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Coordinate regulation of two estrogen-dependent genes in avian liver

(very low density lipoprotein/vitellogenin/serum albumin)

R. WISKOCIL, P. BENSKY, W. DOWER*, R. F. GOLDBERGER, J. I. GORDON, AND R. G. DEELEY†

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT **Livers of egg-laying species contain abundant mRNAs encoded by both estrogen-responsive and constitutively expressed genes. We have recently constructed cDNA clones from three members of the abundant mRNA class of hen liver. One of these mRNA species was identified as serum albumin mRNA, and another as vitellogenin mRNA. In this study we have identified the third member of the group as apoVLDLII mRNA. Hybridization analyses using cloned cDNA probes indicate that expression of the apoVLDLII gene in rooster liver, like that of the vitellogenin gene, is completely dependent upon the administration of estrogen. The apoVLDLII and vitellogenin genes appear to be the only genes capable of high rates of expression in the liver that exhibit such an exceptional response to the hormone. Administration of estrogen resulted in the appearance of both mRNA species within 30 min, followed by a rapid accumulation to several thousand copies per cell. Removal of the hormone caused a marked destabilization of both vitellogenin mRNA and apoVLDLII mRNA. In contrast, the absolute levels of serum albumin mRNA were unaffected by the hormone. Comparative studies on the structure and organization of these three genes may reveal elements involved in determining their rates of expression in the presence and absence of estrogen.**

Avian egg yolk proteins are a complex group of vitamin- and mineral-binding proteins, lipoproteins, and phosphoproteins. They are synthesized in the liver of the laying hen, under the regulation of estrogen (1). There is a wide variation in the rates at which individual proteins within the group are synthesized and also in the extent to which their synthesis is regulated by estrogen. Thus, it appears that the group is composed of some genes that may be regarded as "estrogen-dependent" and other genes whose expression appears to be modulated by, but not dependent upon, the hormone.

It is possible to induce the synthesis of a complete spectrum of egg yolk proteins in mature males in response to a single injection of estrogen. Despite a considerable commitment to yolk protein synthesis, the liver of the estrogen-treated rooster continues to synthesize serum albumin throughout the vitellogenic response, at a rate that is relatively unaffected by the hormone. The constitutive expression of the albumin gene provides a useful control for distinguishing the specific effects of the hormone from its more general effects on the overall transcriptional and translational activities of the tissue.

We are interested in comparing the structure and organization of genes that are estrogen-responsive in the liver with those that are constitutively expressed. In order to facilitate these studies, we recently constructed cDNA clones derived from chicken serum albumin mRNA (2) and two estrogen-inducible mRNA species (3). One of these was vitellogenin mRNA which specifies the precursor of the egg yolk phosphoproteins (4, 5). The other was a much smaller mRNA species of approximately 800 nucleotides; its identity was not known.

The small estrogen-inducible mRNA has now been purified. It has been identified as apoVLDLII mRNA by characterization of its translation product and, more directly, by comparison of nucleic acid and protein sequences. The estrogen-dependent induction of synthesis of very low density lipoprotein (VLDL) in rooster liver has been studied extensively over the past 5 years. In general, immunological techniques have been utilized to estimate rates of VLDL synthesis in tissue slices or *in vivo* (6–8) and for quantifying the levels of apoVLDLII mRNA in cockerel liver after the administration of hormone (8). Recent data on induction of apoVLDLII indicated that a 12-fold increase in the level of apoVLDLII mRNA occurs after the administration of estrogen (9). It has been concluded that the estrogen-mediated regulation of VLDL synthesis involves the modulation of genes that are already expressed at a significant level in the normal rooster.

