.6% agarose gels, 20 mM MOPS, 2 M formaldehyde, 5 mM sodium acetate (pH 7.0), and 1 mM EDTA. Ethidium bromide was added to the samples for visualization of 28S and 18S rRNA under UV illumination. The gels were 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) and labeled with non-radioactive digoxigenin-specific probes as described below. Hybridization conditions and the immunological chemiluminiscence detection procedure were performed as described by Engler-Blum et al. [1993].

LDH-B cDNA Probe

A non-radioactive probe was obtained from a recombinant clone containing 711bp of the coding region of LDH-B by PCR amplification using the forward AGGGTTTTCCCAGTCAC-GACGTT and reverse primers AGGAAACA-GCTATGACCATG of the vector pPCR-Script Amp SK (+), an Expand-High Fidelity DNA polymerase (*PCR DIG Probe Synthesis kit,* Boehringer Mannheim, Germany), and a dNTP mix containing dUTP-digoxigenin (DIG –11-dUTP). PCR conditions were as follow: 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 52°C and 3 min at 72°C, and a final extension of 7 min at 72°C. The probe was purified free of nucleotides with Millipore Ultrafree MC 30,000 filters. For hybridization assays, 5–20 ng/ml of probe were used.

LDH-A cDNA Probe

The primers CCATGTCTCTCAAGGATC-ATCTC and GCACCAGCAGTGACAATGAC deduced from the published sequence [Hirota et al., 1990] and spanning 295 nucleotides of coding region (nucleotides 58-352, Bank accession number X53828) were used to amplify first-strand cDNA from testis. The PCR product was cloned in pCR-Script and sequenced to confirm that it was LDH-A. A non-radioactive probe was obtained by amplifying a LDH-A recombinant clone using the forward and reverse primers from the vector, in the same conditions described for LDH-B cDNA probe.

RNAase H Treatment

The method described by Kleene et al. [1984] was used. RNA was isolated from different tissues: heart, kidney, skeletal muscle, liver, prepuberal testis, and adult testis. Each RNA (20–30 μg) was 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, two units of RNAase H were added (Pharmacia Biothech) and incubated at 37°C for 30 min. The samples were extracted with phenol-chloroform, followed by ethanol precipitation and analyzed in agarose-formaldehyde gels 1.6%, as previously described, in parallel with control samples from the same tissues.

Cell Isolation by Centrifugal Elutriation

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 (18 ml/min flow rate), meiotic and premeiotic cells (36 ml/min flow rate). The purity of the fractions was determined by phase contrast microscopy. Separated cells were centrifuged at 1,000g for 5 min and the pellet was extracted with TRIZOL as mentioned before.

Determination of mRNA Stability

For mRNA stability assays Actinomycin-D (cell culture grade, Sigma), 5 μg/ml, was added to primary cultures of chicken testis cells (1–6 $\times$ 10^5 cells/20 ml) prepared as described in the previous paragraph. Cells were placed in sterilized Petri plates (100 mm) containing MEM (GIBCO/BRL) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. At 2-h intervals cells were harvested and the RNA was isolated and Northern blotted. The blots were hybridized successively with probes for LDH-A and LDH-B, and QKI transcripts. The bands were densitometrically scanned and the rate of decay was determined.

Polysomal Gradients

Fractionation of post-mitochondrial extracts over sucrose gradients was performed as previously described [Mezquita et al., 1999]. The tissue, 1,500 mg, was homogenized at 4°C in 6 ml of buffer containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM HEPES pH 7.5, 1 mg/ml heparin, 0.2 μl/ml diethyl pyrocarbonate and 90 μg/ml cycloheximide with 10 strokes of a Dounce homogenizer. The homogenate was centrifuged (5,000g) for 5 min at 4°C and the supernatant was centrifuged again (12,000g) for 10 min at 4°C in the presence of Triton N101 0.5% v/v. The final supernatant (1 ml) was layered onto a 8 ml 15–40% linear sucrose gradient containing 100 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES pH 7.5, 1 mg/ml heparin, and 0.2 μl/ml diethyl pyrocarbonate and then centrifuged for 110 min at 4°C in a Beckman SW40 rotor at 36,000 rpm. Six fractions of 1.5 ml were collected and precipitated with ethanol overnight at −20°C.

