

Developmental regulation of expression of the lactate dehydrogenase (LDH) multigene family during mouse spermatogenesis

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Summary

Expression of the Lactate Dehydrogenase (LDH) genes during various stages of spermatogenesis was studied by using a combination of Northern blot analyses and *in situ* hybridization techniques. These studies have indicated that developmentally programmed expression of all three functional LDH genes occurs during differentiation of germ cells. The LDH/C (ldh-3) gene was expressed exclusively during meiosis and spermiogenesis, beginning in leptotene/zygotene spermatocytes and continuing through to the elongated spermatids. LDH/C (ldh-3) gene expression was accompanied by transient expression of the LDH/A (ldh-1) gene in pachytene

spermatocytes and round spermatids. The LDH/B (ldh-2) gene was expressed mainly in Sertoli and spermatogonial cells. By using somatic cell hybrids, the LDH/C (ldh-3) gene has been mapped to mouse chromosome 7, establishing that it is syntenic with the LDH/A (ldh-1) gene locus. Experimental observations made in this study provide new insight into the order and sequence of events involved in the regulation of gene expression of the LDH gene family during spermatogenesis.

Key words: spermatogenesis, LDH gene expression, meiosis.

Introduction

Insights into the molecular mechanisms that regulate expression of the Lactate Dehydrogenase (LDH) genes during mammalian spermatogenesis can be helpful in understanding other complex programs controlling gene regulation during cellular differentiation. Studies on a variety of vertebrate species have led to the conclusion that significant evolutionary changes in LDH isoenzyme expression occur at the level of gene regulation (Ohno *et al.* 1968; Market *et al.* 1975; Zuckerman, 1978; Whitt, 1984). In vertebrates Lactate Dehydrogenase (LDH) isoenzymes are encoded by a multigene family. The LDH/A (ldh-1) and LDH/B (ldh-2) genes code for protein subunits that associate to form the five distinctive tetrameric isoenzyme bands that make up the pattern of LDH activity observed after electrophoresis in several somatic tissues. The subunit composition of these bands are A₄, A₃B, A₂B₂, AB₃ and B₄. These characteristic specific isoenzyme patterns correlate with the metabolic conditions (aerobic and anaerobic) prevailing within the respective tissue (Market *et al.* 1975; Nadal-Ginard, 1978).

Expression of the third functional LDH gene (ldh-3), which encodes the testis-specific isoenzyme LDH/C₄, is restricted to the differentiating germ cells within the seminiferous epithelium of the testis (Meistrich *et al.* 1977; Goldberg and Hawtrey, 1967; Weiben, 1981). Attempts to detect heterotetrameric isoenzyme forms that include LDH/C subunits have not been successful (Goldberg and Hawtrey, 1967). Studies detailed in this paper demonstrate that mRNA corresponding to all three functional Lactate Dehydrogenase (LDH) genes are transcribed by germ cells of the mouse seminiferous epithelium. More significantly, these results confirm that stage-specific expression of each of the respective LDH genes occurs during spermatogenesis.

Materials and methods

Isolation of seminiferous epithelium and spermatogenic cells

Seminiferous cords and tubules were prepared from Swiss Webster mice (Charles River) by collagenase treatment. Monodisperse suspensions of spermatogenic cells were pre-

pared from the seminiferous cords/tubules by trypsin digestion (Romrell *et al.* 1976; Bellvé *et al.* 1977). Types A and B spermatogonia were isolated from the testes of day 8 prepubertal mice. Preleptotene spermatocytes, leptotene/zygotene spermatocytes and early pachytene spermatocytes (P₁₇) were isolated from seminiferous epithelia of day 17 mice (Bellvé *et al.* 1977). Pachytene spermatocytes (P₆₀), spermatids (steps 1–8), elongating spermatids (steps 12–16) and residual bodies were isolated from the testes of >60-day-old mice. The germ cells were separated by velocity sedimentation at unit gravity on 2 to 4% BSA gradients (Romrell *et al.* 1976). TM₃ Leydig culture cells were obtained from the American Tissue Culture Collection. TM₄ Sertoli cells were a gift from J. Mather.

Isolation of total RNA

Cytoplasmic RNA was isolated according to Chirgwin *et al.* (1979). Seminiferous cords and tubules, purified germ cells and somatic cells were lysed in 0.5% NP-40, 1.5 mM MgCl₂, 14 mM NaCl, 10 mM vanadyl complexes (BRL) in 10 mM Tris-HCl (pH 7.5). Nuclei and membranes were removed by centrifugation at 4,000 revs min⁻¹ for 15 min. Guanidine thiocyanate was added to 6 M and then CsCl to 0.15 g ml⁻¹ final concentrations. The homogenate was layered over a 2 ml cushion of 5.7 M CsCl and centrifuged in an SW 50.1 rotor, at 35,000 revs min⁻¹ for 18–24 h at 20°C. After the supernatant was removed, the pelleted RNA was resuspended in 0.25% SDS and precipitated with 3 M sodium acetate and absolute ethanol (vol:vol, 0.1:2) at -20°C. RNA for Northern blots was isolated by LiCl precipitation (Cathala *et al.* 1983). The samples were homogenized briefly in 5 M guanidine thiocyanate, 10 mM EDTA, 8% BME, 50 mM Tris-HCl (pH 7.5). RNA was precipitated at 4°C by addition of LiCl to 4.0 M final concentration, centrifuged and then redissolved in 0.1% SDS, 1 mM EDTA in 10 mM Tris-HCl (pH 7.5). The resuspended RNA was extracted with phenol/chloroform (vol:vol; 1:1) and precipitated by addition of 3 M sodium acetate and ethanol (vol:vol; 0.1:2) at -20°C.

Isolation of Poly(A)⁺ RNA

Poly(A)⁺ RNA was isolated by affinity chromatography over oligo(dT)-cellulose (Bantle *et al.* 1976). Total RNA was dissolved in 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), followed by the addition of 9 volumes of DMSO and an equal volume of 1.0 M LiCl, 5 mM EDTA, 2% SDS in 10 mM Tris-HCl (pH 7.5). The RNA was heated to 55°C for 5 min and diluted tenfold with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M LiCl, 0.5% SDS (binding buffer) before loading onto the oligo(dT)-cellulose column. The flowthrough was recycled twice and the column was finally washed with at least five bed volumes of binding buffer. The bound poly(A)⁺ RNA was eluted with 0.5% SDS in 10 mM Tris-HCl (pH 7.5), and precipitated with 3 M sodium acetate and ethanol (vol:vol; 0.1:2) at -20°C.

Preparation of Northern blots

Northern blots were constructed by using either total RNA or poly(A)⁺ RNA. The RNA was denatured in 50% formamide buffer at 55°C for 15 min and electrophoretically separated in 1.4% agarose gels containing 2.2 M formaldehyde (Thomas, 1980). Following blotting onto a nylon membrane Gene Screen [NEN] for 24 h, the transferred RNA was cross-linked to the membrane by exposure to UV for 4 min. The same blots were used for the LDH/A, B and C probes shown in Figs 2, 3, 4. The specific activities of the probes were similar and the exposure time was the same. Thus, relative levels of mRNA can be roughly estimated from the signals observed on these blots.

