Isolation of chicken vitellogenin I and III cDNAs and the developmental regulation of five estrogen-responsive genes in the embryonic liver

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The isolation of cDNA clones that code for portions of the two minor chicken vitellogenin (VTG) genes (VTGI and VTGIII) is reported. These clones represent unique sequences that are expressed exclusively in the livers of estrogenized birds. In the liver of the egg-laying hen, the levels of RNAs encoding VTGI, VTGII, and VTGIII are approximately 11,000, 30,000, and 3,000 molecules per cell, respectively. We have used the newly isolated clones, as well as the yolk protein cDNAs previously available [VTGII, apolipoprotein II (apoVLDLII), and apolipoprotein B], as probes to examine several aspects of the regulation of these genes by estradiol. First, we demonstrate that the capacity of each gene to respond to estradiol is acquired between 8 and 13 days in ovo. The response of four of these genes to estradiol is diminished during late fetal development, but the responsiveness is recovered within a week after hatching. Second, we demonstrate that these genes display distinct kinetic response profiles following the addition of estradiol. Third, as has been described previously for the VTGII and apoVLDLII genes, we demonstrate that a single injection of estradiol effects a long-term reprogramming event (hepatic memory) that allows a faster onset of the rapid accumulation of both VTGI and VTGIII RNAs following a subsequent rechallenge by estradiol. Collectively, these three sets of data suggest molecular parameters that may contribute to both the coordinate and noncoordinate regulation of this set of genes by estradiol.

[Key Words: Vitellogenin; apolipoprotein; yolk protein; regulation by estrogen; liver-specific gene expression; hepatic memory]

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The chicken yolk protein genes provide an excellent model system for studying tissue-specific, steroid-specific, and developmentally regulated programs of gene expression. The yolk proteins are coordinately synthesized in the livers of chickens (and other oviparous species) in response to the elevated circulating levels of estradiol, which also serve to coordinate the production of the egg-white proteins in the chicken oviduct [for reviews, see Chambon et al. 1984; Wahli and Ryffel 1985]. The three vitellogenin (VTG) genes and the apolipoprotein II (apoVLDLII) gene are normally expressed only in hens during periods of egg laying [Wiskocil et al. 1980; Wang et al. 1983]. ApoVLDLII appears unchanged in the egg yolk, whereas the VTG genes are processed proteolytically to yield mature yolk proteins. The precise precursor–product relationship between VTGII and three of the yolk proteins has recently been resolved [van het Schip et al. 1987]. Less is known of these relationships for the other two VTG genes [Deeley et al. 1975; Wang et al. 1983; Wallace and Morgan 1986]. A fifth gene that contributes protein to the egg yolk is constitutively expressed in chickens of both sexes and codes for a protein, apolipoprotein B (apoB), that also serves as a component of circulating very-low-density and low-density lipoprotein particles [Evans and Burley 1987]. The level of apoB RNA in the liver is increased about sixfold following the administration of estrogen [Kirchgessner et al. 1987].

Although the molecular mechanisms by which estrogen regulates these genes remain unknown, sequence comparisons between the 5'-flanking regions of the two previously cloned yolk protein genes (VTGII and apoVLDLII) have revealed a number of shared sequence motifs that could be involved in their coordinate transcriptional regulation [Burch 1984; van het Schip et al. 1986]. In addition, the conserved dyad sequence GGTCANNNTGACC has recently been shown to be an
essential part of the estrogen-response elements (EREs) associated with two frog VTG genes (Klein-Hitpass et al. 1986; Seiler-Tuyns et al. 1986), as well as the chicken VTGII gene (Burch et al. 1988). The significance of these and other DNA sequence elements to the coordinate regulation of the entire set of target genes awaits further functional analysis, as well as comparisons with additional liver-specific genes that are regulated by estrogen. The isolation of cDNA clones for the VTG genes (VTGI and VTGIII) reported in this study represents an important step toward this goal.

One intriguing aspect of the regulation of the estradiol-dependent induction of protein (Jost et al. 1973; Luskey et al. 1974; Capony and Williams 1980) and/or RNA (Gruber et al. 1976; Deeley et al. 1977; Jost et al. 1978) for the various yolk protein genes is the phenomenon of hepatic memory. This concept derives from the observation that there is a faster onset of rapid accumulation of both RNA and protein following a secondary administration of estrogen relative to that observed following the initial exposure to the hormone. In the present report we demonstrate that VTGI and VTGIII also display memory at the level of RNA. In fact, the VTGI gene displays the most pronounced memory effect of any of the chicken and frog yolk protein genes examined to date.