RESULTS

The Expression of LDH-B and LDH-A is Different in Adult Chicken Testis and Somatic Tissues

To characterize the expression of LDH-B and LDH-A in different chicken tissues we have used specific probes from the coding region of both transcripts. RNA was isolated as described in Materials and Methods from prepuberal testis (6-week-old chickens), adult testis (6-month-old chickens), and several somatic tissues from adult animals: heart, liver, kidney, and skeletal muscle. The expression patterns of LDH genes were analyzed by Northern blot and immunological chemiluminescence's procedures.

The results revealed quantitative differences in the expression of LDH-B and LDH-A transcripts between testis and somatic tissues, as shown in Figure 1. The expression of LDH-B was high in heart, kidney, and adult testis and low in liver, skeletal muscle, and immature testis. LDH-A transcripts clearly predominated in skeletal muscle and in adult testis, compared with its weak expression observed in other somatic tissues and immature testis. The results also showed qualitative changes in the expression of both LDH-B and LDH-A between adult testis, immature testis, and somatic tissues (Fig. 1). The pattern of expression of LDH-B in adult testis revealed two bands of similar intensity, while only one band was observed in somatic tissues and immature testis. LDH-A showed in testis a broad band of lower electrophoretic mobility than the corresponding band of somatic tissues.

To identify how polyadenylation contributed to the observed heterogeneity in both transcripts we proceeded to digest the poly (A) tails by treatment with RNase H. As shown in Figure 1, the treatment eliminated the heterogeneity observed in somatic tissues and immature testis but did not affect the pattern observed in adult testis that showed two hybridization bands in contrast with a single band present in other tissues. This indicates that different transcripts of LDH-B exist in germ cells.

In the case of LDH-A, the treatment eliminated the heterogeneity detected in adult testis but not in liver, which showed two hybridization bands (Fig. 1). The level of LDH-A polyad-
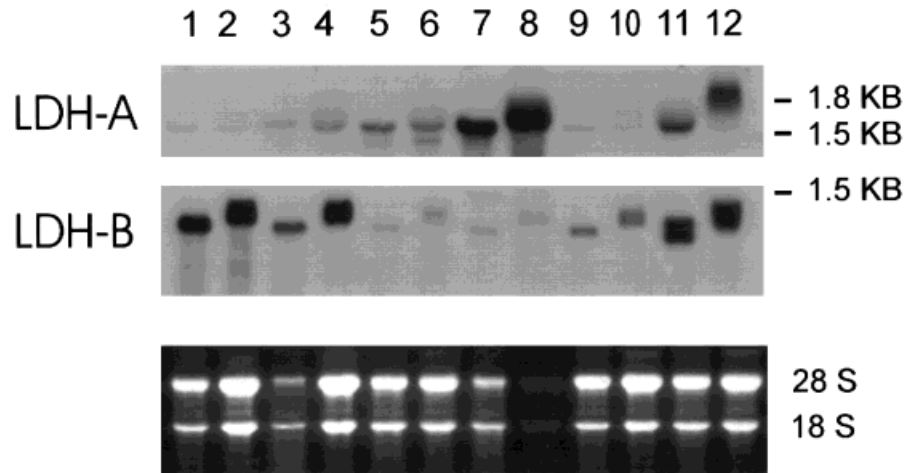
Fig. 1. Northern blotting analysis of LDH-A and LDH-B mRNAs obtained from heart (**1,2**), kidney (**3,4**), liver (**5,6**), skeletal muscle (**7,8**), prepuberal testis (**9,10**), and adult testis (**11,12**) with (1, 3, 5, 7, 9, and 11) and without (2, 4, 6, 8, 10, and 12) RNase H treatment. Probes used are described in Materials and Methods. The lower panel shows the equalization of RNA samples used for analysis.

enylation was very low in heart, kidney, liver and immature testis (Fig. 1). In skeletal muscle, polyadenylated tails are shorter than in the testis, which showed a broad band of highly polyadenylated transcripts.

A Novel Transcript of LDH-B and a Highly Polyadenylated Transcript of LDH-A are Expressed During Spermatogenesis

Previous studies in mammals and columbid birds have demonstrated that LDH-C isoenzymes are found only in mature testis and that both LDH-A and LDH-B are expressed during certain stages of spermatogenesis [Thomas et al., 1990; Zinkham et al., 1964]. In immature chicken testis, enriched in spermatogonia and Sertoli cells, we observed a pattern of expression of LDH-A and LHD-B similar to somatic tissues. However, in adult testis enriched in meiotic and post-meiotic cells, we have detected specific transcripts of LDH-B and high polyadenylated forms of LDH-A. To determine in which spermatogenic cells the LDH-A/B transcripts were expressed, we isolated chicken male germ cells by centrifugal elutriation. Four main fractions were obtained [Roca and Mezquita, 1987]: (1) meiotic and premeiotic cells, (2) round spermatids, (3) elongated spermatids, and (4) testicular spermatozoa and residual bodies. As shown in Figure 2, both LDH-A and LDH-B were expressed in meiotic

