**The Avian Apoprotein II Very Low Density Lipoprotein Gene**

**METHYLATION PATTERNS OF 5' AND 3' FLANKING REGIONS DURING DEVELOPMENT AND FOLLOWING INDUCTION BY ESTROGEN**

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Valerie Colgan‡, Alex Elbrecht, Paula Goldman‡, Catherine B. Lazier‡, and Roger Deeley‡‡

From the ‡Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6 and the Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

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Apoprotein II very low density lipoprotein (apo-VLDLI1) mRNA is one of the major abundant mRNA species present in hen liver. It specifies a major component of the very low density lipoprotein fraction of egg yolk. Expression of the apoVLDLII gene normally begins in the female chick at the onset of vitellogenesis. However, apoVLDLII synthesis can be induced in roosters or embryos by administration of pharmacological doses of estrogenic steroids. We have examined regions flanking the apoVLDLII gene for the presence of other expressed sequences and for alterations in methylation patterns that correlate with its state of activity. We find no evidence of other estrogen-dependent or constitutively expressed genes within a 25-kilobase pair region spanning the apoVLDLII gene. Inverted-repeat sequences were found in the vicinity of the 5' end of the gene that hybridized with polyadenylated RNA from several tissues. However, we could detect no specific transcript, polyadenylated or otherwise, that originated from these sequences. Methylation patterns of 5' and 3' flanking regions of the gene were investigated at different stages of embryogenesis, as well as in both normal and estrogen-treated roosters. These studies revealed that demethylation of an Msp I site, 2.6 kilobase pairs upstream from the 5' end of the gene, and an Xho I site, 1.6 kilobase pairs downstram from the 3' end, occurs between day 7 and 9 of embryogenesis. Hybridization analyses indicate that it is during this period that the embryo also acquires competence to express the apoVLDLII gene. As far as we have been able to determine, the adult methylation pattern of regions flanking the gene is established in embryonic liver by day 9 and remains unchanged following activation of the gene in estrogen-treated roosters.

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Regulation of genes encoding the abundant mRNA class of avian liver differs strikingly from that observed in mammals. Approximately 30% of the protein synthesized in the liver of the hen is destined for deposition in the egg yolk (1). These proteins are encoded by a group of estrogen-responsive genes that exhibit varying degrees of dependence on the hormone and a wide range of rates of expression (2-8). Two of the proteins: vitellogenin, the major component of the high density lipoprotein fraction of yolk, and apoVLDLII, a major constituent of the very low density lipoprotein fraction, are specified by genes that display the most dramatic estrogen dependence (9, 10).

Expression of the apoVLDLII and vitellogenin genes can be induced in roosters, chicks, and embryos by administration of pharmacological doses of estrogen (10-12). Previous hybridization analyses have shown that the concentration of apoVLDLII and vitellogenin mRNA in livers of roosters or chicks is considerably less than 1 molecule/cell (10). Immunocytochemical studies suggest that this extremely low level, at least in the case of apoVLDLII mRNA, may be the result of expression of the gene in a small percentage of "precocious" hepatocytes (13). Induction of expression of the apoVLDLII gene in the general hepatocyte population occurs rapidly following primary administration of estrogen and mature mRNA can be detected at a concentration of 5-10 mol/cell within 30 min after intramuscular injection of hormone.

The rapidity of the primary response in the liver contrasts with the initial induction of egg white protein synthesis in the oviduct of the immature hen. Here, expression of the egg white protein genes is preceded by a period of cellular proliferation and differentiation of the tubular gland cells (14, 15). Although it has not been proved rigorously in birds, studies with metabolic inhibitors suggest that induction of egg yolk protein production can be achieved in the absence of DNA synthesis (16). Induction of expression of vitellogenin genes in primary cultures of Xenopus hepatocytes has demonstrated that this is certainly the case in amphibians (17, 18).

Many aspects of the vitellogenic response elicited by primary stimulation of the rooster with estrogen are transient, lasting between 3 and 7 days. However, the liver does exhibit an enhanced ability to respond to subsequent doses of hormone that persists for many months, suggesting that relatively permanent alterations, perhaps in DNA or chromatin structure, may occur in response to primary stimulation (19, 20).

