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Developmental and nutritional regulation of the messenger RNAs for fatty acid synthase, malic enzyme and albumin in the livers of embryonic and newly-hatched chicks

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Summary

The mRNAs for fatty acid synthase and malic enzyme were almost undetectable in total RNA extracted from the livers of 16-day old chick embryos. Both mRNAs increased in abundance between the 16th day of incubation and the day of hatching. In neonates, fatty acid synthase mRNA level was dependent on nutritional status, increasing slowly if the chicks were starved and rapidly if they were fed. The abundance of malic enzyme mRNA decreased in starved neonatal chicks and increased in fed ones. When neonates were first fed and then starved, starvation caused a large decrease in the abundance of both mRNAs. Conversely, feeding, after a period of starvation, resulted in a substantial increase in both mRNAs. The relative abundances of fatty acid synthase and malic enzyme mRNAs correlated positively with relative rates of enzyme synthesis. Thus, nutritional and hormonal regulation of the synthesis of these two 'lipogenic' enzymes is exerted primarily at a pre-translational level.

The abundance of albumin mRNA decreased significantly between the 16th day of incubation and the day of hatching but did not change thereafter in fed or starved chicks. The relative stability of albumin mRNA levels after hatching attests to the selectivity of the nutritional regulation of fatty acid synthase and malic enzyme mRNAs. The decrease in albumin mRNA which occurred between 16 days of incubation and hatching contrasts with the increase in albumin mRNA sequences which occurred during late gestation in the fetal rat (20). High levels of albumin in the chick embryo may be related to the lack of an analogue of mammalian alpha-fetoprotein in birds.

Abbreviations

PIPES, piperazine-N,N-bis (2 ethanesulfonic acid); SDS, sodium dodecyl sulfate.

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Introduction

Fatty acid synthase, a multifunctional polypeptide, and malic enzyme [L-malate-NADP oxidoreductase (decarboxylating), EC 1.1.1.40] are two of several enzymes involved in the *de novo* synthesis of long chain fatty acids. The activities of these enzymes are correlated with the rate of fatty acid synthe-

sis in the livers of animals in different nutritional and hormonal states. Thus, the activities of both fatty acid synthase and malic enzyme are low in the liver of the embryonic chick, increase greatly when newly-hatched chicks are fed, and decrease when chicks fed for several days are starved (1–3). These changes in enzyme activities are due to changes in the concentrations of enzyme proteins which, in turn, are due to changes in the relative rates of synthesis of enzyme proteins (4–6). The next step in this analysis is to determine whether enzyme synthesis is controlled by regulating the amount of specific mRNA or the efficiency of its translation. In the study reported here, we have used ^{32}P-labeled cDNAs prepared from cloned goose DNAs (7, 8) to measure the abundance of fatty acid synthase and malic enzyme mRNAs in total RNA extracted from the livers of embryonic and newly-hatched chicks. The levels of the mRNAs for fatty acid synthase and malic enzyme correlate closely with the relative rates of synthesis of their respective enzyme proteins, suggesting regulation at pre-translational steps.

Materials and methods

Animal care

Embryonated eggs from white Leghorn chickens were obtained from Squire Valleevue Farm of Case Western Reserve University and incubated in electric forced-draft incubators at 37.5 ± 0.5 °C and 60% relative humidity. Newly-hatched chicks were placed in brooders with heat lamps. Chicks were provided water *ad libitum* and commercial chicken mash as indicated in 'Results'.

Isolation of RNA and quantitation of mRNA levels

Embryonic and hatched chicks were decapitated. Livers were rapidly removed and homogenized in guanidine thiocyanate. Total RNA was isolated by a modification (9) of the method of Chirgwin et al. (10). Yields of RNA were calculated from the absorbance of purified RNA at 260 nm ($1A_{260}$ = 50 μg). The levels of specific mRNAs were determined by a modification of the 'dot-blot' assay (11), using a filtration manifold (Schleicher and Schuell, Keene, New Hampshire, USA). Samples of total RNA (5 μg/dot) dissolved in 32 μl of 25 mM sodium phosphate buffer, pH 6.5, were applied to 'Gene Screen' membranes (New England Nuclear, Boston, Massachusetts, USA) which had been wetted with the same buffer. The 'Gene Screen' membranes so prepared were heated at 80 °C for 2 to 3 hr *in vacuo* and then prehybridized in 50% formamide, 0.8 M NaCl, 2 mM EDTA, 20 mM PIPES, 0.5% SDS and denatured salmon sperm DNA (200 μg/ ml) for 23 to 27 hr at 37 °C.