and premeiotic cells as well as in early spermatids. The LDH-B transcript was also present in elongated spermatids and in the fraction containing testicular spermatozoa and residual bodies. Both the somatic and the testicular specific transcripts of LDH-B persisted until the end of spermatogenesis. The size of the LDH-B transcripts in late spermatids is shorter than the polyadenylated forms of previous stages and longer than deadenylated forms obtained after RNase treatment, indicating that shortening of poly (A) tails is taking place during spermiogenesis (Figs. 1 and 2). It has been proposed that in early spermatids, certain mRNA with long poly (A) tails are translationally repressed and become translationally active when the poly(A) tracts shorten in late spermatids [Kleene, 1996].

Novel LDH-B Transcripts in Adult Testis

In order to characterize the observed heterogeneity of LDH-B in adult testis we performed 5′ and 3′ RACE, and proceeded to clone and sequence the PCR products. Three clones from 3′ RACE and 10 clones from 5′ RACE were sequenced. The sequences obtained were compared with chicken heart LDH-B mRNA (GeneBank, F069771). The results indicated that our 3′ UTR LDH-B sequences were identical to the reported sequence from heart. In
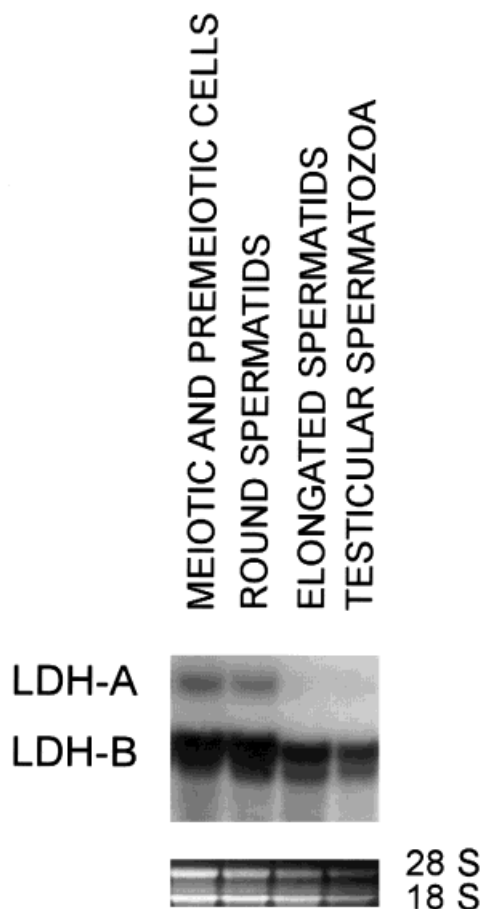
Fig. 2. Northern blotting analysis of LDH-A and LDH-B mR-NAs from testicular cells separated by centrifugal elutriation as previously described [Roca and Mezquita, 1989]. Probes used are described in Materials and Methods. The lower panel shows the equalization of RNA samples used for analysis.

contrast, the 5′UTR LDH-B sequences from chicken adult testis showed marked differences. Two clones, variant LDH-B-T1, expanded the 5′ UTR nucleotide sequence reported in heart with 136 additional nucleotides (Figs. 3 and 4). One clone, variant LDH-B-T2 showed a 5′UTR shorter than T1, and lacked a segment of 37 nucleotides adjacent to the conserved sequence GAGGTT that immediately precedes the ATG translation initiation codon (Fig. 4). The rest of the clones, LDH-B T3, have a completely different 5′UTR, except for the six conserved nucleotides GAGGTT. LDH-B T3 5′UTRs of different lengths (58, 30, 28, 20, and 14 nucleotides) were detected.

The sequence of the LDH-BT1 transcript is identical to the previously reported sequence of heart LDH-B (GeneBank, F069771) except for

two nucleotide changes in the coding region, which do not affect the sequence of the protein product, and two nucleotide changes in the 5′UTR. The extended 5′UTRs of the LDH-B-T1 and LDH-B-T2 showed a 75% GC content and two Sp1 binding sites (Figs. 3 and 4). The typical GC-rich housekeeping promoter of the avian LDH-B gene used in somatic tissues [Kraft et al., 1993] is incorporated in the novel LDH-B transcripts characterized in adult chicken testis. Incorporation of somatic promoters in the testis specific transcripts is the consequence of an alternative initiation of transcription of the gene in meiotic and post-meiotic chicken testis cells. The lengthened GC-rich 5′UTR sequences of LDH-B transcripts may allow the formation of hairpin structures absent in the shorter 5′UTR of somatic cells (Fig. 5A,B). Formation of these potential structures could hinder a premature translation of the transcripts during spermatogenesis. Previously we have observed the same phenomenon in several genes expressed during avian and mammalian spermatogenesis [Mezquita et al., 1999].