Studies on induction of apoVLDLII protein synthesis at various stages of embryogenesis have revealed that the embryo is competent to respond to estrogen by day 10 (12). Since the liver is readily accessible from day 5, this system offers the opportunity to examine the structure of the apoVLDLII gene during acquisition of competence by embryonic liver, as well as during its activation in the adult.

In this manuscript, we describe the organization of the apoVLDLII gene with respect to other expressed sequences present within a 25-kb region of the genome. These studies indicate that transcription within this region appears to be limited to the apoVLDLII gene and that alterations in the degree of methylation of sites flanking the gene correlate with the acquisition of competence by the embryo rather than with the state of activity of the gene itself.

**EXPERIMENTAL PROCEDURES**

The isolation of recombinant bacteriophages and subcloning of...
some of the DNA fragments used in this study have been described (21). All operations involving growth and extraction of DNA from recombinant bacteriophages or plasmids were carried out in accordance with the Medical Research Council of Canada’s “Guidelines for the Handling of Recombinant DNA Molecules and Animal Viruses and Cells.” Published procedures, used without modification, are referenced in the text. Additional experimental details are provided in figure legends.

**Estrogen Treatment**—A vitellogenic response was induced in immature White-Leghorn roosters, 8-18 weeks old, by intramuscular injection of 17β-estradiol (20 mg/kg body weight) dissolved in propylene glycol. A maximal response was obtained by administering three doses, 3 days apart. Birds were killed 2-3 days after the final administration of hormone. Eggs were injected under the air-sac membrane with 17β-estradiol (1.25 mg in 50 µl of propylene glycol) and embryos were removed 48 h later.

**Extraction of High Molecular Weight DNA**—Livers of 7-, 9-, and 12-day embryos were frozen, intact, in liquid N₂, immediately following removal from the embryo. Rooster and hen liver was rapidly diced and rinsed in isotonic saline prior to freezing. Frozen tissue was mixed with dry ice, pulverized, and suspended in 10 volumes of a buffer containing Tris-HCl (0.05 M), NaCl (0.4 M), and EDTA (0.05 M). The suspension was adjusted to pH 8.0, incubated overnight with gentle shaking. The DNA was extracted twice with equal volumes of phenol and chloroform, “spooled” from the aqueous phase, dissolved in one-third of its original volume in a buffer containing Tris-HCl (0.05 M), NaCl (0.05 M), and EDTA (0.01 M), and then incubated with heat-treated pancreatic RNase A (final concentration, 10 µg/ml) previously boiled for 10 min in sodium acetate (0.01 M, pH 5.2) and NaCl (0.15 M) at 37 °C for 1 h. RNase and any other residual protein was destroyed by adjusting the solution to 0.5% SDS, 0.4 M NaCl, and 200 µg/ml in proteinase K, followed by incubation at 37 °C for 2 h. The incubation mixture was then extracted twice with phenol and chloroform. DNA was spooled, dissolved, and dialyzed against 1X EDTA. DNA was extracted from blood in the same fashion except that packed cells were not frozen but were resuspended in extraction buffer immediately following centrifugation.

**RNA Extraction**—Total RNA was extracted from livers of immature chicks or roosters by initial homogenization of fresh tissue in guanidine thiocyanate (4.7 M) and mercaptoethanol (1.0 M), followed by repeated ethanol precipitations from guanidine HCl (8.0 M) (22-24). RNA was isolated from embryonic liver by ethanol precipitation from guanidine HCl (8.0 M) and repeated extractions with phenol:chloroform:isoamyl alcohol (50:50:1 v/v) in the presence of SDS (0.5%). Poly(A)⁺ RNA was purified by chromatography on either oligo(dT)-cellulose (25) or poly(U)-Sephadex (24).

**Restriction Mapping**—A restriction map illustrating some of the sites relevant to this study has been published (21). The locations of additional sites for the enzymes Hha II and Xho I were deduced from appropriate double digests of subcloned Eco RI fragments. The map of Ali I and Hind III sites, in the region extending from Eco RI site, R₃ to Pst I, site F (Fig. 4B), was obtained both by double digestion of the subcloned, purified fragment and by partial digestion with each enzyme. In order to simplify analysis of partial digests, the fragment was labeled asymmetricaly, at the Eco RI site, by incubation with reverse transcriptase and [α-³²P]ATP (26). Partial and complete digests were analyzed on polyacrylamide gels (5% and 10%). Maps were deduced by examining the ladder of labeled fragments visible on autoradiographs of the gels and by comparison of the labeled fragments with the sizes of all partial digestion products visible after staining with ethidium bromide.

