

Regulation of the Synthesis of Lactate Dehydrogenase-X during Spermatogenesis in the Mouse

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ABSTRACT Total mouse testis RNA directs the synthesis of the sperm-specific C subunit of lactate dehydrogenase-X (LDH-X) when translated in a cell-free system derived from rabbit reticulocytes. The newly synthesized C subunits were isolated by immunoprecipitation with antibody specific for this isozyme, and quantitated by electrophoresis on SDS polyacrylamide gels. The amount of radioactivity incorporated into the enzyme subunit was directly proportional to the amount of testis RNA added to the translational system, thereby providing a sensitive and reliable method for assessing relative LDH-X mRNA activity. A combination of sucrose gradient centrifugation and oligo(dT)-cellulose chromatography resulted in a 23-fold purification of LDH-X mRNA over total cytoplasmic testis RNA.

Analysis of LDH-X mRNA activity in the developing testis indicated that the appearance of functional LDH-X mRNA activity coincides with the appearance of LDH-X catalytic activity at 14 d postpartum. Measurement of LDH-X mRNA levels in separated testis cell populations prepared by centrifugal elutriation demonstrated that LDH-X mRNA represents 0.17–0.18% of the total functional mRNA activity in fractions enriched in pachytene spermatocytes and round spermatids, but only 0.09–0.10% of the translation products of elongated spermatids.

The mature spermatozoon is a highly differentiated cell whose function is to transfer the male's chromosome complement to the ovum during fertilization. In support of this specialized function, sperm contain a large number of proteins which are not found in any other cell type (1). In addition to unique basic chromosomal proteins analogous to protamines in fish (2, 3), mammalian sperm contain specific isozymal variants of a number of enzymes including lactate dehydrogenase (LDH), phosphoglycerate kinase, diaphorase and hyaluronidase (1). The best-characterized sperm-specific isozyme is lactate dehydrogenase-X (LDH-X), which is synthesized only in the mature testis during spermatogenesis. In mice, LDH-X is a tetramer composed of four identical polypeptide subunits coded for by the lactate dehydrogenase C gene (hereafter *Ldh-C*), which apparently is active only in the testis (4). In contrast, the genes coding for the A and B subunits, from which the other LDH isozymes are assembled, are active in almost all mammalian tissues. Studies on the activity of the *Ldh-C* gene have been confined largely to measurements of LDH-X enzyme activity. As a first step towards elucidating the mechanisms which underlie the expression of the *Ldh-C* gene, an assay for LDH-X mRNA has been developed. In this paper, this assay is used

to analyze the levels of functional LDH-X mRNA present during specific stages of spermatogenesis in the mouse.

MATERIALS AND METHODS

Materials

L-[³⁵S]methionine (500–600 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). Oligo(dT)-cellulose was purchased from Collaborative Research Inc. (Waltham, Mass.).

Animals

C57B16/J or C3Hf/Bu mice were used in all experiments. No differences in LDH-X mRNA activity were observed between the two strains.

Assay for LDH-X Activity

LDH-X activity was measured by the method of Meistrich et al. (5), using α -ketovalerate as a substrate.

Antibody Preparation

Anti-LDH-X IgG was purified by ammonium sulfate precipitation from the serum of New Zealand White rabbits which had been immunized with mouse LDH-X purified by oxamate-Sepharose 4B affinity chromatography (6). The

specificity of the antibody was established by several criteria. A single precipitin line was observed when anti-LDH-X was tested against purified LDH-X or total testis homogenate on Ouchterlony double diffusion plates. No cross reaction was observed with the other purified mouse LDH isozymes. In addition, anti-LDH-X specifically neutralized LDH-X enzyme activity without affecting the other LDH isozyme activities. By the same criteria, antibodies formed in response to immunization with the somatic LDH isozymes (LDH 1 to 5) did not cross-react with LDH-X. Finally, isozymic specificity of these two antibody preparations was confirmed by the combined immunological-electrophoretic technique of Markert and Holmes (7) (Fig. 1).

