Vol. 100, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1981 Pages 407-412

PURIFICATION OF MESSENGER RNA CODING FOR RAT LIVER ALDOLASE B SUBUNIT

Ken-ichi Tsutsumi and Kiichi Ishikawa

Department of Biochemistry, Yamagata University School of Medicine

Zaoh-iida, Yamagata 990-23, Japan

Received March 31,1981

SUMMARY

Messenger RNA (mRNA) coding for aldolase B subunit was purified from rat liver polysomes through specific immunoadsorption, oligo(dT)-cellulose chromatography and sucrose density-gradient centrifugation. Translation of the purified mRNA in a rabbit reticulocyte system followed by SDS-polyacrylamide gel electrophoresis revealed that almost all of the translation products co-migrated with marker aldolase B subunit. About 85% of the proteins synthesized in vitro were identified as aldolase B subunit by the immunochemical analysis. The activity of the mRNA to synthesize aldolase B was increased to 127 times that of poly(A)-containing RNA from total polysomes. The size of the purified mRNA was around 14.5 S as estimated by sucrose density-gradient centrifugation.

INTRODUCTION

Fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) appears in three isozyme types, i.e., aldolase A (muscle type), aldolase B (liver type) and aldolase C (brain type). In addition to these organ- or tissue-specific expressions, the enzyme changes its isozyme pattern during fetal development or carcinogenesis in the same tissue. Aldolase A is predominant in fetal rat liver, while almost exclusively aldolase B is present in adult rat liver (1). In hepatoma cells, a switch of the isozyme pattern from aldolase B to aldolase A has been reported (2-6). These isozymes are believed to be expressed under some transcriptional control mechanism(s), but not to be expressed as a result of selective translation of particular mRNA. However, little direct evidence concerning the transcriptional control for the isozyme switching has been accumulated. For such an investigation, it may be prerequisite to obtain considerably pure mRNA for

0006-291X/81/090407-06$01.00/0

each isozyme. In this report, we describe the purification and translation of the mRNA coding for aldolase B subunit.

MATERIALS AND METHODS

Preparation of polysomes. Rat livers were homogenized with 2.5 vol. of 20 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$ and 50 mM KCl (TKM buffer). The homogenate was centrifuged at 12,000 xg for 15 min. The resulting post-mitochondrial supernatant was adjusted to 1% each of sodium deoxycholate (DOC) and Triton X-100, and loaded onto 10 ml of TKM buffer containing 1.2 M sucrose. After centrifugation at 50,000 rpm in a Hitachi RP-50 rotor for 90 min, polysomal pellet was collected, washed and resuspended in the TKM buffer. All buffers used contained heparin (0.3 mg/ml).

Preparation of antibodies. Aldolase B from rat livers of Wistar strain was purified by affinity chromatography on P-cellulose as described by others (7). Purified aldolase B (2 to 3 mg) in PBS (20 mM sodium phosphate buffer containing 0.15 M NaCl) mixed with an equal volume of Freund's complete adjuvant was injected subcutaneously into a chicken four times at two weeks intervals. Immune serum was collected by heart puncture 10 days after the last injection. Anti-aldolase B antibody was purified by affinity chromatography through antigen-coupled Sepharose 4B after ammonium sulfate fractionation (8). Monospecificity of the antibody will be described elsewhere (9).

Rabbit anti-chick immunoglobulin antibody was prepared as follows; Chick immunoglobulin was purified by ammonium sulfate fractionation and DEAE-cellulose column chromatography and injected subcutaneously into a rabbit with the Freund's complete adjuvant four times as described above. Purified anti-chick immunoglobulin antibody was obtained by affinity chromatography as above.

Cell-free translation and immunoprecipitation of aldolase B subunit synthesized in vitro. Translation of mRNA in a nuclease-treated rabbit reticulocyte lysate was performed according to the method described by Pelham and Jackson (10). After incubation at 30°C for 90 min, the reaction mixtures were centrifuged at 105,000 xg for 90 min. The supernatants were dialyzed against PBS. To this, about 20 µg of purified anti-aldolase B antibody was added and incubated at room temperature for one hour. Then 120 µg of purified anti-chick immunoglobulin antibody was added and incubated for more two hours. The precipitates formed were collected by centrifugation and washed with PBS and then with PBS containing 0.5% Triton X-100. The washed precipitate was dissolved in 0.5% SDS and counted for its radioactivity.

SDS-polyacrylamide gel electrophoresis of the cell-free translation product was performed as described by Laemmli (11).

RESULTS AND DISCUSSION

In the preliminary experiments (9), we observed that the mRNA coding for aldolase B subunit was localized mainly in free polysomes, however, a considerable amount of aldolase B mRNA was also detected in the membrane-bound polysomes. Therefore, we started to purify it from the total polysomes of rat livers. Polysomes in the post-mitochondrial supernatant were isolated by DOC/Triton X-100 treatment and a discontinuous sucrose-density gradient centrifugation.

