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Isolation and Partial Characterization of Rat LDH A-Genomic Sequences

Mi Young Lee, Sun Young Yim and Seung Ki Lee
College of Pharmacy, Seoul National University, Seoul 151-742, Korea
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Abstract□As a part of the study to elucidate the mechanism by which the transcription of LDH A-gene is regulated by cAMP, we aimed to isolate rat LDH A gene and characterize cAMP-responsive element (CRE). We have screened 1.2×10^6 recombinant phages of rat Charon 4A genomic library and isolated 33 positive clones among which we identified 12 different LDH A gene-related clones. By the results of restriction enzyme mapping, Southern blotting, and nucleotide sequence analyses, we concluded that the 12 LDH A gene-related clones were intronless and frequently mutated LDH A-pseudogenes. In this report, we present the characteristic features of the 12 rat liver LDH A-pseudogenes.

Keywords□Rat liver LDH A-gene, cAMP-responsive element, screening of rat genomic library, rat LDH A-pseudogenes.

It has been known that the expression of lactate dehydrogenase A gene (LDH A-gene) is modulated at the transcriptional level via cAMP as a second messenger during regeneration of rat liver[1, 2].

Study of the promoters of several cAMP-inducible genes has led to the recognition of a cis-regulatory sequence, the cAMP-responsive element (CRE)[3-5], whose presence is absolutely necessary but not sufficient for cAMP regulation to occur[6-8].

As a step toward elucidating the mechanism by which the expression of LDH A-gene is regulated by cAMP during regeneration of rat liver, we have undertaken the investigation of LDH A-gene structure and the promoter sequence containing CRE sequence.

In this study, we aimed to isolate the rat LDH A gene related sequences from rat liver genomic library and illustrate their gene organizations. We have screened 1.2×10^6 phages of rat Charon 4A genomic library and isolated 33 LDH A gene-related clones. In this report, we provide evidence that rat liver contains 12 different LDH A pseudogenes which were identified as intronless and frequently mutated LDH A-pseudogenes by restriction enzyme mapping, Southern blotting, and partial nucleotide sequencing analyses.

MATERIALS AND METHODS

Materials

Rat genomic library in Charon 4A was purchased from Clontech laboratories. Nitrocellulose filters were purchased from Gelman, [$\alpha - {}^{32}$P] dATP from Amersham, restriction endonucleases from KOSCO, T7 Sequencing kit from Pharmacia. Other reagents were purchased from Sigma.

Methods

Screening of rat genomic library

1.2×10^6 phages were screened with rat liver LDH A cDNA probe (0.7 kb coding region probe and 0.5 kb 3' untranslated region probe, ref. 9) according to the method of Benton and Davis[10]. Nitrocellulose filters were hybridized with the ^{32}P-labeled probe (10^7cpm/μg) in the buffer N (10% dextran sulfate, 40% formamide, 4×SSC, 7 mM Tris-Cl (pH 7.4), 1×Denhardt's solution, 0.02 mg/ml salmon sperm DNA) at 42°C overnight. Positive clones were confirmed by secondary and tertiary screening.

Preparation of phage DNA and Southern blot hybridization of LDH A gene-related DNA fragments

Phage DNA was prepared[11] from 10 ml lysates of Charon 4A clones hybridizing to LDH A cDNA and digested with *Eco*RI and electrophoresed on 0.8% agarose gel. *Eco*RI digested DNA fragments were transferred on to nitrocellulose filters and hybridized with LDH A cDNA probe according to

the method of Southern[12]. The phage clones were also digested with *Xba*I and *Hin*dIII enzymes for Southern blotting hybridization.

Subcloning of LDH A gene-related DNA fragments into pWR34 plasmid

Hybridizable DNA fragments were ligated with pWR34 plasmid vector and the ligation mixtures were used to transform competent JM101 cell. The recombinant plasmids were isolated[11] and inserted DNA fragments were confirmed by *Eco*RI digestion and Southern blot hybridization.

Restriction endonuclease (RE) mapping of subcloned DNA fragment

Subcloned LDH A gene-related fragments were digested with *Eco*RV, *Pst*I, and *Bgl*II enzymes and analyzed for LDH A-gene sequence by Southern blotting method using coding and noncoding region of LDH A cDNA as probes. Their RE maps were compared with that of rat LDH A cDNA.

