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LACTATE DEHYDROGENASE-C mRNA: ITS ISOLATION AND IN VITRO TRANSLATION

Aftab A. ANSARI, Masroor A. BAIG and Heinrich V. MALLING

Laboratory of Biochemical Genetics
National Institute of Environmental Health Sciences
P. O. Box 12233
Research Triangle Park, North Carolina 27709 USA

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SUMMARY: Lactate dehydrogenase-C (LDH-C) mRNA was purified from DBA/2 mouse testes and translated in vitro. First, the LDH-C synthesizing polysomes were isolated by double immunoprecipitation using specific anti-LDH-C and anti-horse immunoglobulin antibodies. Extraction of mRNA was made from the isolated polysomes using hot sodium dodecyl sulfate-phenol method at alkaline pH. In a wheat germ cell-free translation system, the mRNA coded for a polypeptide chain that could be immunoprecipitated with specific anti LDH-C antibody and comigrated with authentic LDH-C in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Higher animals contain three distinct types of lactate dehydrogenase (EC 1.1.1.27), the two major A and B isoenzymes, and a third isoenzyme, designated C or X (1-6). While the genes for A and B polypeptide chains are expressed in most somatic as well as germinal tissues, the gene encoding the C polypeptide is expressed only in the male germinal cells. The synthesis of LDH-C correlates with active spermatogenesis. Thus, LDH-C is not detectable in prepubertal gonad in human (2), pigeon (7), rabbit (8), bat (9), gerbil (10), rat (11), and mouse (12). In mammals showing testicular regression during a sexual cycle, e.g., the South American bat, the appearance or disappearance of LDH-C coincides with the initiation or cessation of spermatogenesis (13). Furthermore, the synthesis of LDH-C can be manipulated by sex hormones such as testosterone (14). These features make LDH-C gene an attractive model for studying gene regulation in higher animals. In addition, LDH-C gene offers an

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ABBREVIATIONS: LDH-C, lactate dehydrogenase-C; IgG, immunoglobulin G; SDS, sodium dodecyl sulfate, EDTA, ethylenediaminetetraacetic acid.

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attractive system for studying gene mutation in mammals (15,16). The first step toward these interesting goals is to isolate the mRNA for LDH-C and to demonstrate that it can be translated *in vitro*. This paper describes the isolation and *in vitro* translation of the LDH-C mRNA from mouse testes.

MATERIALS AND METHODS

Sterile Technique. All glassware was acid-washed and autoclaved. All buffers and sucrose solutions were treated with 0.1% diethylpyrocarbonate and autoclaved. Dialysis tubing was boiled in 5% $NaHCO_3$ containing 0.1 mM EDTA, washed in distilled water and autoclaved.

Antibodies. Antiserum against purified mouse LDH-C (17,18) was raised in Shetland ponies. Gamma globulin fraction was isolated from the antiserum by 18% Na_2SO_4 precipitation and the specific anti LDH-C antibody was purified by immunoabsorbent chromatography using a glutaraldehyde crosslinked LDH-C Sepharose immunoabsorbent (19). The antibody was absorbed twice on plain Sepharose 4B to remove any natural anti-saccharide antibodies (20) and then dialyzed against 0.1 M NaCl and stored at -80°C. The purified antibody showed no cross-reaction with either LDH-A or LDH-B as judged by Ouchterlony gel diffusion, immunoprecipitation and enzyme-linked immunoabsorbent assay. When mouse testicular homogenate was immunoprecipitated with the purified anti LDH-C antibody and the resulting precipitate analyzed by SDS-polyacrylamide gel electrophoresis, a band corresponding to LDH-C position was detected; no extraneous testicular protein was detected, further demonstrating the specificity of the antibody preparation.

Specific rabbit anti-horse IgG antibody was purified by first absorbing the antiserum (Cappel Laboratories, PA) on plain Sepharose 4B followed by immunoabsorbent chromatography as described earlier (19).

