Characterization of the Estrogen-responsive Domain of Avian Liver and Cloning of Double-stranded cDNA Derived from Estrogen-inducible RNA Species*

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In the laying hen the egg yolk proteins are synthesized in the liver. Some of these proteins, such as vitellogenin, are synthesized exclusively by the hen, while others, such as low density lipoprotein, are also synthesized by the rooster, but at a very low rate. Treatment of the rooster with estradiol, however, results in synthesis of the complete spectrum of egg yolk proteins at a rate comparable to that characteristic of the hen.

We are interested in mechanisms that coordinate regulation of this group of estrogen-responsive genes and to this end have developed a procedure for selecting and cloning members of the group that exhibit common induction ratios.

Complexity analyses carried out on total RNA isolated from rooster liver at different stages of the vitellogenic response suggested that the family of the estrogen-inducible mRNA species was located primarily in the abundant and intermediate complexity classes of mRNA and that the population of the complex class remained essentially unchanged throughout the vitellogenic response. This conclusion was confirmed by more detailed analyses using isolated cDNA fractions corresponding to individual abundance classes. We have used the information provided by these analyses to isolate cDNA probes specific for estrogen-inducible sequences of a particular abundance and exhibiting a particular induction ratio.

The first probe that we have isolated in this fashion is specific for sequences that are induced by estrogen at least 1000-fold. It is composed predominantly of sequences derived from two species of mRNA, vitellogenin and another, as yet uncharacterized mRNA, approximately 800 nucleotides long. This cDNA probe has been used in the selection and isolation of cDNA clones containing both of these sequences.

The synthesis of avian egg yolk proteins is regulated by estrogenic steroids (1, 2). The protein components of the yolk are synthesized in the liver of the mature female and transported in the serum to the developing oocyte (2, 3). They are a complex group of molecules that includes various vitamin- and mineral-binding proteins (4–6), lipoproteins (7), phosphoproteins (8–10), and proteins normally regarded as typical constituents of the serum, such as serum albumin (11). Some members of this group, for example vitellogenin (12, 13) and riboflavin-binding protein (14, 15), are exclusively egg yolk proteins. They are not found at all in the liver of the mature male. Other members of the group, however, are synthesized in males: some, such as low density lipoprotein, at rates severalfold lower than those found in the female; others, such as serum albumin, at rates that are comparable to those found in the female. Thus, the group is heterogeneous with respect to the sex-dependent rate of synthesis of its individual members, yet it represents a family of proteins that must be synthesized by the hen in a tightly coordinated fashion.

The synthesis of avian egg yolk proteins is an attractive system for studying the mechanisms by which steroid hormones regulate gene expression. Administration of large doses of 17β-estradiol to adult males results in the synthesis of an apparently complete spectrum of yolk proteins at rates similar to those found in the laying hen (7, 16). Recent studies on the effects of estrogen on gene expression in rooster liver have focused primarily on mechanisms involved in regulating expression of a particular yolk protein gene, that specifying vitellogenin (17–20). However, it seems likely that an understanding of the mechanisms that regulate expression of an individual gene will be facilitated by the ability to compare the structure of that gene with others that respond similarly to a common stimulus. Such studies may also shed light on the hierarchy of control mechanisms that coordinate the expression of a given gene family or domain.

It has become possible recently to obtain cDNA clones corresponding to parts of both the vitellogenin' and serum albumin genes (21). The cDNA for these clones was generated by reverse transcription of either purified or partially purified preparations of the relevant mRNA. Such an approach was possible because of the relative abundance of the mRNA species in question and also because the proteins specified by them had been well characterized. Rather than follow this strategy for the isolation and cloning of sequences corresponding to other estrogen-responsive genes, we have developed a procedure that allows the rapid cloning of estrogen-inducible species without purification of mRNA, and that divides the members of the estrogen-responsive domain into subgroups, each characterized by a common induction ratio.