In addition to the memory response, the estrogen-induced expression of the yolk protein genes is subject to a number of developmental constraints. For example, Williams and co-workers demonstrated that the apoVLDLII gene will respond to estradiol earlier in development than the VTGII gene (Elbrecht et al. 1984). Recently, we have shown that the yolk protein genes are also subject to developmental influences between 1 and 6 weeks after hatching (Evans et al. 1987). During both of these periods of development, the changes in expression of the yolk protein genes appear to be correlated with developmental changes in the level of estrogen receptors. In the present study we have extended these data further and demonstrate that there is a third distinct period of development just prior to hatching when the expression of the yolk protein genes is suppressed. Possible explanations for the decrease in estrogen responsiveness that characterizes this developmental period are discussed.

Results and discussion

The isolation of chicken VTGI and VTGIII clones from a cDNA library

We employed the strategy of differential screening as a means of isolating clones of genes that are responsive to estrogen in the avian liver. A λgt11 cDNA library (prepared from RNA that was isolated from the livers of egg-laying hens) was screened sequentially with cDNA probes prepared from liver RNA of estradiol-stimulated and hormone-naive chickens. To identify and exclude clones of the two most abundant liver RNAs that are induced by estradiol, we added a mixture of unique-sequence VTGII (Cozens et al. 1980) and apoVLDLII (Wiszkocil et al. 1980) SP6-derived 32P-labeled RNA to the 32P-labeled cDNA that was prepared against hormone-naive liver RNA. In a primary screen of 4000 recombinant phage, 44 clones were identified that appeared to code for RNA species induced by estradiol. Three of these clones (λcV1-36, λcV3-38, and λcAB-11) were plaque purified, and the inserts (0.9, 0.65, and 0.7 kb, respectively) were subcloned in both orientations into the EcoRI site of the plasmid vector SP65 for further analysis. The λcAB-11 clone was identified as coding for a portion of the apoB gene (Kirchgessner et al. 1987; T. Kirchgessner, pers. comm.)

The sense and antisense strands for pcV1-36 and pcV3-38 were determined by preparing labeled SP6 transcripts and hybridizing these to Northern blots of liver RNA isolated from hormone-naive and estradiol-stimulated birds. Data obtained using the antisense probes are shown in Figure 1 and corroborate that the RNA species homologous to pcV1-36 and pcV3-38 are induced de novo in the liver in response to estradiol. The data also indicate that the two probes hybridize to RNAs that are slightly larger (pcV1-36) and slightly smaller (pcV3-38) than the VTGII mRNA, which runs with an apparent molecular weight of 6.7 kb (Wieringa et al. 1978) and has 5787 bp by sequencing (van het Schip et al. 1987).

Hybrid-selected translation (see Materials and methods) was used to identify the pcV1-36 and pcV3-38 clones as VTGI and VTGIII. RNAs selected by hybridization to pcV1-36 and pcV3-38 were prepared and translated in vitro. As shown in Table 1, when total RNA from the livers of egg-laying hens was translated, the relative synthesis of the three VTG proteins (VTGI : VTGII : VTGIII, 3 : 6 : 1) was similar to the reported in vivo ratio of 3 : 10 : 1 in the chicken liver (Wang et al. 1983). Compared to the translation products of total RNA, the products derived from the pcV1-36 selected message were enriched in VTGI, and those derived from the pcV3-38 selected message were enriched in VTGIII. We conclude that pcV1-36 and pcV3-38 code for portions of VTGI and VTGIII, respectively.

The VTGI and VTGIII cDNA fragments each correspond to unique sequences in the chicken genome

Given the high degree of homology among the family of four frog VTG genes (Wahl and Ryffel 1985), we wondered whether the VTGI and VTGIII cDNA fragments might cross-react similarly, either with each other or with the VTGII gene. To address this, we prepared SP6-derived RNA probes for VTGI and VTGIII and hybridized them under low stringency conditions (see Materials and methods) to Southern blots containing these fragments, as well as fragments covering the entire VTGII gene. The results of this analysis are shown in Figure 2. In each case, the probes are demonstrated to hybridize to their homologous fragments as expected. In contrast, the VTGI and VTGIII probes fail to hybridize to each other or to any portion of the VTGII gene.
Figure 1. pcV1-36 and pcV3-38 correspond to large RNA species that are inducible by estradiol. Northern blots were prepared with RNA from hormone-naive (−) and estrogenized (+) liver and hybridized as described in Materials and methods. The probes used are pcV1-36 (A), pcV3-38 (B), VTGII (C), apoVLDLII (D), and chicken serum albumin (E). The migration of HindIII fragments of λ DNA are indicated in kilobases.

