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INSULIN MEDIATES THE ASYNCHRONOUS ACCUMULATION OF HEPATIC ALBUMIN AND MALIC ENZYME MESSENGER RNAs

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Summary: We have compared the rate of accumulation of hepatic albumin and malic enzyme mRNAs following insulin treatment of diabetic rats to determine whether insulin coordinately increases mRNA levels or specifically induces the accumulation of individual mRNAs. Initially, the quantities of both albumin and malic enzyme mRNAs are reduced in diabetic rats compared to normal rats as determined by RNA blot analysis using complementary DNA probes. Following insulin administration for 12 h, albumin and malic enzyme mRNA levels increase at similar rates. However, after 12 h the rate of malic enzyme mRNA accumulation increases dramatically while albumin mRNA continues to increase at its initial rate. This accelerated rate of accumulation of malic enzyme mRNA continued through 60 h of hormone treatment and was associated with the onset of hepatic lipogenesis. Thus, our results suggest that insulin regulates the accumulation of mRNAs encoding these two inducible proteins in an asynchronous manner directly related to the metabolic requirements of the animal.

Insulin alters the synthesis of many hepatic proteins by effecting an increase in the amount of each of their respective mRNAs (1-12). However, there have been no comparative studies assessing the kinetics of these insulin-mediated accumulations of multiple mRNA species in a single experimental model. Thus, it is unclear whether these increases represent a coordinate regulation of the induced genes, resulting in a proportional increase in each of the mRNAs, or whether they represent a differential induction of specific mRNAs. We examined these possibilities by comparing the kinetics of increases in hepatic albumin and malic enzyme mRNAs following insulin treatment of diabetic rats. These two mRNAs were chosen because they encode different insulin-inducible proteins. Albumin is a major hepatic secretory protein (13) and its mRNA represents a major percentage of hepatic mRNAs (14). In contrast, malic enzyme [L-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating)

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EC1.1.1.40] is an important metabolic enzyme which provides NADPH for de novo fatty acid synthesis (15,16) and its mRNA represents a minor percentage of hepatic mRNAs (17,18). A similarity between these two proteins is that their synthesis has been directly correlated with the nutritional and hormonal status of the experimental animal (1, 9, 19-25). Thus, a detailed comparison of the kinetics of accumulation of these two mRNAs should indicate whether or not the insulin-mediated increase in hepatic mRNAs is a synchronous or asynchronous event.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats with initial body weights of 175-195 g were provided with food and water ad libitum. Diabetes was induced by intravenous injection of 4% (w/v) alloxan monohydrate at a dosage of 45 mg/kg body weight. Rats with plasma glucose levels greater than 300 mg/dl and little or no weight gain were considered diabetic (26). Rats meeting these criteria were maintained for at least 10 days after alloxan administration to stabilize the diabetic condition (27). After 10 days, insulin treatment was initiated which consisted of subcutaneous injections of 4 units of regular insulin and 4 units of NPH insulin every 12 h for 12, 24, or 60 h. Simultaneously, rats receiving insulin were placed on a high carbohydrate, fat-free diet (AIN-76; Zeigler Brothers, Gardners, PA) to provide maximal availability of carbohydrate substrate for de novo lipogenesis. As previously shown in our laboratory, diet alone has no effect on the stimulation of malic enzyme activity in diabetic rats (28). Normal rats were maintained on regular rat chow. At the specified times after the initiation of diet and insulin treatment, all rats were sacrificed at 9:00 a.m. by decapitation. The livers were removed and malic enzyme activity determined (25) to monitor the stage of enzyme induction.

