Characterization of Messenger RNA for Fructose 1,6-Bisphosphate Aldolase A Isozyme of Rat Ascites Hepatoma AH 7974 Cells

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ABSTRACT

The messenger activity for fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) (aldolase) A isozyme has been characterized in the polysome- or the messenger RNA-directed, protein-synthesizing system using the pH 5 fraction of rat liver or wheat germ extracts, respectively. The subunit of aldolase A synthesized in vitro was detected by immunoprecipitation with anti-aldolase A antibody raised in chickens followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The synthesis of the enzyme depended on the addition of polysomes or polyadenylate-containing RNA precipitated by sodium perchlorate. The synthesis of aldolase A mRNA of AH 7974 cells mainly concern the subcellular localization of this mRNA and its size estimation.

INTRODUCTION

The resurgence of fetal-type isozymes of various enzymes such as aldolase, pyruvate kinase, and hexokinase in tumors, especially in hepatomas, has been well reviewed (29, 35). Three distinct isozymes of aldolase, which are different immunologically, were characterized electrophoretically regarding their substrate specificities (23). Aldolase A, muscle type, appears in various hepatomas at the expense of aldolase B, adult liver type, while aldolase C, brain type, is expressed only in some fast-growing hepatomas (20, 28). Very little is known, however, about the mechanism which might operate in switching the isozymic pattern at the molecular level from the adult type to the fetal type and vice versa in tumor tissues and in developing fetal tissues, respectively.

In considering the relationship between fetal-type isozymes and fetal proteins, it should be noted that the synthesis of α-fetoprotein in the mouse liver during its developing stage was found to be under transcriptional control when mRNA was assayed by its ability to synthesize α-fetoprotein in vitro (14).

In a previous paper, we reported the synthesis of aldolase A subunit in vitro under the direction of both polysomes and poly(A)-containing RNA of rat ascites hepatoma AH 7974 cells which show the nearly complete shift of isozymic pattern from aldolase B to aldolase A (27). Meanwhile, Lebherz and Doyle (16) have shown the synthesis of an aldolase A subunit in vitro by polysomal RNA of chicken muscle. Thus far, there is very little knowledge about the metabolism of mRNA for isozymes appearing in tumor cells. Therefore, the isolation and characterization of the mRNA’s coding for isozyme molecules is a prerequisite step and will afford a basis for investigations of the control mechanisms of gene expression in tumor cells.

In the present report, we describe the further characterization of aldolase A mRNA of AH 7974 cells mainly concerning the subcellular localization of this mRNA and its size estimation.

MATERIALS AND METHODS

Chemicals. ATP, GTP, creatine phosphate, and creatine phosphokinase were obtained from Sigma Chemical Co., St. Louis, Mo. Poly(U)-Sepharose was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Formamide, obtained from E. Merck, Darmstadt, Germany, was vacuum distilled at 3 mm Hg (b.p. 78°) by K. Takahashi, Division of Chemotherapy Chiba Cancer Center Research Institute. [4,5-3H]Leucine, [U-14C]leucine, and Protosol were purchased from New England Nuclear, Boston, Mass. Wheat germ was a gift from Nisshin Milling Co., Ltd., Chiba, Japan.

Cells. Rat ascites hepatoma AH 7974 cells, originally induced by 4-dimethylaminoazobenzene by Yoshida (36) were supplied from Sasaki Research Institute, Tokyo, and were maintained by serial transplantation via i.p. injection of ascites fluid to male Donryu rats weighing 250 g. Animals were given food and water ad libitum. About 1 week after the transplantation of AH 7974 cells, rats were killed by cervical dislocation and cells were harvested and washed with 35 mm Tris-HCl (pH 7.6)-0.14 M NaCl until the cells became largely free from RBC. The livers of male Donryu rats weighing 250 g were perfused with the above buffer and served as the source of adult liver.

Preparation of Polysomes. Total polysomes were prepared from adult rat liver or AH 7974 cells according to the method of Falvey and Staehelin (8), except for the addition of Nonidet P-40 as described previously in the case of AH 7974 cells (27).

Free and membrane-bound polysomes of AH 7974 cells

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2 The abbreviations used are: aldolase, fructose 1,6-bisphosphate aldolase (EC 4.1.2.13); poly(A), polyadenylate; poly(U), polyuridylate; mRNP, messenger ribonucleoprotein.

