RATE OF SYNTHESIS OF β_L-LIPOVITELLIN IN THE LIVER OF IMMATURE CHICKS TREATED WITH 17β ESTRADIOL

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Received December 30,1974

Summary: Using a combination of radioimmunoprecipitation and SDS-polyacrylamide gel electrophoresis of the immunoprecipitate we studied the rate of synthesis of the heavy chain of β-lipovitellin in the liver of immature chicks. In male and female chicks the base-line synthesis of β_L-lipovitellin[1] was about 30 molecules per minute and per cell. Four days after a single injection of 40 mg estradiol/kg as many as 48,000 molecules of β_L-lipovitellin were synthesized per minute and per diploid liver cell. The increase in the rate of β_L-lipovitellin synthesis could be correlated with an increase in membrane bound mRNA coding for β_L-lipovitellin.

INTRODUCTION

Together with phosvitin, lipovitellin is one of the main protein of the egg yolk (1). While the magnum portion of the oviduct is the site of synthesis of egg white proteins (2,3), the liver is the site of synthesis of lipovitellin and other yolk proteins (4-9). The main proteins of the yolk granule are phosvitin and α,β-lipovitellin (1). As analyzed by SDS-polyacrylamide gel electrophoresis, the molecular weights of the polypeptides present in α-lipovitellin are about 135,000, 105,000 and 40,000, whereas for β-lipovitellin two polypeptide chains with molecular weights of 135,000 and 30,000 are observed (10,11). Like all secretory proteins, lipovitellin is synthesized on membrane bound polysomes (12) and then secreted into the blood stream. This communication presents the first quantitative study on the *in vivo* synthesis of β_L-lipovitellin in the liver of immature chicks treated with estradiol. Furthermore, we show a

[1] The following abbreviations were used: SDS, sodiumdodecylsulfate. β_L-lipovitellin, heavy chain of β-lipovitellin. DTT, dithiothreitol.

correlation between the high in vivo rate of synthesis of β_L-lipovitellin, and the presence of poly A containing mRNA coding for β_L-lipovitellin and bound to the endoplasmic reticulum.

MATERIALS AND METHODS

Nitrocellulose powder was obtained from Serva, Heidelberg, Germany, and [^{3}H]-leucine (56 Ci/mmole) was purchased from Amersham Radiochemical Centre, England. White Leghorn chicks (150 g) were used throughout all experiments (13).

Purification of lipovitellin. Egg yolk granules were prepared from fresh eggs as described by Bernardi and Cook (14). Lipovitellin was purified and separated in α and β components by TEAE cellulose chromatography (1). The β-lipovitellin fraction was extensively dialyzed against water and lyophilized. β-Lipovitellin was further separated into heavy (L) and light (S) chains by chromatography on Sephadex G-200. About 30 mg of β-lipovitellin was dissolved in 1.5 ml of 5% SDS, 0.3 M β-alanine-acetic acid pH 4.5 buffer. The solution was heated at 50° for 2 minutes and lipids were extracted once with ether. The protein sample was chromatographed at room temperature on a 1.5 x 70 cm Sephadex G-200 column using 1% SDS in 0.07 M β-alanine-acetic acid pH 4.5, flow rate 6.4 ml per hour. The purity of all fractions was tested by electrophoresis on 5% polyacrylamide-SDS gels (10). Figure 1 shows a scan of a purified fraction of β-lipovitellin heavy chain used for immunization of rabbits.

Preparation of liver homogenate. The livers were homogenized for 2 minutes with a high speed Polytron homogenizer in four volumes of 0.15 M NaCl, 0.025 M Tris pH 7.5, 1% Triton X-100. The homogenate was centrifuged for 1 hour at 105,000 x g and the supernatant fraction was tested for β-lipovitellin synthesis by radioimmunoprecipitation as described below. An aliquot of the crude homogenate was kept for the DNA and amino acid pool determinations.