Single-stranded, ^{32}P-labeled, cDNAs were synthesized from DNA sequences for malic enzyme, fatty acid synthase and albumin, all of which had been subcloned into M13 vectors (8). The reactions contained M13 messenger-sense DNA, 100 ng/μl; pentadecamer sequencing primer, 0.38 ng/μl; Klenow fragment of *E. coli* DNA polymerase I, 0.025–0.10 Units/μl; ^{32}P-dCTP, 0.5 μCi/μl (800 Ci/mmol); 50 mM NaCl; 7.6 mM Tris–HCl (pH 7.5); 6.6 mM $MgCl_2$; 6.6 mM dithiothreitol; 100 μM each of dATP, dGTP, dTTP (pH 6.5–7.0) in a final volume of 20 μl. Template, primer, NaCl, Tris–HCl and $MgCl_2$ where mixed in a volume of 7 μl, heated for 3 min at 90 °C followed by 30 min at 65 °C. After cooling to room temperature, the remaining reactants were added and the mixture incubated for 14 or 15 hr at 15 °C. The entire reaction mixture was then subjected to electrophoresis in a 1% Seaplaque agarose gel (FMC Corp., Rockland, Maine, USA) under alkaline conditions (12). The region of the gel containing the labeled DNA strand was located by autoradiography, excised, solubilized by heating in a boiling water bath, and immediately added to the hybridization reaction mixture.

Hybridization was carried out for 65 hr at 37 °C in prehybridization buffer containing about 1 ng of ^{32}P-labeled cDNA probe per ml ($1–2 \times 10^9$ cpm per μg DNA). Two membranes (10 cm × 13 cm) were incubated with 14 ml hybridization buffer in sealed plastic bags. After hybridization, the membranes were washed three times at 37 °C for 40 min per wash in 400 ml of 50% formamide, 750 mM NaCl, 75 mM Na citrate, 0.5% SDS. The membranes were then washed three additional times at 37 °C for 30 min per wash in 400 ml of 15 mM NaCl, 1.5 mM Na citrate, 0.1% SDS. The membranes were blotted dry, sealed in plastic bags and subjected to autoradiography at –70 °C with Kodak XAR-5 film and DuPont Cronex Hi-Plus intensifying screens. The resulting autoradiograms were scanned with an integrating densitometer (Transidyne General Corp.,

Ann Arbor, Michigan, USA: Model 2510). Varying amounts of total RNA extracted from the liver of a fed goose were dotted in duplicate on each filter (0.05 to 4 μg per dot). This preparation of goose RNA contained high levels of the mRNAs for fatty acid synthase, malic enzyme and albumin. Concentration curves generated as described above were used to normalize the results. Abundance of an mRNA in a chicken sample is expressed as being equivalent to that amount of goose RNA which gave an equivalent hybridization signal.

Materials

cDNA clones for goose malic enzyme and fatty acid synthase were isolated from a uropygial gland cDNA library (7, 8) and subcloned into M13mp8 or M13mp9, respectively (13). The fatty acid synthase subclone is a 660 bp Pvu II fragment from pFAS 3 (7). Characterization of these cDNA clones and their authentication as malic enzyme and fatty acid synthase cDNAs has been described (7, 8). The albumin cDNA clone (kindly provided by Dr Roger Deeley, Queen's University, Kingston, Canada) was isolated from a chicken liver cDNA library (14) and subcloned into M13mp8 (13). Characterization and authentication of this clone has been described (14). Klenow fragment of *E. coli* DNA polymerase I and [alpha ^{32}P]dCTP (New England Nuclear (Boston, Massachusetts, USA)), and universal pentadecamer sequencing primer (New England Biolabs, Beverly, Massachusetts, USA) were obtained from the indicated suppliers. All other chemicals were purchased from sources indicated previously (7, 8) or were of the highest quality commercially available.