This 12-fold increase in concentration of apoVLDLII mRNA is in contrast with the several thousand-fold increase in concentration of vitellogenin mRNA that occurs under similar circumstances (10), suggesting that the regulatory characteristics of the two estrogen-responsive genes are quite different. In the study reported here we used cloned cDNA probes to compare the kinetics of induction and decay of apoVLDLII mRNA with those of vitellogenin mRNA. The effect of the hormone on the levels of serum albumin mRNA has also been monitored in an identical fashion. Contrary to published results, we find that, like vitellogenin mRNA, apoVLDLII mRNA is not present in the liver of the normal rooster and that the induction characteristics of both genes are strikingly similar.

MATERIALS AND METHODS

RNA Isolation and *in Vitro* Translation. Total cellular RNA or poly(A)$^+$RNA was isolated from the livers of roosters or hens as described (11). RNA samples were translated, and the polypeptides produced were analyzed (12).

Purification of the "Small Estrogen-Inducible" mRNA. DNA (60 μg) from a chimeric plasmid, PBR322-E18, containing a 350-nucleotide insert was coupled to 15 mg of diazobenzyloxymethyl-cellulose (13). Poly(A)$^+$RNA (200 μg) from hen liver was hybridized to the DNA-cellulose and the specifically bound RNA was eluted as described (14).

Immunoprecipitation. Hen VLDL and rabbit anti-delipidated VLDL antibody were gifts from D. Lane and P. Siuta (15). Goat anti-apoVLDLII was generously provided by L.

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Abbreviation: VLDL, very low density lipoprotein.

* Present address: Dept. of Biological Sciences, Stanford University, Stanford, CA 94305.

† To whom reprint requests should be addressed at: Dept. of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6 Canada.

Chan (9). Aliquots (125 μl) of the polysome-free supernatant from the cell-free translation system were adjusted to a final volume of 300 μl so that each sample contained 1% Triton X-100, 10 mM L-methionine, 0.15 M NaCl, and 50 mM sodium phosphate buffer (pH 7.2). The appropriate antiserum (10 μl) was added and the samples were incubated at 4°C. After 2 hr, 25 μl of a 1:10 (wt/vol) suspension of glutaraldehyde-fixed Cowan strain *Staphylococcus aureus* was added and the samples were processed essentially as described by Kessler (16).

Preparation of Sense-Strand Hybridization Probes from Cloned DNA Fragment. DNA from recombinant plasmids containing serum albumin, apoVLDLII, or vitellogenin DNA sequences was digested with *Pst* I (17). The inserted DNA sequence was purified by electrophoresis in agarose gels (18, 19). Approximately 0.1–0.5 g of DNA was labeled with ^{32}P by nick-translation (20) to a specific activity of 10^7–10^8 cpm/μg. Sense strand was isolated by hybridization of the DNA to a 10-fold sequence excess of the appropriate mRNA in 80% (vol/vol) formamide containing 0.4 M NaCl and 0.1 M 1,4-piperazinediethanesulfonic acid (pH 7.8) at 50°C. The anti-sense strand was digested with nuclease S1 and residual RNA was destroyed by alkaline hydrolysis (11). In order to remove any remaining anti-sense strand, the DNA was allowed to rehybridize and double-stranded DNA was removed from the mixture by chromatography on hydroxyapatite (21). Sense strand-specific DNA prepared in this fashion exhibited less than 5% self-hybridization. RNA-excess hybridization analyses were carried out as described (11). The maximal levels of hybridization reached were typically 65–80% of the input DNA.

RESULTS

Identification of the Small Estrogen-Inducible mRNA. The small estrogen-inducible mRNA was purified from the poly(A)$^+$RNA fraction of hen liver by chromatography on a plasmid DNA-cellulose column (14). Polypeptides synthesized in the wheat germ system from preparations of total poly(A)$^+$RNA from hen and rooster liver or the purified estrogen-inducible mRNA are shown in Fig. 1. The polypeptide specified by the estrogen-inducible mRNA had an apparent mass of 6800 daltons, judged from its migration on a NaDodSO$_4$/15% polyacrylamide gel. A polypeptide of the same size was readily detectable among the translation products synthesized from total poly(A)$^+$mRNA from the livers of estrogen-treated roosters or hens but not among the proteins specified by poly(A)$^+$RNA from the livers of normal roosters.