Probes and hybridization conditions

The LDH 3'-specific probes were obtained from mouse cDNA clones previously identified and characterized by sequencing from M13 subclones. The LDH/A 3' probe was a 0.4 kb *HindIII/Xba* fragment isolated from M13 phage mA 151 (Akai *et al.* 1985). The LDH/B 3' probe was a 0.7 kb *BamHI* fragment isolated from M13 phage mB 162-1 (Hiroaki and Li, personal communication). The LDH/C 3' probe was a 0.4 kb *EcoRI* fragment isolated from M13 phage mC 31-1 (Sakai *et al.* 1987). The 3' LDH fragments were isolated and subcloned into Bluescript (Stratagene). Hybridization probes were prepared from DNA fragments isolated from CsCl-purified plasmid DNA. After restriction by the appropriate enzymes, the DNA fragments were separated on 1% agarose gels. The ethidium bromide (EtBr)-stained DNA fragments were identified by UV, excised from the gel and recovered by precipitation in saturated sodium perchlorate (NaClO₄) at 37°C. The DNA was purified by binding to GF/C glass fiber filters and eluting in 0.1×TE buffer [1 mM EDTA in 1 mM Tris-HCl (pH 7.5)]. Isolated DNA fragments were nick translated in the presence of ³²P-labeled nucleotides. The specific activities of all nick-translated probes were >10⁹ cts min⁻¹ μg⁻¹ DNA. The sensitivity of detection on a calibrated Northern blot assay with the LDH/C probe was estimated to be 160 fg μg⁻¹ total RNA.

Hybridization conditions

Northern hybridization assays were carried out at 42°C in 50% formamide, 0.1% SDS, 5×SSPE (1.1 M NaCl, 5 mM EDTA, 50 mM NaH₂PO₄, pH 7.4), 1×Denhardt's and sheared calf thymus DNA (100 μg ml⁻¹). After hybridization was completed, three washings were undertaken at 15 min intervals initially in 0.1% SDS, 1×SSC at 22°C. If the backgrounds were excessive the filters were rewashed three times at 15 min each with 0.1% SDS, 0.1×SSC at 60°C before exposing to Kodak film with intensifier screens at -70°C (Maniatis *et al.* 1982).

Parental and hybrid cell lines

The mouse chromosome content of the mouse×Chinese hamster and mouse×rat cell hybrids used in this study have been identified by karyotyping, analysis for marker enzymes and Southern hybridizations with cDNA probes (Fournier and Frelinger, 1982; Fournier and Moran, 1983; Killary and Fournier, 1984; Peterson *et al.* 1985; Lem and Fournier, 1985; Lenwand *et al.* 1983; Killary *et al.* 1987).

Genomic mapping by Southern analysis

DNA was isolated from the designated hybrid cell lines according to methods described by Maniatis *et al.* (1982). The isolated DNA samples were digested with the appropriate restriction endonucleases and the fragments separated by agarose gel electrophoresis followed by transfer to nitrocellulose (Southern, 1975). The filters were prehybridized at 65°C in 1% SDS, 6×SSC, 5×Denhardt's and salmon sperm DNA (50 μg ml⁻¹). Hybridizations were done at 65°C in 1% SDS, 6×SSC, 5×Denhardt's, 10% dextran sulfate for 18 h. After hybridization, the filters were washed in 0.1% SDS, 2×SSC for 15 min followed by 0.1% SDS, 0.1×SSC for 15 min at 22°C. If required, a final wash was done in 0.1% SDS, 0.1×SSC for 3 min at 65°C to remove background.

In situ hybridization

Seminiferous tubules were isolated from testes of day 8, day 12, day 17 and day 60 mice. Cells were dispersed nonenzymatically in EKRB buffer (see Materials and Methods; Bellvé *et*

al. 1977). The suspension of cells was cytocentrifuged at 400 g onto glass coverslips. The cells were fixed in 4% paraformaldehyde in PBS, washed in PBS, dehydrated and acetylated by incubation in 2.5% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. Hybridizations were performed in a humidified chamber for 12 h at 50°C, using 4×10^6 cts min^{-1} per slide of ^{35}S -labelled antisense RNA in a buffer containing 50% formamide, 20 mM DTT, 10% dextran sulfate and 1×salts (0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM NaH_2PO_4 (pH 6.8), 5 mM EDTA and 1×Denhardt's solution. Antisense RNAs were obtained by 'in vitro' transcription initiated at the T₃ and/or T₇ promoters of Bluescript (Stratagene) into which 3'-specific cDNA fragments of the LDH-A, -B, and -C were subcloned. After hybridization, the preparations were washed for 4 h in WDTT [50% formamide, 10 mM DTT and 1×salts (0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM NaPO_4 (pH 6.8) 5 mM EDTA, 1×Denhardt's] at 50°C, incubated in 20 mg ml^{-1} RNase A in 1.5 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA at 37°C for 30 min washed in the same buffer for 1 h at 37°C and followed by 16 h in WDTT at 50°C. The slides were dehydrated in ethanol, coated with Kodak NTB-2 emulsion and, after exposure for the appropriate times, they were developed and counterstained with Hoechst 33258 (Gatti *et al.* 1976). The slides were visualized and photographed using a microscope with epifluorescence. Hoechst staining facilitated recognition of different germ cell types by the distinctive morphological patterns of their cell nuclei (del Mazo *et al.* 1986). Nuclear morphological patterns as well as radioactive grains were observed simultaneously by UV fluorescence and bright-field illumination. Nomarski optics were utilized to enhance morphological features.

For quantitative studies of LDH/B expression, the radioactive grains were counted in at least 30 individual type B spermatogonia and Sertoli cells for each designated time point. Radioactive grains in the surrounding cells (excluding type B spermatogonia and Sertoli cells) were also counted and used as controls for background levels of hybridization signals.

Results

1. Probe specificity

In the current studies, expression patterns of the LDH genes were analyzed by hybridization techniques. The relative specificities of the molecular probes for LDH/A, LDH/B and LDH/C transcripts were therefore examined. To prepare specific probes the 3' fragments of the LDH/A, LDH/B and LDH/C, cDNA clones were subcloned as described in Materials and methods. These 3'-specific DNA fragments were isolated after restriction with the appropriate enzymes, labelled by nick-translation in the presence [^{32}P]dNTPs and tested for hybridization against each of the other two DNA samples. No cross-hybridization was detected (data not shown). The specificity of each probe was examined further by analysis of poly(A)⁺ RNA transcripts in samples isolated from various mouse tissues expressing different LDH isoenzyme patterns (Nadel-Ginard, 1978; Allen, 1961). Tissue-specific differences are expected to be reflected in the patterns and levels of LDH mRNAs detected by hybridization with the 3'-specific LDH probes.

