Expression of the cDNA encoding for mouse sperm-specific lactate dehydrogenase C in Chinese-hamster ovary cells

Esther W. HOU, Ikuya SAKAI and Steven S.-L. LI*

Laboratory of Genetics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, U.S.A.

The cloned cDNA encoding for mouse sperm-specific lactate dehydrogenase C (LDH-C) was inserted immediately downstream to the MMTV 5' LTR promoter, and it was shown to synthesize mouse LDH-C polypeptide in Chinese-hamster ovary cells. The mouse LDH-C subunit and the endogenous Chinese-hamster LDH-A subunit formed *in vivo* a heterotetrameric LDH-A₃C₁ isoenzyme, and this novel isoenzyme exhibited enzymic activity utilizing lactate as substrate.

INTRODUCTION

In mammals, the five isoenzymes of tetrameric lactate dehydrogenase (LDH), which are formed *in vivo* by random combination of the LDH-A and LDH-B subunits, are found in various proportions among different somatic tissues, whereas the homotetrameric LDH-C₄ isoenzyme is present only in mature testis and spermatozoa [1]. The enzymic properties, amino acid sequence and three-dimensional structure of mouse LDH-C isoenzyme have been studied extensively [2–4]. The LDH-C protein is highly immunogenic in females as well as in males, because an effective blood–testis barrier isolates LDH-C from the animal's immune system. The active immunization of animals with the LDH-C autoantigen has been shown to reduce fertility in females [5]. In order to study LDH-C gene expression during spermatogenesis and to pursue the development of a contraceptive vaccine using this LDH-C autoantigen, we have undertaken the molecular cloning of the cDNA encoding for the mouse LDH-C polypeptide chain [6]. In the present work the cloned mouse LDH-C cDNA was inserted immediately downstream to the MMTV 5' LTR promoter and was shown to synthesize the LDH-C subunit in CHO cells.

MATERIALS AND METHODS

The cloned mouse LDH-C cDNA consists of the protein-coding sequence (996 bp), the 5' (54 bp) and 3' (116 bp) non-coding regions, and poly(A) tail (70 bp) described previously [6], and this cDNA was inserted immediately downstream to the dexamethasone-inducible MMTV 5' LTR promoter (Fig. 1) of the mammalian expression vector pMSG developed from pMDSG [7]. The plasmid pMSG also contains the *Escherichia coli* *gpt* gene expressed by SV40 early promoter as a selection marker. The orientation of the LDH-C cDNA relative to the LTR promoter in the recombinant plasmid DNAs was determined by restriction-endonuclease cleavage and Southern blotting with the LDH-C cDNA probe [8].

The CHO cell line X3/5 is a well-characterized transfection host [9], and is useful because these cells appear to synthesize only the LDH-A₄ isoenzyme (Fig. 2). CHO X3/5 cells were seeded at a density of 5×10^5 per 100 mm dish with 10 ml of Ham's F-12 medium

supplemented with 10% (v/v) heat-inactivated fetal-calf serum under 10% CO₂ in air at 37°C, and were transfected with 10 µg of supercoiled plasmid DNA coprecipitated with calcium phosphate [10]. To elicit the expression of the *gpt* gene, a selective medium including aminopterin (1.3 µg/ml), mycophenolic acid (10 µg/ml), xanthine (250 µg/ml), adenine (25 µg/ml) and

Fig. 1. Structural map of the recombinant plasmid pMSG-mC1

The *EcoRI* fragment containing mouse LDH-C cDNA was inserted at the *XhoI* site of the mammalian expression vector pMSG (Pharmacia), and the inserted cDNA of 1242 bp can be recovered either by *EcoRI* or *XhoI* cleavage. In plasmid pMSG-mC1, the mouse LDH-C cDNA is oriented properly relative to the MMTV 5' LTR promoter for expressing LDH-C, and its protein-coding sequence is indicated by solid block with the 3' untranslated region hatched. The pMSG vector also contains SV40 early promoter–*E. coli* *gpt* gene as a selection marker. The splice site and polyadenylation signal (An) provide proper processing and transport of poly(A)⁺ RNAs. The numbers of base-pairs between restriction sites are also given. Plasmid pMSG-mC2 possesses the LDH-C cDNA insert in the reverse direction.