**Hybridization to Nitrocellulose and Diazoitized Cellulose**—The basic hybridization solution used for all blots consisted of formamide (50%), SSC (6X), Denhardt’s solution (5X), SDS (0.2%), yeast RNA (100 µg/ml), and calf thymus DNA (100 µg/ml) (27). The solutions used for hybridization of cDNA probes to RNA bound to diazotized cellulose were supplemented with glycine (15% w/v), polyadenylate (25 µg/ml), and additional yeast RNA (0.5 µg/ml). Dextran sulfate (6% w/v) was included in the hybridization solution when examining genomic DNA digests. In most instances, hybridization was carried out at 42 °C for 36-48 h. Blots were washed twice in formamide (50%), SSC (2X), and 0.1% SDS at 42 °C, followed by 4-6 washes in 2X SSC and repeated washes in formamide (50%) and 2X SSC. The exposure of this track revealed hybridization of hen liver cDNA to the other strand, at a level comparable to that detected with oviduct cDNA. Hybridization experiments carried out with a nick-translated preparation of a “Cot 50” fraction of genomic DNA, indicated that the 5.4-kb fragment also contains moderately repeated DNA sequences (Fig. 3).

Rather than implying that the 5' flanking region is transcribed in all of the tissues tested, the ability of oviduct and normal rooster liver cDNA to hybridize equally well with both DNA strands was used to demonstrate which of the DNA strands is the antisense strand of the apoVLDLII gene. Prolonged exposure of this track revealed hybridization of hen liver cDNA to the other strand, at a level comparable to that detected with oviduct cDNA. Hybridization experiments carried out with a nick-translated preparation of a “Cot 50” fraction of genomic DNA, indicated that the 5.4-kb fragment also contains moderately repeated DNA sequences (Fig. 3).

**RESULTS**

**Expression of Regions Flanking the ApoVLDLII Gene**—We have used three recombinant bacteriophages that contain overlapping DNA fragments spanning 24.3 kb of the chicken genome, in order to assess the disposition of the apoVLDLII gene relative to other expressed sequences. The position of the apoVLDLII gene within this region is illustrated in Fig. 1, as are the positions of sites for restriction enzymes that are relevant to studies described in the text. As a preliminary screening, nitrocellulose blots of Eco RI digests of all three clones were hybridized, both with large quantities of cDNA (5.0-10.0 × 10⁵ cpm/blot, specific activity = 10⁵ cpm/µg) prepared by reverse transcription of total RNA isolated from hen liver, oviduct, rooster liver, and embryonic muscle, or directly with ³²P-labeled nuclear RNA (10⁵ cpm/blot, specific activity = 3 × 10⁵ cpm/µg), generated by partial alkaline hydrolysis and incubation with polynucleotide kinase (28). As expected, hen liver cDNA and RNA hybridized strongly with the 4.0-kb Eco RI fragment containing the bulk of the apoVLDLII gene, and to a lesser extent with the 5.4-kb fragment containing only the small leader exon. Hybridization to additional DNA fragments was not detected. All of the other probes tested also exhibited a low, but quite detectable, level of hybridization with only the 5.4-kb fragment, raising the possibility that the apoVLDLII gene was adjacent to a constitutively expressed sequence.

This fragment was subjected to electrophoresis through a strand separation gel in order to determine whether one or both DNA strands were involved in the hybridization. Duplicate nitrocellulose blots were prepared and hybridized with either hen liver or oviduct cDNA. The results are summarized in Fig. 2. Oviduct cDNA hybridized equally well with both DNA strands. Exposure of the track hybridized with hen liver cDNA was adjusted to demonstrate which of the DNA strands is the antisense strand of the apoVLDLII gene. Prolonged exposure of this track revealed hybridization of hen liver cDNA to the other strand, at a level comparable to that detected with oviduct cDNA. Hybridization experiments carried out with a nick-translated preparation of a “Cot 50” fraction of genomic DNA, indicated that the 5.4-kb fragment also contains moderately repeated DNA sequences (Fig. 3).