To determine whether VTGI and VTGIII themselves might be members of larger gene families unrelated to VTGII, we analyzed the complexity of each of our new cDNA probes by hybridizing them to genomic Southern blots. A parallel genomic blot was hybridized with a VTGII probe for comparison. The autoradiograms from this analysis are presented in Figure 3 (A–C) [low stringency wash] and Figure 3 (D–F) [high stringency wash]. It is clear that even under conditions of low stringency, the VTGI [Fig. 3A] and VTGIII [Fig. 3B] cDNA probes yield very simple hybridization patterns. The fact that only single bands are apparent in several lanes of each blot indicates that VTGI and VTGIII are very likely each single-copy genes [Fig. 3 D and E, respectively]. In contrast, at least two genomic bands are apparent in each lane probed with the VTGII cDNA fragment [Fig. 3C, F]. The complexity of this VTGII pattern cannot be explained by the restriction map of the cloned VTGII gene, suggesting that there probably is a VTGII-related gene or pseudogene, as has been noted previously [Wilks et al. 1981]. At present there is no evidence that this VTGII-related genomic sequence is either VTGI or VTGIII, but a definitive conclusion cannot be made until we achieve the isolation of complete sets of overlapping clones for the two minor VTG genes.

The three VTG genes are each expressed exclusively in the livers of estrogenized birds and all display hepatic memory at the RNA level

Whereas it has been demonstrated previously that the three chicken VTG proteins are synthesized and se-

Table 1. Identification of clones for VTGI and VTGIII by hybrid selection, in vitro translation, and specific immunoprecipitation

| RNA translated | anti-VTGI | anti-VTGI | anti-VTGI | I:II:III ratio |
|----------------|----------|----------|----------|---------------|
| Total          | 2850     | 6140     | 680      | 29:64:07      |
| pcV1-36 selected | 1630     | 470      | 490      | 63:18:19      |
| pcV3-38 selected | 290      | 320      | 980      | 18:20:62      |

RNA was isolated from the livers of egg-laying hens and enriched for either pcV1-36 or pcV3-38 sequences by hybrid selection, as described in Materials and methods.

Figure 2. Cloned VTGI and VTGIII cDNA fragments lack homology with VTGII and apoVLDLII sequences. Cloned DNAs were digested with EcoRI and subjected to electrophoresis through 1% agarose gels. The lanes contain an entire apoVLDLII genomic clone (1), portions of the VTGII gene which, together, represent the entire VTGII gene (2,3,4), an apoVLDLII cDNA clone in SP6 vector (5), a VTGI cDNA clone in SP6 vector (6), and a VTGII cDNA clone in SP6 vector (7). (Lane S) HindIII fragments of λ DNA, the sizes of which are indicated in Fig. 1. The gels were stained with ethidium bromide (A), transferred, and hybridized (see Materials and methods) with SP6-derived antisense probes of VTGII (B), VTGI (C), and VTGIII (D). The large fragment in lanes 5, 6, and 7 is the SP6 vector. The filters were washed for 1 hr at low stringency (2× SSC, 0.1% SDS, 25°C) and exposed to Kodak XAR film.
Figure 3. Cloned VTGI and VTGIII cDNA fragments appear to represent single-copy genes. Chicken genomic DNA was digested with BamHI (lane 1), EcoRI (lane 2), HindIII (lane 3), PstI (lane 4), and PvuII (lane 5). Southern blots were prepared as described in Materials and methods and hybridized with SP6-derived antisense probes of VTGI (A,D), VTGIII (B,E), and VTGII (C,F). The filters were washed for 1 hr at low stringency (2x SSC, 0.1% SDS, 25°C), exposed to Kodak XAR film (A–C), washed for 1 hr at high stringency (0.1 x SSC, 0.1% SDS, 65°C), and reexposed to film (D–F). (Lane S) HindIII fragments of λ DNA; their sizes are indicated in Fig. 1.
creted from the liver in response to estradiol [Wang et al. 1983], it has not been determined whether the VTG genes are expressed in other tissues as well. To address this, the amounts of RNA coding for each of the three VTG genes were directly compared in a variety of tissues exposed to estradiol in vivo. RNA from hormone-naive liver was included among the samples as a reference. A probe for mouse α-actin RNA [Minuty et al. 1981] was used to indicate the amount of hybridizable RNA in each dot. Of the 10 tissues examined [Fig. 4], the only one that contains detectable amounts of VTG RNAs is the estrogen-stimulated liver. Overexposure of these blots indicates that if VTG RNAs are expressed in any of the other tissues, the levels must be less than 1% of those observed in liver [data not shown].