Statistical significance of the data was tested where appropriate by the Mann-Whitney, signed-ranks test (15). Standard errors of the mean are provided to indicate the degree of variance in the data.

Results and discussion

Fatty acid synthase

Relative synthesis of fatty acid synthase increased 100-fold between 19th day of incubation and 4 days after hatching in unfed chicks. If neonates were fed,

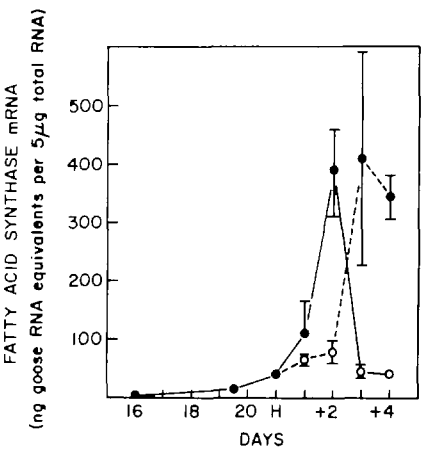

Fig. 1. Relative abundance of fatty acid synthase mRNA in total RNA extracted from the livers of embryonic and neonatal chicks.

Total RNA was extracted, fixed to 'Gene Screen' membranes and hybridized to a single-stranded, ^{32}P-labeled, fatty acid synthase cDNA as described in 'Materials and methods'. Each sample (5 μg) was assayed in duplicate. Seven concentrations of total RNA from goose liver (0.05 to 4 μg) were dotted in duplicate on every filter. The hybridization signals from the goose RNA samples were used to construct a standard curve. The concentration of fatty acid synthase mRNA in the unknown samples is expressed as the equivalent of that amount of goose RNA giving the same hybridization signal. Embryonic and fed, hatched chicks are represented by the solid circles. Starved, hatched chicks are represented by the open circles. Data points for embryonic and hatched chicks which were fed and then starved are connected by a solid line; those for hatched chicks which were starved and then fed are connected by a dashed line. Each point represents the mean ± SEM of 3 to 7 separate experiments except for the chicks which were fed 48 hr and starved 48 hr. In this case, the point is the mean ± deviation of two experiments. Points without error bars had standard errors equal to or smaller than the symbol. At or before hatching, two to four livers were pooled for each experiment. After hatching, one or two livers were used for each experiment.

enzyme synthesis increased an additional 10-fold (5). Similar results were obtained by Zehner et al. (6). With a cloned cDNA for fatty acid synthase, we assessed the level at which synthesis of fatty acid synthase was regulated both during hatching when feeding was not involved and during cycles of starvation and feeding after hatching. Fatty acid synthase mRNA was barely detectable in RNA extracted from the livers of embryos incubated for 16 days (Fig. 1). By 19 days of incubation, abundance of this mRNA had increased about 5-fold but still was relatively low. Fatty acid synthase mRNA level increased throughout the hatching period and the

post-hatching period in starved chicks so that by 2 days after hatching, it was 30-fold higher than at 16 days of incubation. If the birds were fed for 2 days instead of being starved, there was an additional 5-fold increase in mRNA abundance (Fig. 1). Feeding caused a 6-fold increase in the level of fatty acid synthase mRNA when neonatal chicks were starved for 2 days and then fed for 2 days. Thus, relative synthesis of fatty acid synthase is positively correlated with the relative abundance of its mRNA.

The reciprocal experiment also indicated a strong correlation between synthesis of fatty acid synthase and the abundance of its mRNA. When 9-day old chicks were starved for 2 days, relative synthesis of the enzyme decreased by almost 90% (5). The level of fatty acid synthase mRNA in the livers of fed, 2-day old chicks was decreased by 80% with 2 days of starvation (Fig. 1). The decrease in fatty acid synthase mRNA caused by starvation occurred very rapidly. By 24 hr, fatty acid synthase mRNA was decreased to the same level as that at 48 hr of starvation (Fig. 1). In one experiment, RNA was extracted from the livers of two chicks which had been fed 48 hr and starved 12 hr. Fatty acid synthase mRNA level was lower than in the extracts from birds starved for 24 or 48 hr (data not shown). Thus, in starved chicks, fatty acid synthase mRNA may have a half-life of 6 hr or less.