Preparation of RNA

Total testis RNA was prepared by a modification of the procedure of Chirgwin et al. (8). Testes of 10 mice (4 g) were homogenized in 10 ml of 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 4 mM MgCl₂. A postmitochondrial supernate was prepared from the homogenate by centrifugation at 20,200 *g*_{max} for 10 min. This postmitochondrial supernate was then mixed with 2 vol of 6 M guanidine-HCl, 10 mM dithiothreitol in 20 mM sodium acetate, pH 5.2, and layered over 10 ml of a 5.7 M cesium chloride cushion in 0.1 M EDTA in a 40-ml centrifuge tube at room temperature. After centrifugation at 25°C for 16 h at 105,000 *g*_{max} in an IEC SB-110 rotor (Damon/IEC Div., Damon Corp., Needham Heights, Mass.), the RNA pellet was dissolved in H₂O, adjusted to 2% potassium acetate, pH 5.2, and precipitated by the addition of 2 vol of ethanol. After precipitation at -20°C for 16 h, the RNA pellet was collected by centrifugation at 17,300 *g*_{max} for 10 min, dissolved in H₂O, and precipitated again with ethanol as described above. The RNA precipitate was washed three times with 80% ethanol, dried under a stream of N₂, and dissolved in a minimum volume of H₂O. RNA from tissues other than testis was prepared by direct homogenization of ~1 g of tissue in 25 ml of 6 M guanidine-HCl, 10 mM dithiothreitol in 20 mM sodium acetate, pH 5.2, followed by centrifugation through CsCl and washing as described above. RNA concentrations were measured by absorbance at 260 nm, taking an absorbance of 1.0 to represent a concentration of 50 µg/ml.

Poly(A)⁺ and poly(A)⁻ RNA were prepared by two cycles of oligo(dT)-cellulose chromatography as described by Aviv and Leder (9), with all buffers containing 0.5% SDS. "Poly(A)⁺" and "poly(A)⁻" are purely operational and refer to RNA that binds and does not bind to oligo(dT)-cellulose, respectively.

Sedimentation analysis of testis RNA was performed on linear 5–20% sucrose gradients in 70% formamide, 10 mM Tris-HCl, pH 7.5, 3 mM EDTA. RNA samples were dissolved in gradient buffer and heated to 65°C for 3 min before

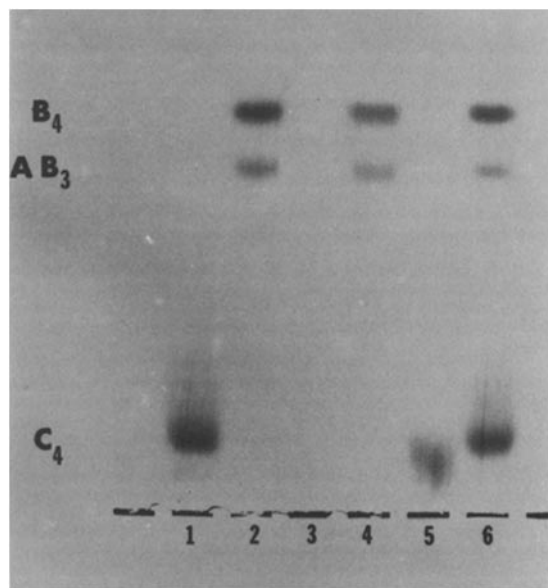


FIGURE 1 Isozymic specificity of anti-LDH-X and anti-LDH 1 to 5 antisera. Antigen-antibody combinations were incubated for 2 h at 25°C, and centrifuged for 5 min at 11,000 *g* before electrophoresis. The supernates were electrophoresed on starch gels for 16 h at 250 V, and gels were stained for LDH activity. Lane 1, purified LDH-X; lane 2, purified testis LDH 1 to 5; lane 3, 45 µl anti-LDH-X + 45 µl anti-mouse LDH 1 to 5 + 10 µl testis homogenate; lane 4, 90 µl anti-LDH-X + 10 µl testis homogenate; lane 5, 90 µl anti-mouse LDH 1 to 5 + 10 µl testis homogenate; lane 6, 10 µl testis homogenate.

centrifugation. They were centrifuged for 44 h at 40,000 rpm in an IEC SB-283 rotor.