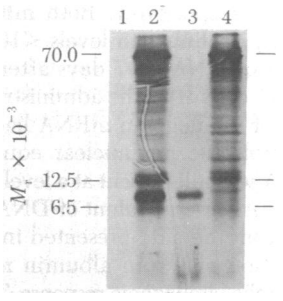

FIG. 1. Cell-free translation of the small, estrogen-inducible mRNA. Samples of poly(A)$^+$RNA from the livers of hens and roosters or of the purified estrogen-inducible mRNA were translated in a nuclease-treated wheat germ protein-synthesizing system. The [^{35}S]methionine-labeled polypeptides produced during the incubation were analyzed on a 0.1% NaDodSO$_4$/15% polyacrylamide slab gel. The figure shows a fluorograph of the dried gel. An aliquot (5 μl) from each incubation mixture (50 μl) was applied to the gel. Lanes: 1, endogenous synthesis; 2, polypeptides synthesized from poly(A)$^+$mRNA (10 μg/50-μl incubation) from hen liver; 3, polypeptides synthesized from the purified small estrogen-inducible mRNA (0.1–0.2 μg/50-μl incubation); 4, polypeptides synthesized from poly(A)$^+$RNA (10 μg/50-μl incubation) from rooster liver.

The polypeptide encoded by the estrogen-inducible mRNA was considerably smaller than anticipated. Its size suggests that between 500 and 600 nucleotides of the 800-nucleotide-long mRNA represent untranslated regions. Other abundant mRNA species from hen liver, such as chicken serum albumin mRNA (2) and vitellogenin mRNA (11), also contain untranslated regions of similar length despite an almost 10-fold variation in overall size.

Preliminary data on the induction characteristics of the mRNA isolated in this study suggested a much greater response to estrogen than that estimated previously for apoVLDLII (9). However, the polypeptide specified by the mRNA was of a size that might be expected for a precursor of this protein (9). Antibody raised against delipidated holoVLDL was used to check the possibility that the polypeptide was in fact a VLDL component. Fig. 2*A* shows the polypeptides that were synthesized from hen or rooster liver total RNA and bound to anti-delipidated holoVLDL or to antibody raised specifically against the apoVLDLII component. Antiserum raised against delipidated holoVLDL bound several polypeptides present among the translation products obtained from both rooster and hen liver poly(A)$^+$mRNA. Antibody specific for apoVLDLII, on the other hand, selectively bound a 6800-dalton polypeptide that was quite abundant among the translation products of hen liver RNA and not detectable, by this assay, among the polypeptides produced from rooster liver RNA.

A purified preparation of the 800-nucleotide-long estrogen-inducible mRNA was also translated in the wheat germ system. Aliquots of the translation mixture were treated with rabbit anti-chicken serum albumin antiserum, rabbit anti-rat serum antiserum, and rabbit anti-chicken VLDL antiserum. The polypeptide specified by the estrogen-inducible mRNA bound specifically to the anti-chicken VLDL antiserum (Fig. 2*B*).