Stability of Testicular LDH-A and LDH-B Transcripts

Tissue-specific differences in RNA levels can be due to differences in transcriptional activity or differences in the rate of RNA turnover. To estimate the stability of the LDH-A and LDH-B transcripts in primary cultures of testicular cells, transcription was blocked by adding Actinomycin D and the rate of decay of LDH-A and LDH-B mRNAs was determined by Northern blotting. As shown in Figure 6, treatment with Actinomycin D did not affect markedly the level of LDH-B mRNA. Most of the LDH-B mRNA (85%) and only a 45% of LDH-A remained after 9 h of treatment with Actinomycin D. In contrast, the level of the 6kb quaking transcript QKI, coding for a signal transduction and RNA-binding protein expressed during spermatogenesis [Mezquita et al., 1998a], dropped more rapidly (90% in 9 h). The stability of chicken testis LDH-A mRNA is higher that the previously reported in C6 glioma cells [Jungmann et al., 1983] and similar to the stability measured in Sertoli cells [Boussouar and Benahmed, 1999].

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   1 GAATGCGGATTGAACGCGGAGGCCGCCGCCTCCCGTCGACGCTGTGCCCGCCCCGAGGGC
  61 GCGGTGAGGGGCTCCTCTACGCATCCCAGCCCGCCCCTTCCGCTGCGGAGCGCAGATTTC
 121 CCGAGCCCCGCCACGGTCACGGTACTGCTCCCGGTTCTCCTTTCACCGCACCGATCCGGA
 181 CGTTATGGCGACCCTGAAGGAGAAGCTGATCACCCCCGTGGCCGCGGGCAGCACGGTTCC
        M   A   T   L   K   E   K   L   I   T   P   V   A   A   G   S   T   V   P
 241 CAGCAACAAGATCACCGTGGTGGGGGTCGGGCAGGTGGGGATGGCGTGTGCCATCAGCAT
        S   N   K   I   T   V   V   G   V   G   Q   V   G   M   A   C   A   I   S   I
 301 CCTCGGCAAGGGTCTTTGTGATGAGCTTGCTCTGGTTGATGTTTTGGAAGACAAGCTAAA
        L   G   K   G   L   C   D   E   L   A   L   V   D   V   L   E   D   K   L   K
 361 AGGAGAAATGATGGATCTACAGCATGGCAGCTTGTTCCTTCAGACTCATAAGATTGTGGC
        G   E   M   M   D   L   Q   H   G   S   L   F   L   Q   T   H   K   I   V   A
 421 AGACAAAGATTATGCTGTCACAGCCAACTCCAAGATTGTGGTAGTAACTGCAGGTGTTCG
        D   K   D   Y   A   V   T   A   N   S   K   I   V   V   V   T   A   G   V   R
 481 TCAGCAAGAGGGGGAGAGTCGTCTCAACCTGGTTCAGAGGAATGTGAACGTCTTCAAATT
        Q   Q   E   G   E   S   R   L   N   L   V   Q   R   N   V   N   V   F   K   F
 541 CATCATTCCTCAGATTGTGAAATACAGCCCCAATTGCACTATCCTTGTGGTTTCCAACCC
        I   I   P   Q   I   V   K   Y   S   P   N   C   T   I   L   V   V   S   N   P
 601 AGTGGATATATTAACCTATGTCACATGGAAGTTGAGTGGCCTGCCAAAGCACCGTGTGAT
        V   D   I   L   T   Y   V   T   W   K   L   S   G   L   P   Q   H   R   V   I
 661 TGGAAGTGGCTGCAATCTAGACACAGCTAGATTCCGCTACCTGATGGCTGAGAGACTTGG
        G   S   G   C   N   L   D   T   A   R   F   R   Y   L   M   A   E   R   L   G
 721 TATCCACCCAACCAGCTGCCATGGCTGGATTTTAGGAGAACATGGTGATTCTAGTGTGGC
        I   H   P   T   S   C   H   G   W   I   L   G   E   H   G   D   S   S   V   A
 781 TGTTTGGAGCGGAGTTAATGTGGCAGGTGTTTCTCTCCAGGAGCTGAATCCTGCCATGGG
        V   W   S   G   V   N   V   A   G   V   S   L   Q   E   L   N   P   A   M   G
 841 AACTGACAAAGACAGCGAGAACTGGAAGGAAGTTCACAAGCAGGTTGTTGAAAGTGCCTA
        T   D   K   D   S   E   N   W   K   E   V   H   K   Q   V   V   E   S   A   Y
 901 TGAGGTAATCAGACTCAAGGGGTATACGAACTGGGCCATTGGTCTTAGCGTTGCTGAGCT
        E   V   I   R   L   K   G   Y   T   N   W   A   I   G   L   S   V   A   E   L
 961 CTGTGAGACAATGCTGAAGAACTTGTACCGAGTTCATTCTGTGTCAACACTGGTAAAGGG
        C   E   T   M   L   K   N   L   Y   R   V   H   S   V   S   T   L   V   K   G
1021 CACATATGGCATTGAGAATGATGTCTTCCTGAGCCTGCCTTGTGTCCTGAGTGCCTCTGG
        T   Y   G   I   E   N   D   V   F   L   S   L   P   C   V   L   S   A   S   G
1081 ATTGACAAGTGTCATCAACCAAAAGCTGAAGGATGATGAAGTGGCTCAGCTGAAGAAGAG
        L   T   S   V   I   N   Q   K   L   K   D   D   E   V   A   Q   L   K   K   S
1141 TGCAGACACATTGTGGAGCATCCAGAAAGATCTTAAAGATCTGTAATTCAAATGTTAGAT
        A   D   T   L   W   S   I   Q   K   D   L   K   D   L
1201 TGCAGCAATAGAAAAACAGCGTGTTGTGCACAAATGTGGGCTCTCTACTCACTATACATC
1261 TCTGTGGTTAACATTTAATGCTCTTCCAGACTGAGCTTTTGTCCACGGTAGCTAAACATA
1321 AGCTTGCTGTAACGCACAGACCTTATGAACAAATAAAGCAACTTTCAGGC(A)ₙ
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Fig. 3. Nucleotide and amino acid sequence of the LDH-BT1. This sequence has been deposited in the GeneBank database under the accession number AF218799. The polyadenylation signal is underlined.