In the first application of this approach we have cloned cDNA sequences derived from the two RNA species in rooster liver that exhibit the most dramatic increase in concentration in response to treatment with estrogen. As was expected, one of these RNA species is vitellogenin mRNA while the other is a mRNA approximately 800 nucleotides long that specifies an as yet unidentified protein.

**EXPERIMENTAL PROCEDURES**

*Isolation of RNA—Total RNA was isolated from the livers of roosters or hens by extraction with guanidine HCl (22). The RNA

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obtained by this procedure contained less than 0.1% by weight contamination with DNA, as judged by the diphenylamine assay (23). Synthesis of cDNA for Hybridization Studies—DNA complementary to total liver RNA was synthesized using conditions similar to those described by Monahan et al. (24) for the preparation of cDNA probes with high specific activity. Reaction mixtures (100 μl) contained total RNA (100 μg), oligo(dT)12-18 (0.25 μg), avian myeloblastosis virus reverse transcriptase obtained from Dr. J. W. Beard, Life Sciences, Inc., St. Petersburg, Fla., through the courtesy of the Virus Cancer Program, National Cancer Institute (129 units), [5,3H]dCTP (22 Ci/mmol), and [3,5-3H]dCTP (26 Ci/mmol) or [γ-32P]dCTP (250 to 300 Ci/mmol). The specific activity of [3H]- and [32P]-labeled cDNA was, respectively, 100 and 50 dpm/pg. RNA Excess Hybridization Reactions—Hybridizations were carried out in 0.5 M NaCl at 68°C, as previously described (22). Each analytical hybridization reaction contained 0.066 ng of cDNA (2000 cpm) and a 1 × 106- to 4 × 107-fold weight excess of total RNA. The mRNA hybridization period used was 48 h. The fraction of cDNA in hybrid was estimated by its resistance to digestion with S1 nucleases (22).

Fractionation of cDNA—Preparative-scale incubations contained 0.3 to 1.0 μg of radiactive cDNA and 0.3 to 2.0 μg of total RNA. Sequences in hybrid were isolated after digestion of unhybridized mRNA with S1 nuclease. S1 nuclease was then removed by treatment with proteinase-K and phenol extraction as described by Gordon et al. (21).

When unhybridized cDNA sequences were required, they were selected as that material bound to hydroxypatite in 0.12 M sodium phosphate at 60°C. One gram of hydroxypatite was used for every 5 μg of RNA. A specific ratio of the RNA was mixed with 0.5 μg of native and denatured sea urchin DNA/g of hydroxypatite (25). Samples containing unhybridized cDNA were concentrated by partitioning against 2-butanol and desalted by chromatography on Sephadex G-50.

Residual RNA was removed from fractionated cDNA probes by hydrolysis in NaOH (0.1 M) containing EDTA (10 mM) at 68°C for 1 h. Following treatment with alkali the cDNA was passed over Sephadex G-75. Only material in the excluded volume was used for subsequent hybridization studies.

Size Estimation of Estrogen-inducible RNA Species—RNA from the livers of hens, normal roosters, and estrogen-treated roosters was subjected to electrophoresis in 1.5% agarose gels containing 5 mM methylmercuric hydroxide, as described by Bailey and Davidson (26). RNA was then transferred from the gel to sheets of diazotized paper using the procedure developed by Alwine et al. (27). Blocks of RNA were hybridized with various amounts (1.0 × 106 to 5.0 × 107 cpm) of estrogen-inducible cDNA for 36 to 96 h, under the hybridization conditions described by Alwine et al. (27). Blocks were washed for 4 h in six changes of 0.1 M sodium citrate (15 mM) and 5 × SSC (0.15 M sodium chloride, 0.015 M sodium phosphate, pH 7.0) at 42°C, followed by four washes (15 min each) in 0.1× SSC containing 0.1% sodium dodecyl sulfate at 52°C and finally two washes in 0.1× SSC (15 min each) (28). The paper was blotted briefly, covered with plastic wrap, and subjected to autoradiography with an intensifier screen for periods between 10 and 90 h.