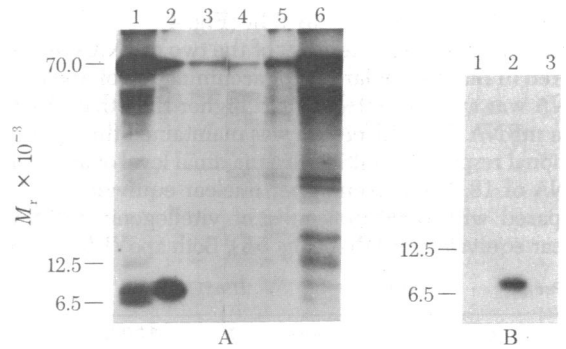

FIG. 2. Immunoprecipitation of VLDL components specified by poly(A)$^+$RNA from rooster or hen liver. Poly(A)$^+$RNA from rooster or hen liver or the purified small estrogen-inducible mRNA were translated in a nuclease-treated wheat germ cell-free protein-synthesizing system. Samples were incubated with various antisera, and the antigen–antibody complexes were adsorbed to glutaraldehyde-treated *S. aureus*. Adsorbed peptides were analyzed in a NaDodSO$_4$/15% polyacrylamide gel which was then fluorographed. (*A*) Polypeptides specified by poly(A)$^+$RNA. Lanes 1–3: polypeptides specified by RNA from hen liver that bind to rabbit anti-chicken holoVLDL antiserum (lane 1), goat anti-chicken apoVLDLII antiserum (lane 2), or rabbit anti-rat antiserum (lane 3). Lanes 4–6: Polypeptides specified by RNA from rooster liver that bind to rabbit anti-rat antiserum (lane 5), goat anti-chicken apoVLDLII (lane 5), or rabbit anti-chicken holoVLDLII antiserum (lane 6). (*B*) Immunoadsorption of the polypeptide specified by the small estrogen-inducible mRNA to rabbit anti-rat antiserum (lane 1), goat anti-chicken apoVLDLII (lane 2), or rabbit anti-chicken albumin antiserum (lane 3).

The immunological data strongly suggested that the small estrogen-inducible mRNA specified a component of VLDL, probably apoVLDLII. Because the amino acid sequence of White Leghorn apoVLDLII has been determined by Jackson *et al.* (22), it was possible to confirm its identity by comparing the sequence of a DNA fragment from one of the recombinant plasmids with the known amino acid sequence of the polypeptide. The nucleotide sequence of part of this fragment corresponds to a sequence of 18 amino acids leading to the carboxy terminus of apoVLDLII (Fig. 3). The sequence differs from the amino acid sequence at two sites, indicating a glutamine rather than a glutamate residue at position 66 and an asparagine rather than an aspartate at position 74. Both differences may be explained by partial deamidation of the polypeptide preparation used for sequence determination. The nucleotide sequence also indicated that tyrosine at position 83 should be the original carboxy-terminal residue rather than glycine at position 82. Tyrosine-83 is followed immediately by a termination codon (UAG). Interestingly, emu apoVLDLII has two additional residues following glycine-82, both tyrosine (24).

Comparison of the Induction Characteristics of apoVLDLII and Vitellogenin mRNA. Recombinant plasmids that contained serum albumin, vitellogenin, or apoVLDLII DNA sequences provided a source of specific DNA probes for use in RNA excess hybridization analyses. Neither apoVLDLII mRNA nor vitellogenin mRNA was detectable in total RNA isolated from the livers of normal roosters, indicating that there was less than one molecule of either mRNA per nuclear equivalent of DNA prior to administration of estrogen. Within 30 min after injection of hormone, both apoVLDLII mRNA and vitellogenin mRNA were found in the RNA preparation at 5 to 10 molecules per nuclear equivalent of DNA. As reported (10), vitellogenin mRNA exhibited nonlinear kinetics of accumulation characterized by a 6- to 7-fold increase in rate about 4 hr after hormonal stimulation. apoVLDLII mRNA displayed similar nonlinear induction kinetics except that the period of slow accumulation lasted only 2 hr (Fig. 4).