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were prepared as described by Adelman et al. (1). The polysomes recovered from 16 g of cells were 368 and 180 A\textsubsc{260} units for free and membrane-bound polysomes, respectively.

Crude polysomes and the postmicrosomal supernatant of AH 7974 cells were prepared according to the method described by Zahringer et al. (38).

**RNA Extraction.** RNA's were isolated from total or crude polysomes or from the postmicrosomal supernatant fraction of AH 7974 cells by phenol-chloroform-isomyl alcohol at pH 9.0 (3). Poly(A)-containing RNA was recovered by affinity column chromatography on poly(U)-Sepharose as described previously (27). RNA concentration was determined on the assumption that 1 mg/ml equals 25 A\textsubsc{260} nm.

**In Vitro Protein Synthesis.** Polysome-directed protein synthesis was carried out as described in a previous paper (27). The incubation mixture (1 ml) contained per ml: 30 \mu mol Tris-HCl (pH 7.6), 70 \mu mol monovalent cations (KCl plus NH\textsubsc{4}Cl), 4 \mu mol magnesium acetate, 1 \mu mol ATP, 0.4 \mu mol GTP, 20 \mu mol creatine phosphate, 100 \mu g creatine phosphokinase, 1 \mu mol diithiothreitol, 40 nmol 19 amino acids, 1 \mu Ci [U-\textsupsc{14}C]leucine (300 \mu Ci/\mu mol), 0.2 ml of pH 5 fraction prepared from adult rat liver and polysomes as indicated. The incubation was carried out at 35° for 60 min.

RNA-directed protein synthesis was carried out as described in a previous paper (27), except that the concentrations of some ingredients were changed. The incubation mixture (0.2 ml) contained per ml: 20 \mu mol N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.6), 98 \mu mol potassium acetate, 3.0 \mu mol magnesium acetate, 1 \mu mol ATP, 0.1 \mu mol GTP, 8 \mu mol creatine phosphate, 6 \mu g creatine phosphokinase, 2 \mu mol diithiothreitol, 20 nmol 19 amino acids, 96 \mu g wheat germ tRNA, 320 \mu Ci [4,5-\textsupsc{3}H]leucine (50 mCi/\mu mol), 0.3 ml wheat germ extracts prepared as described previously (19), and RNA as indicated. The incubation was carried out at 30° for 60 min.

**Product Analysis.** In either the polysome- or the mRNA-directed system, 1 \mu l of the supernatant obtained after centrifugation of the reaction mixture at 180,000 x g for 60 min was spotted on filter papers, and total messenger activity was measured after trichloroacetic acid wash (27).

The details of the method of identification of one of the in vitro products with aldolase A were described in a previous paper (27). Briefly, after incubation, the reaction mixture was centrifuged at 180,000 x g for 60 min, and the supernatant containing released polypeptides was fractionated by ammonium sulfate precipitation between 40- and 60% saturation. After dialysis and the addition of 14 \mu g of aldolase A purified from AH 7974 cells as a carrier, aldolase A was precipitated by chicken anti-aldolase A \gamma-globulin. Immunoprecipitates formed were analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (7.5%) (34). The gels were then sliced, solubilized with Protosol, and then counted with a counting efficiency of 35 and 60% for [3H]- and [3C]leucine-labeled products, respectively. The values for the samples on filter papers were 10 and 60%, respectively.

The messenger activity for aldolase A was quantitated by adding the radioactivity which appeared in the region of the subunit molecule of aldolase A minus background determined by averaging the radioactivity on both sides of the peak on the gel.

**Sucrose Density Gradient Centrifugation.** Total polysomal RNA (400 \mu g) of AH 7974 cells was dissolved in 3 mM Tris-HCl (pH 7.6), 3 mM EDTA, and 70% formamide and heated for 3 min at 70° (12). Linear 5 to 20% sucrose gradients were made in the above buffer. Following centrifugation in a Beckman SW 27 rotor at 26,000 rpm for 72 hr, the gradients were fractionated from the top of the tube. Each fraction (0.4 ml) was adjusted to 0.2 M with respect to NaCl, and 2 volumes of ethanol were added. After the RNA was washed twice with 2 volumes of ethanol in the presence of 0.2 M NaCl, it was finally dissolved in 15 \mu l of H\textsubsc{2}O, and 10-\mu l aliquots were used for the in vitro assay for protein synthesis.