Abbreviations used: CHO cells, Chinese-hamster ovary cells; LDH, lactate dehydrogenase; SV40, simian virus 40.

* To whom correspondence and reprint requests should be addressed.

Fig. 2. Agarose-gel analysis of LDH isoenzymes

The LDH isoenzymes present in soluble protein fractions extracted from mouse heart, Chinese-hamster liver and CHO cell line X3/5 (CHO) were analysed on an agarose-gel electrophoresis system [12]. Only a single LDH-A₄ isoenzyme was found in CHO cells.

thymidine (12 µg/ml) was used [11]. Only CHO X3/5 cells expressing *E. coli gpt* gene will grow and form colonies under this selection regime. After 2–3 weeks in selective medium, a mixed population was pooled from three dishes and re-plated. The cells were grown without selection for 2–3 days followed by 7 days under selection conditions to ensure that most of them contained the SV40 promoter-*gpt* gene as well as LTR promoter-LDH-C cDNA fusion gene. In order to induce the expression of the LTR promoter-mouse LDH-C cDNA, CHO X3/5 cells were treated with 0.3 µM-dexamethasone for 2–3 days before they were harvested.

The cellular DNAs were isolated from the CHO cells transfected with plasmid pMSG, pMSG-mC1 or pMSG-mC2 (Fig. 1), and subjected to restriction-endonuclease cleavage and Southern-blot analysis [8]. The LDH isoenzymes present in the soluble protein fraction were analysed on an agarose-gel system [12], and stained for LDH enzymic activity with lactate as substrate. The proteins separated on agarose gels in non-denaturing buffer as well as SDS/polyacrylamide gels [13] were also transferred on to nitrocellulose filter, and the mouse LDH-C protein was detected immunochemically by rabbit antiserum prepared against mouse LDH-C isoenzyme [3].

RESULTS

Characterization of recombinant plasmids containing mouse LDH-C cDNA

The mouse LDH-C cDNA of 1242 bp was inserted at the *Xho*I site immediately downstream to the LTR promoter of mammalian expression vector pMSG. The recombinant plasmid pMSG-mC1 contains LDH-C cDNA in the proper orientation for expressing LDH-C polypeptide chain (Fig. 1), whereas plasmid pMSG-mC2 possesses the LDH-C cDNA insert in the reverse direction. The restriction DNA fragments obtained from the CHO cells stably transfected with either plasmid pMSG-mC1 or pMSG-mC2 were hybridized to the LDH-C cDNA probe, and the results are presented in Fig. 3. The *Hind*III fragment of 3.9 kb, *Xho*I fragment of 1.2 kb and *Eco*RI-*Pst*I fragment of 1.1 kb were recovered from the CHO cells transfected with either plasmid pMSG-mC1 or plasmid pMSG-mC2. The *Pst*I fragments

Fig. 3. Southern-blot analysis of the cellular DNAs

The cellular DNAs were isolated from CHO X3/5 cells transfected with plasmids pMSG (CHO), pMSG-mC1 (mC1) or pMSG-mC2 (mC2) and cleaved with restriction endonucleases *Hind*III (H), *Pst*I (P), *Eco*RI (E) and/or *Xho*I (X). These DNA fragments were separated on 1% agarose gels and Southern-blotted on to nitrocellulose membrane. The filter was hybridized at 65 °C overnight with ³²P-labelled *Eco*RI 1242 bp fragment of mouse LDH-C cDNA as a probe and washed with 0.1 × SSC at 65 °C for 4 h [8]. The estimated sizes in kilobases of major DNA fragments are indicated. The disparate levels of hybridization signal in different lanes might be due to different amounts of DNA used and/or various extents of partial cleavage.

of 4.1 kb and 4.7 kb were observed from those of pMSG-mC1 and pMSG-mC2 respectively. The *Hind*III-*Pst*I fragments of 2.5 kb and 1.4 kb were present in the cells transfected with pMSG-mC1, and the *Hind*III-*Pst*I fragments of 2.3 kb and 1.6 kb (weak signal) were from those of pMSG-mC2. These results indicate that the stably transfected DNAs of pMSG-mC1 and pMSG-mC2 were most probably integrated into the CHO chromosomes in head-to-tail tandem array. Those DNA fragments whose sizes are larger than *Hind*III of 3.9 kb, *Pst*I of 4.1 or 4.7 kb and *Hind*III-*Pst*I of 2.5 or 2.3 kb were probably derived from the integrated plasmid DNA fragments containing host DNA and/or partial restriction cleavage. The mouse LDH-C cDNA probe did not cross-hybridize with the DNA fragments isolated from CHO cells transfected with pMSG vectors under the conditions used in this experiment.