The induction characteristics of the two mRNA species also differed in that the molar rate of accumulation of apoVLDLII mRNA was approximately 3-fold higher than that of vitellogenin mRNA. This difference was maintained throughout the hormonal response, resulting in a maximal level of apoVLDLII mRNA of 18,000 molecules per nuclear equivalent of DNA, compared with 6000 molecules of vitellogenin mRNA per nuclear equivalent of DNA (Fig. 5*B*). Both apoVLDLII mRNA and vitellogenin mRNA accumulated with kinetics expected for stable mRNA species reaching maximal levels at 3 days after injection of the hormone. The half-lives estimated from the accumulation data are 26 and 22 hr for apoVLDLII mRNA and vitellogenin mRNA, respectively. Both mRNA species also decayed in concert, declining to levels <100 molecules per nuclear equivalent of DNA by 7 days after injection of the hormone. Within 17 days after the administration of estrogen, the concentration of vitellogenin mRNA had dropped to approximately 1.0 molecule per nuclear equivalent of DNA; apoVLDLII mRNA was still present at a level of approximately 15 molecules per nuclear equivalent of DNA.

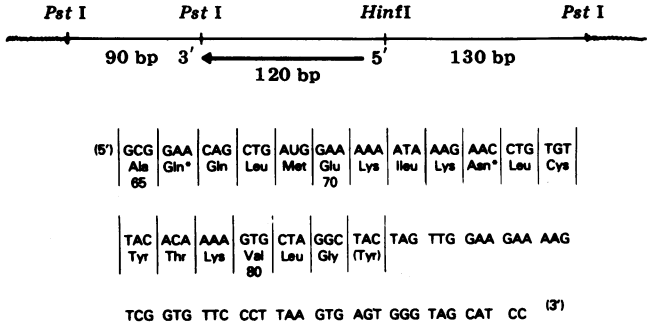

FIG. 3. Partial nucleotide sequence of the double-stranded cDNA insert in chimeric plasmid pBR322-E18. The plasmid was digested with *Hin*fI and *Pst* I. An internal 120-nucleotide fragment was subjected to Maxam and Gilbert (23) sequence determination procedure. The nucleotide sequence begins 15 nucleotides from the *Hin*fI site. The sequence has been matched with the carboxyterminus of White Leghorn apoVLDLII (24). Gln* (66) and Asn* (74) are positions at which the nucleotide sequence specified the amide but the peptide sequence data indicated the free acid. bp, Base pairs.

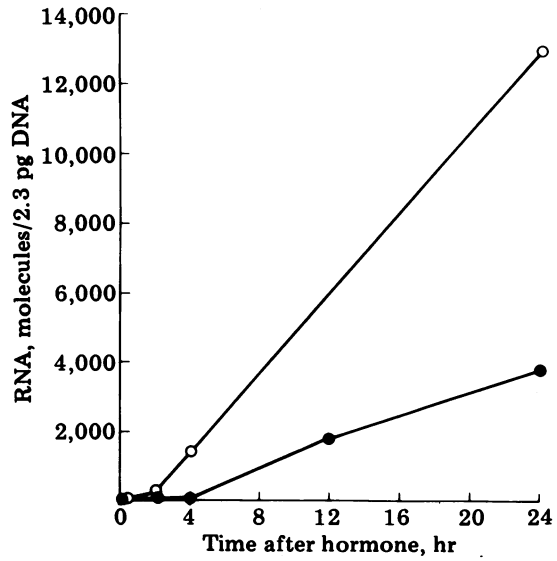

FIG. 4. Initial rates of accumulation of apoVLDLII mRNA (○) and vitellogenin mRNA (●) after primary stimulation with 17β-estradiol. White Leghorn cockerels (6–8 weeks old) were injected intramuscularly with 17β-estradiol (20 mg/kg body weight) dissolved in propylene glycol (20 mg/ml). Total RNA was prepared from the livers of two birds at each of the time points indicated. RNA-excess hybridization analyses were carried out with ^{32}P-labeled, sense strand DNA (10^7–10^8 cpm/μg) isolated from chimeric plasmids. The amount of a specific mRNA in each sample was determined by comparison of the $R_0t_{1/2}$ value obtained with that of purified vitellogenin mRNA hybridized under identical conditions. The data were corrected for the 8.75-fold difference in complexity between apoVLDLII mRNA and vitellogenin mRNA. The number of molecules of each mRNA species per nuclear equivalent of DNA (2.3 pg) was calculated from the hybridization data, the molecular weight of each mRNA, and the yield of RNA (5.0–6.0 mg) and DNA (2.0–2.5 mg) per g of tissue. Incubation of liver RNA from normal roosters with apoVLDLII cDNA resulted in approximately 5% of the cDNA becoming resistant to nuclease S1 at a R_0t value of 10^4 mol·sec/liter. This value was indistinguishable from that obtained during hybridization of the cDNA with yeast RNA. Accumulation rates: apoVLDLII mRNA, 0.159 molecule per sec per cell; vitellogenin mRNA, 0.062 molecule per sec per cell.