Rather than implying that the 5' flanking region is transcribed in all of the tissues tested, the ability of oviduct and normal rooster liver cDNA to hybridize equally well with both strands of the 5' flanking region could be due to the presence of related sequences in transcribed regions elsewhere in the genome. In this case, hybridization with both strands would indicate that the sequences are transcribed in both orientations. An alternative possibility, that does not exclude the one just considered, is that the sequences involved in the hybridization are present as inverted repeats in the 5' flanking region.

**FIG. 1.** Restriction map of the apoVLDLII gene and flanking regions. Eco RI (R) sites and the sizes of fragments generated by this enzyme are indicated for the entire 24.3 kb. Bam HI (B), Msp I/ HpII II (M), and Xho I (X) sites are shown only for the region to the right of site 1. Positions of Eco RI, Rfl II (Rf), and Xho I (H) sites present in the Eco RI fragments of 5.4 and 4.0 kb are also indicated. The structure and location of the apoVLDLII gene is illustrated above the map.
of the apoVLDLII gene. The latter possibility was examined by constructing a heteroduplex between pBR322 and a recombinant plasmid containing the 5.4-kb Eco RI fragment bounded by R3 and R4 (Fig. 1). In order to be able to determine the distance of structural features of the DNA from the 5' end of the gene, the position of the leader exon was identified by hybridizing the heteroduplex with apoVLDLII mRNA. A representative electron micrograph is shown in Fig. 4. Two inverted repeats can be seen in the region that hybridizes with the cDNA and repeated DNA probes. These are indicated in the figure. The smaller of the two is situated 3.3-3.5 kb from the start of the gene and involves 180-200 nucleotides. It is separated from the larger duplex of 350-400 nucleotides by 90-100 nucleotides.

A more detailed restriction map of the region in which the inverted-repeats are situated is shown in Fig. 4B. Hybridization experiments carried out with HindIII and Alu I digests indicate that the sequences which hybridize with cDNA and repeated DNA probes are confined to Alu I fragments of 640 and 400 nucleotides. We have attempted to detect a specific transcript that may originate from this region, but thus far have not succeeded. When the 1.8-kb R1-R4 fragment illustrated in Fig. 4B was nick-translated and hybridized with diazotized cellulose blots of RNA isolated from hen liver and oviduct, no specific hybridization was detected. The fragment did hybridize with a broad spectrum of RNA molecules present in the polyadenylated RNA fraction of both tissues. (Fig. 5, track C). As positive controls for these experiments, duplicate blots were hybridized with a nick-translated fragment, Bg3-R4, that includes the leader exon of the apoVLDLII gene and 800 nucleotides of the adjacent (Fig. 5, track A) intron, and also with the entire 5.4-kb R1-R4 fragment (Fig. 5, track B).

Hybridization of the Bg3-R4 fragment to a blot of total polyadenylated RNA from hen liver revealed intense hybridization with mature apoVLDLII RNA and at least three precursors (Track A, Fig. 5). The largest of these, 3200 nucleotides, is very close to the size estimated for the apoVLDLII gene. When the entire R1-R4 fragment was used as a probe, a background of hybridization was observed extending from 800 to approximately 7000 nucleotides (Track b, Fig. 5). No specific hybridization was detected when only the R3-R4 fragment was used, with either hen liver or oviduct RNA (Track C, Fig. 5). It is clear from the results that the RNA preparations used
were intact and that nuclear RNA species were quite detectable even though total polyadenylated RNA was applied to the gels. Similar experiments, carried out with the poly(A') RNA fraction of hen liver and preparations of small RNA located 3.0 kb upstream from the 5' end of the gene, and Xho I sites are present 1.2 kb (Xb) and 3.3 kb (Xc) downstream from the 3' end. Numerous sites for the enzyme Taq I are scattered throughout the apoVLDLII gene region. This enzyme will cleave at the sequence TCAGA or T2CGA. However, since there are no sites for the methylation-sensitive Sma I (restriction sequence cleaved, GTCGAC) that would allow this enzyme pair to be used to probe alterations in methylation, the Taq I sites have not been included in the figure.