Distribution of Testicular LDH Transcripts in Polysomal and Non-Polysomal Fractions

Since it has been reported that more than 70% of all mRNA in testis is mRNP particle associated [Iatrou and Dixon, 1977; Kleene, 1993; Tafuri et al., 1993] and also that testis-specific mRNAs with extended 5′UTRs are translationally less active [Yiu et al, 1994; Gu et al., 1995; Fautsch et al., 1997], we have determined the relative translational utilization of LDH-B mRNA isolated from chicken adult testis. Post-mitochondrial extracts were fractionated by sucrose gradient sedimentation, and purified RNAs were hybridized to LDH-B probe. The majority of the LDH-B was present in non-polysomal fractions (Fig. 7, lane 2). A similar distribution of the testis specific LDH-C in mammals has been reported [Fujimoto et al., 1988; Alcivar et al., 1991; Mezquita et al., 1999]. We have used the distribution of the 6kb QKI mRNA in the same sample as a control. The QKI transcript was present in the rapidly sedimenting polysomal fraction excluding the possibility that the slowly sedimenting
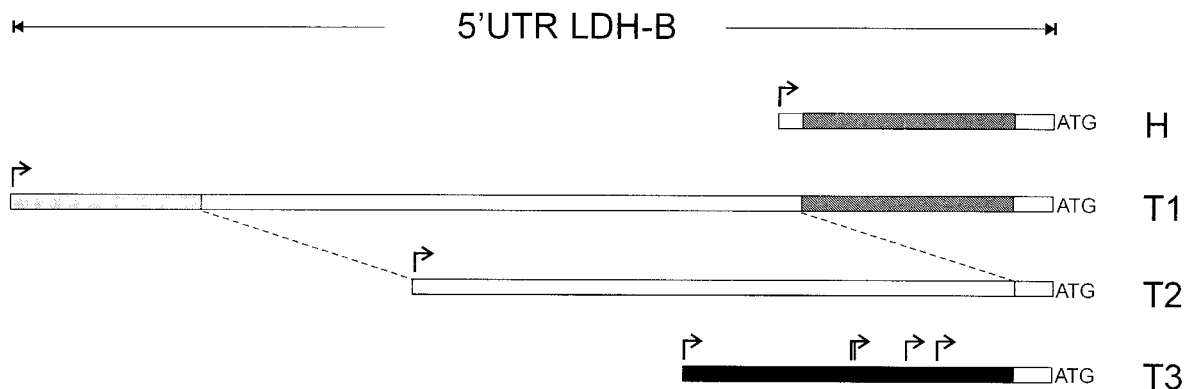
LDH-B HEART