DNA-sequencing of plasmid subclones

The nucleotide sequences of subcloned plasmids were partially analyzed by Sanger's dideoxy chain termination method[13] from the RE sites of *Eco*RV, *Pst*I, and *Bgl*II and their nucleotide sequences were compared with those of LDH A cDNA.

RESULTS AND DISCUSSION

Isolation of rat LDH A-genomic sequences

Using rat liver LDH A cDNA as a probe, we have screened 1.2×10^6 recombinant phages from rat Charon 4A genomic library and isolated 33 LDH A gene-related phage clones. After *Eco*RI digestion of the positive clones and Southern blotting hybridization analysis of the *Eco*RI digested DNA fragments (Fig. 1), we identified 12 different LDH A gene-related clones of which sizes for DNA fragments hybridizable to LDH A cDNA probe were in the range of 1-11 kb (Table I).

Restriction enzyme mapping of rat LDH A gene-related genomic sequences

In order to compare the genomic arrangements of the phage clones, the 12 LDH A genomic clones were digested with *Eco*RI, *Xba*I, and *Hin*dIII restriction enzymes and analyzed by Southern blotting method using rat liver LDH A cDNA probe. The results from the restriction enzyme mapping of the genomic phage clones show that λ RLA_1 and λ RLA_3 are overlapping clones, and λ RL8-2 shares the same

Table I. Rat LDH A gene-related Charon 4A clones and the results of the Southern blotting hybridization

Ch4A clones	*Eco*RI-DNA fragments hybridized with	
	LDH A coding region probe[a]	LDH A 3'-UT region probe[b]
λ RLA1	8 kb	8
λ RLA2	2.7	8
λ RLA3	3	3
λ RL3	8	8
λ RL5	Not hybridized	2
λ RL8-2	3	3
λ RL8-3	3	3
λ RL18	11	11
λ RL41	4.8	4.8
λ RL42	2.7	2.7
λ RL54	4	4
λ RL94	1.1	1.1

[a] 0.7 kb in Fig. 1 for λ RLA1- λ RL42, 0.52 kb in Fig. 1 for λ RL54 and λ RL94
[b] 0.5 kb in Fig. 1.

*Eco*RI fragment coding for LDH A genomic sequence with λ RL8-3. The sizes of the RE fragments that hybridized with LDH A cDNA probes were reduced to the range of 1 to 6 kb. This result suggests that the LDH A gene-related sequences may be too short to contain functional LDH A gene according to the previous reports on mouse and human LDH A gene (9 kb-long). The *Eco*RI fragments that hybridized with the probes were subcloned into pWR34 plasmid vector for further structural characterization.

Subcloning of LDH A genomic sequences and their restriction enzyme mapping

The subcloned LDH A genomic sequences were analyzed for *Eco*RV, *Pst*I, and *Bgl*II cutting sites, which are characteristic RE sites on the LDH A cDNA map (Fig. 2) and the RE fragments were hybridized with the LDH A cDNA probes. Restriction enzyme maps of the subcloned DNA fragments exhibited similar pattern of that of rat liver LDH A cDNA as shown in Fig. 2.

Additionally, the sizes of the RE fragments of the LDH A genomic sequences that hybridized with LDH A CDNA probes were almost the same as that of rat liver LDH A cDNA indicating that the LDH A gene-related genomic sequences may be intronless pseudogenes. For this possibility we have