Synthesis and Cloning of Double-stranded cDNA—The procedures used for synthesis and cloning of double-stranded cDNA have been described previously in Gordon et al. (21). Briefly, double-stranded cDNA was synthesized from total RNA isolated from the livers of estrogen-treated roosters, using avian myeloblastosis virus reverse transcriptase for the synthesis of both strands. The cDNA was then passed through a nuclease to remove single-stranded loops and tails. Tracts of polydeoxyctydilic acid, approximately 15 to 20 nucleotides long, were then synthesized on the 3′-ends of the cDNA, using calf thymus terminal transferase. This cDNA was annealed to DNA from the plasmid PBR322 that had been digested with the restriction enzyme Pst I and similarly tailed with deoxyguanosine residues.

The annealed chimeric plasmid was used to transform the approved Escherichia coli EK2 host, x1776, under the P3 level of containment. Clones containing recombinant plasmids were isolated by virtue of their resistance to tetracycline and identified initially by their sensitivity to ampicillin. Clones containing sequences of interest were then identified by a modification of the colony hybridization procedure of Grunstein and Hogness (29), introduced by Tiemeyer et al. (28). Hybridization reactions were carried out in sealed plastic bags (Kapak Industries) each containing filters from as many as four 9.00-cm diameter Petri plates. The hybridization mixture contained 1 to 3 × 106 cpm of [3P]-labeled cDNA (specific activity 30 cpm/pg of cDNA) in a volume of 20 ml. Hybridization conditions, washing, and autoradiography of the filters were as described by Tiemeier et al. (28). Filters were also screened with vitellogenin [3P]mRNA. This probe was prepared by mild alkaline hydrolysis of purified vitellogenin mRNA followed by terminal labeling of RNA fragments with [γ-32P]ATP with a specific activity > 3000 Ci/mmol, exactly as described by Doel et al. (30). The RNA probe generated in this fashion had a modal size of 190 nucleotides and a specific activity of 25 to 40 cpm/pg.

RESULTS AND DISCUSSION

Synthesis of cDNA from Total RNA—Complementary DNA was synthesized by reverse transcription of total RNA isolated from rooster liver by extraction with guanidine HCl (22). We used total liver RNA as a template for AM virus reverse transcriptase rather than a fraction enriched in polyadenylated RNA, to avoid the risk of discriminating against mRNA species that may not bind well to oligo(dT) or poly(U) chromatography matrices (22). Only 2% of the RNA used was polyadenylated we were concerned that high concentrations of nonpolyadenylated RNA might interfere with the efficiency of the reverse transcriptase reaction. This was not found to be the case. The efficiency of using total RNA as a template was comparable to that obtained with purified mRNA, providing that a correction was made for the nonpolyadenylated component of the RNA. Complementary DNA was synthesized under two different incubation conditions. When cDNA of high specific activity was required, the conditions described under “Materials and Methods” were used. The yield of cDNA obtained from 100 μg of total RNA varied between 0.3 and 0.4 μg. This represents a 15 to 20% efficiency of conversion of the polyadenylated RNA fraction into cDNA. If the cDNA was intended for cloning purposes and was of low specific activity, it was synthesized using conditions described by Gordon et al. (21). Under these conditions as much as 1.2 μg of cDNA was synthesized from 100 μg of total RNA. In both instances, the reaction was dependent on the presence of an oligo(dT) primer and nonpolyadenylated RNA, prepared by passing total RNA repeatedly over poly(U)-Sephadex, supported less than 3% of the normal level of cDNA synthesis.