Immunization of rabbits and immunoprecipitation. For the immunization of rabbits we used either total β-lipovitellin or purified heavy chain of β-lipovitellin. Rabbits were immunized by Bos et al. (12). Three weeks after immunization, blood samples were collected from the lateral ear vein and the serum tested for antibody reaction. The gamma globulin fraction of pooled serum was obtained by ammonium sulfate precipitation (15). Immunoprecipitation with a threefold excess of antibody was carried out as previously described (16). After centrifugation at 2,000 x g for 20 minutes, the immunoprecipitate was resuspended in 2-5 ml of 0.15 M NaCl and centrifuged as above. The immunoprecipitate was further purified over 1 M sucrose as described by Palmiter (17). The purified antibody-antigen precipitate was either dissolved in formic acid and directly counted for radioactivity or it was solubilized by boiling for 5 minutes in 50 µl of a SDS-DTT solution as described by Palmiter et al. (18) and analyzed electrophoretically on 5% polyacrylamide-SDS gels (10). Gels were stained with Coomassie brilliant blue, the β_L-lipovitellin band was cut out and oxidized as described

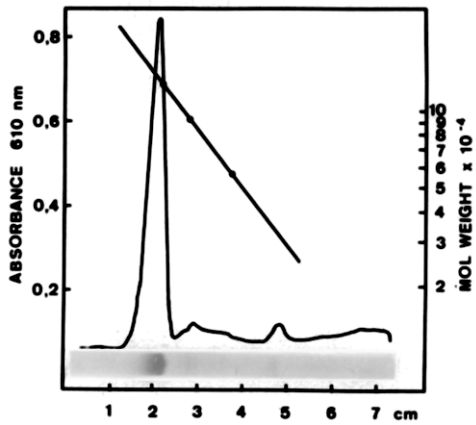

Figure 1: Electrophoresis of 75 μg of β_1-lipovitellin (post Sephadex G-200 fraction) on 5% polyacrylamide-SDS gels. The calibration curve was established with the following standards: β-galactosidase, M.W. 130,000, phosphorylase A, M.W. 92,500, and catalase, M.W. 57,000.

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by Dierks-Ventling and Jost (19).The specific activity of the leucine pool was determined as previously described (19).

Extraction of RNA and partial purification of lipovitellin mRNA. Throughout the entire procedure, buffers and liver extracts were kept at 0° in crushed ice. All buffers and glassware were sterilized. We found it essential to use ribonuclease-free sucrose and 500 μg of purified yeast RNA and 100-500 μg of heparin were added per ml of all buffers. The livers were minced in 10 volumes of 0.25 M sucrose containing 0.025 M Tris pH 7.2, 0.001 M $MgCl_2$ and homogenized in a glass teflon Potter homogenizer. After filtration of the homogenate through sterile cheese cloth, nuclei, cell debris and mitochondria were sedimented at 5,000 x g for 10 minutes. The microsomal fraction was then sedimented by centrifugation at 105,000 x g for 40 minutes. The pellet was resuspended with a homogenizer in 8 volumes of 0.25 M sucrose containing 0.025 M Tris pH 7.2, 0.05 M KCl, 0.005 M $MgCl_2$, 5% Triton X-100, 500 μg yeast RNA and 500 μg heparin per ml. The polysomes suspension was overlayed on 6 ml of 20% sucrose Tris-$MgCl_2$ buffer and centrifuged at 105,000 x g for 90 minutes in a Spinco 30 angle rotor.

The RNA was extracted from the polysomes with 0.1 M Tris pH 9.0, 0.5% SDS and cold phenol as described by Brawerman et al. (20). RNA was precipitated at minus 30° for 3 hours with 2 volumes of ethanol in the presence of 0.2 M sodium acetate pH 5.0. The separation of total poly A containing mRNA was carried out on a nitrocellulose column. RNA precipitate was first dissolved in a minimum amount of sterile water and then diluted with 0.5 M KCl buffer to a concentration of 20-30 OD 260 nm per ml. The RNA solution was adsorbed on the nitrocellulose column preequilibrated with 0.5 M KCl, 0.01 M Tris pH 7.6, 0.001M $MgCl_2$. The column was washed with 0.5 M KCl buffer at 6° until the UV absorption of the

effluent dropped below 0.01. The second elution was made with about 4 bed volumes of the same buffer at 19° and the third elution was carried out at 6° with 0.1 M Tris pH 7.6 0.0025 M EDTA. The first two eluates contained mainly ribosomal RNA and tRNA, all mRNA (about 2-3% of total RNA) was eluted in the last fraction. The maximum binding capacity of the commercial nitrocellulose for poly A containing mRNA was about 50-100 µg per g of nitrocellulose (dry weight). By that method a 30 to 40 fold enrichment of lipovitellin mRNA, as judged by its translation in a cell-free system, could be observed.