The 3'-specific LDH/A probe detected 1.6 kb tran-

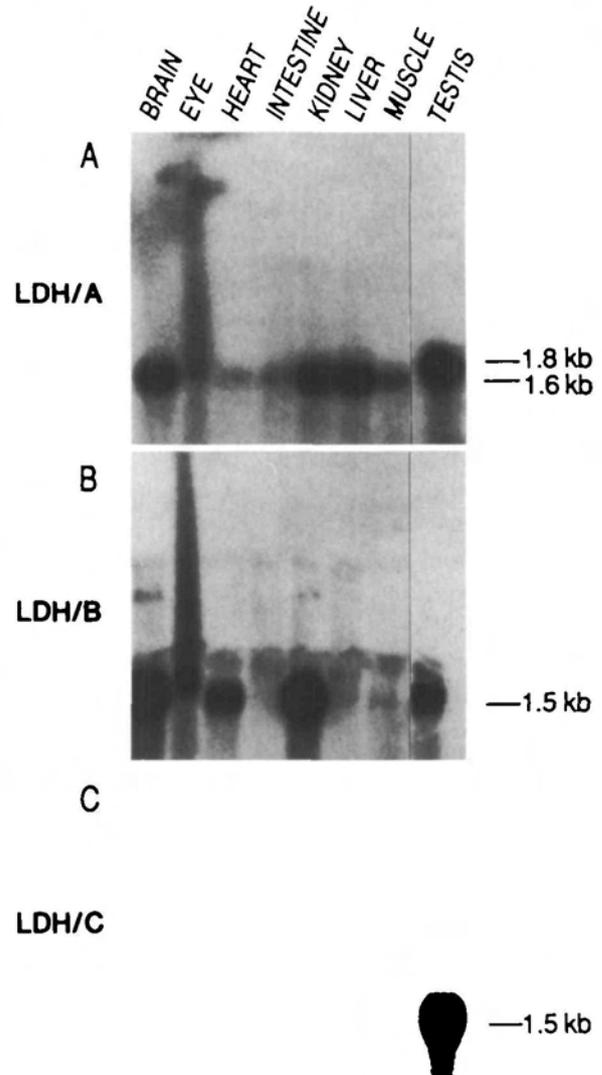


Fig. 1. LDH gene expression in various mouse tissues. Northern analysis of poly(A)⁺ RNA (10–20 μg /lane) isolated from mouse brain, eye, heart, intestine, kidney, liver, muscle and testis with 3'-specific cDNA probes LDH/A (1A), LDH/B (1B) and LDH/C (1C).

scripts that were abundant in RNA isolated from brain, kidney, liver and muscle, and less abundant in RNA isolated from eye, heart and intestine. A slightly larger, 1.8 kb transcript was also observed in RNA isolated from testis (Fig. 1A). Hybridization with the 3'-specific LDH/B probe showed a different pattern, this probe detected 1.5 kb transcripts present at high levels in RNA isolated from brain, eye, heart, kidney and testis (Fig. 1B). It was clear from direct examination of these hybridization results that the relative levels of mRNA transcripts detected with the LDH/A probe are different than those found with the LDH/B probe. In addition, the tissue distributions of LDH/A and LDH/B mRNA transcripts correspond to the expected distribution of LDH/A and LDH/B polypeptides. For example, in heart tissue predominantly high levels of the LDH/B transcripts correlates with the presence of

tetrameric LDH/B₄ isozyme, which is primarily involved in aerobic metabolism. In skeletal muscles, high levels of LDH/A transcripts correlate with the accumulation of LDH/A₄ isoenzyme, which is involved in the anaerobic metabolism. The LDH/C₄ isozyme is found only in testis, thus hybridization with the LDH/C probe demonstrated the presence of the corresponding transcripts only in RNA derived from testis (Fig. 1C).

2. Developmental regulation of LDH transcript accumulation

Previous studies have demonstrated that LDH/C isozymes are found only in mature differentiating germ cells within the seminiferous epithelium of the mouse testis (Meistrich *et al.* 1977; Hintz and Goldberg, 1977; Weiben, 1981). Since data in Fig. 1A, 1B and 1C indicated that all three LDH gene transcripts were detected in the testis, an attempt was made to determine their distribution patterns among morphologically distinguishable cells. In the developing seminiferous epithelium, germ cells at more advanced stages of differentiation appear at well defined times after birth (Bellvé *et al.* 1977). As a result, RNA isolated at different periods of maturation allowed correlation of the presence of specific messages with the appearance of specific cell types.

RNA samples isolated from prepuberal mice were separated by electrophoresis, transferred to nitrocellulose paper and hybridized with radioactive probes. These will be referred to as "developmental Northern blots". Type A and B spermatogonia constitute the predominant germ cell population in 6- to 8-day-old mice. Leptotene and zygotene spermatocytes appear after day 10, pachytene spermatocytes at day 14 and spermatids at day 20 (Bellvé *et al.* 1977). Hybridization with the 3'-specific LDH/C probe first detected LDH/C transcripts in germ cells derived from 12-day-old prepuberal mice (Fig. 2A). The level of expression increased until day 20, after which consistently high levels of LDH/C transcripts were observed in all germ cell differentiation stages up to elongating spermatids. This profile of developmental expression suggests activation of LDH/C gene expression during early stages of meiosis, concomitant with the accumulation of leptotene and zygotene spermatocytes. The temporal accumulation of LDH/C transcripts was confirmed by using RNA derived from purified germ cells (Fig. 2B). Strong signals for LDH/C were found in pachytene spermatocytes and spermatids, whereas weak signals were detected in RNA derived from leptotene/zygotene spermatocytes. LDH/C transcripts were not detected in RNA isolated from spermatogonial cells. The levels of LDH/C RNA appeared to be decreasing in elongating spermatids and very little or no transcripts were detected in RNA isolated from residual bodies.

By utilizing the developmental Northern blot, hybridization studies with the LDH/A 3'-specific probe identified significantly lower levels of the 1.6 kb LDH/A mRNA transcript in RNA samples isolated from the seminiferous epithelium from day 6 through to day 20. By day 24, however, two LDH/A mRNA

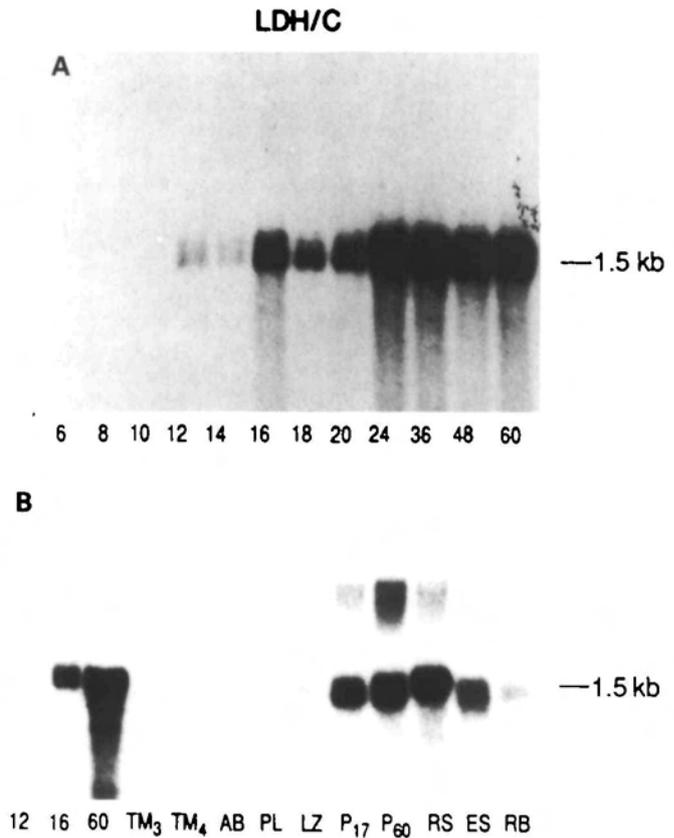


Fig. 2. (A) Pattern of LDH/C transcript accumulation within cells of the developing mouse seminiferous epithelium. Poly(A)⁺ RNA (6 µg/lane) isolated from the seminiferous epithelium at days 6–60 after birth was analyzed by using the LDH/C 3'-specific cDNA probe. (B) Expression of the LDH/C genes in purified cell types. Northern analysis of total RNA (30–40 µg/lane) isolated from the seminiferous epithelium at days 12 (12), 16 (16), 60 (60); from a Leydig cell line (TM₃); from a Sertoli cell line (TM₄); from type A and B spermatogonia (AB); from preleptotene spermatocytes (PL); from leptotene and zygotene spermatocytes (LZ); from day 17 pachytene spermatocytes (P₁₇); from day 60 pachytene spermatocytes (P₆₀); from spermatids, steps 1–8 (RS); from elongating spermatids, steps 12–16 (ES); from residual bodies (RB). This purified-cell-type Northern blot was hybridized with the LDH/C 3'-specific cDNA probe.