Table 1. Purification of Aldolase B mRNA

RNA fraction	Specific activities[a] (cpm/μg RNA)	Purification[b] (fold)	Aldolase B[c] Total protein (%)
1. Total poly(A)$^+$RNA from polysomes	550	1.0	0.48
2. Poly(A)$^+$RNA from immunoadsorbed polysomes	62,230	113	74.6
3. Sucrose density-gradient centrifugation	69,680	127	86.3

a) ^{35}S-methionine incorporated into aldolase B subunit in the rabbit reticulocyte system in response to 1 μg of RNA from each fraction, which was assayed by immunoprecipitation with anti-aldolase B antibody.
b) Increase of aldolase B synthesizing activity per 1 μg of RNA.
c) The ratio of immunoprecipitated aldolase B subunit synthesized in the reticulocyte translation assay to the total TCA-insoluble radioactivities in the assay mixture. Endogenous incorporations were subtracted.

band which migrated slower than marker aldolase subunit may be the product of the endogenous mRNA in reticulocyte lysate (Fig. 1, A). About 85% of the newly-

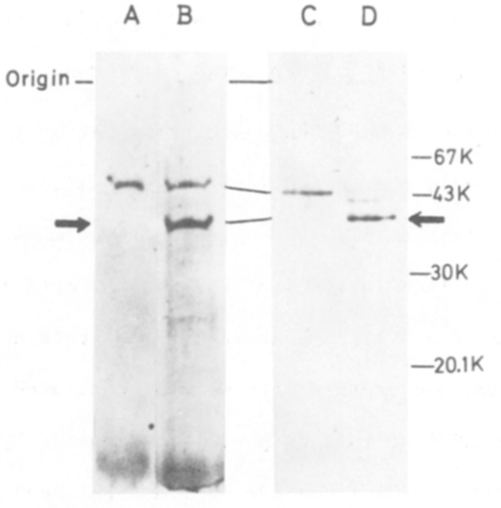

Fig. 1 SDS-polyacrylamide gel electrophoresis of the proteins synthesized in vitro in the reticulocyte lysate system. 0.4 μg of purified aldolase B mRNA was incubated at 30°C for 90 min in reticulocyte lysate containing 10 μCi of ^{35}S-methionine. An aliquote of the reaction mixture was run on 15% polyacrylamide gel and the bands detected by autoradiography. A: No added mRNA, B: Translation products of purified aldolase B mRNA.
Another aliquote was incubated with anti-aldolase B antibody and resulting immunoprecipitate(D) and supernatant(C) were run on acrylamide gels. The position of the molecular weight markers are shown on the right.

Immunoadsorption of the specific polysomes was carried out essentially according to the method described by Schutz et al (8) with some modifications. Polysomes suspended in TKM buffer containing 0.2 M NaCl (70 to 100 A_{260} units/ml) were added with purified anti-aldolase B antibody to the ratio of 1.5 μg per A_{260} unit of polysomes. After standing for one hour in an ice-bath with occasional stirrings, the mixture was added to Sepharose 4B which was coupled with purified rabbit anti-chick immunoglobulin antibody and left for another hour. The amount of the anti-antibody-coupled Sepharose added was excessive to arrest the chick antibody. The Sepharose was washed successively with TKM buffer, TKM buffer containing 0.5% Triton X-100, and then TKM buffer until absorbance at 260 nm of the supernatant was less than 0.05. The washed Sepharose was poured into the column and messenger ribonucleoproteins were eluted from the column by dissociation of the trapped polysomes with 10 mM Tris-HCl, pH 7.5 containing 20 mM EDTA. RNAs were extracted by the phenol/chloroform/isoamyl alcohol method and poly(A)-containing RNA was isolated by chromatography on oligo(dT)-cellulose column (12). Usually, 0.5 to 0.6 A_{260} units of poly(A)-containing RNA were recovered from 5,000 A_{260} units of the total polysomes.