Poly(A^+)RNA Preparation. Total cellular RNA was extracted from pools of at least 5 livers in each experimental group. Livers, frozen in liquid nitrogen, were homogenized in 10 volumes of 0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 5 mM EDTA, 0.5% $NaDodSO_4$ and 10 volumes of buffer-saturated phenol, followed by the addition of 5 volumes of chloroform (29). The aqueous phase was extracted 3 additional times with an equal volume of a mixture of phenol-chloroform-isoamyl alcohol (24:24:1). Following ethanol precipitation of the final aqueous layer, the nucleic acids were washed 3 times with 3 M sodium acetate, pH 5.5, 5 mM EDTA to remove DNA (30). The total cellular RNA was reprecipitated in ethanol and suspended in 20 mM Hepes, pH 7.4, 0.5 M KCl. Poly(A^+)RNA was prepared by affinity chromatography on oligo(dT)cellulose (31).

RNA Blot Analyses. Quantities (50 ng to 500 ng) of poly(A^+)RNA were denatured in 7.4% formaldehyde, 6X SSC at 60°C for 15 min for the preparation of RNA dot blots (32). The samples were applied with suction to nitrocellulose (Schleicher and Schuell BA85, 0.45 µm) using a Hybrid-Dot manifold (Bethesda Research Laboratories). Each sample well was washed with 6X SSC and the nitrocellulose was baked at 80°C for 2 h under reduced pressure.

For electrophoretic analysis, 10 µg samples of poly(A^+)RNA, along with 10 µg of 28S and 18S markers, were denatured in 6.6% formaldehyde, 50% formamide at 60°C for 15 min and size fractionated by electrophoresis on 1% agarose gels containing 6.6% formaldehyde (33). Following electrophoresis, the marker lanes were removed from the gel, stained, and photographed. The RNA samples were transferred directly to nitrocellulose using 10X SSC as the blotting buffer (34). The nitrocellulose was baked at 80°C for 2 h under reduced pressure.

Hybridization of Immobilized RNA. The plasmid prME, containing the 1250 base pair rat hepatic malic enzyme cDNA (34, hybridization kindly performed by Drs. M. Magnuson and V. Nikodem), or the plasmid pmalb2, containing the 700 base

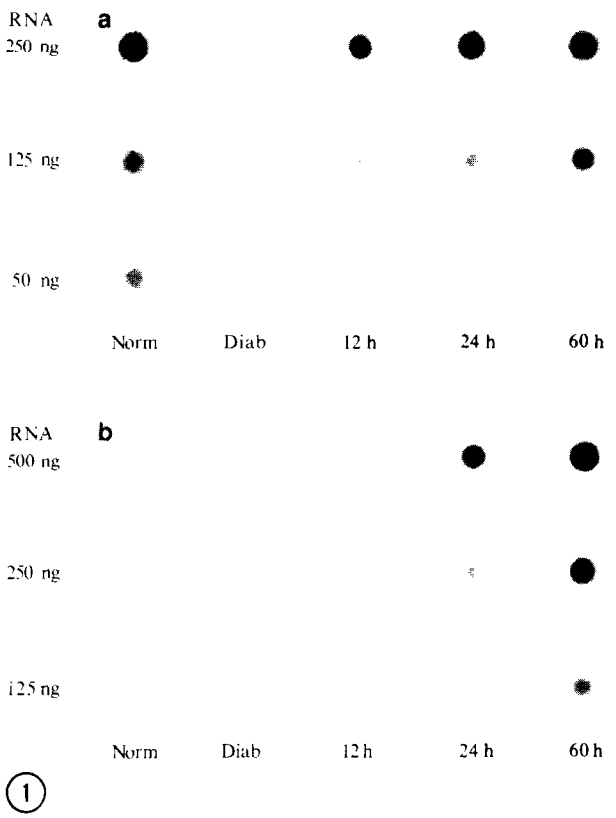
pair mouse albumin cDNA (36, kindly provided by Dr. S. Tilghman), was labelled with [α-^{32}P]dCTP by nick translation (37) to specific activities of 4 x 10^8 and 2 x 10^8 cpm/μg DNA, respectively. The nitrocellulose filters were prehybridized at 68°C for a minimum of 2 h, followed by hybridization in the same solution containing the nick translated probe (35). After hybridization, the filters were washed under stringent conditions (35), dried and exposed to Kodak XAR5 film.

