*ORGAN-SPECIFIC ESTROGEN-INDUCED RNA SYNTHESIS RESOLVED BY DNA-RNA HYBRIDIZATION IN THE DOMESTIC FOWL**

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Abstract and Summary.—In the domestic fowl and other oviparous vertebrates, estrogens induce hepatic synthesis of yolk proteins. The oviduct also increases in size and produces ovalbumin when estrogens are administered. DNA-RNA hybridization assays indicate that within 105 minutes following treatment with estrone the liver of immature pullets contains most, if not all, of the liver RNA species that are present in the livers of the laying hen. Because of limitations of the nucleic acid hybridization technique, which remain to be clearly defined, it is not known whether the hepatic RNA populations in estrone-treated pullets and laying hens are completely homologous or differ in some important way. The results indicate that the genomic response in the avian liver to exogenous estrone is "normal" (relative to the laying hen) and further suggest that the hepatic response to estrogen is primarily pertinent to vitellinogenesis.

DNA-RNA hybridization assays on total RNA also indicate that estrogen-induced RNA species in the liver are not homologous to estrogen-evoked RNA species of the oviduct. Therefore, in these two target organs estrogen-evoked RNA synthesis appears to be in part organ-specific. These data indicate that the specificity of hormone action can be explained in part on the basis of induced synthesis of specific RNA molecules. Whether these alterations in transcription are due to indirect hormonal action or are the result of a primary action of estrogens or estrogen-binding site complexes on the genome and/or its repressors remains a basic question.

It is well established that RNA synthesis in specific tissues and organs is regulated in part by certain hormones.[1] Generally, when hormones have any effect on nucleic acid metabolism, they stimulate RNA synthesis in respective target cells or organs, but inhibition has been found in several instances. Hydrocortisone, for example, decreases RNA synthesis in cultured osteocytes.[2] Also, thyroxine appears to inhibit RNA synthesis, at least qualitatively, in the tail skin of larval anurans.[3] Although the actual mechanism(s) of hormone-induced RNA synthesis is not elucidated for any hormone, the fact that the nucleic acid system is activated by hormones (direct or indirect action?) is clear.[1] Among the basic remaining questions are those that inquire into the qualitative characterization of endocrine-evoked RNA synthesis, especially in regard to possible hormone- and organ-specific transcription.

We have shown earlier[4] that estrone and progesterone induce the synthesis of new species of RNA in the avian oviduct.[5] The estrone-induced species can be shown by DNA-RNA hybridization analyses to be distinct, at least in part, from progesterone-induced species.[4] The synthesis of certain oviduct RNA

species is therefore "hormone-specific" with regard to these two steroids which are known to induce the synthesis of specific proteins.[6-8] Estrogens also evoke yolk protein synthesis (vitellinogenesis) in the livers of oviparous vertebrates.[9-12] Prior to the synthesis of yolk proteins, estradiol-17β induces the transcription of new liver RNA species in the oviparous lizard, *Uta stansburiana*.[13] Since new RNA species are synthesized in both the oviduct and the liver in response to estrogens, we examined RNA synthesis in estrogenized pullets to determine whether the *same* hormone can induce the synthesis of different RNA species in these two organs. In this report we present evidence which indicates that organ-specific (oviduct and liver) estrone-induced transcription does occur. Evidence which indicates that the population of liver RNA species in the estrone-treated pullet is similar to that of the laying hen is also presented.

Materials and Methods.—Animals and treatments: Ten-week-old White Leghorn pullets were injected intramuscularly with 10 μg of estrone (Estrogenic Substances, Ayerst Co.) per gram body weight (bw) 1 hr prior to intraperitoneal injection of 5 mc of H^3-uridine (Swartz BioResearch Corp.). The pullets were sacrificed 45 min later and the livers were removed, rinsed in saline, and frozen on dry ice. Control livers were similarly obtained from pulse-labeled (H^3-uridine-injected) animals which did not receive estrone. Unlabeled livers were also obtained from pullets pretreated with estrone for 105 min and from untreated pullets.