Further purification of the mRNA was carried out by a sucrose density-gradient centrifugation in the presence of 70% formamide. The RNA peak, slightly smaller than 18S rRNA (about 15S) was collected. The mRNA at each purification step was translated in vitro in 10 μl of the reticulocyte lysate containing 10 μCi of ^{35}S-methionine (Table 1). About 75% of the proteins newly-synthesized by the mRNA at the immunoadsorption step were precipitated with anti-aldolase B antibody. When the mRNA at this step was fractionated by sucrose density-gradient centrifugation in 70% formamide, a minor RNA peak slightly larger than 18S rRNA was observed in addition to the main peak. This RNA also synthesizes exclusively aldolase B subunit, however, we cannot rule out the possibility of aggregation at this point (data not shown). Figure 1 shows the SDS-polyacrylamide gel electrophoresis of the proteins synthesized by the mRNA at the final purification step. The main band co-migrated with marker aldolase B subunit. The

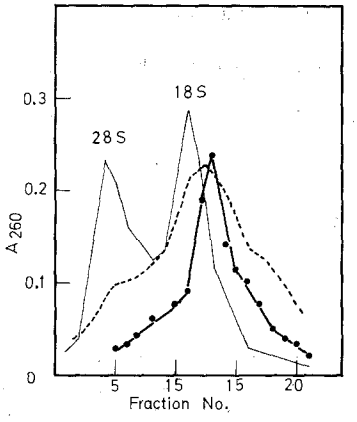

Fig. 2 Sucrose density-gradient centrifugation of the purified mRNA. An aliquote of the purified aldolase B mRNA (●—●) was centrifuged at 40,000 rpm for 16 hrs in the presence of 70% formamide in a Hitachi RPS-50 rotor. Four drops fractions were collected from the bottom of the tube. Marker rRNAs(rat liver)(——) and total poly(A)-containing RNA from polysomes (----) were centrifuged in separate tubes.

synthesized proteins were precipitated with anti-aldolase B antibody (Fig. 1 and Table 1). The results also demonstrated that aldolase B subunit was not synthesized via a significantly larger precursor protein by reticulocyte lysate system.

The activity of the purified mRNA to synthesize aldolase B subunit was increased to about 127 times that of total poly(A)-containing RNA from the polysomes (Table 1). About 20 μg of the purified mRNA was obtained from 5,000 A_{260} units of the polysomes by the purification described above. The size of the purified aldolase B mRNA, as determined by sucrose density-gradient centrifugation in the presence of 70% formamide, was around 15S, which may correspond to about 1,500 nucleotides (Fig. 2). Since aldolase B subunit has its molecular weight of 40,000 daltons (about 360 amino acid residues) (13), the coding region of the mRNA can be calculated to have 1,080 nucleotides. Therefore, the size of the mRNA we obtained was sufficiently large enough for coding aldolase B subunit

As expected from the analysis of the newly-synthesized proteins in polysome-dependent or mRNA-dependent translation systems (9), mRNA coding for aldolase B subunit is not so abundant as those of serum albumin (14), ovalbumin (15), or

globin (16) in the cell. Using immunoadsorption with double antibody method, we could isolate such a non-abundant class of mRNA with considerable purity.

Purification of another mRNA, which codes for aldolase A, is now in progress Availability of the pure mRNAs for each aldolase isozyme will enable us to study in detail the control mechanisms of the isozyme expression.

ACKNOWLEDEMENT

We thank Dr. T. Tanaka for valuable discussions, and also Drs. T. Ohno and S. Hidaka for technical assistance. This work was supported in part by a scientific grant from the Ministry of Education, Science and Culture of Japan.

REFFERENCES

1. Horecker, B.L., Tsolas, O. and Lai, C.Y. (1972) The Enzymes vol. 7 pp. 213-258 Academic Press, New York
2. Endo, H., Eguchi, M. and Yanagi, S. (1970) Cancer Res. 30, 743-752
3. Matsushima, T., Kawabe, S., Shibuya, M. and Sugimura, T. (1968) Biochem. Biophys. Res. Commun. 30, 565-570
4. Ikehara, Y., Endo, H. and Okada, Y. (1970) Arch. Biochem. Biophys. 136, 491-497
5. Shapira, F., Hatzfeld. and Reuber, M.D. (1971) Cancer Res. 31, 1224-1230
6. Sakiyama, S., Yoda, K. and Sugimura, S. (1976) Biochem. Biophys. Res. Commun. 72, 1271-1277
7. Lebhertz, H.G. (1975) J. Biol. Chem. 250, 5976-5978
8. Schutz, G., Kieval, S., Groner, B. Sippel, A.E., Kurtz, R.C. and Feigelson, P. (1977) Nucleic Acids Res. 4, 71-84
9. Tsutsumi, K. and Ishikawa, K. (1981) J. Biochem. submitted
10. Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256
11. Laemmli, U.K. (1970) Nature 227, 680-685
12. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1408-1412
13. Gracy, R.W., Lacko, A.G., Brox, L.W., Adelman, R.C. and Horecker, B.L. (1970) Arch. Biochem. Biophys. 136, 480-490
14. Taylor, J.M. and Tse, T.P.H. (1976) J. Biol. Chem. 251, 7461-7467
15. Shapiro, D.J. and Schimke, R.T. (1975) J. Biol. Chem. 250, 1759-1764
16. Lochard, R.E. and Lingrel, J.B. (1969) Biochem. Biophys. Res. Commun. 37, 204-212