**RESULTS**

Since aldolase A is known to be entirely absent in adult rat liver, studies were carried out to investigate whether this absence is associated with that of messenger activity at the polysomal level. Total polysomes prepared from adult rat liver were compared with those from AH 7974 cells for the ability to synthesize aldolase A in vitro. The results shown in Chart 1 and Table 1 clearly demonstrate that there is no detectable messenger activity for aldolase A on rat liver polysomes in spite of the presence of a significant amount of total messenger activity. When RNA extracted from polysomes of adult rat liver was investigated for aldolase A messenger activity in extracts derived from wheat germ, no activity was detected (data not shown).

It has been widely believed that mRNA can exist in various forms as regards intracellular sites, i.e., polysome bound (10, 17, 32, 33, 37, 38) or as free mRNP particles in the cytoplasm (4, 5, 38). The former can be further divided into membrane-bound and free polysomes for the synthesis of proteins for export and nonsecreted proteins, respectively.

![Chart 1. Electrophoretic profiles of in vitro products synthesized by AH 7974 cells and rat liver total polysomes. Polypeptides synthesized and released were partially purified for aldolase A by ammonium sulfate fractionation and then precipitated by anti-aldolase A chicken \gamma-globulin as described under "Materials and Methods." Sodium dodecyl sulfate-polyacrylamide gel (7.5%) electrophoresis was carried out according to the method of Weber and Osborn (34). Electrophoretic migration is from left to right. Arrows, relative distance of migration of aldolase A subunit as well as marker proteins. Polypeptides synthesized by 32.0 A\textsubsc{260}/ml of AH 7974 polysomes (•) and 36 A\textsubsc{260}/ml of rat liver polysomes (○).](chart1.png)
containing RNA prepared from total polysomes of AH 7974 cells to synthesize aldolase A in the wheat germ system. The synthesis of aldolase A was saturated at the concentration of 100 µg/ml. In this experiment, leucine incorporation into aldolase A represented about 0.02% of the total incorporation into released polypeptides. This number is approximately one-fifth of that obtained from experiments using polysomes (Table 1; Ref. 27).

Since a number of mRNA’s are known to carry the nontranslatable nucleotides to a certain degree (12, 21, 24), the molecular weight of aldolase A mRNA was measured by formamide-containing sucrose density gradient centrifugation. As shown in Chart 3, aldolase A mRNA appeared as a

Therefore, we performed the following experiments to see which subcellular fraction is enriched with mRNA for aldolase A. Since the above method for the isolation of total polysomes from AH 7974 cells included the use of deoxycholate which solubilizes membrane, hence membrane-bound polysomes, a method without the use of the detergent should be introduced, and the method of Adelman et al. (1) used in the present experiment was found to be most suitable for the preparation of an active subfraction of polysomes of AH 7974 cells. Table 2 indicates that the messenger activity for aldolase A is located exclusively on free polysomes, although overall messenger activity was within the same range between these two polysomal subfractions. It is conceivable that a trace amount of the messenger activity for aldolase A present on membrane-bound polysomes might be due to the contamination of this fraction by free polysomes.

As a next step, we investigated whether mRNA for aldolase A is present as free mRNP particles in the cytoplasm. AH 7974 cells were fractionated into crude polysomes and cell sap according to the method of Zähringer et al. (38), and RNA extracted from each fraction was assayed for the aldolase A messenger activity in the wheat germ extract after partial purification of RNA by passage through a poly(U)-Sepharose column. There was no detectable messenger activity for the synthesis of aldolase A in RNA obtained from the supernatant fraction (Table 3). The results shown in Table 3 also suggest the presence of a significant quantity of poly(A)-containing RNA which possesses messenger activity in the supernatant fraction as shown previously (37, 38).