In order to detect alterations in methylation in the vicinity of the apoVLDLII gene, high molecular weight DNA was prepared from the livers of hens, normal and estrogen-treated roosters, as well as 7-, 9-, and 12-day embryos. We also prepared DNA from hen blood, both to assess the tissue specificity of any alterations in methylation that might be detected and also because the recombinant bacteriophage library from which the apoVLDLII gene was isolated, was constructed from blood DNA (29). The risk of incomplete

**Alterations in Methylation in the Vicinity of the apoVLDLII Gene**—As indicated in Fig. 1, the VLDLII gene does not contain sites for the most commonly used methylation-dependent isoschizomers, *Msp* I and *Hpa* II, nor for the enzymes *Ava* I, *Xor* II, and *Sma* I. However, the gene is bracketed by *Msp* I sites, one (M1) located 2.6 kb transcriptionally upstream from the 5' end of the gene, the other (M2) 3.6 kb downstream. M1 and M2 are 2.5 kb apart and are situated on either side of the repeated DNA sequences described above. In addition to sites for *Msp* I, *Hha* II sites are located 3.0 kb (H1) and 0.4 kb (H2) upstream from the 5' end of the gene, and *Xho* I sites are present 1.2 kb (Xa) and 3.3 kb (Xb) downstream from the 3' end. Numerous sites for the enzyme *Taq* I are scattered throughout the apoVLDLII gene region. This enzyme will cleave at the sequence TCAGA or T2CGA. However, since there are no sites for the methylation-sensitive *Sal* I (restriction sequence cleaved, GTCGAC) that would allow this enzyme pair to be used to probe alterations in methylation, the *Taq* I sites have not been included in the figure.

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digested was minimized by using a 5-10-fold sequence excess of enzyme and incubation times of 16-20 h. Under these conditions, digestion of λ-DNA, mixed with the genomic DNA, was complete within 2-3 h.

Initial digestions were carried out with Msp I and Hpa II. The fragments generated were separated on 0.8% agarose gels, transferred to nitrocellulose, and hybridized with a nick-translated DNA fragment (2.0 \times 10^4 cpm, specific activity = 10^7 cpm/μg) extending from an Eco RI site 850 nucleotides transcriptionally downstream from the 5' end of the gene to an Eco RI site 1700 nucleotides past the 3' end. B, digestion of blood DNA with Msp I (M) or Hpa II (H): DNA (15 μg) from hen blood or 7-day embryonic liver was digested with Msp I or Hpa II. The fragments were separated, transferred to nitrocellulose, and hybridized as described above. C, location of the Msp I site resistant to cleavage in liver DNA from 7-day embryos. DNA (30 μg) from livers of 7 (7d) and 9 (9d)-day embryos, normal (US) and estrogen-treated roosters (SR, 20 mg of 17β-estradiol/kg, body weight administered 3 days prior to removal of the liver), was digested with Msp I (M) or Hpa II (H). Half of each sample was subsequently digested with Eco RI (R) and the fragments produced by both single and double digests were separated on a 0.8% agarose gel. A nitrocellulose blot of the gel was then hybridized with a nick-translated DNA fragment, Bg-RI (2.0 \times 10^4 cpm, specific activity = 10^7 cpm/μg). This fragment contains 500 nucleotides flanking the 5' end of the apoVLDLII gene and 850 nucleotides of structural sequence.