Isolation of LDH-C mRNA. Testes were aseptically removed from DBA/2J mice (Jackson Laboratory, ME) and frozen immediately in liquid nitrogen. The conditions for homogenization and isolation of total polysomes were the same as described by Palacios *et al*. (21). Isolation of the specific LDH-C polysomes from the total polysomes was carried out by immunoprecipitation as described by Schechter (22). The total polysome suspension was supplemented with sodium deoxycholate and Triton X-100 to a final concentration of 0.4% each and centrifuged at 9500 g for 20 min at 2°C. The antibody solutions were supplemented with heparin (10 units/ml), sodium deoxycholate (0.4%) Triton X-100 (0.4%) and magnesium acetate (5 mM) and clarified by centrifugation at 12,000 gfor 20 min at 2°C. To the polysome suspension were added purified horse anti-mouse LDH-C antibody (6 µg antibody/1 A_{260} unit of polysome) and, 6 min later, purified rabbit anti-horse IgG (72 µg antibody/1 A_{260} unit of polysome). After 1 hr incubation at 4°C, the mixture was centrifuged at 9000 g for 20 min at 2°C. The precipitated polysomes were washed twice with 25 mM Tris-HCl, pH 7.6 containing 100 mM sucrose, 50 mM KCl, 5 mM magnesium acetate, 0.2% Triton X-100, and 10 units/ml of heparin.

Extraction of mRNA from the specific polysomes was achieved by SDS-phenol extraction at alkaline pH essentially as described by Schechter (22). The immunoprecipitated polysomes were suspended in 10 mM Tris-HCl, pH 9.0, containing 100 mM sucrose, 10 mM KCl, 2 mM magnesium acetate and 3% SDS, and extracted with an equal volume of water-saturated phenol at room temperature for 10 min and in ice for 5 min. After centrifugation at 12,000 g for 10 min, the aqueous phase was collected and the phenol phase was re-extracted for 10 min at room temperature with an equal volume of 10 mM Tris-HCl, pH 9.0, containing 0.5%

sodium deoxycholate. The aqueous phases from the two extractions were combined and re-extracted three times with phenol at 4°C. The aqueous phase from the last extraction was made 0.2 M in NaCl and 0.2 M in sodium acetate, pH 5.5, and the mRNA was precipitated with 2 volumes of ethanol at -20°C.

Removal of heparin from the mRNA preparation was carried out at this stage by LiCl precipitation (23). The ethanol precipitated RNA was dissolved in 1 ml of 10 mM EDTA, pH 7.3, and an equal volume of 4.0 M LiCl was added. After overnight incubation at 2°C, the mixture was centrifuged at 12,000 g for 10 min. The pellet was washed twice with 2 M LiCl-10 mM EDTA, pH 7.3, and then dissolved in 0.2 M potassium acetate, pH 5.5. Ethanol precipitation of RNA was carried out twice from potassium acetate solution to remove LiCl. The final pellet was dissolved in water and then lyophilyzed to get rid of ethanol. The residue was dissolved in water and distributed in aliquots.

Translation of LDH-C mRNA. Wheat germ cell-free system (Bethesda Research Laboratories, MD) was used to assay the translational activity of the purified mRNA. [^{35}S]Methionine (1010 Ci/mmole, New England Nuclear, MA) was used as the labeled amino acid. A 60 µl assay mixture contained the following: HEPES (27 mM), magnesium acetate (2.3 mM), KCl (96 mM), β-mercaptoethanol (1.7 mM), creatine kinase (6 µg), creatine phosphate (5.5 mM), spermidine (80 µM), 19 unlabeled amino acids (50 µM each), [^{35}S]methionine (4 µM, 82 µCi) and 0.7-5 µg of mRNA. The reaction was allowed to proceed for 60 min at 25°C. To estimate total protein synthesis, 15 µl of the mixture was transferred to a tube containing 15 µl of 5% bovine serum albumin followed by the addition of 0.7 ml of 5% trichloroacetic acid containing 10 mg/ml unlabeled methionine. Samples were incubated at 95°C for 15 min and in an ice bath for 45 min before centrifuging at 2000 g for 5 min. The precipitate was washed three times with 5% cold trichloroacetic acid, dissolved in 0.5 ml NCS tissue solubilizer and counted in a Beckman 7000 scintillation counter.