transcripts were recognizable, the original 1.6 kb transcript and a new 1.8 kb transcript. The appearance of 1.8 kb transcripts coincided with the differentiation of round spermatids (Fig. 3A). Hybridization analyses with RNA isolated from purified germ cells (Fig. 3B) show that the 1.6 kb LDH/A transcripts accumulated in purified pachytene spermatocytes and the 1.8 kb LDH/A transcripts were present in round and elongating spermatids. The 1.6 kb transcripts was just detectable in RNA isolated from the cell lines TM₃ (derived from Leydig cells) and TM₄ (derived from Sertoli cells). The basis for differences in LDH/A transcript length are not yet known.

The expression patterns of LDH/A and LDH/C showed transcript accumulation in meiotic and post-

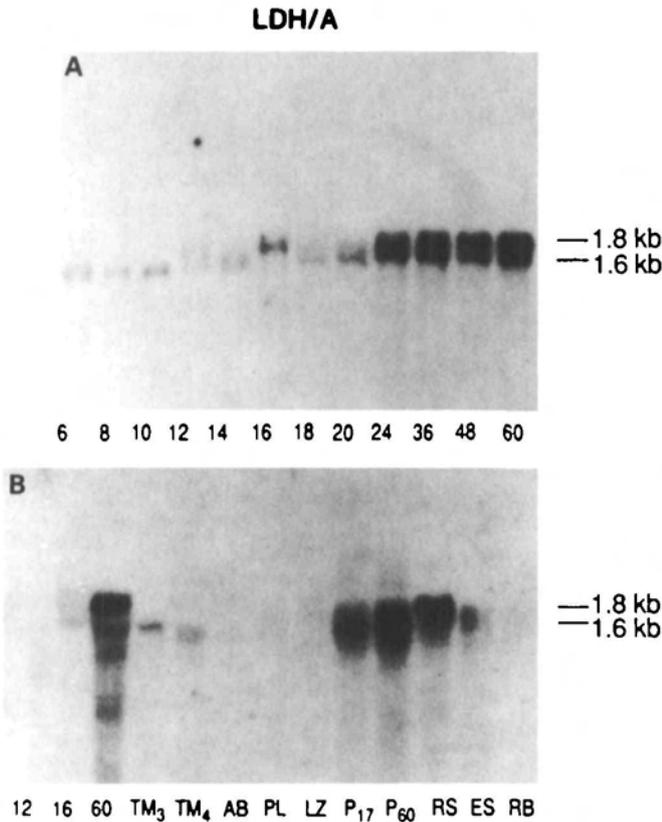


Fig. 3. (A) Pattern of LDH/A transcript accumulation within cells of the developing mouse seminiferous epithelium. Northern analysis of poly(A)⁺ RNA isolated from seminiferous epithelium at days 6–60 after birth using the LDH/A 3'-specific cDNA probe. (B) Expression of the LDH/A genes in different seminiferous tubule cell types. Purified-cell-type Northern blot as described in Fig. 2B.

meiotic cells; by contrast, LDH/B transcripts were found in high levels in RNA isolated from day 6 and 8 seminiferous epithelium. A significant increase in LDH/B gene expression was observed between day 10 and 14, with levels steadily decreasing at subsequent developmental times (Fig. 4A). These observations suggested that LDH/B transcripts were present in Sertoli and/or spermatogonial cells, since both cell types are represented in RNA isolated from the developing seminiferous epithelium. Hybridization studies with a Northern blot containing RNA isolated from purified germ cells (Fig. 4B) confirmed that LDH/B transcripts were present in spermatogonia, and in TM₄ Sertoli cells. Note, however that LDH/B gene expression was also observed in highly enriched germ cells corresponding to leptotene, zygotene and day 17 pachytene spermatocytes, but not in adult pachytene spermatocytes. No LDH/B transcripts were detected in round or elongating spermatids. Since minor cross-contamination of these germ cell preparations by earlier or later staged cell types could affect the interpretation of the Northern hybridization data, *in situ* hybridization was utilized to investigate LDH gene expression in individual cells.

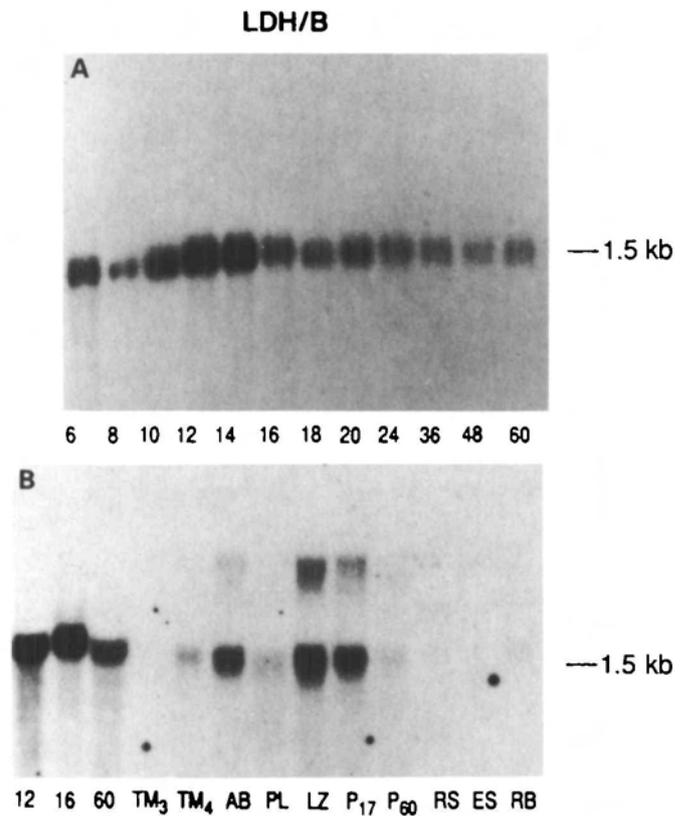


Fig. 4. (A) Pattern of LDH/B transcript accumulation within cells of the developing mouse seminiferous epithelium. Northern analysis of poly(A)⁺ RNA isolated from the seminiferous epithelium at days 6–60 after birth using the LDH/B 3'-specific cDNA probe. (B) Expression of the LDH/B genes in different seminiferous tubule cell types. Purified-cell-type Northern blot as described in Fig. 2B.