Fig. 6. Methylation of embryonic liver DNA in the vicinity of the apoVLDLII gene. A, DNA (15 μg) from 7-, 9-, and 12-day embryonic liver was digested with either Msp I or Hpa II and subjected to electrophoresis through a 0.8% agarose gel. A nitrocellulose blot of the gel was hybridized with a nick-translated DNA fragment (2.0 \times 10^4 cpm, specific activity = 10^7 cpm/μg) extending from an Eco RI site 850 nucleotides transcriptionally downstream from the 5' end of the gene to an Eco RI site 1700 nucleotides past the 3' end. B, digestion of blood DNA with Msp I (M) or Hpa II (H): DNA (15 μg) from hen blood or 7-day embryonic liver was digested with Msp I or Hpa II. The fragments were separated, transferred to nitrocellulose, and hybridized as described above. C, location of the Msp I site resistant to cleavage in liver DNA from 7-day embryos. DNA (30 μg) from livers of 7 (7d) and 9 (9d)-day embryos, normal (US) and estrogen-treated roosters (SR, 20 mg of 17β-estradiol/kg, body weight administered 3 days prior to removal of the liver), was digested with Msp I (M) or Hpa II (H). Half of each sample was subsequently digested with Eco RI (R) and the fragments produced by both single and double digests were separated on a 0.8% agarose gel. A nitrocellulose blot of the gel was then hybridized with a nick-translated DNA fragment, Bg-RI (2.0 \times 10^4 cpm, specific activity = 10^7 cpm/μg). This fragment contains 500 nucleotides flanking the 5' end of the apoVLDLII gene and 850 nucleotides of structural sequence.
Methylation Patterns Flanking the ApoVLDLII Gene

Fig. 7. Methylation at Hha I and Xho I sites flanking the apoVLDLII gene. Samples of DNA (15 μg) from embryonic (7- and 9-day) or stimulated rooster liver were digested with Barn HI and subsequently by either Hha I or Xho I. The digests were subjected to electrophoresis, transferred to nitrocellulose, and hybridized exactly as described in the legend to Fig. 6. Digestion with Barn HI is expected to generate a fragment of 14 kb that should hybridize with the Bg-R4 fragment. Cleavage at the Hha I site proximal to the gene, H2, would reduce this to approximately 9.0 kb, while cleavage at H1 would yield a fragment of 11.5 kb. The results indicate that neither site is susceptible to digestion. Similarly, cleavage at Xho I site, X1, should produce a fragment of 9.5 kb. Such a fragment was detected in liver DNA from day 9 embryos and stimulated roosters. However, this site was resistant to cleavage in DNA from 7-day embryonic liver.

Fig. 8. Poly(A⁺) RNA (6 μg) from the livers of control (C) or estrogen-treated (E) embryos was subjected to electrophoresis through a 2.0% agarose gel containing methylmercury hydroxide (30) and transferred to a sheet of nitrocellulose (31). The blot was hybridized with a nick-translated, cloned apoVLDLII cDNA fragment (10² cpm, 10² cpm/μg) characterized previously (10). The size of the RNA species that hybridized with the probe was estimated from the mobilities of 28 S and 18 S rRNA, visible after staining the gel with ethidium bromide. Its size (800 nucleotides) is consistent with previous estimations of the length of apoVLDLII mRNA using this technique (9, 10).

in 7-day embryos and remain methylated in the estrogen-treated rooster (Fig. 7). The Xho I sites, X1 and X2, situated 1.2 and 3.3 kb downstream from the gene, also appear to be methylated in the embryo at day 7. However, site X2 has been demethylated by day 9 and remains so in both normal and estrogen-treated roosters. At the moment, we have not examined alterations in methylation at site X1.

Induction of Expression of the apoVLDLII Gene in Embryonic Liver—Previous studies on induction of the apoVLDLII gene in embryos have relied upon immunological quantitation of apoVLDLII polypeptide. The earliest stage at which induction was detected was at day 10 of embryo development (12). Since these measurements required that the liver be competent, not only to express the gene, but also to translate apoVLDLII mRNA, we have re-examined induction of the gene by hybridization analysis with a cloned apoVLDLII cDNA fragment (10).

Embryos were injected with 17β-estradiol on days 5, 7, 9, and 13 of development and liver RNA was extracted 48 h later. Aliquots of the poly(A⁺) RNA fraction were analyzed by electrophoresis through agarose gels (2%) containing methylmercury hydroxide (5 mM) (30), transferred to nitrocellulose filters (31), and hybridized with nick-translated, cloned apoVLDLII cDNA. The results of these experiments are illustrated in the form of an autoradiograph in Fig. 8. ApoVLDLII mRNA was not detected in the livers of control embryos at day 7, 9, 11, or 15, nor in the livers of embryos injected on day 5 and analyzed on day 7. However, a significant response was obtained by day 9. Although the results obtained by this procedure are only semiquantitative in nature, it is clear that the magnitude of the response increases through day 15. This observation is consistent with the increase in estrogen-dependent accumulation of estradiol receptor that is known to occur in the liver during this period of embryogenesis (32).