Identification of LDH-C in the Translation Mixture. The reaction mixture was centrifuged at 183,000 g for 10 min in a Beckman Airfuge. The supernatant was either directly mixed with an equal volume of sample buffer (0.1 M Tris containing 2% SDS, 20% glycerol and 0.2 M dithiothreitol, pH 7.0) for SDS-polyacrylamide gel electrophoresis or the LDH-C was first immunoprecipitated using the double antibody method. For immunoprecipitation, the high speed supernatant (40 µl) was added to 130 µl of a mixture containing 1 µg of purified mouse LDH-C and 6 µg of purified horse anti-mouse LDH-C in 50 mM phosphate buffered saline, pH 7.2-10 mM methionine-1% Triton X-100. After shaking the mixture for 30 min at room temperature and for 60 min at 4°C, 75 µg of the purified rabbit anti-horse IgG was added and the mixture was again shaken in the cold for 2 hr. The immune precipitate was collected by centrifugation for 2 min in a Beckman Microfuge and washed three times by suspending in 500 µl of 50 mM phosphate buffered saline, pH 7.2-10 mM methionine-1% Triton X-100. The final pellet was dissolved in 30 µl of the sample buffer and heated in boiling water bath for 5 min before electrophoresis. Controls for the immunoprecipitation experiment included (a) replacing the purified horse anti-mouse LDH-C antibody with normal horse gamma globulin, (b) immunoprecipitation of the supernatant from a reaction mixture lacking LDH-C mRNA and (c) immunoprecipitation with horse anti-mouse LDH-C that had been preincubated with purified mouse LDH-C. Electrophoresis in 10% polyacrylamide slab gels in the presence of 0.1% SDS was carried out according to the procedure of Laemmli (24). Purified mouse LDH-C was also electrophoresed simultaneously. For autoradiography the gel slab was treated with an autoradiographic enhancer, "Enhance" (New England Nuclear, MA) for 30 min after the destaining step, washed with a mixture of 10% acetic acid-2% glycerol and then dried on Whatman 3 MM paper using slab gel dryer (BioRad Laboratories, CA). It was then exposed to Kodak X-Omat-R film.

TABLE I

EFFECT OF LiCl PRECIPITATION OF LDH-C mRNA ON ITS TRANSLATIONAL ACTIVITY IN THE WHEAT GERM CELL-FREE TRANSLATION SYSTEM

Exogenous mRNA	TCA Precipitable Radioactivity
	cpm x 10^{-4}
None	5.02
LDH-C mRNA before LiCl precipitation (0.7 µg)	0.79
LDH-C mRNA after LiCl precipitation (0.7 µg)	12.75

RESULTS AND DISCUSSION

In order to isolate specific LDH-C mRNA, the polysomes involved in the synthesis of the enzyme were first isolated by specific immunoprecipitation using a double antibody method. The hot SDS-phenol procedure at alkaline pH (25) was used to extract mRNA from these polysomes. Extraction at the basic pH and at elevated temperature was necessary for the quantitative recovery of the mRNA because most of the eukaryotic mRNAs contain relatively large segments of adenylic acid residues and this makes the mRNA refractory to the phenol extraction carried out in cold at neutral or acidic pH (26). The A_{260}/A_{280} ratio for the purified mRNA was in the range 2.0-2.1 indicating its purity.

We have included LiCl precipitation of mRNA in our purification protocol for a specific reason. Table 1 shows the data on translational activity of purified LDH-C mRNA before and after precipitation with LiCl. Since wheat germ cell-free system has some intrinsic translational activity, trichloroacetic acid precipitable radioactivity was observed even when no exogenous mRNA was added (blank). Surprisingly enough, the amount of trichloroacetic acid precipitable radioactivity decreased when purified LDH-C mRNA was added to the translation mixture before precipitation with LiCl. The decrease in the

translational activity of the wheat germ system, upon the addition of mRNA, was indicative of the presence of some inhibitor of protein synthesis in our mRNA preparation. It was later discovered that the mRNA preparation was contaminated with heparin, which is a potent inhibitor of protein biosynthesis (23). Heparin was used in the isolation procedure to inhibit ribonuclease activity. During the phenol extraction, heparin stayed with mRNA in the aqueous phase and was precipitated with mRNA during ethanol precipitation. Therefore, the ethanol precipitated mRNA was further precipitated and washed with LiCl solution, a medium in which heparin is soluble. LiCl was then removed from mRNA by repeating the ethanol precipitation. When this mRNA preparation was translated in the wheat germ system, high amounts of trichloroacetic acid precipitable radioactivity was obtained (Table I) demonstrating the translational activity of the purified mRNA.

Identification of LDH-C Synthesized In Vitro. Purified LDH-C mRNA was translated in the wheat germ system in the presence of [^{35}S]methionine and the products were analyzed in SDS-polyacrylamide gels followed by autoradiography. The results are shown in Fig. 1. Several radioactive protein bands were seen (Fig. 1, lane 2), including a band that co-migrated with authentic mouse LDH-C. In an attempt to determine whether or not this band was due to LDH-C, we employed an immunological technique: (a) the polypeptide chains in the high speed supernatant were reacted with purified horse anti-mouse LDH-C antibody; (b) the LDH-C-anti-LDH-C complex was precipitated by purified rabbit anti-horse IgG; and (c) the precipitate was washed free of extraneous proteins, dissolved and denatured in SDS, and subjected to SDS-polyacrylamide gel electrophoresis. Only a single radioactive band was seen in the autoradiogram that co-migrated with mouse LDH-C (Fig. 1, lane 6). In a control where LDH-C mRNA was not added to the translation mixture, no radioactive band was observed after immunoprecipitation (Fig. 1, lane 5). Also, no radioactive band was seen in another control where the horse anti-mouse LDH-C antibody was substituted by normal horse IgG at the immunoprecipitation step (Fig. 1, lane 7). Appearance of the radioactive band co-migrating with LDH-C was