Complexity Analyses—We have carried out complexity analyses, under conditions of RNA excess, with cDNA obtained by transcription of total RNA isolated from livers of both normal and estrogen-treated roosters. Such analyses, introduced by Bishop et al. (31), have been used extensively for several years to calculate the approximate complexity of the mRNA population in a variety of tissues. However, as pointed out by these investigators, calculations of total complexity using this approach suffer from practical difficulties encountered in measuring the complexity of the RNA of the lowest frequency (i.e. most complex) class. The sensitivity of these analyses as a means of detecting and quantifying changes in a RNA population has been extended in two ways: 1) The kinetic data of hybridization of a cDNA with the RNA from which it was transcribed (homologous hybridizations) can be compared with those obtained when the cDNA is hybridized to RNA isolated from the tissue at a different stage of the hormonal response (heterologous hybridizations); and 2) total cDNA can be fractionated so that it contains only sequences from a selected frequency class and this isolated fraction can then be used for both homologous and heterologous hybridizations.

Homologous and Heterologous Hybridizations with Total cDNA—It is possible to detect shifts in the relative abundance...
Fig. 1 (left). Hybridization of cDNA prepared from total RNA from the liver of a normal rooster to its homologous RNA and to total RNA from the liver of an estrogen-treated rooster. Total liver cDNA (0.066 ng) was incubated with various amounts of total RNA from the liver of either a normal rooster, \( \circ \) (0.5 to 350 \( \mu \)g) or an estrogen-treated rooster, \( \bullet \) (0.5 to 350 \( \mu \)g). Incubations were carried out at 68°C in capillaries containing 10 or 20 \( \mu \)l of 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.0, NaCl (0.6 M), and Na\_EDTA (0.02 M). The \( R_{ot} \) values shown are those reached during the experiment and have not been corrected for ribosomal RNA content. The line drawn is a theoretical curve derived solely from the homologous hybridization data. The curve was generated using a Texas Instruments printing calculator, programmed to fit the data to a curve describing a population consisting of three abundance classes as described in the legend to Fig. 1. The hybridization curves shown in a arc: \( \circ \), total RNA from the livers of estrogen-treated roosters; and \( \bullet \), total RNA from the livers of normal roosters. The hybridization curves shown in b are: \( \bigcirc \), total RNA from the livers of estrogen-treated roosters; \( \bigcirc \), total RNA from the liver. The curves shown for the homologous hybridization reactions are curves calculated to fit three abundance classes as described in the legend to Fig. 1. Any curves describing heterologous hybridization reactions were simply drawn through the data.

With this exception, the kinetic data of the homologous and heterologous hybridization reactions do not differ significantly, suggesting that within the limits of the analysis the entire mRNA population of the normal liver is maintained following treatment with estradiol.

The data obtained from the homologous hybridization reaction shown in Fig. 2a indicate that following treatment with estradiol the fraction of the RNA population in the abundant class increased to approximately 50%. The \( R_{ot1/2} \) value calculated for this class, after correction for its fractional representation in the total RNA population, was 2.2 \( \times 10^3 \) nucleotides\-s\-liter\(^{-1}\). Thus, treatment with the hormone resulted in a 6- to 7-fold increase in the complexity of the abundant mRNA species. This increase can be accounted for in part by the addition of vitellogenin mRNA (7000 nucleotides) to the abundant class (18). The increased complexity of this class is reflected in data from the heterologous hybridization reactions shown in Fig. 2a which show that 60% of the cDNA derived from the abundant class of mRNA found following treatment with estrogen, does not hybridize with this class of RNA normally present in rooster liver, although it may hybridize to RNA in a lower frequency class. These results are in contrast to those obtained when hen liver RNA is used in such an analysis. As shown in Fig. 2b the kinetic data of hybridization of both the homologous and heterologous reactions are extremely similar. It would appear from this type of analysis that treatment with estrogen effectively shifts the RNA population found in rooster liver to a distribution of RNA species indistinguishable from that found in the hen.

Hybridization with "Complex Class" cDNA—It is impossible to determine, from the heterologous hybridization in Fig. 2a, whether or not treatment with estrogen affects only RNA...
Analysis of the data indicated that approximately 10% of the fractionated cDNA probe used was derived not from the class of 8.7 nucleotides. A calculated nucleotides per liter was determined for the complex class. When this was considered, an observed RO$_{1/2}$ of 850 was obtained. The hybridization reaction showed no significant difference between any of the RNA samples tested.