Assay of LDH-X mRNA Activity

LDH-X mRNA activity was quantitated by its ability to direct the synthesis of the LDH C subunit in the mRNA-dependent reticulocyte cell-free translation system described by Pelham and Jackson (10), using 60–70 µCi of [³⁵S]methionine in a final reaction volume of 172.5 µl. After a 90-min incubation at 30°C, the reaction mixtures were chilled to 4°C and centrifuged at 105,000 *g* for 75 min. Further analysis revealed that the preparation of released polypeptide chains was not necessary for the quantitative immunoprecipitation of LDH-X. Hence, the 105,000 *g* centrifugation was omitted in some experiments. The amount of [³⁵S]methionine incorporated into total protein or released polypeptide chains was quantitated for a 5-µl sample by trichloroacetic acid precipitation. [³⁵S]methionine-labeled LDH-X was immunoprecipitated with anti-LDH-X IgG after the addition of 10 µg of pure unlabeled LDH-X as carrier. Immunoprecipitations and washing procedures were performed as described (11), except that 50 mM methionine was added to all buffers. The washed LDH-X immunoprecipitates were solubilized by boiling in 2-mercaptoethanol and SDS and then subjected to electrophoresis in 12.5% SDS polyacrylamide gels (12). After electrophoresis, gels were sliced and counted in 3% Protosol in Econofluor (New England Nuclear). The amount of [³⁵S]methionine incorporated into LDH-X was quantitated by summing the radioactivity of the four gel slices containing the enzyme peak and subtracting a background estimated from the radioactivity of the slices on both sides of the peak.

Gel Electrophoresis and Autoradiography

For analysis of total translation products, aliquots of [³⁵S]methionine-labeled translation products were mixed with an equal volume of double-strength sample buffer and electrophoresed on SDS polyacrylamide gels according to the procedure of Lefebvre et al. (13).

Separation of Testis Cells by Centrifugal Elutriation

The preparation of testis cell suspensions, separation of testis cells by centrifugal elutriation, and the counting of testicular cells were performed as described by Meistrich et al. (5). At least 400 cells were counted during the analysis of each fraction. After elutriation, the cells were washed one time with phosphate-buffered saline, then quick-frozen for later analysis.

RESULTS

Cell-free Synthesis of LDH-X

Total testis RNA stimulates the incorporation of [³⁵S]methionine into protein when added to the nuclease-treated, rabbit reticulocyte lysate translation system of Pelham and Jackson (10). The rate of incorporation was linear for 90 min and was directly proportional to RNA concentration up to 30 µg/ml (see Fig. 3, *inset*). At saturation, testis RNA consistently stimulated protein synthesis at least 40-fold over the endogenous background.

The cell-free synthesis of LDH C subunits in this system is demonstrated in Fig. 2. Total testis RNA directed the synthesis of a product which was precipitated by antibody specific for LDH-X and which co-migrated with authentic LDH-X subunits (*M*_r = 32,000) during SDS polyacrylamide gel electrophoresis of the immunoprecipitate (Fig. 2*b*). In the absence of added testis RNA, the synthesis of LDH C subunits was not detected. Furthermore, repeated analysis revealed that the amount of radioactivity incorporated into the LDH subunit peak was directly proportional to the concentration of testis RNA added to the translational system. Fig. 3 depicts a representative example of such an experiment and reveals that the linear relationship between RNA input and the *in vitro* synthesis of LDH is maintained at RNA concentrations as high as 26 µg/ml. A similar response was observed for total protein synthesis with respect to RNA concentration under these con-

ditions (Fig. 3, inset). Hence, cell-free translation provides a sensitive and reliable method for assessing LDH-X mRNA activity.

Subunit Composition of LDH Synthesized In Vitro

Total testis cytoplasmic RNA also directs the synthesis of somatic LDH subunits in the reticulocyte lysate. However, these mRNAs represent a much smaller proportion of the functional mRNA activity in the mouse testis than the mRNAs coding for the C subunit (Table I, exp 1). Random association of the LDH subunits produced in vitro into tetramers should produce primarily C₄ tetramers, given the preponderance of C subunits produced in vitro in response to total testis RNA (~15–20 C subunits per somatic [A or B] subunit, Table I).