It can be seen from the data presented in Fig. 5*A* that the relative concentration of serum albumin mRNA decreased temporarily during the vitellogenic response. This decrease may result simply from dilution of serum albumin mRNA by other abundant, relatively stable mRNA species, such as vitellogenin mRNA and apoVLDLII mRNA. Correction of the data for the overall RNA and DNA content of the tissue indicates that, following hormonal treatment, the absolute amount of serum albumin mRNA remained relatively constant at 16,000–18,000 molecules per nuclear equivalent of DNA.

mRNA Stability in the Presence and Absence of Hormone. The induction kinetics of apoVLDLII mRNA and vitellogenin mRNA are consistent with the idea that both of these mRNA species are relatively stable in the presence of hormone. Their

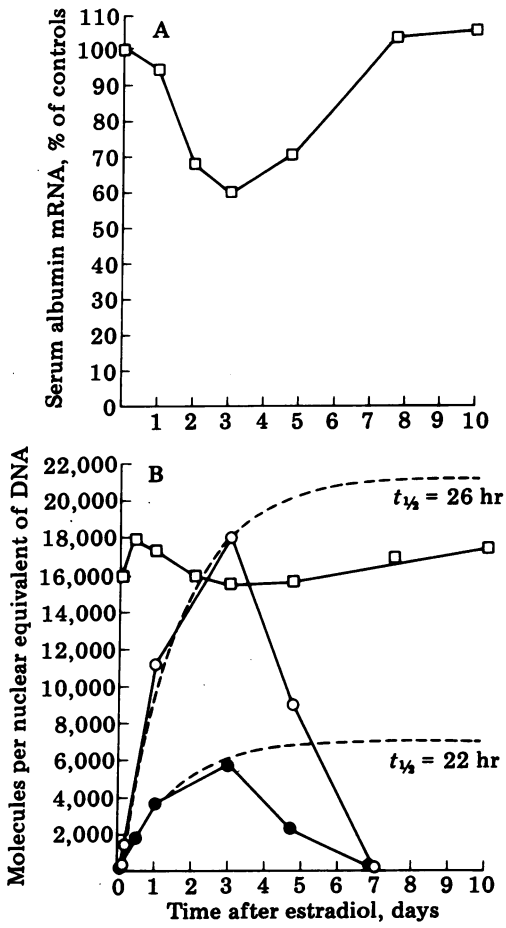

FIG. 5. Levels of apoVLDLII mRNA, vitellogenin mRNA, and serum albumin mRNA during 10 days after primary stimulation with 17β-estradiol. Experimental procedures and treatment of data were as described in the legend to Fig. 4. (*A*) Relative concentration of serum albumin mRNA in total RNA isolated from cockerel liver expressed as a percentage of the concentration of serum albumin mRNA found in RNA isolated from the livers of untreated cockerels. (*B*) Absolute levels of serum albumin mRNA (□), apoVLDLII mRNA (○), and vitellogenin mRNA (●) after injection of hormone. The data are expressed as molecules of mRNA per nuclear equivalent of DNA. The theoretical accumulation curves (---) were calculated as described by Kafatos (25) from the data in Fig. 4, for the period between 4 and 8 hr. It was assumed that degradation during this period was negligible and that the rate of accumulation was equivalent to the rate of synthesis.