In situ hybridization

The distribution of LDH transcripts in different cell types in the seminiferous epithelium was determined by *in situ* hybridization with antisense RNAs corresponding to 3'-specific probes for each LDH transcript. The cell types could be distinguished in dispersed cell preparations by specific staining and nuclear morphology. *In situ* hybridization analyses indicated both the presence of mRNAs and their distribution in the cytoplasm and/or nucleus of the cell. Hybridization with 3'-specific LDH/C antisense probes detected relatively low levels of LDH/C transcripts in leptotene and zygotene spermatocytes. Higher levels of LDH/C expression were observed in pachytene spermatocytes and even in the metaphase I and metaphase II spermatocytes. LDH/C transcripts were also detected in round spermatids (steps 1–8) but little was found in elongating spermatids (steps 9–16) and residual bodies (Figs 5B, 6B and 6D). No LDH/C transcripts were detectable in Sertoli and spermatogonial cells. The density of silver grains associated with individual leptotene, zygotene, pachytene and spermatid cells correlated with the levels of LDH transcripts present. These results were consistent with data obtained by using Northern hybridization

of RNA isolated from different ages of prepuberal mice (developmental blot) and from specific germ cells (purified-cell-type blot).

LDH/A transcripts were detected by *in situ* hybridization with the LDH/A 3'-antisense probe in pachytene spermatocytes and in some but not all spermatids (steps 1-8) (Fig. 5A). These results, also, were consistent with those obtained with the developmental and purified-cell-type Northern blots. Finally, *in situ* hybridization studies with the LDH/B 3'-antisense probe detected low levels of LDH/B transcripts in type B spermatogonial and Sertoli cells. However, LDH/B transcripts were not detected in leptotene, zygotene, pachytene spermatocytes nor in spermatids (Fig. 7A and 7C).

Initial *in situ* hybridization experiments were carried out with cells isolated from adult tubules (day 60). To determine whether the cell-type specificity of LDH expression was similar during germ cell development in both prepuberal and adult mice, parallel *in situ* hybridization experiments were carried out on cells isolated from prepuberal tubules. In all cases, identical LDH expression patterns were observed in prepuberal and in adult cells, even for developing Sertoli cells in which LDH/B signals were found. A quantitative analysis of LDH/B mRNA accumulation was carried out by utilizing *in situ* hybridization techniques, in an effort to confirm the cell-type specificity of developmental changes in LDH/B gene expression. Fig. 8 is a representation of the average number of autoradiographic

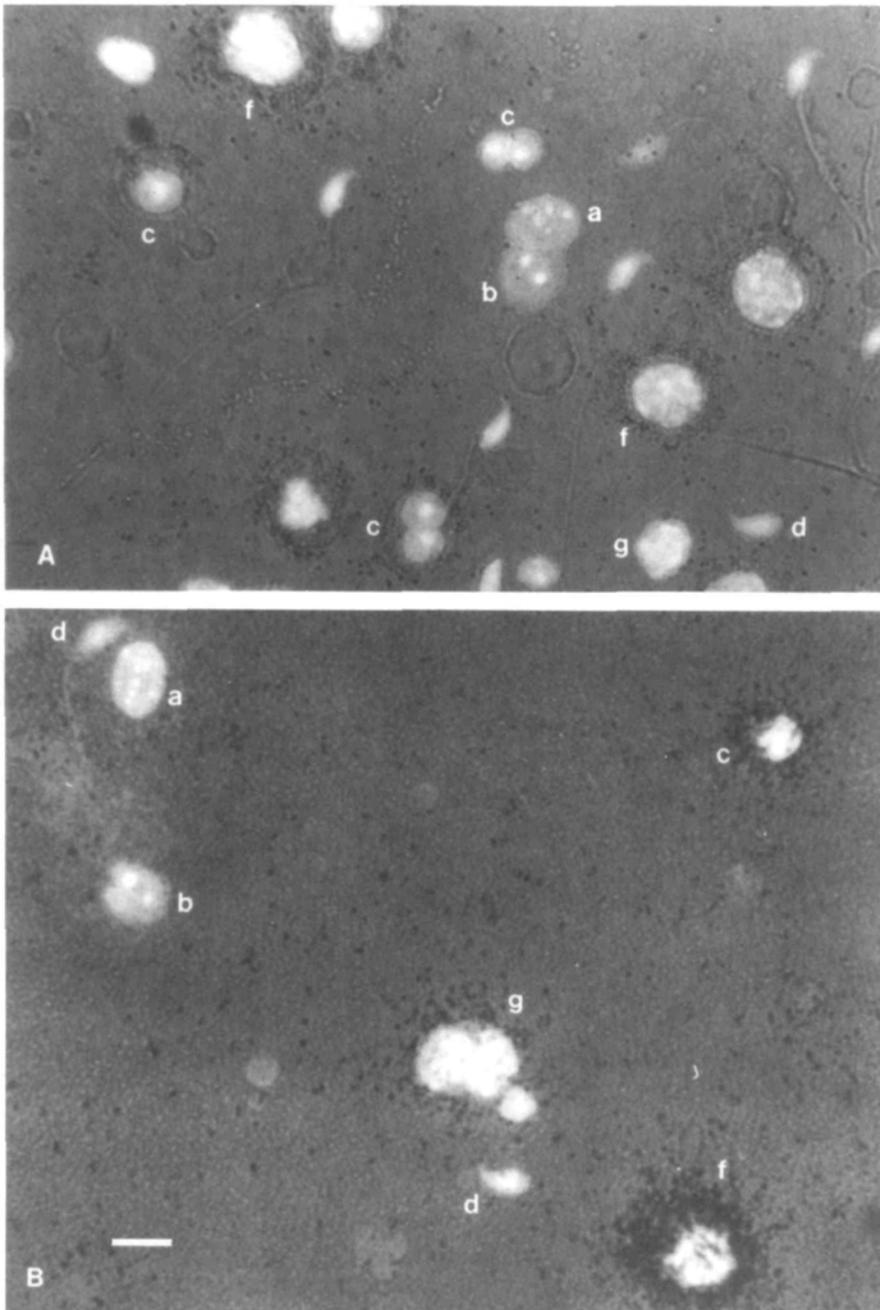


Fig. 5. *In situ* localization of LDH mRNAs in different seminiferous tubule cell types from day 60 mice: (a) spermatogonias; (b) Sertoli cells; (c) round spermatids; (d) elongating spermatids; (f) pachytene spermatocytes; (g) leptotene-zygotene spermatocytes. (A) Hybridization with antisense LDH-A RNA. (B) Hybridization with antisense LDH-C RNA. Note: i) Differences in the level of expression; ii) Cytoplasmic localization of the transcripts. Simultaneous fluorescence and bright-field illumination. Autoradiographic exposure: 11 days. Bar represents 10 μ m.