Taking this into consideration, an observed RO$_{1/2}$ of 100 nucleotides per liter was obtained. DNA sequences in hybridized and subsequently removed all the sequences that had hybridized with total liver RNA from a normal rooster by RO$_{1/2}$ 100 nucleotides per liter. Details of the isolation procedure are given under “Experimental Procedures.” However, their hybridization reactions were carried out in 0.4 M NaCl while those in this study were carried out in 0.6 M NaCl resulting in a 1.46-fold increase in rate of hybridization.

Fig. 4 (center). Hybridization of abundant and intermediate class cDNA from the livers of estrogen-treated roosters with total RNA from the livers of normal, estrogen-treated, or hormone-withdrawn roosters. Complex class cDNA was isolated as that fraction of total cDNA that had not hybridized with its homologous RNA by a RO$_{1/2}$ value of 100 nucleotides per liter. When this cDNA was hybridized with total liver RNA from a normal, a hormone-treated, and a “withdrawn” rooster, the curve shown in Fig. 3 was obtained. The hybridization reaction showed no significant difference between any of the RNA samples tested. Analysis of the data indicated that approximately 10% of the fractionated cDNA probe used was derived not from the complex class but from the intermediate abundance class. Taking this into consideration, an observed RO$_{1/2}$ of 850 nucleotides per liter was determined for the complex class. When this RO$_{1/2}$ was corrected for the presence of rRNA (98%) and for the fractional representation of this abundance class (30%), a calculated RO$_{1/2}$ of 5.0 nucleotides per liter was obtained. Axel et al. (33), have determined a corrected RO$_{1/2}$ of 8.7 nucleotides per liter for the comparable abundance class of hen liver RNA, based on total complexity analyses.

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Hybridization with “Abundant Class” cDNA—The preliminary total complexity analyses indicated that hormonally induced changes in the RNA population would be much more apparent when a cDNA probe specific for abundant mRNA species was used for homologous and heterologous hybridization studies. Abundant class cDNA complementary to RNA isolated from an estrogen-treated rooster was prepared by hybridizing total cDNA to its homologous RNA to a RO$_{1/2}$ value of 10 nucleotides per liter. DNA sequences in hybridized and subsequently removing all the sequences that had hybridized with total liver RNA from a normal rooster by RO$_{1/2}$ 100 nucleotides per liter. Details of the isolation procedure are given under “Experimental Procedures.” The conditions used for hybridization are described in the legend to Fig. 1. Curves shown are: total RNA from the livers of estrogen-treated roosters; and total RNA from the livers of normal roosters.

However, their hybridization reactions were carried out in 0.4 M NaCl while those in this study were carried out in 0.6 M NaCl resulting in a 1.46-fold increase in rate of hybridization.
nucleotides·s·liter⁻¹.

Isolation of ‘Estrogen-inducible’ cDNA—The substantial difference observed between the kinetics of hybridization of abundant class cDNA with its homologous RNA compared to RNA from a normal rooster, suggested that it would be possible to fractionate the cDNA still further so that it contained sequences corresponding only to estrogen-inducible mRNA species. The first cDNA probe of this type that was prepared was isolated by selecting those sequences that had hybridized to liver RNA from an estrogen-treated rooster by Rot 10 nucleotides·s·liter⁻¹ and then removing all those sequences that hybridized to RNA from normal rooster liver by Rot 100 nucleotides·s·liter⁻¹. Such a selection procedure should have completely removed cDNA sequences corresponding to mRNA species not induced at least 10-fold by estrogen and also reduced to a variable degree, the proportion of sequences corresponding to mRNA species induced between 10- and 30-fold.