half-lives are similar to those of other major estrogen-inducible mRNA species such as chicken ovalbumin mRNA (26). Recent experiments on mRNA stability in the acutely withdrawn chicken oviduct indicate that, in the absence of hormone, these abundant mRNA species become quite unstable (27). Because the liver, unlike the oviduct, remains biosynthetically active after withdrawal of hormone treatment, we were interested in determining whether or not a similar destabilization of mRNA occurred and whether or not the destabilization was confined to hormonally responsive mRNA species.

When estradiol was administered by injection, as it was in the induction studies already described, the hormone reached a maximal serum concentration of >160 μM about 12 hr after injection. It took approximately 10 days to drop to 0.05 nM, the concentration normally present in the untreated male (28). During this period of declining hormone levels, vitellogenin mRNA and apoVLDLII mRNA disappeared from the liver relatively slowly. Their decay kinetics are consistent with both mRNA species having half-lives similar to those estimated during the induction phase. Interpretation of the results, however, is complicated by the significant concentration of hormone present during this period.

A more abrupt withdrawal from hormone treatment can be obtained by the use of Silastic implants containing diethylstilbestrol. In a second series of experiments, Silastic implants were placed subcutaneously in the backs of 5- to 7-day-old chicks and left in position for 14 days. The steady-state levels of apoVLDLII mRNA and vitellogenin mRNA reached at the end of this period were found to be essentially the same as the maximal levels obtained following a single large injection of hormone (Fig. 6). Removal of the implants, however, resulted in the rapid loss of both estrogen-inducible mRNA species from the liver. Both apoVLDLII and vitellogenin mRNA disappeared from the liver at rates consistent with them having half-lives of <3 hr. The level of serum albumin mRNA during this period remained essentially unchanged, indicating that withdrawal of hormone did not cause a generalized destabilization of abundant mRNA species.

DISCUSSION

Immunological studies and nucleic acid sequence data demonstrate that the small estrogen-inducible mRNA we have isolated specifies apoVLDLII. However, we failed to detect this mRNA in rooster liver, prior to treatment with estrogen. The discrepancy between these results, based on nucleic acid hybridization data, and earlier reports, based on immunological studies, raised the interesting possibility that a polypeptide immunologically related to apoVLDLII might be produced in rooster liver prior to administration of the hormone. Antibody raised against holoVLDL does crossreact with polypeptides specified by total RNA from both rooster and hen liver. Although some of the immunoreactive polypeptides specified by rooster liver RNA are similar in size to apoVLDLII they do not appear to crossreact with antibody raised against purified apoVLDLII. It appears from these results that the most dramatic effect of estrogen on the production of VLDL in the rooster is to induce the synthesis of a "female-specific" com-

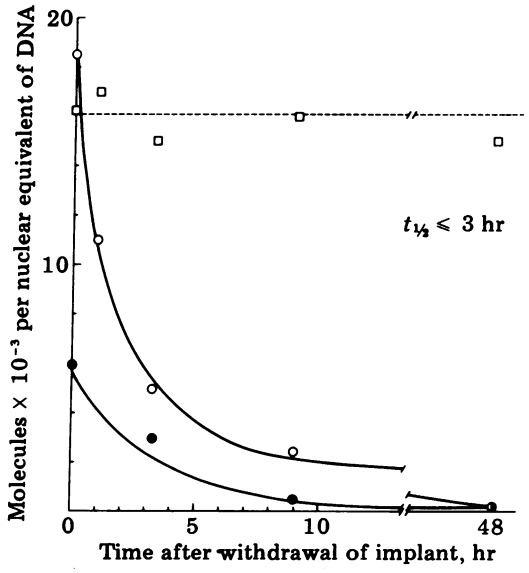

FIG. 6. Levels of apoVLDLII mRNA (○), vitellogenin mRNA (●), and serum albumin mRNA (□) after acute withdrawal of estrogen. Silastic pellets containing 14 mg of diethylstilbestrol were placed subcutaneously in the backs of 1-month-old chicks and left in place for 2 weeks. The levels of serum albumin mRNA, apoVLDLII mRNA, and vitellogenin mRNA were determined at the end of this period and at various times after removal of the implants, as described in the legend to Fig. 1.