DISCUSSION

The lack of a requirement for DNA synthesis and the rapidity with which it is possible to elicit expression of the apoVLDLII gene in rooster liver following primary stimulation with estrogen, raises the possibility that many of the structural alterations thought to be involved in gene activation have taken place prior to administration of the hormone. One type of organization that could facilitate induction might involve location of the gene in a chromosomal domain containing regions that are already active in both roosters and hens. The results of initial hybridization experiments suggested that this might be the case, since sequences in the 5' flanking region of the gene hybridized with several different cDNA probes.

We have now shown that the region concerned contains inverted-repeat sequences and hybridizes with a broad spectrum of poly(A⁺) RNA species from various different tissues. Similar hybridization characteristics have been observed with some mammalian repeated DNA sequences, such as the human Alu I family and its rodent equivalent (33-36). In this instance, it could reflect dispersion of the sequences throughout the genome or their complementarity with small RNA species, such as 4.5 S RNA, that are capable of hybridizing with a variety of poly(A⁺) RNA molecules. In this study, we have not attempted to distinguish between hybridization with nuclear and cytoplasmic polyadenylated RNA species because of technical difficulties associated with preventing hnRNA and mRNA degradation in avian liver during subcellular fractionation. However, small RNA species were prepared from both nuclei and cytoplasm. Again, hybridization experiments did not reveal a discrete species complementary to a
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site or sites in the 5' flanking region of the gene. In experiments not described here, we have used an RNA polymerase III transcription system as an additional check on the possibility that the 5' flanking region of the apoVLDLII gene contains sites that could act as RNA polymerase III promoters, again with negative results. In view of these data, it seems most probable that the 25-kb region we have examined is dormant prior to administration of estrogen and that, within it, only the apoVLDLII gene responds to the hormone.

Alterations in methylation patterns in and around developmentally regulated genes, such as those specifying chicken ovalbumin and the globins in a variety of species, have been correlated with changes in the state of activity of these genes. The studies have shown, in some cases, that general demethylation of the transcribed region is associated with activation and expression of the gene (36). Most frequently, however, only specific sites appear to be affected and these are often outside the gene in question. In studies on the ovalbumin gene, for example, the most striking correlation of undermethylation with expression occurs at a Hha I site 1200 nucleotides downstream from the 3' end of the gene and to a lesser extent with an Msp I/Hpa II site approximately 2.8 kb upstream from the 5' end (37). In this instance, the correlation between undermethylation of these sites and activity of the gene was drawn from a comparison of the degree of methylation found in DNA from oviduct, with that found in DNA from tissues where the gene was not expressed. Since administration of estrogen triggers both differentiation of the tubular gland cell and expression of the ovalbumin gene, it is difficult in this system to determine whether demethylation accompanies differentiation of the cell and/or induction of the gene.

Our experiments have focused primarily on alterations in methylation patterns of liver DNA in regions flanking the apoVLDLII gene during different stages of development and states of activity of the gene. Interpretation of the data is simplified by the fact that the liver of the chicken embryo, unlike mammalian embryos, is not a major site of hematopoesis and nonparenchymal cells make up a very minor component of the tissue (38, 39). Hybridization analyses with a cloned apoVLDLII cDNA fragment demonstrated that embryonic liver acquires the ability to express the apoVLDLII gene, in response to treatment with estrogen, between days 7 and 9 of incubation. Similar analyses indicate that, at day 7, the serum albumin gene is already being expressed. Digestion of liver DNA from embryos at day 7 showed that an Msp I/Hpa II site, present in all three apoVLDLII clones, was approximately 90% resistant to cleavage by both enzymes. Forty-eight hours later, this site is approximately 90% sensitive to Msp I but remains resistant to digestion with Hpa II in the embryo and adult, regardless of the state of activity of the apoVLDLII gene. The site is situated 2.6 kb upstream from the apoVLDLII gene and approximately 0.5 kb downstream from the nearest inverted-repeat sequence. As far as we have been able to determine, it is not situated in a region that is transcribed in the liver. However, at the moment, we cannot exclude the possibility that demethylation may reflect a developmental pulse of transcription in this region.