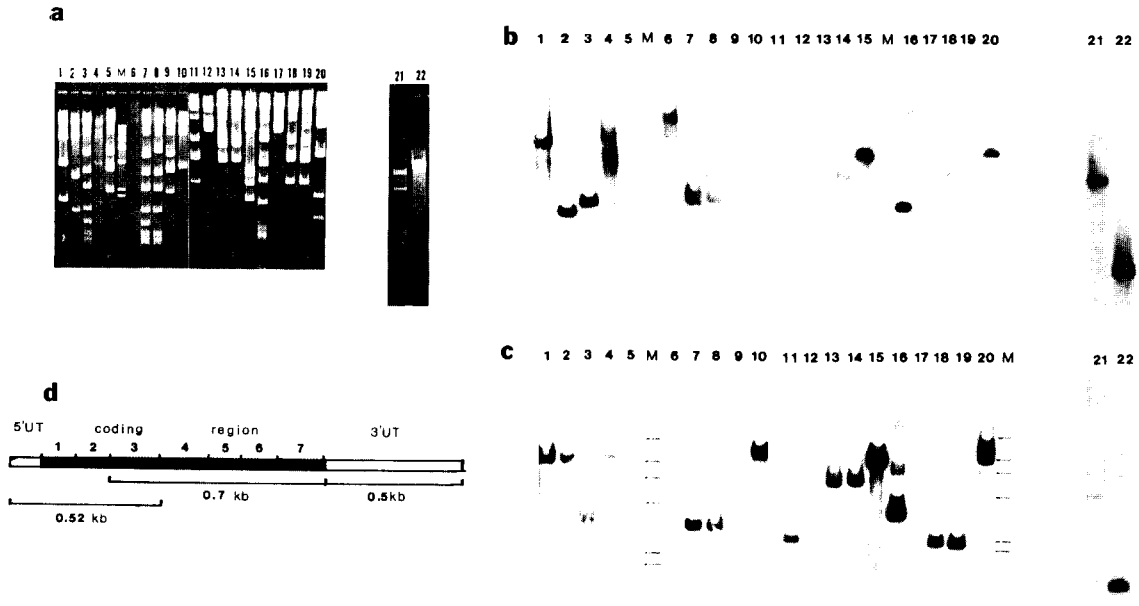

Fig. 1. *Eco*RI digestion pattern and Southern blot hybridization of LDH A gene-related genomic clones.

a. *Eco*RI digestion pattern of the clones.

b. hybridization of each clones with LDH A cDNA coding region probe.

c. hybridization of each clones with LDH A cDNA 3'-untranslated region probe.

d. rat LDH A cDNA probes for Southern blot hybridization.

lane	clone	lane	clone	lane	clone
1	λRLA1	8	λRL8-3	15	λRL42
2	λRLA2	9	λRL17	16	λRL47
3	λRLA3	10	λRL18	17	λRL49-2
4	λRL3	11	λRL19	18	λRL49-3
5	λRL5	12	λRL25	19	λRL49-4
6	λRL8-1	13	λRL41	20	λRL51
7	λRL8-2	14	λRL41-2	21	λRL54
				22	λRL94

5'UT: 5'-untranslated region

3'UT: 3'-untranslated region

M: Molecular weight markers are *Hin*dIII-cut λ DNA fragment. The sizes of the fragments are 23.1, 9.4, 6.6, 4.4, 2.3 kb and 2.0 kb.

further analyzed the nucleotide sequences of the LDH A gene related genomic clones according to the DNA sequencing strategy as shown in Fig. 2.

Nucleotide sequencing of the subcloned LDH A genomic sequences

By Sanger's dideoxy chain termination method, we have partially analyzed the nucleotide sequences of the subcloned LDH A gene-related sequences starting from one of the characteristic restriction enzyme cutting sites (*Eco*RV, *Pst*I, and *Bgl*II) of rat LDH A cDNA (Fig. 2), and found the nucleotide sequences that match with LDH A cDNA sequences at the corresponding RE sites of the genomic clones. To find out whether the genomic clones were intronless pseudogenes or not, we have read the nucleotide sequences of the genomic clones about 100-200 nucleotides long to the regions which are expected to contain exon-intron junction sites on the basis of the mouse and human LDH A gene structures[14, 15], and found no intron sequences at the expected region. Furthermore, the coding sequences of the LDH A gene-related genomic clones contained frequently mutated se-