We quantitated the levels of the VTG RNAs in the livers of egg-laying hens by solution hybridization (see Materials and methods) and found that VTGII is expressed at approximately 30,000 molecules of RNA per cell. This estimate for the abundance of VTGII RNA falls between the two determinations reported previously [Gruber et al. 1976; Jost et al. 1978]. Our results indicate that VTGI RNA is expressed at 11,000 molecules per cell and VTGIII RNA at 3,000 molecules per cell. Thus, the relative levels of VTGI : VTGII : VTGIII RNA in the liver of the laying hen are approximately 4 : 10 : 1, respectively. These relative values are similar to the ratio of the VTG proteins synthesized in adult estrogenized roosters [Wang et al. 1983], indicating that there is no appreciable differential translational control among this set of coordinately regulated genes.

Hepatic memory has been described for the chicken VTGII [Deeley et al. 1977; Jost et al. 1978] and apoVLDLII [Codina-Salada et al. 1983] genes, as well as the four frog VTG genes [Wolffe and Tata 1983; Wahli and Ryffel 1985]. To determine whether the VTGII and VTGIII genes also display hepatic memory at the level of RNA, we examined the kinetics of the accumulation of VTGI and VTGIII RNA following the administration of primary and secondary injections of estradiol to 1-week-old birds. Figure 5 shows that both VTGI and VTGIII display lag times for the onset of rapid accumulation of RNA following a primary exposure to estradiol and that these lag times are reduced in a secondary response. Interestingly, whereas the primary and secondary lags observed prior to the rapid accumulation of VTGIII RNA [Fig. 5B] are very similar to those described previously for VTGII [Jost et al. 1978; Evans et al. 1987], the lag times for VTGI [Fig. 5A] are significantly longer.

It is also worth noting that the maximal level of VTGIII RNA in these week-old birds is only half that observed in laying hens. For VTGI, the difference is even greater, with the maximal level of VTGII RNA at this age reaching less than one-fifth the level achieved in the fully mature birds. The relationship between such age-dependent differences in the expression of the various yolk protein genes and developmental changes in the expression of the estrogen receptor is described in detail elsewhere [Evans et al. 1987].

Estradiol-induced expression of five target genes during liver ontogeny

It has been reported previously that the expression of the apoVLDLII gene can be induced by estradiol in the liver earlier in development than that of the VTGII gene [Elbrecht et al. 1984]. To determine the inducibility of the other two VTG genes prior to hatching, we injected estradiol into embryos on different days of development and assayed for the induction of specific yolk protein gene RNAs later [see Materials and methods]. The estradiol-induced expression measured for each of five target genes examined as a function of development is shown in Figure 6. It is apparent from these data that the estrogen responsiveness of the individual yolk genes is achieved at different times during the development of the liver. The apoB gene, whose expression is increased approximately sixfold in response to estradiol [Kirchgessner et al. 1987; M.I. Evans et al., unpubl.], can be maximally expressed as early as day 9 of embryonic development [12 days prior to hatching]. Although not maximal, apoVLDLII RNA is also readily detected in response to estradiol at day 9, whereas the three VTG genes are barely inducible at this stage. The estrogen-induced expression of apoVLDLII and the three VTG genes increases progressively for the next 4–6 days, in agreement with the apoVLDLII and VTGII results of Williams and co-workers [Elbrecht et al. 1984]. These data are reminiscent of the developmental regulation of the estrogen-dependent expression of the four frog VTG RNAs that accumulate at different rates and to different extents during different stages of development [Ng et al. 1984]. The observed increase in the levels of chicken yolk gene RNAs coincides with a prehatching period of increasing levels of estrogen receptors [Gschwendt and Kittstein 1977; Elbrecht et al. 1984].