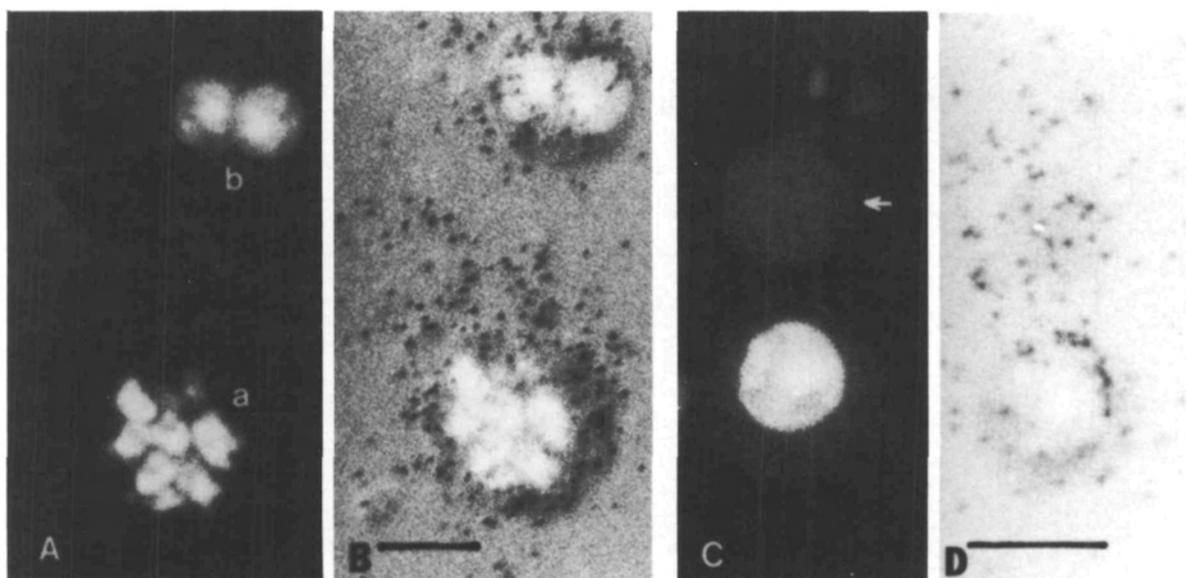


Fig. 6. (A, B) *In situ* localization of LDH-C mRNA in germ cells: (a) at meiotic metaphase I; (b) in round spermatids. (A) Fluorescence observed after Hoechst staining. (B) Fluorescence/Nomarski microscopy. Autoradiographic exposure: 9 days. (C, D) *In situ* localization of LDH/C mRNA in spermatid and residual body (arrow). (C) Fluorescence; (D) Fluorescence/bright-field microscopy. Autoradiographic exposure: 6 days. Bars represent 10 μm .

grains observed in Sertoli and type B spermatogonial cells compared to other seminiferous tubule cells present in the surrounding area on the slides (used as controls in the Figure). Only slight increases in accumulation of LDH/B mRNA were detected in both cell types during postnatal cellular differentiation (day 8 to 60).

Mapping of the LDH/C gene

The LDH/A (*ldh-1*) and the LDH/B (*ldh-2*) genes have been mapped to mouse chromosomes, 7 and 6 respectively (Soares, 1978; O'Brien *et al.* 1978; Peters and Andrews, 1985). It was important to establish the chromosomal location of the LDH/C (*ldh-3*) gene, since its genomic position may give some clues to its restricted expression. Genomic DNA was isolated from somatic cell hybrids, including mouse–Chinese hamster and mouse–rat cell hybrids that have segregated various mouse chromosomes. DNAs isolated from these cell lines were analyzed by Southern hybridization using the LDH/C 3'-specific cDNA probe. All hybrid cell lines utilized have been characterized with different cDNA molecular probes used to map genes to specific mouse chromosomes (Fournier and Moran, 1983; Lem and Fournier, 1985; Killary *et al.* 1987).

Hybridization with the 3'-specific LDH/C probe to restriction endonuclease-digested DNAs, detected only a single 5.2 kb *Eco*RI fragment and 3.0 kb *Hind*III fragment in the mouse parental and some somatic cell hybrid DNAs. No hybridization occurred to the hamster or rat parental DNAs. The 3.0 kb genomic *Hind*III fragment was observed only in DNA isolated from mouse parentals and the ABM-30 and ABM-31 hybrid cell line (Fig. 9). The only mouse chromosomes present in these cell lines and absent from the other DNA

samples were mouse chromosomes 2 and 7. To determine which of these two chromosomes carried the LDH/C (*ldh-3*) gene, Southern blots containing additional somatic cell hybrid genomic DNAs were employed (Fig. 10). Hybrid cell line F2-8 contained a Robertsonian translocation resulting in fused mouse chromosomes 2 and 8. No hybridization of the LDH/C probe was observed in *Eco*RI-digested DNA isolated from the F2-8 somatic cell hybrids, suggesting the LDH/C (*ldh-3*) gene is not present on mouse chromosome 2. This result confirmed the assignment of the LDH/C gene to mouse chromosome 7, which is present in both the ABM-30 and ABM-31 somatic cell hybrids (Table 1; Fig. 9).

Discussion

The sequence of developmental events occurring within the mouse seminiferous epithelium provided an experimental system to examine the temporal expression of Lactate Dehydrogenase (LDH) genes during cellular differentiation. By utilization of LDH/C isoenzyme activity and *in vitro* translation assays, previous studies indicated that expression of the LDH/C gene product is restricted to mature gametogenic cells of the testis (Meistrich *et al.* 1977; Hintz and Goldberg, 1977; Goldberg and Hawtery, 1967; Weiben, 1981). Heterotetrameric isoenzyme forms that included the LDH/C subunit, were not found in the mouse seminiferous epithelium (Goldberg and Hawtery, 1967) though detected in some mammals, including humans (Skude *et al.* 1984), rats (Ansari, 1981) and guinea pigs (Goldberg, 1973). Significant changes in the relative concentrations of the LDH/A and LDH/B subunits in testicular extracts with the onset of LDH/C subunit synthesis

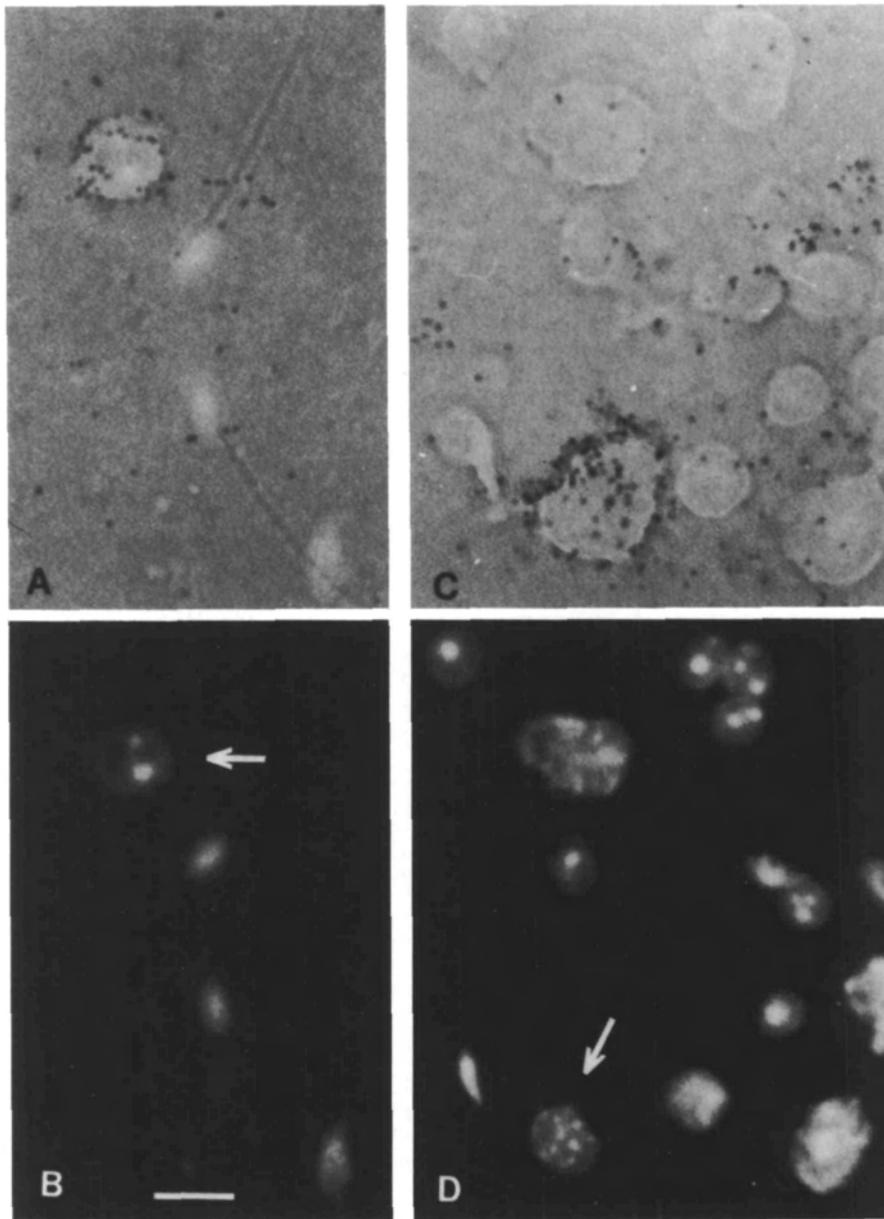
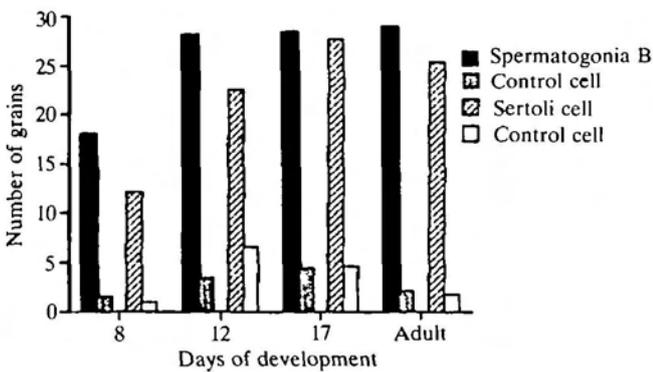