The kinetic data with which cDNA isolated in this fashion hybridized with rooster liver RNA are shown in Fig. 5. The cDNA hybridized to 95% completion with liver RNA from estrogenized roosters as a single abundance class with a Rot 1/₂ of 0.5 nucleotide·s·liter⁻¹. In contrast, only 15% of this cDNA hybridized to liver RNA from a normal rooster by a Rot value of 900 nucleotides·s·liter⁻¹. Thus, approximately 85% of the cDNA corresponded to species of RNA that increased in concentration at least 1000-fold following treatment with estrogen.

Cloning of Estrogen-inducible Sequences—We were interested in using cDNA fractionated in the fashion described above to assist in cloning members of the estrogen-responsive domain of the liver that share similar induction ratios. Rather than use fractionated cDNA directly for cloning purposes, we found it more practicable to construct a “cDNA clone” library from total RNA and to use fractionated cDNA, labeled with ³²P, for screening purposes only. Previous data obtained during the cloning of serum albumin cDNA (21) suggested that the frequency distribution of the original cDNA population was maintained during the cloning process. This information was combined with an estimate of the abundance and sequence complexity of the fractionated cDNA probe in order to calculate the minimum size of “library” that would be expected to include clones containing the sequences of interest.

It appeared from the hybridization data obtained during fractionation of the cDNA that the sequences selected constituted approximately 15% of the total cDNA. Rather than rely solely on Rot analyses to estimate the complexity of the cDNA probe we also determined the number of different cDNA species present by the blotting technique of Alwine et al. (27). Samples of RNA from hen liver and from the livers of normal or estrogen-treated roosters were subjected to electrophoresis in 1.5% agarose gels containing methymercuric hydroxide. The separated RNA was then transferred from the gel to diazotized filter paper and hybridized with ³²P-labeled cDNA (specific activity 30 cpm/pg) as described in the legend to Fig. 6. The RNA blot was hybridized with nick-translated plasmid DNA (5×10⁶ cpm, specific activity 70 cpm/pg) isolated from one clone containing a nonvitellogenin, estrogen-inducible sequence.
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strongly reacting, non-vitellogenin clones and labeled with $^{32}$P by nick translation. An RNA blot similar to the one that had been hybridized with estrogen-inducible cDNA shown in Fig. 6 was challenged with nick-translated plasmid DNA. Both of the plasmids tested hybridized with a RNA species approximately 800 nucleotides long. The results obtained with nick-translated plasmid DNA from one of these clones, designated E18, are shown in Fig. 7. The recombinant plasmid isolated from E18 was found to contain a fragment of DNA that could be excised by digestion with Pst 1. The cloned sequence contained a Pst 1 site within it and was cleaved by the enzyme to yield two fragments, one 270 nucleotides and another 190 nucleotides long.

At the moment, a polypeptide specified by the 800-nucleotide-long, estrogen-inducible mRNA has not been identified. The only egg yolk protein, in addition to vitellogenin, that is known to be totally dependent upon estrogen for its synthesis, is riboflavin-binding protein (14, 15). This is a glycosphosphoprotein containing a polypeptide of approximately 25,000 to 30,000 daltons (34). The time course of induction of riboflavin-binding protein following administration of estrogen to the rooster, is similar to that observed for vitellogenin. In fact, both proteins appear to be present in serum during the vitellogenic response at approximately molar equivalence. A protein, immunologically identical with egg yolk riboflavin-binding protein, is also found in egg white (35). Both the egg yolk and egg white proteins are believed to be products of the same gene (34). The egg white protein, however, is thought to be produced in the oviduct (34). Riboflavin-binding protein mRNA would appear to be a possible candidate for the small estrogen-inducible RNA species found in rooster liver. However, preliminary hybridization experiments using a cloned cDNA fragment have failed to detect the sequence in total RNA from oviduct. Further studies on the characterization of this RNA species and the protein that it may specify are in progress.

The procedure we have described has allowed us to select two RNA sequences from rooster liver that are related both by their induction ratio and their molar abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance. Whether or not parameters of induction ratio and abundance.

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