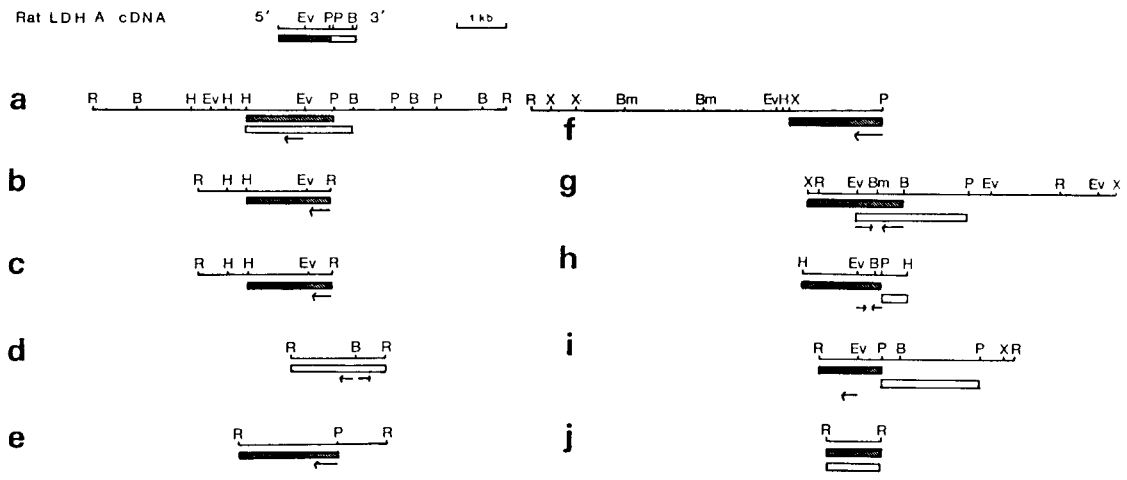

Fig. 2. Restriction enzyme maps and DNA sequencing strategy for LDH A genomic subclones.

a. λ RLA1 *Eco*RI 8 kb, b. λ RLA2 *Eco*RI 2.7 kb, c. λ RLA3 *Eco*RI 3 kb, d. λ RL5 *Eco*RI 2 kb, e. λ RL8-2 *Eco*RI 3 kb, f. λ RL18 *Eco*RI-*Pst*I 7 kb, g. λ RL41 *Xba*I 6.2 kb, h. λ RL42 *Hin*dIII 2.2 kb, i. λ RL54 *Eco*RI 4 kb, j. λ RL94 *Eco*RI 1.1 kb

▨ region hybridizing with LDH A cDNA coding region probe

□ region hybridizing with LDH A cDNA 3'-UT region probe

⟷ region whose nucleotide sequences were partially analyzed

R: *Eco*RI, X: *Xba*I, *Hin*dIII, Ev: *Eco*RV, P: *Pst*I, B: *Bgl*II, Bm: *Bam*HI.

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LDH A          910        920        930        940 ▼      950        960
c DNA  CCTTAGGCGG GTGCATC---CCA TTTCCACCAT GATTAAGGGT CTCTATGGAA TCAAGGAGGA
λ RLA2 -.......A. .......---... ..-..... .........A. .......... ..---..T.C
λ RLA3 .T......A. .......---... .......... .........A. .......... ..G.T..T..
λ RL8-2                        A.......... ....C.A... .........G ....T..T..
λ RL18 .........A. .......CAT... ..-..... ...-....-. .......... ....T..T..

               970        980        990       1000       1010       1020
       TGTCTTCCTC AGCGTCCCAT GTATCCTGGG ACAAAATGGA ATCTCAGATG TTGTGAAGGT
λ RLA2 A....-.--- ..T.....G. .......... .......... .......... ..........
λ RLA3 C....C.... ..T.....G. .......... .......... .......... ..........
λ RL8-2..C....... ..T....TG. .......-.. .....G.... T.......C. ..........
λ RL18 C......... ..T.....G. .........A -.......-. .......... ..........
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Fig. 3. Partial nucleotide sequences of LDH A gene-related genomic clones.

The partial nucleotide sequences of rat LDH A pseudogene clones λ RLA2, λ RLA3, λ RL8-2 and λ RL18 are compared with that of rat LDH A cDNA, and only those nucleotide differences are given. Identical residues are indicated by dots, and deletions are denoted by hyphens. The solid triangle indicates the positions of introns of mouse LDH A gene.

quences and the sequence change of some of the positions were common for some of the genomic clones. The results were compared and summarized in Fig. 3.

Our data demonstrate that the 12 LDH A gene-related sequences we have isolated from the rat genomic library are intronless and processed LDH A pseudogenes. It is not suprising, however, to see at least 12 different LDH A pseudogenes in rat liver genome since it has been reported that other gene family exhibited many pseudogenes in animal cells[16].

Additional experimentation is needed to isolate functional rat LDH A genomic sequence. For this study, we have undertaken a different approach for the isolation of functional LDH A genomic sequence. Because there are so many processed LDH A pseudogenes in rat gemone, we have designed a PCR method for construction of a intron probe to

preclude the chance of isolation of intronless pseudogenes. Taking advantage of the intron probe, we will undergo screening of rat genomic library to isolate functional rat LDH A gene and the results of this study will be reported in some other time.