With respect to the ease with which these genes can first be activated as a function of development, the five
genes are ordered in the following way: apoB > apoVLDLI > VTGIII > VTGI = VTGI. Interestingly, a similar order is apparent with respect to the time in development at which these genes gain the capacity to be maximally expressed in response to estradiol. For the three VTG genes, this occurs during the period between 1 and 6 weeks after hatching and coincides with a period when the maximal level of nuclear estrogen receptors increases from 1500 to 5000 sites per hepatocyte [Evans et al. 1987]. As a working model, we suggest that the ordering of the individual genes with respect to estrogen responsiveness may reflect differences in the abilities of the respective receptor binding site[s] to productively bind activated estrogen receptors.

What then might determine how efficiently a gene is able to respond to activated estrogen receptors? The recent delineation and characterization of the EREs associated with three different VTG genes [two from the frog and one from the chicken] may provide some hints as to how variable estrogen responsiveness could be encoded. Although extensive data are not yet available, it seems reasonable that variations within the ERE core sequence [GGTCANNTGACC] might affect the ability of the respective EREs to bind receptors and/or effect transcriptional activation. Additionally, the particular sequence context in which this 13-bp element resides, the distance of this element from the promoter, and the number of functional elements can each clearly influ-
protein genes during embryonic development. RNA was iso-
hybridization with SP6-derived antisense probes to VTGI (A),
ficed 48 hr after a single injection of 17-estradiol. Following
VTGII (I), VTGIII (o), apoVLDLI (+), and apoB (O), the specific
RNAs were quantitated as described in Materials and methods.

translated from the livers of embryos and hatchlings that were sacri-
mary and secondary injections of estradiol (Evans et al.
contrast, the rapid accumulation of yolk protein RNA
based on our analysis of the kinetics of the accumula-
tion of RNA of the five major yolk protein genes, it is
striking that the order in which the yolk protein genes are
activated following a single injection estradiol (Fig.
similar to the order in which the respective genes can
be expressed in response to estradiol during develop-
ment (Fig. 6; Evans et al. 1987). Whereas the latter or-
dering could be related to the efficiency with which each
gene can be expressed in response to a particular level of
nuclear estrogen receptors (see above), the lag times in-
volved prior to the onset of expression of the respective
genes do not correlate with changing receptor levels. We
have shown previously that in the livers of 1-week-old
birds (the age used in these experiments), the level of
nuclear receptors becomes maximal within an hour of
the administration of estradiol (Evans et al. 1987). In
contrast, the rapid accumulation of yolk protein RNA
commences 4 (apoVLDI) to 20 hr (VTGI) after a pri-
mary injection of estradiol. Moreover, whereas the ki-
netics of the accumulation of RNA are more rapid fol-
lowing a secondary presentation of estradiol for each of
the four yolk protein genes that are induced de novo in
response to estradiol, the kinetics of the accumulation
of nuclear estrogen receptors are the same following pri-
mary and secondary injections of estradiol (Evans et al.
Whatever the basis is for the different lag times be-
tween the addition of estradiol and the rapid accumula-
tion of the respective yolk protein RNAs, we think it is
significant that these ordered differences are apparent in
both primary and secondary responses to estradiol [Fig.
5]. We speculate that the differences in secondary re-
ponse profiles may reflect inherent DNA sequence dif-
fences in the respective cis-regulatory regions asso-
ciated with each gene (see above). Epigenetic events (e.g.,
changes in chromatin structure) may then be superim-
posed on these different primary DNA sequences to ac-
count for the slower initial responses to estradiol. Altem-
atively, critical changes in the activities and/or availa-
bilities of trans-acting factors could be brought about as
a consequence of the initial exposure to estradiol, and
this may be relevant to memory.

In addition to the two periods of development noted
above in which the expression of the yolk protein genes
increases, we have identified a third period of develop-
ment in which a striking dissimilarity in expression of
the individual yolk protein genes is evident. This occurs
during the late embryonic period when estradiol-in-
duced expression is diminished for four of the five genes
examined (Fig. 6). Whereas this is the first study of VTGI
and VTGIII expression during late fetal development,
other labs previously examined apoB, apoVLDII, and
VTGII expression at the protein level during this period of
development (Nadin-Davis et al. 1980; Elbrecht et al.
1981). Curiously, whereas the RNA and protein syn-
thesis data appear to be qualitatively similar for both the
VTGII and apoVLDI genes, the 40% decrease that we
observe in apoB RNA expression is not reflected by a
comparable decrease in apoB protein synthesis. The
basis for this difference is not apparent. Although a com-
parable phenomenon has not been observed during
normal frog development, Tata and co-workers have
shown that the expression of the frog VTG genes is tran-
siently depressed when liver cells are placed into cul-
ture. They also demonstrated that this result can be
mimicked by heat shock and that nuclear estrogen re-
ceptor levels are reduced as part of a general stress re-
ponse (Wolffe et al. 1984). The depression in the expres-
sion of the chicken yolk protein genes that we observe
occurs during a period when the embryos are very likely
subject to the stress of anoxia. However, since receptor
levels do not decrease during this period of development
(Lazier 1978) additional complexities are suggested. This
is also indicated by the observation that the VTGII gene
is much less refractile than the other yolk protein genes.
Further experiments will be required to deduce the basis
for the observations.