Expression of the mouse LDH-C subunit in CHO cells

The CHO cells transfected with plasmid pMSG-mC1 containing the mouse LDH-C cDNA in the proper

Fig. 4. Expression of mouse LDH-C subunit in CHO cells

The CHO cells transfected with either plasmid pMSG, pMSG-mC1 (mC1) or pMSG-mC2 (mC2) were treated with and without dexamethasone (DEX), and the LDH isoenzymes from these CHO cells as well as from mouse testis were electrophoresed on a Beckman Paragon agarose gel in non-denaturing buffer, pH 8.2 [12]. The Chinese-hamster LDH-A subunit is labelled as 'a' in order to distinguish it from the mouse LDH-A subunit.

orientation relative to the LTR promoter were able to synthesize the mouse LDH-C subunit, and this LDH-C subunit and the endogenous Chinese-hamster LDH-A subunit formed a heterotetrameric LDH-A₃C₁ isoenzyme (Fig. 4). The expression of the LTR promoter-mouse LDH-C cDNA in CHO cells was induced by dexamethasone, as expected. The mouse LDH-C subunit was not synthesized in the CHO cells transfected with plasmid pMSG-mC2, because the LDH-C cDNA was inserted in the reverse direction relative to the LTR promoter. Also, only homotetrameric LDH-A₄ isoenzyme was present in the CHO cells transfected with the vector pMSG containing no cDNA insert. The native proteins present on the agarose gel were transferred on to nitrocellulose filter, and the LDH-C subunit present in heterotetrameric LDH-A₃C₁ isoenzyme was detected immunochemically (Fig. 5). The presence of the denatured mouse LDH-C as well as the endogenous LDH-A polypeptide chain were also detected immunochemically on the protein blot transferred from an SDS/polyacrylamide gel (Fig. 6). The mouse LDH-C polypeptide of 35 kDa appeared to have a little faster mobility on SDS/polyacrylamide-gel electrophoresis than did the Chinese-hamster LDH-A subunit.

DISCUSSION

The mouse LDH-C cDNA promoted by MMTV 5' LTR was shown to synthesize the LDH-C subunit in CHO cells. Thus the LTR promoter-LDH-C cDNA fusion gene appeared to be transcribed and translated properly, although the extent of its expression was relatively low compared with that of the endogenous

Fig. 5. Immunodetection of the mouse LDH-C subunit in a heterotetrameric LDH-A₃C₁ isoenzyme

The soluble proteins separated on the agarose gel (Fig. 4) were transferred on to nitrocellulose membrane by a simple capillary procedure. The nitrocellulose membrane was laid on the agarose gel with several sheets of absorbing paper above the filter, and a weight was put on the top for 30 min to facilitate the protein transfer. The LDH-A₃C₁ as well as LDH-A₄ isoenzyme was detected immunochemically with rabbit anti-(mouse LDH-C) serum [3] and a Promega immunoblotting system.

Fig. 6. Immunodetection of the denatured LDH-C polypeptide

Discontinuous-polyacrylamide-(10 %)-slab-gel electrophoresis with the Tris/glycine/SDS buffer [13] to separate proteins of different molecular masses. The cellular proteins along with protein markers were either stained with Coomassie Brilliant Blue R250 for estimating molecular masses of LDH polypeptides (not presented) or Western-blotted on to a nitrocellulose filter for immunodetection with rabbit anti-(mouse LDH-C) serum [3].