TCACGGCACTGTTCCCGGTTCTCCTTTCACCGCACCGATCCGGACGTT

LDH-BT1

GAATGCGGATTGAACGCGGAGGCCGCCGCCTCCCGTCGACGCTGTGC**CCGCCCC**GAGGGC
GCGGTGAGGGGCTCCTCTACGCATCCCAGC**CCGCCCC**TTCCGCTGCGGAGCGCAGATTTC
CCGAGCCCCGCCACGGTCACGGTACTGCTCCCGGTTCTCCTTTCACCGCACCGATCCGGA
CGTT

LDH-BT2

GTCGACGCTGTGC**CCGCCCC**GAGGGCGCGGTGAGGGGCTCCTCTACGCATCCCAGC**CCGC
CCC**TTCCGCTGCGGAGCGCAGATTTCCCGAGCCCCGCCACGGTCACGGACGTT

LDH-BT3

GGGGCGCGCGCTGGAATGCCGGCCCGTTAAACCGAACCTCCTCCAATCCCTTCTTGTAGG
ACGTT

Fig. 4. Diagram and sequences of the 5'-untranslated regions (5'UTRs) of heart (H) and testicular (T1, T2, and T3) LDH-B mRNAs. The conserved sequence GAGGTT that immediately precedes the ATG translation initiation is underlined. Two Sp1 binding sites are shown in bold. Alternative start sites are indicated by squares. These sequences have been deposited in the GeneBank database under the accession numbers AF218799, AF218800, and AF218801. The 5'UTR from heart LDH-B is from GeneBank, F069771.
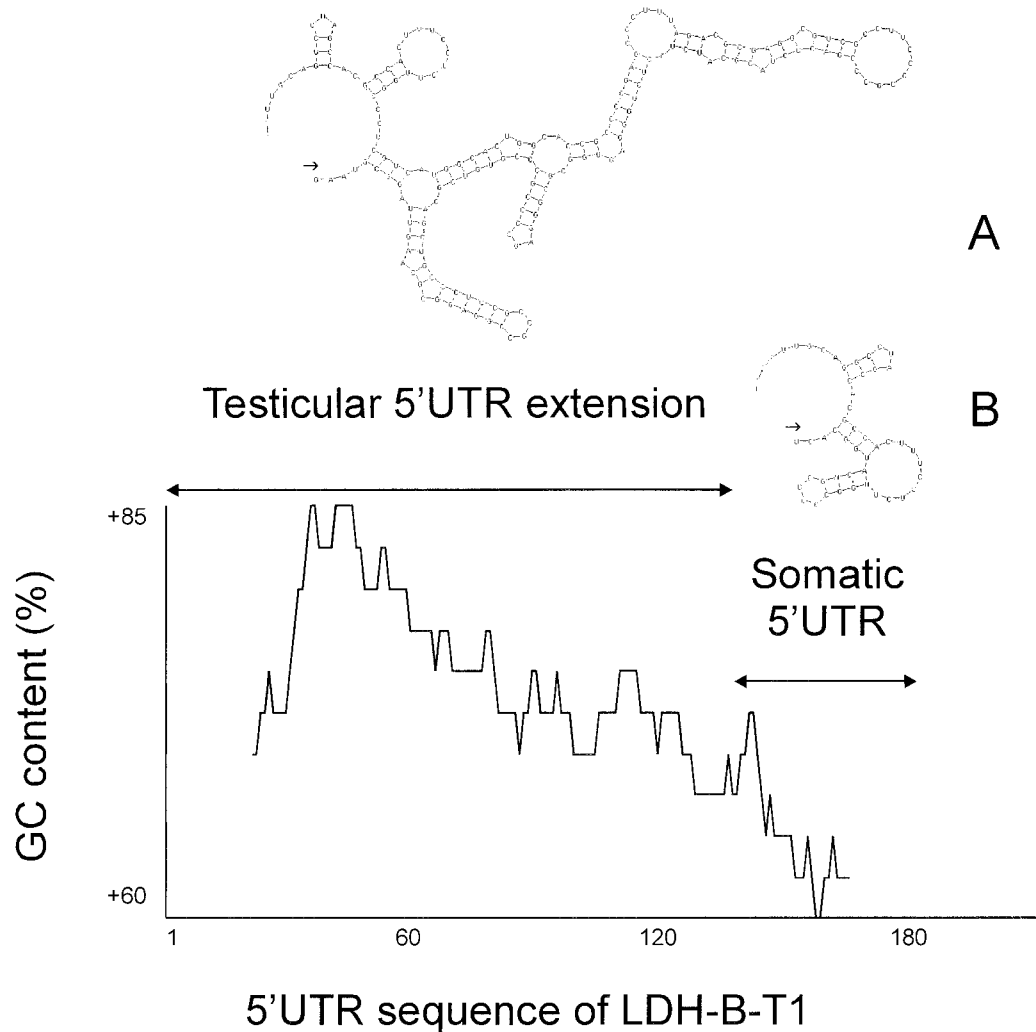
Fig. 5. GC content and predicted secondary structures of the 5'UTR of testicular LDH-BT1 (**A**) and the somatic LDH-B form (**B**). Calculated free energy values for the structures at 37°C were −59.59 Kcal/mol (A) and −4.76 kcal/mol (B). The RNA folding pattern was determined using the method described by [Zuker et al., 1999]. Arrows indicate the 5' ends.