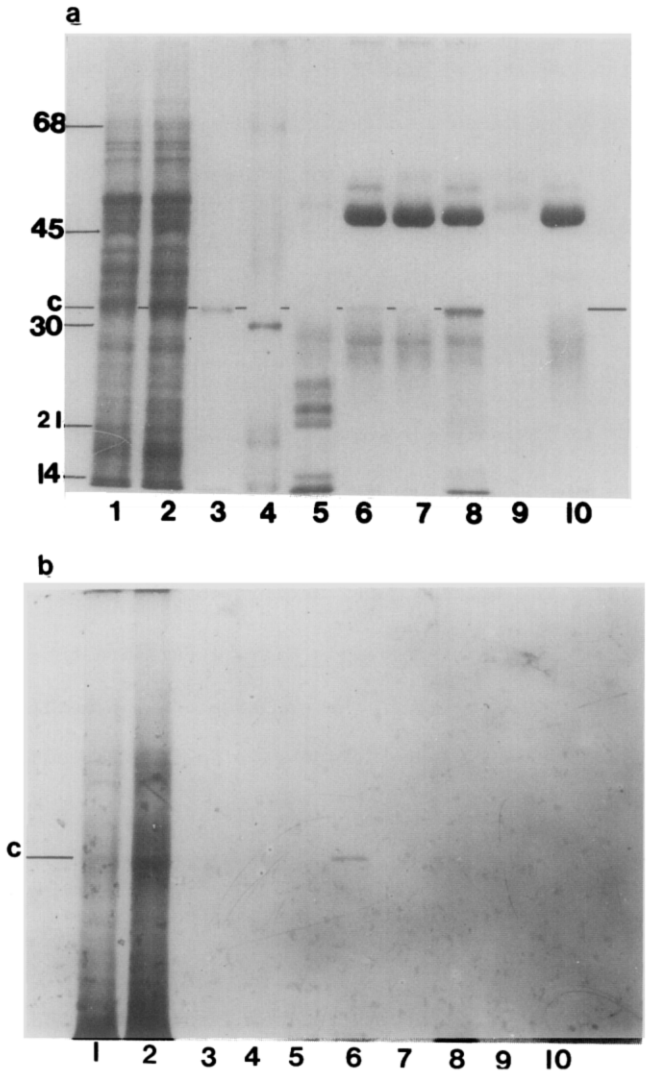

Fig. 1. SDS-polyacrylamide gel electrophoretic analysis of the in vitro synthesized polypeptide products of mouse LDH-C mRNA in a wheat germ translation system.

(a) Polypeptides stained with coomassie brilliant blue.

(b) Autoradiogram of the electrophoretogram showing the ^{35}S label.

Numbers on the horizontal axis represent lane numbers; numbers on the vertical axis are molecular weights in thousands, c = LDH-C. Lane 1, no exogenous mRNA added, total protein; Lane 2, LDH-C mRNA added, total protein; Lane 3, authentic LDH-C; Lane 4, protein molecular weight standards; Lane 5, no exogenous mRNA added, immunoprecipitation with horse anti-LDH-C and rabbit anti-horse IgG antibodies; Lane 6, LDH-C mRNA added, immunoprecipitation with the antibodies; Lane 7, LDH-C mRNA added, anti-LDH-C antibody replaced with normal horse IgG; Lane 8, LDH-C mRNA added, immunoprecipitation with the anti-LDH-C antibody preincubated with 100 µg of purified LDH-C; Lane 9, normal horse IgG; Lane 10, mixture of horse anti-LDH-C and rabbit anti-horse IgG antibodies showing the positions of light and heavy chains of the antibodies.

completely inhibited when the anti-LDH-C antibody was incubated with 100 μg of LDH-C prior to immunoprecipitation (Fig. 1, lane 8). These data demonstrated that the specific LDH-C mRNA purified from mouse testes was faithfully translated in the wheat germ cell-free system. Also, since the in vitro translated, labeled LDH-C co-migrated with authentic mouse LDH-C, presequence of LDH-C, if any, must be short.

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