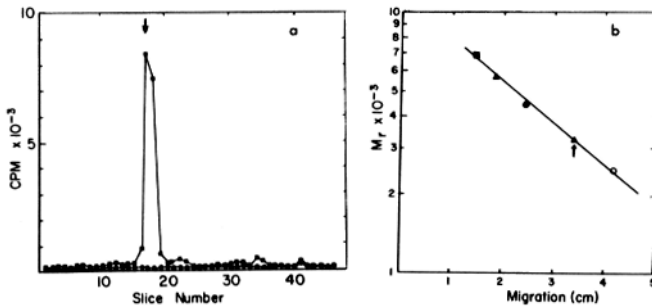


FIGURE 2 (a) SDS¹polyacrylamide gel electrophoresis of immunoprecipitated LDH-X synthesized in vitro. Radioactive LDH-X was immunoprecipitated from 450 μ l of released polypeptide chains synthesized in the reticulocyte system containing 58.5 μ g of testis RNA (■) or without added RNA (●). The arrow marks the position of authentic LDH-X. (b) Molecular weight of LDH C subunits synthesized in vitro. Purified LDH-X, molecular weight standards, and immunoprecipitated radioactive LDH C subunits were electrophoresed on parallel 12.5% SDS polyacrylamide gels as described. After electrophoresis, the position of the unlabeled proteins was determined by staining with Coomassie Blue. The position of the radioactive LDH subunit (arrow) was determined by slicing the gel and counting as described. The position of the radioactive LDH subunit was corrected for a 5% shrinkage of the gel during freezing. The molecular weight markers used were BSA (■), pyruvate kinase (\blacktriangle), ovalbumin (●), chymotrypsinogen (○), and purified LDH-X (Δ).

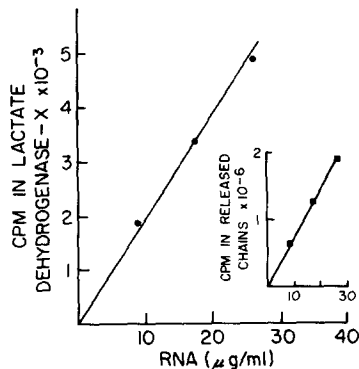


FIGURE 3 RNA concentration dependence for the in vitro synthesis of LDH-X. The counts per minute incorporated into the LDH-X immunoprecipitate peak by the reticulocyte system were calculated as described. Inset: Total radioactivity incorporated into 135 μ l of released chains was determined as described. Endogenous incorporation was subtracted from each of the values shown.

TABLE I
Isozymic Specificity of the Translational Assay for LDH-X mRNA Activity

RNA sample	Antibody directed against	cpm in Total protein ($\times 10^{-3}$)	cpm in LDH peak of immunoprecipitate
exp 1			
Total cytoplasmic testis RNA	LDH-X	970	1,620
Total cytoplasmic testis RNA	LDH 1 to 5	970	110
Total cytoplasmic testis RNA	LDH 1 to 5 and LDH-X	970	1,660
exp 2			
5 μ g Cytoplasmic RNA from elutriation fraction No. 1	LDH-X	445	250
5 μ g Total mouse heart RNA	LDH 1 to 5	470	480
5 μ g Cytoplasmic RNA from elutriation fraction No. 1 + 5 μ g total mouse heart RNA	LDH-X	911	230