It is more difficult to estimate half-life of the mRNA in fed chicks. When neonates are allowed immediate access to food they do not start eating immediately, possibly accounting for the small increase in level of the mRNA for fatty acid synthase in chicks fed for 24 hr immediately after hatching. When chicks are starved for 48 hr before feeding, they start to eat rapidly and synchronously. There was no change in mRNA level between 24 hr and 48 hr after feeding previously starved chicks, suggesting that the steady-state level of fatty acid synthase mRNA may have been achieved at 24 hr. Since the time required to achieve a new steady-state is a function of the degradation rate constant (16) fatty acid synthase mRNA may have a half-life of 12 hr or less in fed animals.

The strong correlation between relative synthesis of fatty acid synthase and abundance of its mRNA indicates that both the pre-feeding increase in enzyme synthesis and the nutritional regulation of enzyme synthesis were due to regulation of the amount of fatty acid synthase mRNA in liver.

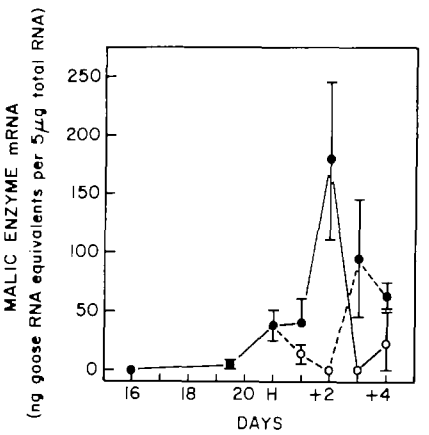

Fig. 2. Relative abundance of malic enzyme mRNA in total RNA extracted from the livers of embryonic and neonatal chicks.

Experimental details and definition of symbols are the same as in Fig. 1 except that the single-stranded, ^{32}P-labeled probe was malic enzyme cDNA.

Malic enzyme

Malic enzyme mRNA was virtually undetectable in total RNA extracted from the livers of embryos at 16 and 19–20 days of incubation (Fig. 2). Despite considerable variability from sample to sample, the level of malic enzyme mRNA on the day of hatching was significantly higher ($p = 0.02$) than that at 19–20 days of incubation. In a much smaller set of experiments, the same pattern of development of malic enzyme mRNA was observed in the liver of embryonic ducklings (17). Thus, abundance of malic enzyme mRNA during development paralleled that of fatty acid synthase mRNA despite the fact that activities of the two enzymes do not increase co-ordinately during the same period (2, 3). The synthesis rate for malic enzyme has not been measured in embryonic chicks or in neonatal chicks on the day of hatching. The discrepancy between mRNA abundance and enzyme activity may be due to a long half-life for malic enzyme at this point in development (cf. Ref. 4). This would mean that steady-state with respect to malic enzyme level was not achieved at hatching, making it improper to equate changes in enzyme activity with changes in enzyme synthesis. Alternatively, malic enzyme activity in the neonate may be regulated at a translational or post-translational level.

Malic enzyme mRNA decreased to undetectable

levels when neonatal chicks were starved (Fig. 2). If the neonates were fed, mRNA for malic enzyme increased slowly during the first 24 hr and then more rapidly during the ensuing 24 hr. The relative increase in abundance of malic enzyme mRNA between neontal chicks starved for 2 days and those fed for two days cannot be calculated because the mRNA was not detectable over background in the starved birds. Nevertheless, feeding the starved chicks or starving the fed chicks caused rapid and extensive increases or decreases, respectively, in malic enzyme mRNA level. Thus, like fatty acid synthase mRNA, the mRNA for malic enzyme may turn-over rapidly. The strong positive correlation between abundance of malic enzyme mRNA and relative synthesis of malic enzyme in fed or starved chicks (4) suggests that nutritional state regulates synthesis of malic enzyme at a pre-translational level.