RESULTS AND DISCUSSION

Dot blot hybridization of liver poly(A^+)RNA with radiolabeled cDNA probes was used to quantitatively assess albumin and malic enzyme mRNA levels. In normal rats fed ad lib, hepatic albumin and malic enzyme mRNA levels reflect the physiology of this animal. Albumin mRNA is present in relatively large amounts (Fig. 1a) which clearly indicates the importance of albumin as a major product of hepatic protein synthesis (13). In contrast, hepatic malic enzyme mRNA is present in levels barely detectable at the quantity of poly(A^+)RNA blotted (Fig. 1b). This result confirms previous studies showing the low amount of malic enzyme protein (25) and its mRNA (17,18) in normal rat livers.

Compared to normal levels, the amount of mRNA per ng of total mRNA that hybridizes to either the albumin cDNA probe (Fig. 1a) or the malic enzyme cDNA probe [as determined using an RNA blot not shown which contained up to 1250 ng of poly(A^+)RNA] is substantially reduced in alloxan-induced diabetes. These depressed mRNA levels correlate directly with the decreased rate of albumin synthesis (1) and the decreased activity and quantity of malic enzyme (25) in the diabetic rat. Furthermore, they strongly argue against the presence of cytoplasmic pools of untranslated mRNA which could become actively involved in protein synthesis following hormonal stimulation. This post-transcriptional regulatory process has been identified as a possible control mechanism for other hepatic proteins (38).

The insulin-mediated accumulation of these hepatic mRNAs was assessed by quantitating albumin and malic enzyme mRNA levels in samples of total cellular poly(A^+)RNA isolated from insulin-treated diabetic rats at specific intervals after hormonal stimulation. The levels of both of these mRNAs increase following insulin treatment. However, there is a significant difference in the rate at which this process occurs. The accumulation of albumin mRNA (Fig. 1a)



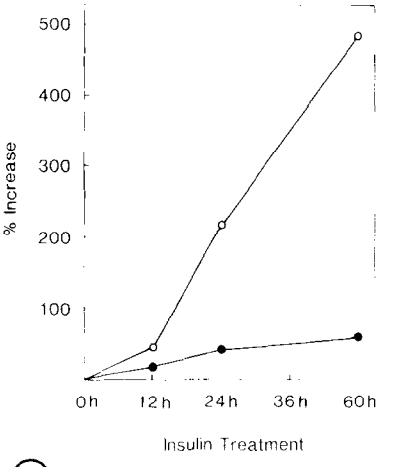


Figure 1. Estimation of hepatic albumin (a) and malic enzyme (b) mRNA levels by dot blot hybridization. Varying amounts of hepatic total cellular poly(A^+)RNA isolated from normal rats, diabetic rats, and diabetic rats treated with insulin for 12 h, 24 h and 60 h were spotted onto nitrocellulose membranes and hybridized with radiolabeled pmalb2(a) and prME(b).

Figure 2. The relative rates of accumulation of albumin mRNA (●) and malic enzyme mRNA (o) at each time interval was determined by cutting out and counting the radioactive spots. The radioactive spots were cut out so as to be centered in a 1 cm square to assure that the entire hybridized dot as well as the surrounding area was counted. The results were expressed as percent increase in cpm relative to diabetic levels.

occurs at a moderate rate throughout the entire treatment period of 60 h and amounts to a 60% increase over diabetic levels (Fig. 2). Rather than this being classified as a specific event, it might be suggested that increased albumin mRNA levels reflect the generalized influence of insulin on RNA transcription (39,40). In contrast, the accumulation of malic enzyme mRNA (Fig. 1b), which initially occurs at a rate similar to albumin mRNA, is specifically enhanced after 12 h of insulin treatment (Fig. 2). By 24 h of hormonal stimulation, malic enzyme mRNA is present in amounts greatly exceeding what would have been expected according to the initial rate of accumulation. Furthermore, this

increased rate of mRNA accumulation continues through 60 h of insulin treatment and amounts to a 480% increase over diabetic levels (Fig. 2). This specific stimulation parallels increases in the amount and activity of malic enzyme (25) and the onset of hepatic lipogenesis (41).