The observed difference in Msp I sensitivity obviously cannot be explained by polymorphism, since DNA was prepared from many embryos at each time point. The most likely explanation for the alteration in sensitivity to Msp I digestion is that the recognition sequence is converted from $^{5}$C$^{4}$mCGG to C$^{4}$mCGG (40). It is difficult to completely rule out the possibility of a mechanism involving mutation at this site. However, the latter explanation seems particularly unlike in this case, in view of the fact that the site is also resistant to cleavage in DNA from hen blood (the source of DNA used for construction of the genomic library), but not in recombinant bacteriophages propagated in bacterial strains lacking modification systems. Doubly methylated sites have been identified in the 5' flanking regions of human $\gamma$-globin genes (41). These sites are resistant to cleavage with Msp I in sperm and adult liver, but are completely unmethylated in the erythroid component of fetal liver and in K562 erythroid cells capable of expressing embryonic and fetal globin genes following induction by hemin. Similar sites may exist in the 5' flanking region of the ovalbumin gene, possibly explaining some of the variations in Msp I sensitivity that have been attributed to polymorphism (37).

During the same period of development, a Xho I site, 1.3 kb downstream from the 3' end of the apoVLDLII gene, also becomes demethylated. Again, demethylation of this site correlates with the acquisition of competence rather than induction of the gene. While it is clear that demethylation of these two sites occurs within 48 h, it is conceivable that it may be accomplished more rapidly. Chicken erythroid progenitor cells isolated from the 26-23 h blastoderm do not synthesize globin and contain extensively methylated globin genes. The suggestion has been made that these genes are demethylated by the time they begin to be expressed, at 35 h, although direct measurements were not made at this stage (42).

The changes we observe could be the consequence of a lack of maintenance methylation at specific sites, perhaps subsequent to the interaction of effector molecules with DNA or chromatin (43). However, in order for such a mechanism to explain the alteration in sensitivity of the Msp I and Xho I sites (assuming conversion from 90% methylated to 90% demethylated in 48 h), it would require a doubling time of 11 h, if the DNA is fully methylated at day 7 or 15 h if it is hemimethylated. This calculation assumes that conversion from 90% methylated to 90% demethylated takes place over 48 h and reflects only the modification pattern of parenchymal cell DNA. If the methylated sites present in 9-day DNA originate from residual blood or other nonparenchymal cells, then cell division times of 5–6 h would be necessary. During this period of embryogenesis, the DNA content of the liver doubles approximately once every 20 h. If this figure correlates with the division time of the parenchymal cell, the data suggest that enzymatic demethylation of these sites may be involved.

At the moment, the molecular stimulus for these events is not known. The role of thyroid hormones in regulating differentiation in amphibians is well established (44) and has been implicated in the development of competence to express the vitellogenin gene in Xenopus laevis (45). In the chicken, secretion of thyroid hormones does not begin until the 10th day of incubation (46), apparently too late to play a causative role in determining inducibility of the apoVLDLII gene. Synthesis of 17beta-estradiol begins considerably earlier, between days 4 and 6, while the gonadal tissue is still in the indifferent state (47). High affinity, estradiol-binding proteins are readily detectable by day 8 in the Mullerian duct (48) and day 10 in the liver (32). More recent experiments indicate that these proteins are present in liver cytosol at a low but significant level by day 9. Thus, increase in endogenous estrogen levels may be a stimulus for alterations in the state of the apoVLDLII gene. However, this would imply that the threshold required for determination is considerably lower than that needed for induction of expression.

The modifications we observe suggest that conversion of...
the apoVLDLII gene from an inactive to a preinduction state involves a region of chromatin that extends at least 3.0 kb upstream and 2.0 kb downstream from the gene. We are currently interested in determining whether or not the region involved extends beyond or ends in the vicinity of the repeated DNA sequences in the 5' flanking region of the apoVLDLII gene. In parallel studies, we have detected related sequences at comparable locations near the 5' end of the chicken serum albumin gene. It seems likely that discrete signals must exist in DNA that confer tissue specificity upon the selection of domains destined to become transcriptionally active. Inverted-repeat sequences possess characteristics that make them appealing candidates for such a role (49, 50).

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