Albumin

Abundance of albumin mRNA also was measured in RNA extracted from the livers of embryonic and newly-hatched chicks (Fig. 3). The level of albumin mRNA was relatively unaffected by short-term starvation of rats (18) and, therefore, represents a control on the specificity of the effects of alimentation on the levels of fatty acid synthase and malic enzyme mRNAs. As expected, the level of albumin mRNA was not significantly different in fed or starved birds nor did the level change significantly between 1 and 4 days after hatching. However, the level of albumin mRNA was some 5-fold higher in RNA from the livers of embryos incubated for 16 days than in that from 1-day old chicks. To our knowledge, albumin synthesis has not been measured during late development of the chick embryo. The concentration of albumin in the plasma increased almost 3-fold between 14 days-of-incubation and 1 day-of-age (19). The discrepancy between concentration of albumin in the plasma and of albumin mRNA in the liver could be due to translational or post-translational regulation of hepatic albumin production or to changes in the stability of plasma albumin during embryonic development. Whatever the mechanism for regulating albumin production in the embryo, the decrease in albumin mRNA in the chick embryo contrasts markedly with the large increase in albumin mRNA level which occurs in the final stages of development of the fetal rat (20). The high concentration of albumin mRNA in the livers of embryonic chicks may relate to the absence in birds of an analogue of alpha-fetoprotein (19), the embryonic 'serum albumin' in mammals.

Total RNA, and DNA

The hybridization assays used to measure specific mRNA levels actually measure relative abundance of the mRNA in total RNA (primarily ribosomal plus messenger RNA). Changes in the total RNA content of liver could influence interpretation of the results. The yield of RNA per liver increased throughout the incubation and post-hatching period (Fig. 3). The state of alimentation of the hatched birds had little or nor effect on RNA content with the exception of chicks fed for 48 hr and starved for 48 hr where RNA yield per liver decreased by almost 40% with respect to fed animals. Although liver weight was not determined in these experiments, other measurements of liver weight in embryos and young chicks of the White Leghorn strain (Goodridge, unpublished results) indicate that recovery of RNA per gram of liver was constant.

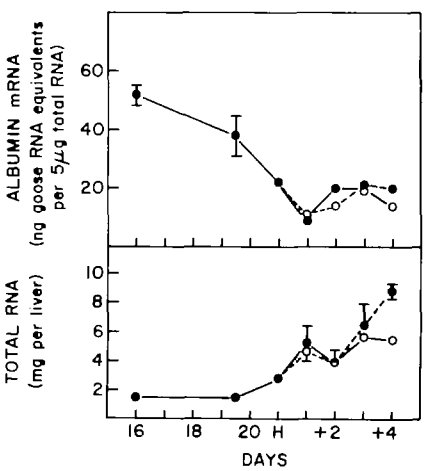

Fig. 3. Relative abundance of albumin mRNA (upper panel) and yield (lower panel) of total RNA extracted from the livers of embryonic and neonatal chicks.

For the upper panel, experimental details and definition of symbols are the same as in Fig. 1 except that the single-stranded, ^{32}P-labeled probe was albumin cDNA. For the lower panel, RNA was extracted and quantified as described in 'Materials and methods'. The definition of the symbols is the same as in Fig. 1.

Therefore, the yield of total RNA did not differ sufficiently to influence the interpretation of the hybridization assays. Furthermore, the fact that the relative levels of the mRNAs for fatty acid synthase, malic enzyme and albumin varied in different directions and/or to different degrees rules out any systematic error in recovery of total RNA which could have influenced the results.

Another potential source of error is DNA contamination of total RNA. A significant amount of DNA in the RNA extract would cause less RNA to be dotted onto the paper. Each of the samples of total RNA assayed for specific sequences was also assayed for DNA content by the diphenylamine procedure (21). Our samples had less than 1% DNA, indicating that this was not a significant source of error (data not shown).

In sum, our results suggest that the relative rates of synthesis of fatty acid synthase and malic enzyme are controlled by regulating the levels of their respective mRNAs during development and during nutritional manipulations. The one possible exception to this conclusion is the unexpectedly high level of malic enzyme mRNA at hatching. The disparate enzyme activity may have a kinetic explanation or be due to translational or post-translational regulation. In any event, this exception suggests that the regulation of the expression of these two genes may be more co-ordinate than measurements of enzyme activity had indicated. Future experiments will determine whether the levels of the mRNAs for fatty acid synthase and malic enzyme mRNA are regulated at the level of transcription, processing or stability of the specific mRNAs.

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