Another group of pullets was treated with estrone for 5 days (injections of 10 μg per gm bw were given on day 1 and 3), after which the livers and oviducts were removed and frozen on dry ice. Oviducts from several untreated pullets of the same age and source were also collected. Livers from White Leghorn vitellinogenic laying hens receiving either 12 mc of H^3-uridine in saline solution or saline only, by intraperitoneal injection 45 min prior to sacrifice, were also obtained and frozen on dry ice.

Preparation of RNA and DNA: RNA was prepared from the frozen livers and oviducts by a hot phenol method in the presence of bentonite as previously described.[4] All RNA preparations were incubated with deoxyribonuclease (DNase, 100 μg/ml) in the presence of 0.01 *M* $MgCl_2$ in 0.01 *M* sodium acetate, pH 6.0, for 1 hr at 32°C. DNase was removed with two phenol extractions at room temperature and the RNA preparation was then passed through G25-coarse Sephadex. RNA was precipitated with 2 vol of ethanol at −10°C. Before use, RNA was dissolved in TES (*N*-tris methyl-2-amino ethane sulfonic acid) buffered 2 × SSC (SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate).

DNA was extracted from liver nuclei with chloroform-octanol (10:1) as described by Hoyer, McCarthy, and Bolton.[14] DNA was purified until all detectable contamination with glycogen, protein, or RNA was removed. The DNA was dissolved in 0.1 × SSC (200 μg/ml), heat-denatured (95°C for 10 min), and quickly cooled in an acetone-ice bath. The denatured DNA was diluted to 10 μg/ml in 4 × SSC and immobilized on a 145-mm nitrocellulose membrane filter, according to the method of Gillespie and Spiegelman[15] as modified by Denhardt.[16] Units 7 mm in diameter were cut from the large membrane filter. A random sample of these 7-mm filters was checked for uniformity of DNA content by "hydrolyzing off" the DNA in boiling 0.1 *N* HCl and reading the absorbance at 260 mμ.

Hybridization procedures: Labeled RNA was hybridized to filter-bound single-strand DNA in the presence of increasing amounts of unlabeled "competitor" RNA's. Hybridization reactions were conducted in 2 × SSC for 18 hr. Following incubation, the filters were removed, washed three times in 2 × SSC at 68°C, dried, and the radioactivity of the DNA-RNA hybrids was then determined in a liquid scintillation counter. Further details are given in the legends to the figures.

Results.—Nucleic acid hybridization analyses indicate that after treatment with estrone, new liver RNA species are transcribed (Fig. 1). When H^3-labeled liver RNA molecules from estrone-treated pullets are hybridized to immobilized,