However, because the spatial and temporal restrictions that limit heterotetramer formation in vivo do not apply to the subunits produced in vitro, a significant number of A-C and B-C heterotetramers could also be formed in the reticulocyte lysate. Because antibodies to LDH-X are capable of reacting with LDH tetramers containing even a single C subunit, a significant number of radioactively labeled A or B subunits might be co-precipitated from the total translation products, leading to an overestimation of the amount of LDH-X mRNA present in an RNA sample. The degree of error introduced by such heterotetramer formation would be minimal in the analysis of total RNA from the mature testis because comparatively few newly synthesized A and B subunits would be available for combination with the C subunits (Table I). Nevertheless, random association of newly synthesized subunits could produce significant error in the estimation of the LDH-X mRNA content of RNA samples from immature mice, which contain lower proportions of LDH-X. To test for the production of such heterotetramers, a combination of RNA samples from mouse heart (which is relatively rich in somatic LDH [14]) and elongated spermatids (which should contain mRNAs for LDH-X but not somatic LDHs) was translated in the reticulocyte lysate. Although there was a clear additive effect of the two RNAs on total protein synthesis, no radioactive LDH in excess of that produced in response to spermatid RNA alone was immunoprecipitated by the anti-LDH-X antibody (Table I, exp 2). The quantity of heart RNA used in the combined translation does direct the synthesis of a significant amount of somatic LDH subunits, as determined by the assay of a parallel translation for somatic LDH mRNAs. Therefore, the fact that none of these A or B subunits co-precipitated with the C subunits produced in response to the spermatid RNA indicates that the assay for LDH-X mRNA is not affected by the production of somatic LDH subunits. In addition, this result suggests that heteropolymers between newly synthesized C and (primarily) B subunits are not formed in the reticulocyte lysate.

Partial Purification of LDH-X mRNA

Total cytoplasmic RNA from mouse testis was fractionated by oligo(dT)-cellulose chromatography into an unbound fraction and a fraction that bound at high ionic strength but was eluted when the ionic strength was reduced. This latter fraction consistently contained 10–15% of the RNA applied to the column, in good agreement with previous analyses of testis RNA (15). Greater than 90% of the recoverable LDH-X mRNA activity was located in the bound and eluted fraction, indicating that most of this mRNA is polyadenylated. In contrast to the quantitative recovery of total RNA from the oligo(dT)-cellulose column, recoveries of LDH-X mRNA activity from the column typically amounted to only 50–60% of the total activity present in the original sample, indicating that some RNA was either degraded or remained bound to the column after elution with low ionic strength buffer. Because sedimentation of either total cytoplasmic or polyadenylated RNA on denaturing sucrose gradients did not reveal any significant amount of low molecular weight RNA species, the latter explanation appears to be the most likely. Similar recoveries have been documented by others (16).

As a preliminary step towards the purification of LDH-X mRNA, poly(A)⁺-enriched RNA was fractionated further by sedimentation through denaturing sucrose gradients. The specific activity of the peak of LDH-X mRNA activity from such a gradient was 23-fold higher than total cytoplasmic testis RNA. However, this represents a limited purification as only 0.8% of the total cell-free translation products directed by the gradient fraction most highly enriched for LDH-X mRNA activity are identifiable as LDH-X subunits.

Regulation of the Synthesis of LDH-X

The tissue specificity of LDH-X mRNA was investigated by preparing RNA from mouse heart, liver, kidney, ovary, and testis. Although each mRNA preparation stimulated [³⁵S]methionine incorporation into total protein, only testis RNA directed the synthesis of the LDH-X subunit as shown in Table II.

The electrophoretic analysis of LDH-X immunoprecipitates was sufficiently sensitive to have detected approximately one-fortieth of the LDH-X mRNA activity present in mouse testis. Therefore, LDH-X mRNA represents <0.007% of the functional mRNA activity in these other tissues, which is consistent with the presence of LDH-X enzyme activity only in the testis. This finding suggests that the synthesis of LDH-X is determined by the level of functional LDH-X mRNA in the testis. To investigate this possibility in greater detail, LDH-X mRNA activity was measured at various stages of spermatogenesis.

The synthesis of LDH-X during spermatogenesis begins at the mid-pachytene stage of meiotic prophase and continues throughout spermiogenesis. Thus, LDH-X activity is first detectable in the mouse testis 14 d after birth, coinciding with the progression of the first wave of spermatogenesis to the pachytene stage (17, 18). Fig. 4 demonstrates that the appearance of functional LDH-X mRNA activity in the testis coincides with the appearance of LDH-X catalytic activity. However, LDH-X mRNA accumulates much more rapidly than the specific activity of the enzyme, reaching half-maximal activity by day 20, as compared to day 25 for the enzymes. Because even the immature testis contains a number of cell types, it is difficult to correlate this rapid increase in LDH-X mRNA activity with an increase in the percentage of particular cell types. Therefore,

TABLE II
Tissue Specificity of LDH-X mRNA Activity

Source	Total cpm incorporated ($\times 10^{-3}$)/ μ g RNA	cpm in Total released chains ($\times 10^{-6}$)*	cpm in LDH-X*
Testis	424	1.27	3,400
Ovary	114	1.58	<100
Kidney	151	1.59	<100
Liver	189	1.59	<100
Heart	289	1.59	<100

Total RNA was isolated from each of the mouse tissues indicated and translated as described in Materials and Methods. All of the RNA concentrations used were below saturation levels for this system. Incorporation of [³⁵S]methionine into total protein and LDH-X was measured as described in Materials and Methods.