mRNAs resulted from partial RNA degradation (results not shown).

DISCUSSION

The present study reveals qualitative and quantitative changes in LDH transcripts expressed in chicken adult testis. LDH-A mRNA is abundant in mature testis in comparison with immature testis and most somatic tissues with the exception of skeletal muscle where LDH-A is the predominant isoform [Markert et al., 1975; Markert, 1984; Li et al., 1983; Thomas et al., 1990; Jungmann et al., 1998]. A characteristic feature of the testicular LDH-A
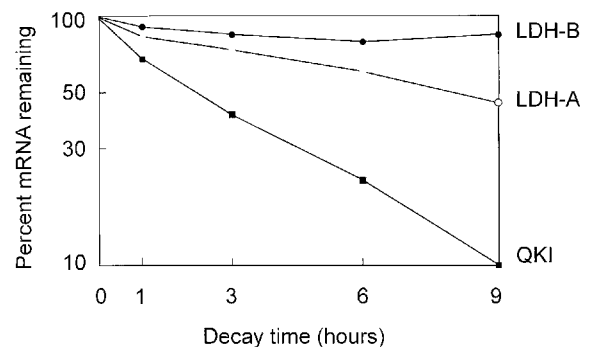
Fig. 6. Decay of LDH-B, LDH-A, and 6kb QKI mRNAs in primary cell cultures of adult chicken testis cells in the presence of Actinomycin-D.
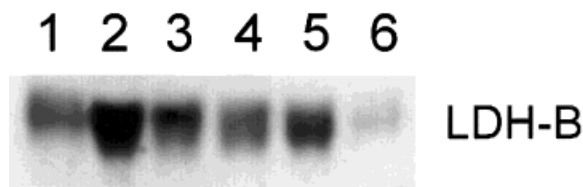
Fig. 7. Distribution of LDH-B mRNA in fractionated postmitochondrial extracts from adult chicken testis. **Lanes 1–6** represent sequential fractions of the gradient from top (fraction 1) to bottom (fraction 6). Much of the LDH-B mRNA is nonpolysomal and may be therefore translationally repressed.

transcript is its high degree of polyadenylation, not observed in any other tissue. We have previously reported the existence of high polyadenylated forms of ubiquitin, Hsp70 and GAPDH transcripts in adult chicken testis [Mezquita et al., 1998, 1998b]. In addition, we have reported that polyadenylation markedly increased upon heat shock during chicken spermatogenesis [Mezquita et al., 1998b]. It has been proposed that polyadenylation enhances the stability of the transcripts, the efficiency of translation, and the mRNA export from the nucleus [Kleene, 1996; Sachs et al., 1997; Huang and Carmichael, 1996].