ponent, apoVLDLII, rather than to enhance the rate of synthesis of a polypeptide already being made.

The primary induction characteristics of apoVLDLII mRNA and vitellogenin mRNA show some striking similarities. Neither mRNA species is detectable in the livers of normal roosters. Both become detectable within 30 min after an intramuscular injection of estrogen and exhibit nonlinear accumulation kinetics. Their half-lives during the accumulation phase of the response are quite similar and are shortened by a comparable factor after acute withdrawal of the hormone. There are some indications, however, that elements involved in regulating the transcription of these genes, or in stabilizing the mRNA species they encode, may exhibit differential sensitivities to estrogen. For example, the low initial rate of accumulation that characterizes the response of both genes lasts only 2 hr in the case of apoVLDLII compared with 4 hr in the case of vitellogenin. apoVLDLII mRNA also accumulates at a molar rate approximately 3-fold higher than that of vitellogenin mRNA.

The increasing rates of accumulation of apoVLDLII mRNA and vitellogenin mRNA in the early stages of primary induction do not show a direct proportionality with the increasing levels of total nuclear estrogen receptor during this period. Data published by Gschwendt and Kittstein (29) indicate that the level of nuclear receptor in rooster liver increases rapidly after an intramuscular injection of 17β-estradiol, reaching 80% of maximum within 2 hr. If the mRNA accumulation data do reflect changes in the rate of transcription of the apoVLDLII and vitellogenin genes, correlation of changes in transcription rate with the levels of nuclear estrogen receptor suggest that a highly cooperative step is involved in the induction process. Varying degrees of cooperativity might then explain the differences between the lag periods found for the two mRNA species (30).

Rather than resulting solely from changes in the rate of transcription, it is possible that the primary induction kinetics could be due in part to an increase in mRNA stability, or in the efficiency of mRNA processing, during the 4 hr following injection of hormone. We have presented evidence that the half-lives of both mRNA species are 7–8 times longer in the presence of hormone than in its absence. A change in half-life of this magnitude would be adequate to account for the 6- to 7-fold increase in accumulation rate that occurs 2–4 hr after injection of hormone. However, in order for such a mechanism to explain the absence of a lag period upon secondary induction, it would require that the stabilizing elements persist after estrogen withdrawal and that they are effective only in the presence of hormone. Alternatively, primary exposure to estrogen could result in the induction of a nuclear factor, possibly a small nuclear RNA (31), that facilitates the processing of both estrogen-inducible mRNA species. If such a factor were rate limiting during the first few hours of the primary response, it might be anticipated that processing of the smaller mRNA species would be favored and thus account for the earlier accumulation of apoVLDLII mRNA.

Estrogen is known to affect the apolipoprotein composition of mammalian VLDL (32). The hormone has been used as an effective treatment for type III hyperlipoproteinemia (33). It is not clear, in these instances, whether estrogen exerts its effect at the level of peripheral metabolism or directly on the biosynthesis of specific apolipoproteins. In the avian system, administration of estrogen to males causes a qualitative change in the apolipoprotein composition of VLDL by inducing the formation of a component, apoVLDLII, that normally is produced only in mature females. In the hen, the induction of apoVLDLII occurs concomitantly with the development of a new deposition site for serum lipoprotein, the egg yolk. The relative simplicity of the avian system should facilitate studies on the estrogen-dependent biosynthesis and metabolism of serum lipoproteins.

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