* Based upon analysis of 135 μ l of released chains from testis, 256 μ l from ovary, 193 μ l from kidney, 154 μ l from liver, and 102 μ l from heart.

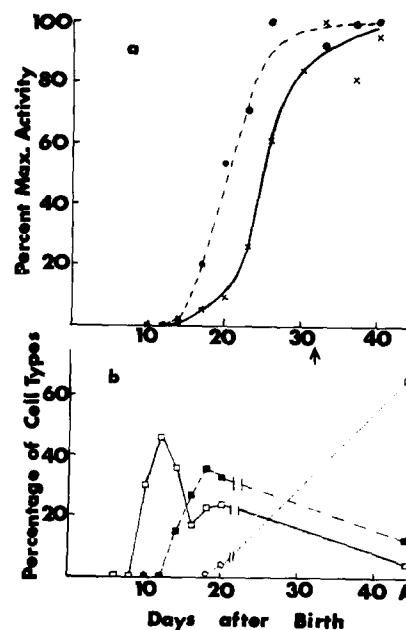


FIGURE 4 LDH-X mRNA activity during postnatal testis development. (a) Comparison of the accumulation of LDH-X enzyme activity (X) and LDH-X mRNA activity (●) during the postnatal development of the mouse. The arrow denotes the time of completion of the first wave of spermatogenesis. (b) Temporal appearance of specific cell types in the seminiferous epithelium of the mouse (drawn from data compiled by Bellive et al. [17]). (□) pre-pachytene spermatocytes; (■) pachytene spermatocytes; (○) spermatids. The percentage of Sertoli cells and spermatogonia in the seminiferous epithelium are not shown.

fractionated cell populations enriched in cells at specific stages of spermatogenesis were analyzed for functional LDH-X mRNA activity. The compositions of the cell populations used in this analysis are presented in Table III.

Although the translation efficiency of RNA samples from different cell fractions varies considerably, Table IV shows that LDH-X mRNA constitutes a constant proportion of total mRNA activity in the cell populations enriched in both pachytene spermatocytes and round spermatids (fractions 3–6 in Table III). In contrast, the populations enriched in elongated spermatids (fractions 1 and 2 in Table III) exhibited a considerably lower relative LDH-X mRNA activity. The reduction in LDH-X mRNA activity in these fractions (1 and 2) is

TABLE III
Percentage Cellular Composition of Fractions of Adult Mouse Testis Cells Separated by Elutriation

	Fraction					
	1	2	3	4	5	6
Rotor speed, rpm	3,000	3,000	3,000	2,000	2,000	2,000
Flow rate, ml/min	11.33	18.8	38.1	17.68	23.8	36.5
Cells/fraction ($\times 10^6$)	120	48	220	48	28	42
	Percent of total cells					
Cell type						
Spermatogonia and early primary spermatocytes	0	3	5	3	4	3
Pachytene spermatocytes	0	1	0	12	60	84
Secondary spermatocytes	0	0	0	4	0	0
Spermatids						
Steps 1-8	1	8	62	55	19	8
Steps 9-13	18	35	21	12	6	0
Steps 14-16	63	11	0	0	1	0
(Spermatid) cytoplasmic fragments*	18	42	12	14	7	3
Nongermlinal cells	0	0	0	0	3	2

* Most of the cytoplasmic fragments that were counted had the size and appearance of the cytoplasmic body of elongated spermatids.

paralleled by a noticeable shift in the pattern of total translation products from these fractions (Fig. 5). At least five major translation products present in each of the other four fractions are not detectable in fractions 1 and 2 (►). The appearance of three other major products coincides with an increasing percentage of spermatids in fractions 5-1 (<◄). However, each of the major translation products present in fractions 1 and 2 is also represented in fractions 3 and 4, although the relative proportions may be significantly altered.