Fig. 7. *In situ* localization of LDH-B mRNA in Sertoli cell (A and B) and type B spermatogonia (C and D). Fluorescence observed after Hoechst staining (B, D). Fluorescence/bright-field microscopy (A, C). Autoradiographic exposure: 11 days. Bars represent 10 μ m.



were observed by Goldberg and Hawtrey (1967). In a subsequent study, these investigators were able to demonstrate that experimental conditions promoting a decline in LDH/C expression resulted in a corresponding increase in LDH/A expression (Goldberg and Hawtrey, 1968). These studies were unable, however, to determine the cell-type specificities of the observed changes in LDH gene expression. More recently, however, Li *et al.* (1989) have found evidence for LDH/A

Fig. 8. Developmental analysis of LDH/B expression by *in situ* hybridization. Radioactive grains present in Sertoli and Type B spermatogonia were compared with other testicular cells (as controls) in samples isolated from 8-, 12-, 17-, and 60-day-old mice.

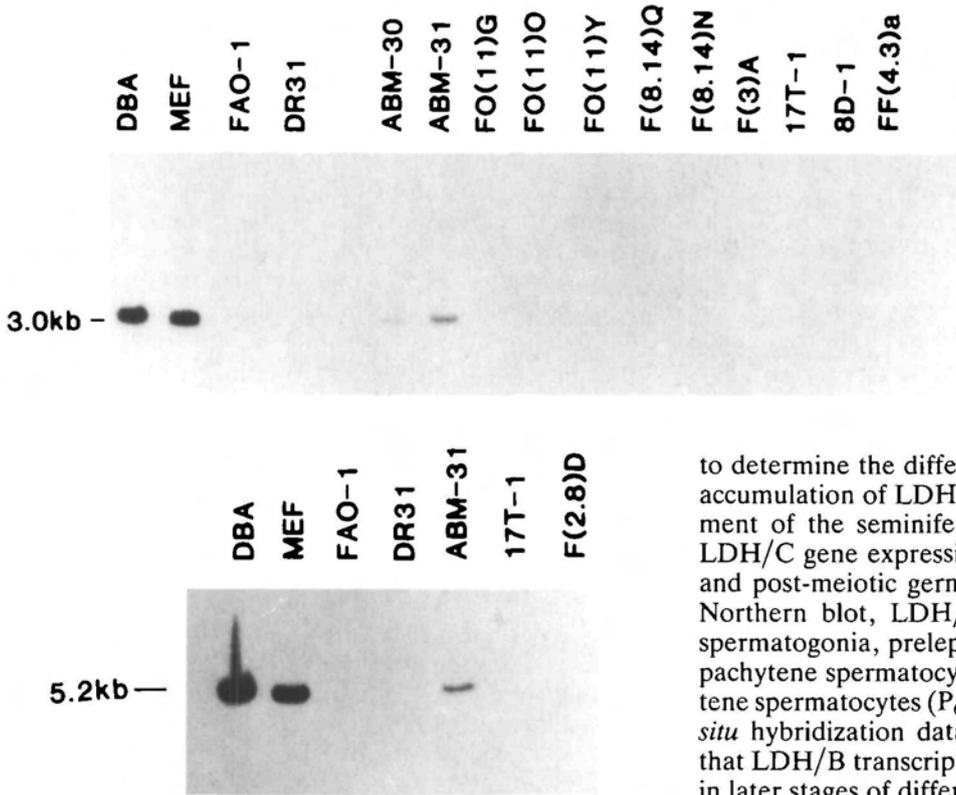


Fig. 10. Southern blot analysis of *EcoRI* restricted genomic DNAs isolated from hamster (DR31), rate (FAO-1), mouse (DBA and MEF) and mouse×hamster/rat somatic cell hybrids using the LDH/C 3'-specific cDNA probe. Note: Only the ABM 31 cell hybrid and the mouse parental DNAs scored positive for the LDH/C (*ldh-3*) gene.

and LDH/B isoenzyme activity in purified mouse germ cells.

The availability of unique molecular LDH probes has facilitated examination of cell-type-specific LDH gene expression at the RNA level. By using both *in situ* hybridization and Northern blot analyses, we were able

Fig. 9. Southern blot analysis of *HindIII* restricted genomic DNA isolated from hamster (DR31), rat (FAO-1), mouse (DBA and MEF) and various mouse×hamster/rat somatic cell hybrids using the LDH/C 3'-specific cDNA probe. Note: Only the ABM 30 and ABM 31 cell hybrids and the mouse parental DNAs scored positive for the LDH/C (*ldh-3*) gene.

to determine the different patterns of cell-type-specific accumulation of LDH gene transcripts during development of the seminiferous epithelium. It is clear that LDH/C gene expression is restricted solely to meiotic and post-meiotic germ cells. On the purified-cell-type Northern blot, LDH/B transcripts were detected in spermatogonia, preleptotene, leptotene/zygotene, and pachytene spermatocytes (P₁₇), but not in adult pachytene spermatocytes (P₆₀) nor in spermatids (Fig. 4B). *In situ* hybridization data have independently confirmed that LDH/B transcripts were absent from all germ cells in later stages of differentiation. Further *in situ* hybridization studies with dispersed seminiferous tubule cells of 8-, 12-, 17-, and 60-day-old mice demonstrated the presence of detectable levels of LDH/B transcripts only in type B spermatogonia and Sertoli cells (Figs 7A, 7C and 8).