CONCLUSION

As a part of the study toward elucidating the mechanism by which the expression of LDH A gene is regulated by cAMP as a second messenger during regeneration of rat liver, we aimed to isolate rat liver LDH A gene-related sequences from rat liver Charon 4A genomic library and characterize their gene organizations. Among 33 LDH A gene-related phage clones isolated from the genomic library we identified that rat liver contains 12 different LDH A gene-related sequences.

The results of restriction enzyme mapping, Southern blotting, and nucleotide sequence analyses demonstrate that the 12 LDH A gene-related sequences isolated from the rat genomic library are intronless and processed LDH A pseudogenes. In order to isolate functional rat LDH A gene, we are in the process of the screening of rat chromosomal DNA utilizing a probe for LDH A-intron I which was synthesized by PCR method.

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LITERATURE CITED

1. Yhu, S.K., Park, H.S., Kim, H.Y., Kim, A.K. and Lee S.K.: Expression of Lactate Dehydrogenase A gene in regenerating rat liver. *Rec. Prog. Mol. Biol. and Genet. Eng. Korea* **2**, 21 (1987).
2. Kim, H.Y. and Lee, S.K.: Transcriptional control of lactate dehydrogenase A gene expression during the pre-replicative phase of regenerating rat liver, *J. Pharm. Soc. Korea* **32**, 4, 239 (1988).
3. Shinomiya, T., Scherer, G., Schmid, W., Zentgraf, H. and Schutz, G.: Isolation and characterization of the rat tyrosine aminotransferase gene, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1346 (1984).
4. Cooke, N.E. and Baxter, J.D.: *Structure and evolution of the prolactin family of genes, Nature (London)* **292**, 603 (1982).
5. Goodwin, R.G., Moncman, C.L., Rottman, F.M. and Nilson, J.H.: Characterization and nucleotide sequence of the gene for the common α subunit of the bovine pituitary glycoprotein hormones, *Nucleic Acids Res.* **11**, 6873 (1983).
6. Montminy, M.R., Sevarino, K.A. and Goodman, R.H.: Identification of a cyclic AMP responsive element within the rat somatostatin gene, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6682 (1986).
7. Short, J.M., Wynshaw-Boris, A., Short, H.P. and Hanson, R.W.: Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region II. Identification of cAMP and glucocorticoid regulatory domains, *J. Biol. Chem.* **261**, 9721 (1986).
8. Silver, B.J., Bokar, J.A., Virgin, J.B., Vallen, E.A. and Nilson, J.H.: Cyclic AMP regulation of the human glycoprotein hormone α subunit gene is mediated by an 18 base pair element, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2198 (1987).
9. Matrisian, L.M., Rautmann, G., Magun, B.E. and Breathnach, R.: Epidermal growth factor or serum stimulation of rat fibroblasts induces an elevation in mRNA levels for lactate dehydrogenase and other glycolytic enzymes, *Nucleic Acids Res.* **13**, 711 (1985).
10. Benton, W.D. and Davis, R.W.: Screening λ gt recombinant clones by hybridization to single plaques in situ, *Science* **196**, 180 (1977).
11. Davis, L.G., Dibner, M.D. and Battey, J.F.: *Basic Methods in Molecular Biology*, 1st ed., Elsevier, New York (1986).
12. Southern, E.: Detection of specific sequences among DNA fragments separated by gel electrophoresis, *J. Mol. Biol.* **98**, 503 (1975).
13. Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
14. Li, S.S.-L., Tiano, H.F., Fukusawa, K.M., Yagi, K., Shimizu, M., Sharief, F.S., Nakashima, Y. and Pan, Y.E.: Protein structure and gene organization of mouse lactate dehydrogenase A isozyme, *Eur. J. Biochem.* **149**, 215 (1985).
15. Chung, F., Tsujibo, H., Bhattacharyya, U., Scharief, F.S. and Li, S.S.-L.: Genomic organization of human lactate dehydrogenase A gene, Biochem. J. **231**, 537 (1985).
16. Wilde, C.D.: Pseudogenes, *CRC Critical Reviews in Biochemistry*, **19**, 323 (1986).