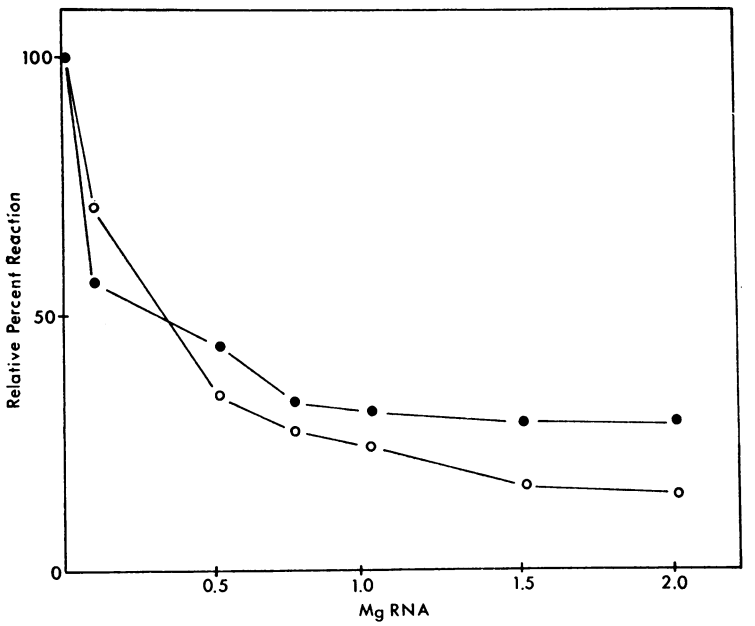

FIG. 1.—Comparison of the liver RNA populations from estrone-pretreated and untreated pullets. 30 μg of H^3-labeled liver RNA from estrone-treated pullets and 27 μg of filter-bound single-strand DNA were placed in each of a series of 10 × 40-mm vials to which increasing amounts of unlabeled liver RNA (given on the abscissa) from estrone-treated (O—O), or from untreated pullets (●—●) were added. Total reaction volume was 0.42 ml. Nucleic acid mixtures in TES-buffered 2 × SSC were incubated for 18 hr at 68°C. Filters were then removed, washed in three aliquots of 2 × SSC at 68°C, and dried; the hybrid radioactivity was then measured in a liquid scintillation counter. All values were corrected for background "noise" with reference to the annealing of labeled liver RNA to *B. subtilis* DNA; this "noise" reaction was 2% or less of the uncompeted homologous system. The ordinate is a relative per cent reaction calculated from counts per minute in which the uncompeted system (i.e., no unlabeled competitor RNA added) was arbitrarily set at 100% reaction.

single-strand DNA in the presence of unlabeled liver RNA from untreated (control) pullets, the degree of competition for complementary (or closely related) base sequences on DNA is less than when unlabeled RNA from estrone-treated pullets (homologous RNA) is used. In view of the fact that unlabeled RNA from hormone-treated pullets yields full competition against labeled RNA from untreated pullets, it appears that estrone evokes the synthesis of new liver RNA species, but it does not detectably inhibit synthesis of rapidly labeled RNA species that are being synthesized prior to estrogenization (see ref. 13 for similar results with a reptilian species). The crossing-over of the competition curves that results from greater initial competition by "control RNA" (Fig. 1) appears not to be an artifact, since it was a constant feature in repeated assays. This suggests that the synthesis of certain common RNA species, which are present both in estrone-treated and control animals, may be somewhat inhibited during the initial or early period of estrogen action in the liver. However, differences found in the initial portions of competition curves are difficult to interpret; thus, this should be considered only a tentative possibility.

Estrone-induced liver RNA synthesis in the pullet seems to be similar to liver RNA synthesis in the laying hen. When H^3-labeled liver RNA from vitellinogenic laying hens is hybridized to DNA in the presence of unlabeled liver RNA from pullets treated with estrone for 105 minutes or 5 days, or from laying hens (homologous RNA), similar competition plateaus are obtained from each of these RNA populations (Fig. 2). Competition between labeled liver RNA from laying hens and unlabeled RNA from untreated pullets indicates that there are RNA species present in the laying hen which either are absent in untreated pullets or present in very small quantities (Fig. 2). This difference is similar in degree to that between treated and untreated pullets (Fig. 1). Since the unlabeled liver RNA from pullets pretreated with estrone for two hours competes essentially as fully as does homologous RNA against the labeled liver RNA from laying hens, it appears that in a qualitative sense most, if not all, of the RNA species that are present in the liver of the laying hen are present in the pullet liver within 105 minutes or less following estrogenization. However, as shown in Figure 2, the slope of the competition curve was greater when unlabeled homologous (laying hen) RNA was used. Since this was a consistent finding in repeated assays, it suggests that there may be quantitative differences between the liver RNA populations of estrogenized pullets and laying hens.

As previously mentioned, estrone also induces the synthesis of new RNA species in the oviducts of chicks.[4] It is of interest, therefore, to determine to what extent estrone-evoked liver and oviduct RNA species are similar or differ-