Chart 2 indicates the dose-response curve of poly(A)-

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<th>Table 1</th>
<th>Messenger activity for aldolase A on total polysomes of AH 7974 cells and rat liver</th>
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<tr>
<td>Source of polysomes</td>
<td>Polysomes added (A_{260}/ ml)</td>
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<tr>
<td>AH 7974 cells</td>
<td>32.0</td>
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<tr>
<td>Rat liver</td>
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<th>Table 2</th>
<th>Distribution of aldolase A messenger activity between free and membrane-bound polysomes of AH 7974 cells</th>
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<tr>
<td>Source of polysomes</td>
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<td>Free</td>
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<tr>
<td>Membrane bound</td>
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<th>Table 3</th>
<th>Distribution of poly(A)-containing RNA and its messenger activity for aldolase A between crude polysomal and postmicrosomal fractions of AH 7974 cells</th>
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<tr>
<td>Source of RNA</td>
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<td>Crude polysomes</td>
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<tr>
<td>Postmicrosomal fraction</td>
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| Chart 2 | Synthesis of total released polypeptides and aldolase A by poly(A)-containing RNA extracted from total polysomes of AH 7974 cells. Protein-synthesizing assay using extracts from wheat germ was carried out with varying amounts of RNA. One µl of the supernatant after centrifugation at 180,000 x g for 60 min was processed for the measurement of total messenger activity (○). The rest of the incubation mixture served for the measurement of aldolase A synthesized as described under "Materials and Methods" (●).
Evidence from numerous studies reveals that secretory proteins are synthesized primarily on membrane-bound polysomes as exemplified by albumin (37), other plasma proteins (10), and amylase (17), whereas cytosol proteins such as arginine (32) and catalase (33) are synthesized on free polysomes. In certain cases, however, this distinction is not strict. Aldolase A can be considered, from its catalytic nature to be located in the cytoplasm. Recently, it has been shown that aldolase A localizes in the cytoplasm of rat hepatoma cells from immunoperoxidase studies (11). The predominant presence of aldolase A messenger activity on free polysomes could be related to the location of the aldolase A molecule. On this point, there is an argument about the localization of aldolase A inside the cell, i.e., the association with endoplasmic reticulum (9) and the presence in the soluble supernatant fraction (2).

Although the precise nature of cytoplasmic mRNP particles still remains to be determined, a number of mRNA's coding for various proteins such as ferritin (38), actin (4), and myosin (5) are present in this state. One possible explanation for the presence of mRNP particles could be a storage function of the precursor of polysomal mRNA. The absence of messenger activity for aldolase A in the postmitochondrial supernatant fraction suggests that there is no regulatory mechanism at this step for the synthesis of aldolase A. This evidence, taken together with the above finding that there is no messenger activity for aldolase A on polysomes of adult rat liver, could imply that the expression of aldolase A gene is under a transcriptional control. This speculation is based on the assumption that the messenger activity observed in vitro protein-synthesizing systems reflects the quantity of mRNA as reported previously (6, 25).

Although it is difficult to make a direct comparison between values of translation efficiency observed at the different laboratories, the fact that the incorporation of leucine into aldolase A represents only 0.02% of the total incorporation by poly(A)-containing RNA shows a significant contrast with the value obtained in the case of the synthesis of aldolase A in vitro by chicken muscle polysomal RNA (approximately 1%) (16). This difference might be due to the predominant synthesis of the aldolase A subunit molecule in chicken muscle, which represents about 6% of the total protein synthesis in vivo (16). On the other hand, the figure of 0.02% is within the same order of magnitude as that observed in the case of translation in vitro of tryptophan oxygenase (13) and tyrosine aminotransferase (22). The lower efficiency of translation of aldolase A by mRNA than by polysomes (0.1%) might be caused by partial degradation of aldolase A mRNA during RNA extraction and storage.

There always exists the technical problem of how to avoid completely the aggregation of mRNA's with other mRNA's or rRNA's. As observed in the present study and others (12, 18), the use of formamide in the sucrose density gradient enables one to make an estimation of the chain length of mRNA. The molecular weight of aldolase A mRNA has been calculated at 5.8 x 10^6, corresponding to approximately 1650 nucleotides. After subtracting 150 nucleotides, on the average, for the poly(A) segment, 1500 nucleotides could represent the transcribed region. Since rabbit muscle aldolase A, the molecular weight of which (40,000) is the same as that of aldolase A of AH 7974 cells (27), has been shown to be composed of 361 amino acid residues (15), the minimum length for the coding region is 1100 nucleotides. Therefore, the noncoding region of aldolase A mRNA is about 400 nucleotides in length, which is longer than that reported for mRNA's for globin (24) or the immunoglobulin light chain (21), but quite comparable with that obtained for mRNA's for enzyme proteins such as tyrosine aminotransferase and tryptophan oxygenase (12).

As a next approach, a model is required in which the isozymic pattern can be modulated during the course of cellular transformation by oncogenic viruses (26, 30) or of carcinogenesis by chemicals (7, 31). In this connection, the study on mRNA coding for aldolase B, which decreases or disappears during hepatocarcinogenesis (31), will be necessary to investigate the control mechanism of biosynthesis and the degradation of both isoyzyme mRNA's and isozymes in tumor cells.

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