DISCUSSION

LDH-X is one of a number of testis-specific enzymes that first appear during the pachytene stage of spermatogenesis. Recent evidence has demonstrated that the appearance of LDH-X activity in these cells results from the *de novo* synthesis of LDH C subunits in these cells, and that the active synthesis of this enzyme continues throughout spermatid development (5). The experiments presented here indicate that the initiation of LDH-X synthesis coincides with the appearance of functional LDH-X mRNA in the cytoplasm of these cells on day 14 postpartum. There is no detectable lag between the first appearance of LDH-X mRNA and the synthesis of functional LDH-X tetramers. However, the accumulation of LDH-X enzyme activity during the development of the testis lags ~4.5 d behind the accumulation of LDH-X mRNA activity. Although the delay between the accumulation of the mRNA and the accumulation of enzyme activity could be indicative of some form of post-transcriptional regulation of LDH-X synthesis, the relatively slow accumulation of enzyme activity is probably a reflection of the stability of the LDH-C₄ tetramer. Because LDH-X has a relatively long half-life, the rate of accumulation of the enzyme during development does not reflect the rate of synthesis (19). Thus, using separated cell fractions similar to those reported here, Meistrich et al. (5) found that although LDH-X specific activity was highest in elongated spermatids, the rate of synthesis of LDH-X in pachytene spermatocytes and round spermatids was twice that of elongated spermatids. This pattern of synthesis agrees favorably with the levels of LDH-X mRNA activity in these cell types. LDH-X mRNA represents 0.17-

TABLE IV
LDH-X mRNA Activity in Separated Testicular Cells *

Cell fraction no.	(a) Translational activity (cpm $\times 10^3$ / μ g RNA)	In vitro synthesis of LDH-X	
		(b) cpm/ μ g RNA*	% (b/a)
1	160	140	0.09
2	241	220	0.09
3	563	990	0.18
4	399	720	0.18
5	336	560	0.17
6	516	930	0.18
Unseparated control	306	470	0.15

* Average of two determinations.

0.18% of the total functional mRNA activity in fractions enriched in pachytene spermatocytes and round spermatids, but only 0.09-0.10% of the translation products of elongated spermatids. Hence, the rate of synthesis of LDH-X is directly correlated with the level of functional LDH-X mRNA activity. This finding suggests that the rate of synthesis of LDH-X is determined largely by the availability of functional LDH-X mRNA in the cytoplasm.

It is not clear whether the availability of translationally active LDH-X mRNA during spermatogenesis is regulated at the level of transcription or by some post-transcriptional event (e.g., processing of the primary transcript or temporary sequestration of the completed mRNA from the sites of protein synthesis in the cytoplasm). The binominal distribution of LDH-X isozymes in pigeons and humans heterozygous at the *Ldh-C* locus has traditionally been interpreted as an indication that the synthesis of LDH-X mRNA terminates before the meiotic divisions (20, 21). Indeed, the abundance of such evidence for a number of genetic markers has led to the hypothesis that much, if not all, of the mRNA utilized by spermatids is synthesized before the meiotic divisions and stored in some fashion until required during spermiogenesis