Chinese-hamster LDH-A gene. The exogenous mouse LDH-C subunit and Chinese-hamster LDH-A subunit were able to form a heterotetrameric LDH-A₃C₁ isoenzyme, indicating that the tertiary structures of mouse LDH-C and Chinese-hamster LDH-A subunits are similar and compatible with each other. The LDH-A₃C₁ isoenzyme exhibited enzymic activity with lactate as substrate. However, preliminary results seemed to suggest that this LDH-A₃C₁ isoenzyme was unable to utilize α -hydroxyvalerate as a substrate and was not stable at 65 °C for 20 min, as is the mouse LDH-C₄ isoenzyme [14].

In the present study the rabbit antiserum prepared against mouse LDH-C protein [3] exhibited some cross-reactivity on the protein blots to Chinese-hamster LDH-A protein in addition to mouse LDH-C subunit. Similar immunochemical cross-reactivity has been observed previously between anti-(mouse LDH-C) serum and LDH-A, as well as the LDH-B antigen [15,16]. It may be noted that there are at least 60% sequence homologies among mammalian LDH-A, LDH-B and LDH-C proteins [17]. The previously reported non-cross-reactivity of the anti-LDH-C serum with native LDH-A₄ and LDH-B₄ isoenzymes [3,18,19] may be explained by the fact that the antibodies directed against the antigenic determinants of the native LDH-C₄ isoenzyme have already formed immunocomplexes with the cross-reactive LDH isoenzymes present in rabbit serum. Since the amino acid sequence of the loop region of LDH-C is remarkably different from the corresponding sequences of somatic LDH-A and LDH-B proteins [2], the genetically engineered or chemically synthesized loop peptide instead of the whole LDH-C protein may be used in preparing an effective contraceptive vaccine, thus avoiding the potential problems caused by immunocross-reactivity [3].

We thank Dr. B. H. Judd for this interest in and support of this project, Dr. K. R. Tindall for CHO X3/5 cell line, Dr.

D. O'Brien and Dr. K. R. Tindall for reading the manuscript, and Ms. N. Gore for typing it.

REFERENCES

1. Markert, C. L. Shakelee, J. B. & Whitt, G. S. (1975) *Science* **189**, 102–114
2. Pan, Y.-C. E., Sharief, F. S., Okabe, M., Huang, S. & Li, S. S.-L. (1983) *J. Biol. Chem.* **258**, 7005–7015
3. Li, S. S.-L., Feldmann, R. J., Okabe, M. & Pan, Y.-C. E. (1983) *J. Biol. Chem.* **258**, 7017–7028
4. Musick, W. D. L. & Rossmann, M. G. (1979) *J. Biol. Chem.* **254**, 7611–7620
5. Goldberg, E. (1973) *Science* **181**, 458–459
6. Sakai, E., Sharief, F. S. & Li, S. S.-L. (1987) *Biochem. J.* **242**, 619–622
7. Lee, F., Mulligan, R., Berg, P. & Ringold, G. (1981) *Nature (London)* **294**, 228–232
8. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor
9. Tindall, K. R., Stankowski, L. F., Machanoff, R. & Hsie, A. W. (1984) *Mol. Cell. Biol.* **4**, 1411–1415
10. Parker, B. A. & Stark, G. R. (1979) *J. Virol.* **31**, 360–369
11. Mulligan, R. C. & Berg, P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2072–2076
12. Beckman Instruments (1981) *Paragon Electrophoresis Manual*, section 5, pp. 1–8, Beckman Instruments, Palo Alto
13. Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
14. Goldberg, E. (1972) *J. Biol. Chem.* **247**, 2044–2048
15. Wright, L. L. & Swofford, J. H. (1984) *Scand. J. Immunol.* **19**, 247–254
16. Holmes, R. S. & Scopes, R. K. (1974) *Eur. J. Biochem.* **43**, 167–177
17. Li, S. S.-L., Fitch, W. M., Pan, Y.-C. E. & Sharief, F. S. (1983) *J. Biol. Chem.* **258**, 7029–7032
18. Goldberg, E. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 349–352
19. Liang, Z.-G., Shelton, J. A. & Goldberg, G. (1986) *J. Exp. Zool.* **240**, 377–384

Received 19 May 1987/8 July 1987; accepted 16 July 1987