Materials and methods
Isolation of cDNA clones
A kgt11 cDNA library prepared from the livers of egg-laying
hens [kindly provided by Todd Kirchgessner, UCLA] was
screened according to the procedure of Huynh et al. [1985].
Plaques were purified [Maniatis et al. 1982], and the inserts
were subcloned into the EcoRI site of an SP65 vector [Promega
Biotech].
Southern blots

Ten micrograms of chicken genomic DNA or 1 μg of cloned DNA was digested with restriction enzymes, separated by electrophoresis through 1% agarose gels, and transferred to Nytran membranes (Schleicher & Schuell). The blots were prehybridized, as described by Meinkoth and Wahl [1984], for 2 hr at 42°C and hybridized with SP6-derived antisense RNA transcripts [Melton et al. 1984] for 16 hr at 42°C followed by 16 hr at 37°C. SDS (1%) was added to all hybridization solutions. The blots were washed as described in the figure legends.

Northern blots

RNA was prepared using the guanidinium/cesium method of Chirgwin et al. [1979]. Total RNA (300 ng/lane) was separated by electrophoresis through 0.7% agarose/formaldehyde gels and transferred to Nytran membranes.

Hybrid selection, in vitro translation, and immunoprecipitation

Total RNA was isolated from the livers of egg-laying hens and hybrid-selected as described [Neilsen et al. 1985]. In vitro translations were carried out using a reticulocyte lysate kit (American) according to the directions therein. The translation products were incubated with preimmune rabbit serum at 22°C for 2 hr, staphylococcus protein A was added and the incubation was continued for 2 additional hr at 22°C. The preimmune complexes were removed by centrifugation, then specific antibodies were added to the supernatant and incubated for 16 hr at 4°C. The immune complexes were precipitated with protein A-Sepharose beads for 2 hr at 22°C and centrifuged. The pellets were washed 3 times in buffer [1% Triton X-100, 0.1% SDS, 15 mM Tris at pH 7.5, 120 mM NaCl, 2 mM EDTA, 2 mM EDTA, 0.1 mM diithiothreitol (DTT)] filtered, and counted. Antibodies to VTGI, VTGII, and VTGIII were a gift from Dr. David Williams (SUNY, Stony Brook).

Quantitation of RNA

RNA was applied to nitrocellulose filters and hybridized as described [Burch and Evans 1986], using 300 ng total RNA per dot for quantitation of the yolk RNAs. Serial dilutions of RNA prepared from the livers of egg-laying hens were included on each filter for reference. The molecules per cell of VTGI, VTGII, and VTGIII were quantitated by solution hybridization [Evans et al. 1981], using cold sense SP6 transcripts for standards and assuming 6.25 pg total RNA per cell [Evans et al. 1987].

Animals and hormone treatment

Fertilized eggs from a white leghorn breeding flock were obtained from Truslow Farms (Chester, Maryland). Eggs were injected with 1 mg of 17β-estradiol dissolved in propylene glycol (Burch and Weintraub 1983), and the livers were dissected at the times indicated in the figures. For the analysis of primary responses in young birds, untreated eggs were allowed to hatch, and the birds were injected with 20 mg/kg 17β-estradiol prior to sacrifice at the times indicated. For the analysis of secondary responses, the eggs were injected with 1 mg of 17β-estradiol on day 12 of embryonic development and allowed to hatch, and the birds were subsequently injected with 20 mg/kg of 17β-estradiol as indicated. For each time point, pieces of liver from three birds were pooled for the isolation of RNA, and the analysis was done in duplicate. To provide tissues for the preparation of RNA for analysis of the tissue distribution of yolk RNAs, a 3-week-old bird, hatched from an estrogen-treated egg, was given daily injections [20 mg/kg] of 17β-estradiol for 3 days, sacrificed, and dissected. The yolk sac was dissected from a 5-day-old embryo.

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