Cell-free system. The RNA fractions were assayed for mRNA activity by use of the Krebs ascites cell-free lysate system as described by Schutz et al. (21). The product of translation was characterized by immunoprecipitation in the presence of carrier β_L-lipovitellin and the radioimmunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis as described above.

RESULTS AND DISCUSSION

Kinetics of β-lipovitellin induction. Figure 2 shows the kinetics of induction of β-lipovitellin following a single injection of 40 mg estradiol per kg body weight. All chicks received estradiol at time zero and at the time indicated in Figure 2, groups of 3 chicks were pulse-labeled for 15 minutes with [^{3}H]-leucine (100 µCi/150 g chicks). The pulse-labeling of 15 minutes was chosen because it was the time necessary to label β-lipovitellin in the

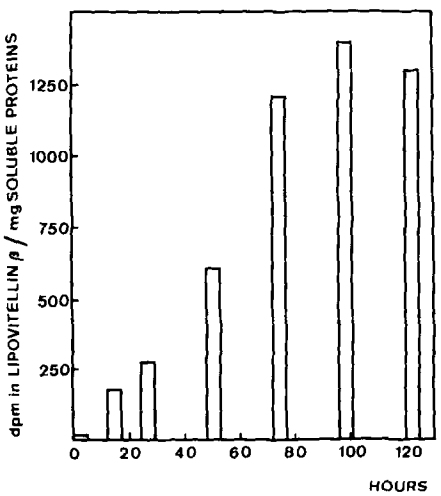

Figure 2: Kinetics of β-lipovitellin induction in immature chicks following a single injection of 40 mg 17β estradiol/kg. At each time point 3 chicks were pulse-labeled with [^{3}H]-leucine (100 µCi/150 g) for 15 minutes and the incorporation of the label into β-lipovitellin was determined as described in Materials and Methods

liver before it was secreted into the plasma (data not shown). The livers were homogenized and β-lipovitellin was immunoprecipitated as outlined in Materials and Methods.

Already 12 hours after estradiol treatment we could observe a significant increase in the rate of incorporation of [^{3}H]-leucine into β-lipovitellin. The rate of incorporation was maximal 4 days after estradiol treatment and within 8 days gradually declined to the base-line (data not shown). Consequently, we chose the 4th day after the initial estradiol injection for our study on the rate of β_L-lipovitellin synthesis.

Groups of three chicks were used; the control received propylenglycol only and the experimental chicks received 40 mg estradiol per kg. Four days later the two groups were pulse-labeled with [^{3}H]-leucine for 15 minutes. The livers of each group were pooled and processed as indicated above. The incorporation of leucine into β_L-lipovitellin present in the immunoprecipitate was separated by electrophoresis as outlined in Materials and Methods. In Table 1 it can be seen that there is a striking difference in the specific activity of the leucine pool between control and hormone-treated animals. This large difference in specific activity could also be observed two hours after the initial injection of estradiol (19). Consistant with our previous findings (13) estradiol had also a general anabolic effect as shown by the 4 fold increase in the incorporation of leucine into total soluble proteins. Under our experimental conditions the incorporation of leucine into β_L-lipovitellin did not exceed 2.4% of the total labeled soluble proteins while the base-line of β_L-lipovitellin in controls was about 0.01%. Knowing the mole ratio of leucine per mole of β-lipovitellin (22,23) and taking a value of 2.5 pg of DNA per diploid chicken liver cell (24), we could calculate that in the control chicks about 30 molecules of β_L-lipovitellin were synthesized per minute and per cell, whereas four days after a single injection of estradiol about 48,000 molelcules were synthesized per minute and per cell. No difference in the base-line synthesis of β_L-lipovitellin could be observed between male and female chicks. Furthermore, radioimmuno-precipitation experiments carried out with labeled chicken brain and muscle homogenate gave no trace of