LDH-B expression is higher in adult testis than in prepuberal testis and somatic tissues where the expression is in general low. Two known exceptions are the kidney and particularly the heart, were the LDH-B is the predominant isoform [Markert et al., 1975; Markert, 1984; Li et al., 1983; Thomas et al, 1990; Jungmann et al., 1998]. In birds and reptiles LDH-A and LDH-B are the only isoforms reported [Stock et al., 1997; Mannen et al., 1997], however in mammals and columbid birds, a testis specific isoform, LDH-C has been characterized [Burgos et al., 1995; Cooker et al., 1993, Bradley et al., 1996; Ambhaikar and Goldberg 1999]. During chicken spermatogenesis both LDH-A and LDH-B are expressed in meiotic and premeiotic cells, but only the LDH-B transcript persists in elongated spermatids until the end of spermiogenesis. A similar situation has been described for the testis specific LDH-C in mammals [Thomas et al., 1990]. The temporal correlation of expression of chicken LDH-B and mammalian LDH-C during spermatogenesis, together with the observation that columbid birds posses a testis specific LDH-C arising from a recent duplication of the LDH-B gene, suggest that the LDH-B mRNA

could perform in chicken testis the same functions that the LDH-C transcript in mammalian spermatogenesis.

Interestingly, we report here for the first time, the presence of testis specific transcripts of the LDH-B isoform. The testis specific transcripts arise from an alternative transcription initiation site and a possible alternative splicing. The novel LDH-B transcripts incorporate in the 5′UTR the GC-rich promoter of the avian LDH-B gene used in somatic tissues [Kraft et al., 1993]. The transcription factor Sp1 bound to this promoter is an abundant protein in testis [Yang and Thomas 1997; Bonny et al., 1998]. Bonny et al. [1998] have proposed that the high level of Sp1 found in testis is necessary for full expression of LDH-C and other testis specific genes. We have reported the presence of Sp1 binding sites and other DNA promoter sequences used in somatic tissues in the 5′UTR of several testis specific transcripts such as polyubiquitin UbII [Mezquita et al., 1993], polyubiquitin UbI [Mezquita et al., 1997], Carbonic anhydrase II [Mezquita et al., 1994, 1999] and GAPDH [Mezquita et al., 1998]. The use of testis specific promoters could offer new possibilities of regulation of expression at the level of transcription and translation. The presence of promoter sequences in the 5′UTR of several testis specific transcripts suggest the possibility that those sequences could be involved in translational regulation under the control of the same regulators that recognize these sequences on the DNA. A precedent of such mechanisms has been reported in the Drosophila male germ line [Kempe et al., 1993].

The testis specific mouse LDH-C mRNA is stable with no detectable decay of mRNA up to 20 h [Salehi-Ashtiani and Goldberg, 1993]. In addition mouse LDH-C is translationally repressed. LDH-C mRNA, fractionated by sucrose gradient sedimentation from adult testis postmitochondrial extracts, is present in both non-polysomal and polysomal fractions, with a substantial amount in the non-polysomal fraction [Fujimoto et al., 1988; Alcivar et al., 1991]. Chicken testis LDH-B is stable like LDH-C and most of the transcript is in the non-polysomal fraction as many other genes expressed in meiotic and postmeiotic stages of spermatogenesis. Translational repression can be an efficient way to localize and concentrate proteins in subcellular domains. Transport of mRNA in a

translationally repressed state, followed by activation of translation when the mRNA reaches its destination has been reported during oogenesis in Drosophila [Saunders, 1999]. The precise localization of LDH and GAPDH in a specific region of spermatozoa close to the motile apparatus of the sperm flagellum [Thomas et al., 1990; Burgos et al., 1995; Bradley et al., 1996; Bunch et al., 1998] may require similar mechanisms.

The non-polysomal mRNA particles stored in spermatocytes and spermatids contain Y-box proteins [Kwon et al., 1993; Tafuri and Wolffe, 1993; Sommerville and Ladomery, 1996; Hebert and Hecht, 1999; Schmidt, 1999]. These proteins are also components of maternal mRNA synthesized and stored through oogenesis and translated later during oocyte maturation and early embryogenesis [Richter, 1991]. The binding of Y-box proteins is sequence independent and is involved in the general repression of translation of paternal mRNAs [Schmidt, 1999]. Dephosphorylation of Y-box proteins in response to the appropriate signals may result in decreased affinity for RNA and unmasking of the mRNAs. Additional proteins that recognize specific sequences on the 5′ or 3′UTRs of mRNAs could be necessary to regulate the recruitment of specific RNAs to ribosomes. The reported presence of helicases in the nonpolysomal mRNA particles [Ladomery et al., 1997] could be also essential to unwind the secondary structures of testis specific transcripts with an extended 5′UTR, such the LDH-B reported in this paper and other testis specific transcripts [Mezquita et al., 1993, 1994, 1997, 1998, 1999; Yiu et al., 1994; Gu et al., 1995a; Fautsch et al., 1997].

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