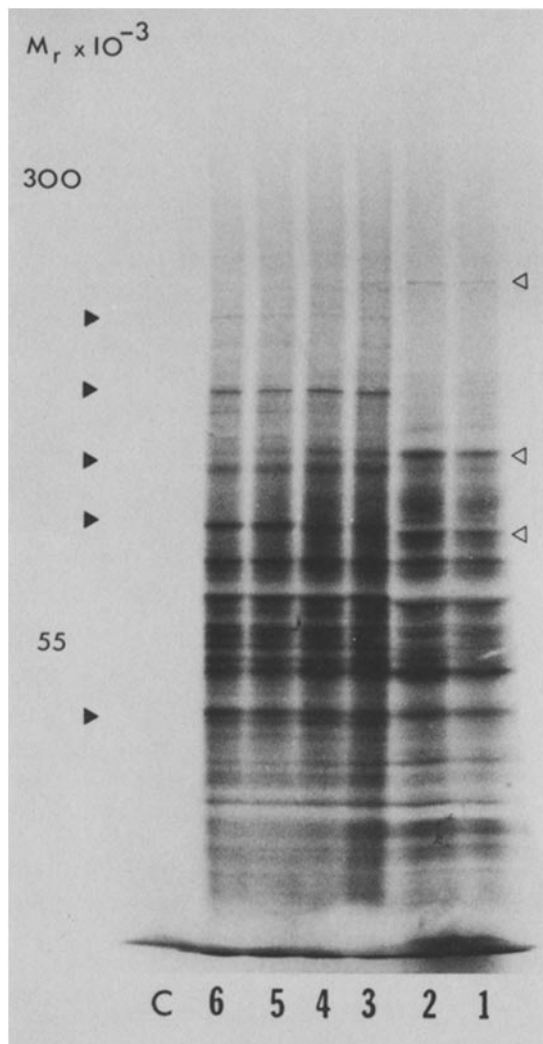


FIGURE 5 Analysis of translation products from separated cell fractions. Translation products of total cytoplasmic RNA (70,000 cpm/sample) from the separated cell populations described in Table IV (1-6) were electrophoresed on SDS polyacrylamide gels and autoradiographed as described in Materials and Methods. The endogenous products of the mRNA-dependent reticulocyte lysate (no added mRNA) are displayed in lane C.

(22-29). In this context, it is interesting to note that analysis of total translation products from separated cell populations reveals that the cytoplasm of round and elongated spermatids contains functional mRNAs not found in the diploid spermatocytes. Of several possible explanations for this finding, the simplest is that these mRNAs are first synthesized postmeiotically. Alternatively, it remains possible that some mRNAs are stored in the nuclei of spermatocytes until the meiotic cell divisions or that preexisting mRNAs are somehow activated in spermatid cytoplasm. However, the report of nuclear storage of mRNA (30) has not been confirmed, and it is difficult to reconcile the cytoplasmic storage of pre-meiotically synthesized mRNA sequences with the appearance of new functional mRNA species in spermatids. mRNA molecules "stored" in spermatocyte cytoplasm by simple sequestration from the sites of active protein synthesis could presumably be translated once these topographical restrictions were eliminated (by disruption of the cell and purification of the RNA, for example). In such a case, the translation products from round spermatids would

be identical to those from spermatocytes, which is inconsistent with the results depicted in Fig. 5. Hence, more complex mechanisms would have to be invoked to explain the appearance of new species of functional mRNA in spermatids in the absence of new mRNA synthesis. In light of these considerations, it seems more likely that synthesis of mRNA (including LDH-X mRNA) continues in round spermatids, and that functional diploidy is maintained by the extensive network of cytoplasmic bridges between spermatogenic cells (31, 32). This hypothesis is supported by recent studies of RNA metabolism during spermatogenesis. Using high-resolution electron microscopic autoradiography, Kierzenbaum and Tres (33) found that RNA precursors were incorporated at steadily decreasing rates by both round and elongating spermatids. Because they found no ultrastructural evidence of nucleoli in spermatids, they concluded that the primary product of this synthesis was hnRNA. Other studies have confirmed this pattern of synthesis but have indicated that 28S and 18S RNA (presumptive rRNA) is synthesized in round spermatids (34, 35). These same authors concluded that no RNA synthesis was detectable in the later stages of spermiogenesis. If the synthesis of mRNA is indeed terminated during the period of spermatid elongation, then the observed decrease in relative LDH-X mRNA activity in late spermatids could be attributed to the slow degradation of this mRNA. However, even as the activity of some mRNA sequences is declining in these cells, other mRNAs (such as those coding for the protamines [3]) must be synthesized (or activated), and the possibility that the observed decrease in the amount of LDH-X mRNA in these cells is caused by the increased synthesis of other mRNA species cannot be eliminated. The complete purification of LDH-X mRNA and the preparation of a cDNA probe should greatly facilitate the study of this and other questions pertaining to the regulation of LDH-X synthesis during spermatogenesis.

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