There are two possible explanations for differences between 17 day and adult pachytene cells in LDH/B expression observed by Northern blot and *in situ* hybridization: (a) expression of LDH/B gene transcripts is very low in individual type A spermatogonia and prepuberal primary spermatocytes and therefore were below the limit of detection by *in situ* hybridization; (b) alternatively, the day 17 germ cell used for isolating RNA on the Northern blots were contami-

Table 1. Karyotypic analysis of hamster/rat hepatoma×mouse fibroblast cell hybrids

Clone	Parentals	Mouse chromosomes retained (fraction of cells)																		No. of cells karyo-typed	LDH/C hybridization		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18			19	X
ABm-30 ^b	(Hamster)	-	0.9	-	-	-	0.56	0.38	-	0.46	0.31	-	0.51	-	-	0.31	0.51	-	-	-	-	39	+
ABm-31 ^b	(Hamster)	-	0.95	-	-	-	0.40	0.51	-	0.59	-	-	0.65	-	0.43	0.27	-	0.73	-	-	-	37	+
17T-1 ^a	(Hamster)	-	-	-	-	-	-	-	0.92	-	-	-	-	-	-	-	-	0.92	-	-	-	25	-
F(2.8)D ^c	(Rat)	-	0.93	-	-	-	-	-	0.93	-	-	-	-	-	-	-	-	-	-	-	-	21	-
F0(11)G ^c	(Rat)	-	-	-	-	-	0.66	-	-	-	-	0.86	-	0.71	0.79	0.83	-	-	-	-	-	24	-
F0(11)O ^c	(Rat)	-	-	-	-	-	-	-	-	-	-	0.80	-	0.74	-	0.50	-	-	-	-	-	24	-
F0(11)Y ^c	(Rat)	-	-	-	-	-	-	-	-	0.76	-	0.83	-	-	-	-	-	-	-	-	-	29	-
F(8.14)Q ^d	(Rat)	0.23	-	-	-	-	-	-	0.95	-	-	-	0.55	0.59	0.95	-	-	-	-	-	-	22	-
F(8.14)N ^d	(Rat)	-	-	-	-	-	-	-	0.81	-	-	-	-	-	0.81	-	-	-	-	-	-	31	-
F(3)A	(Rat)	-	-	0.81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	25	-
8D-1 ^a	(Hamster)	-	-	-	-	-	-	-	0.96	-	-	-	-	-	-	-	-	-	-	-	-	25	-
FF4-3a ^d	(Rat)	-	-	-	-	-	-	-	0.47	0.47	0.33	-	0.47	0.40	0.53	0.67	0.27	-	0.60	0.40	0.67	15	-

^aFournier, R. E. K. and Frelinger, J. A. (1982). *Mol. Cell. Biol.* 2: 526.
^bFournier, R. E. K. and Moran, R. G. (1983). *Somatic Cell Gen.* 9: 69.
^cKillary, A. M. and Fournier, R. E. K. (1984). *Cell* 38: 523.
^dPeterson, T. C., Killary, A. M. and Fournier, R. E. K. (1985). *Mol. Cell. Biol.* 5: 2491.
^eLem, J. and Fournier, R. E. K. (1985). *Somatic Cell and Mol. Gen.* 11: 633.

nated by type B spermatogonia cells and/or Sertoli cells. Cytological studies have observed no more than 1% contamination of leptotene, zygotene and pachytene spermatocytes isolated from prepubertal tubules by type B spermatogonia (Bellvé *et al.* 1977). Furthermore, hybridization studies with Sertoli cell-specific gene product, sulfated glycoprotein-2 (SGP-2) (Thomas *et al.* 1989), indicated that none of the purified cell-type RNA samples contained Sertoli cell RNA, suggesting that contamination of the day 17 RNA preparations with Sertoli cell RNA is an unlikely explanation. The most favored explanation for the LDH/B expression data appears to be as in (a) above; however, possibility (b) though equally likely, can only be further tested when specific molecular markers for type B spermatocytes are available.

The LDH/C (*ldh-3*) gene was expressed only after differentiation of the leptotene and zygotene spermatocytes had occurred around day 11 in the development of the seminiferous epithelium (Figs 2A, 2B and 5B). Expression increased significantly to higher levels by day 16 with differentiation of pachytene spermatocytes. Relatively abundant levels of LDH/C transcripts are detected in round spermatids, but found to decline in elongating spermatids and are barely detectable within residual bodies (Figs 2B, 5B, 6B and 6D).

It is interesting to speculate about the significance of the transient expression of the LDH/A (*ldh-1*) gene coincident with that of LDH/C (*ldh-3*) (Figs 3B and 6). The LDH/C isoenzyme with its much wider substrate specificity can certainly partially substitute for any metabolic functions served by the LDH/A isoenzyme (Blanco *et al.* 1976; Battelino *et al.* 1968; Battelino and Blanco, 1970; Everse and Kaplan, 1973; Blanco, 1980). The LDH/A (*ldh-1*) gene has been mapped to mouse chromosome 7 (Soares, 1987; O'Brien *et al.* 1978). By using somatic cell hybrids, the LDH/C (*ldh-3*) gene also maps to mouse chromosome 7 (Figs 9 and 10, Table 1). Thus, these two LDH gene loci are syntenic on mouse chromosome 7. Similar results have been obtained by mapping studies in humans (Edwards *et al.* 1987). One plausible explanation therefore, for coexpression of the LDH/A (*ldh-1*) and LDH/C (*ldh-3*) gene loci in pachytene spermatocytes involves the possibility of regulatory 'crosstalk', i.e., interactions of regulatory molecules at upstream promoter/enhancer regions of both genes. These two LDH genes loci may have retained common promoter/enhancer subelements that could recognize and respond to germ-cell-stage-specific transcription factors. Unfortunately, although the promoter sequences are available (Fukusawa *et al.* 1986), so far stage-specific DNA-binding proteins have not been identified or characterized. LDH/B transcripts were not detected in any later differentiated germ cells assayed by Northern blot analysis and by *in situ* hybridization. LDH/B transcripts were, however, found in spermatogonia and primary spermatocytes (Figs 4A, 4B, 7A and 7C). Studies by Brinster and Harstad (1977) have demonstrated that the LDH/B (*ldh-2*) gene is expressed in primordial germ cells.

The functional basis for expression profiles of the LDH genes during spermatogenesis is unknown. These studies, however, suggest that temporal LDH gene expression correlate with the stage of germ cell differentiation and thus their spatial position in the seminiferous epithelium. The LDH/B (*ldh-2*) gene is expressed mainly in germ cells which are situated close to the basement membrane. These germ cells have access to a readily available supply of metabolic nutrients from both the lymph and vascular systems. However, differentiating germ cells eventually migrate into an anaerobic adluminal microenvironment, physically separated from this source of nutrient supply by the Sertoli cell junctions. Later stages of differentiated germ cells were therefore found to express, appropriately, both the anaerobic Muscle (M)-type LDH/A (*ldh-1*) and germ-cell-specific LDH/C (*ldh-3*) gene products.

The various molecular mechanisms involved in regulating these complex patterns of expression observed for the LDH multigene family in differentiating germ cells are currently a matter of speculation. However, since all of the LDH genes have been isolated and sequenced, it should be feasible to utilize the respective promoter/enhancer sequences, to identify and further characterize promoter/enhancer subelements and any *trans*-acting factors involved in the temporal regulation of LDH gene expression during spermatogenesis.

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