This substantial increase in malic enzyme mRNA could be due to increases in either the transcription and/or the stabilization of the message. While an increase in message stabilization could account for small increases in mRNA levels, the magnitude of the response we observe strongly suggests that increased transcription contributes to the insulin-mediated accumulation of malic enzyme mRNA. Furthermore, while one might speculate that insulin is not directly responsible for this dramatic elevation of malic enzyme mRNA due to the timing of the response, a direct effect of insulin on malic enzyme activity has been demonstrated in primary cultures of normal hepatocytes (42) and during the insulin-induced differentiation of 3T3-L1 preadipocytes (43). Thus, while this does not eliminate the influence of other cellular or hormonal factors, it does demonstrate that insulin plays a critical role in malic enzyme induction.

The existence of multiple albumin and malic enzyme mRNA species was investigated by electrophoretically fractionating hepatic poly(A^+)RNA, transferring the RNA to membranes, and hybridizing the membrane-bound mRNA with the radiolabeled cDNA probes. As shown in Fig. 3, hybridization of immobilized mRNA with the malic enzyme cDNA probe identified two mRNA species that possibly encode malic enzyme. The estimated length of these mRNAs is 3900 bases and 2300 bases, which is similar to size estimations described by Sul *et al.* (44). Furthermore, our results demonstrate that the two malic enzyme mRNA species are present in normal and diabetic rats (data not shown) and are regulated in parallel during hormone induction (Fig. 3). We also note that malic enzyme mRNA levels in each experimental group are consistent with our dot blot results. The albumin cDNA probe hybridized to a single species of electrophoretically separated poly(A^+)RNA with an estimated length of 2200 bases (data not shown).

In conclusion, our results demonstrate that the insulin-mediated accumulation of hepatic mRNAs is an asynchronous event related to the metabolic

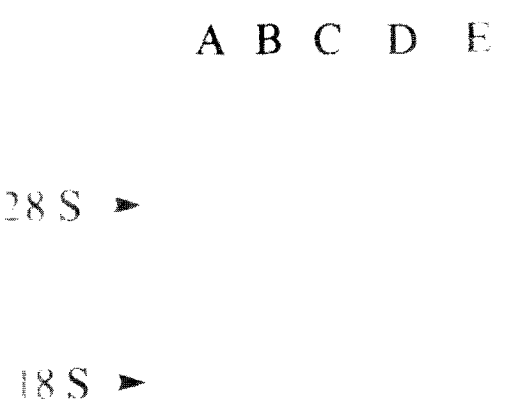


Figure 3. RNA blot analysis of hepatic total cellular poly(A^+)RNA hybridized with the malic enzyme cDNA probe. Poly(A^+)RNAs, isolated from livers of rats in the various experimental groups, were fractionated by electrophoresis on formaldehyde/agarose gels. Samples were 10 µg of poly(A^+)RNA from normal rats (A), diabetic rats (B), and diabetic rats treated with insulin for 12 h (C), 24 h (D) and 60 h (E). Size standards were 28S rRNA and 18S rRNA.

changes occurring in the experimental animal. In support of this contention, the mRNA encoding hepatic glucokinase, a key enzyme in glucose metabolism, increased within 1.5 h following the administration of insulin to diabetic rats (6,12). Thus, insulin's influence on the accumulation of mRNAs encoding insulin-inducible proteins involves additional regulatory processes occurring at the level of transcription.

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