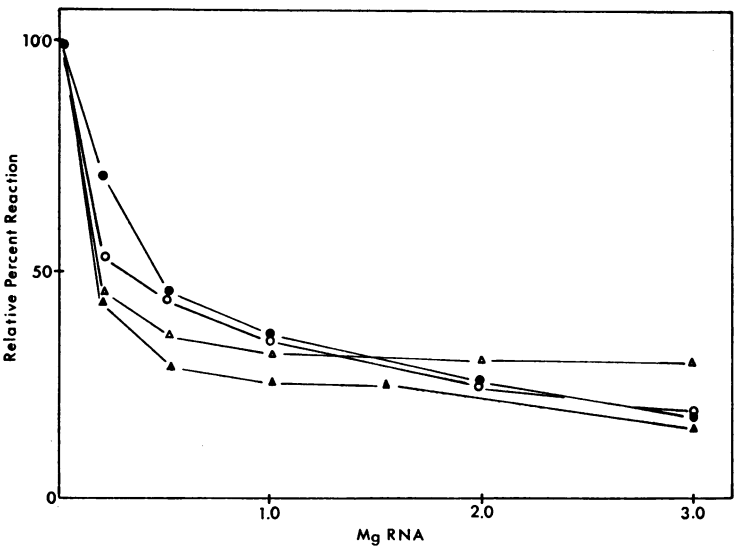

FIG. 2.—Comparison of RNA populations prepared from livers of the laying hen, 105-min estrone-treated pullets, 5-day estrone-treated pullets, and untreated pullets. 35 μg of H^3-labeled laying hen liver RNA and 27 μg of filter-bound DNA were placed in each reaction vial in the presence of increasing amounts of unlabeled liver RNA (*abscissa*) from untreated pullets (△—△), 105-min estrone-treated pullets (○—○), 5-day estrone-treated pullets (●—●), and laying hen (▲—▲) (homologous RNA). Reaction volume was 0.36 ml. Conditions for the annealing reaction were the same as given in Fig. 1. See Fig. 1 for definition of the ordinate.

ent. In other words, is part of the estrone-induced RNA synthesis organ-specific? To examine this question we compared the effectiveness of unlabeled oviduct RNA from estrone-treated and untreated pullets in competition with labeled liver RNA from estrogenized pullets. As shown in Figure 3, similar competition curves were obtained when oviduct RNA's from estrone-treated and untreated pullets were used as competitors against labeled liver RNA from estrone-treated pullets. If estrone evoked the production of some of the same or similar uncommon RNA species in the liver and oviduct, oviduct RNA from estrone-treated pullets would be expected to yield greater competition against labeled liver RNA from estrone-treated pullets than would oviduct RNA from untreated pullets. Since this is not the case, it appears that most of the estrone-evoked RNA species of these two responding organs are different, and hence *organ-specific*. Competition curves (Fig. 3) also show that a majority of the RNA species present in the liver are also present in the oviduct.

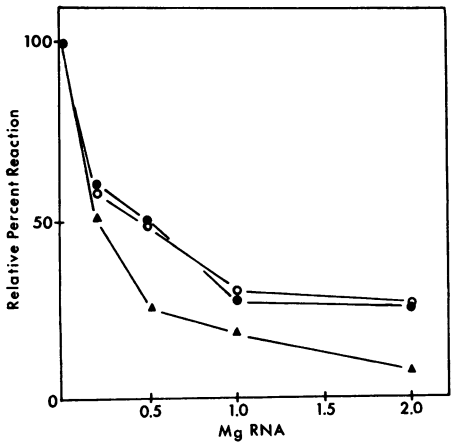

FIG. 3.—Comparison of oviduct RNA from 5-day estrone-treated pullets and untreated pullets as competitors for hybridization sites on DNA against H^3-labeled liver RNA from 105-min estrone-pretreated pullets. 15 μg of H^3-liver RNA from estrone-treated pullets were placed in reaction vials containing 27 μg of filter bound DNA. Unlabeled oviduct RNA from estrone-treated pullets (O—O), untreated pullets (●—●), or unlabeled liver RNA from estrone-treated pullets (▲—▲) were added in increasing amounts as indicated (*abscissa*). Reaction volume was 0.45 ml. Conditions for hybridization were the same as in Fig. 1. Definition of the ordinate is the same as for Fig. 1.

Discussion.—Estrogen-evoked liver RNA synthesis in the domestic fowl is comparable to that observed in another oviparous vertebrate, the lizard *Uta stansburiana*.[13] RNA synthesis in both these oviparous vertebrates is promptly accelerated after estrogen administration, and evidence of the formation of new gene products can be obtained by use of nucleic acid annealing techniques. It appears that the genes involved in vitellinogenesis in oviparous vertebrates are conservative in the sense of evolution because the yolk proteins are similar. Cross-species DNA-RNA hybridization studies, which are currently in progress, may provide evidence supporting this possibility.