Table 1

	Leucine pool dpm / pmole	pmoles leucine incorporated/mg protein into total soluble proteins	β_L-lipovitellin	% of total	Molecules of β_L-lipovitellin synthesized/ min / cell
Control	3.7	2591	0.3	0.01	31
Estradiol treatment	1.0	11542	277	2.40	48000

Table 1: Study on the in vivo synthesis of β_L-lipovitellin in immature chicks (150 g) receiving either propylenglycol only (controls) or 40 mg 17β estradiol/kg. Four days later, groups of 3 chicks were pulse-labeled with [^{3}H]-leucine (100 μCi/150 g) for 15 minutes. Livers were pooled, homogenized and the incorporation of leucine into β_L-lipovitellin determined in the radioimmunoprecipitate as described in Materials and Methods. For each value triplicates were run and the standard variation did not exceed 10%.

Table 2

	Incorporation of [^{3}H]-leucine into		
	total proteins dpm/sample	β_L-lipovitellin dpm/sample	% of total proteins
Control	17015	4	0.02
Estradiol treatment	30000	301	1.00

Table 2: Assay of mRNA fractions for β_L-lipovitellin mRNA activity. All fractions were tested in the Krebs ascites cell-free system and the product of translation was analyzed by radioimmunoprecipitation followed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. In each assay 10 µg of mRNA were included per 100 µl incubation mixture and the incubation was performed at 34° for 80 minutes. In that particular system the reaction was linear up to 25 µg of total mRNA per 100 µl of reactions mixture. Each value represents the average of triplicates and the standard variation did not exceed 10%.

radioactivity corresponding to β_L-lipovitellin, indicating that the base-line synthesis is probably real.

From the above results we conclude that in the liver of 150 g immature chicks there is a small base-line of β_L-lipovitellin synthesis, and the injection of estradiol brings about a massive increase in the rate of synthesis of that protein. That the massive increase in the synthesis of β_L-lipovitellin corresponds to an increase in translatable mRNA bound to the endoplasmic reticulum was studied in the following experiment.

In vitro translation of β_L-lipovitellin mRNA. This experiment was designed similarly to the one summarized in Table 1. Rough endoplasmic reticulum, polysomes, RNA and total poly A containing mRNA were prepared as outlined in Materials and Methods. Aliquots of

the mRNA fractions were tested in a Krebs ascites cell-free system and the product of translation was analyzed by radioimmunoprecipitation and electrophoresis. Table 2 shows that the mRNA from control chicks codes for trace amount of cross reacting material corresponding to β_L-lipovitellin. Incubation with an equal amount of total mRNA from estradiol treated chicks resulted in a 75 fold increase in the translation of proteins crossreacting with anti-β-lipovitellin. From the above results we conclude that estradiol injection of immature chicks not only increases the rate of synthesis of β_L-lipovitellin in the liver but also greatly increases the quantity of mRNA coding for β_L-lipovitellin. Whether that high mRNA activity represents a true increase in the rate of transcription of β_L-lipovitellin gene(s) or a decrease in turnover of the mRNA is not known. Regarding the last possibility it is noteworthy that the higher concentration of seryl-tRNA found in the liver of estradiol treated chicks could be explained by an apparent decrease in the turnover of tRNA (25,26). Since in control chicks a low base-line of β_L-lipovitellin synthesis is observed, it is questionable whether estradiol increases the rate of transcription of "open" lipovitellin gene(s) rather than activates or derepresses β_L-lipovitellin gene(s). Moreover it is conceivable that in the immature chicks there is already sufficient 17β estradiol to stimulate the synthesis of trace amount of β_L-lipovitellin.

Acknowledgments: We should like to thank Drs. J. Gordon, I. Levitan, D. Monard and M. Staehelin for valuable criticism and discussion.

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