Studies in which actinomycin D has been administered to estrogenized cockerels indicate that a large population of templates for phosphoprotein synthesis is established within the first few hours following treatment.[17] Actinomycin D when given four to six hours after treatment with diethylstilbestrol has much less effect on plasma protein P^{32} levels than when it is given simultaneously with the hormone. Our findings, which indicate that the livers of immature 105-minute estrone-pretreated pullets contain (qualitatively) most, if not all, of the RNA species that are present in the livers of laying hens, are compatible

with results from actinomycin D experiments. In our studies, even though estrone was given in what must be considered tentatively as nonphysiological or pharmacological doses (the level of estrone in the blood is not known in our animals), the nucleic acid system response seems to be at least qualitatively "normal" relative to the laying hen, which has a comparatively high endogenous estrogen level.[18]

Qualitative differences between the liver RNA populations of estrogenized and untreated pullets, shown by competition experiments in this study, should be considered as minimum estimates of the actual differences. As McCarthy has pointed out,[19] the base-sequence redundancy of related genes in the genomes of higher organisms allows for the formation of a variety of partially mismatched hybrids. Thus, if some of the newly evoked RNA molecules form stable hybrids (under the conditions used in our experiments) with DNA base sequences of related genes that were being actively transcribed prior to hormone treatment, their presence may be obscured as a result of cross-hybridization.

Some of the estrone-evoked RNA species that are unique for the liver are no doubt necessary for the synthesis of yolk proteins. Since vitellinogenesis is the dominant hepatic biosynthetic process (at least in a quantitative sense) that is induced by estrogen in oviparous vertebrates, it is tempting to suggest that most of the changes in the liver RNA population are associated with yolk protein synthesis. Indeed, the close similarity of the liver RNA populations of the 105-minute estrone-pretreated pullets and laying hens supports this possibility (Fig. 2). Some cell division does occur in livers of estrogenized pullets. However, it is unlikely that many of the new RNA species which appear following estrogen administration are associated with this process, since the liver RNA population closely resembles that of the laying hen in which cell division is slight. A variety of templates are probably necessary in addition to those for the yolk proteins themselves, because vitellinogenesis is a complex process involving enzymatic changes, alteration in lipid metabolism, etc.[9, 11]

DNA-RNA hybridization analyses indicate that estrone evokes the transcription of RNA species in the liver which are, at least in large part, unrelated to certain estrone-induced RNA species in the oviduct. Thus the specificity of RNA synthesis with respect to these two estrogen target organs is in part organ-dependent rather than hormone-dependent. This is not surprising, since a steroid hormone is intrinsically a "low-information" molecule. Since it appears that precise endocrine information does not reside in the steroid alone, it is likely that specificity is generated when the hormone interacts with some yet-to-be identified molecules which are organ- or cell-specific. Stereospecific estrogen-receptor molecules are present in the uterus, but their roles in the formulation of endocrine information and subsequent hormone action remains to be determined.[20] Density-gradient centrifugation analyses indicate that H^3-estradiol is bound to a protein component of uterine chromatin and it is apparently not metabolized as long as this association is maintained.[21] Studies on pea nuclei indicate that the enhancement of RNA synthesis (exogenous gibberellin) is dependent on some cytoplasmic factor, but that the intact cell is not necessary for a specific response.[22] However, studies on RNA synthesis in thyroid nuclei

exposed to thyroid-stimulating hormone suggest that in the case of this polypeptide hormone, cytoplasmic factors may not be necessary.[23] It is likely that a number of organ-specific hormone-receptor molecules will be found. If a single hormone induces the synthesis of RNA species that are unique to the responding cells, it is apparent that the specificity of the response resides in the responding cells of the respective organs or tissues, and it is likely that the mechanisms necessary for specific responses involve stereospecific receptors and perhaps certain yet-to-be-identified rapid-turnover nuclear proteins.

The synthesis of all classes of liver RNA is accelerated by estrogens.[13] For example, the synthesis of ribosomal RNA is markedly increased. Thus, estrogens, in addition to inducing the synthesis of organ-specific RNA species, also stimulate the synthesis of many common (non-organ-specific) RNA species that may be necessary for the augmentation of protein synthesis.

Recent studies have indicated that in higher organisms more of the genome is transcribed than is actually translated.[24, 25] Hence hormonal action at the level of messenger transport and translation is a strong possibility. Recent data indicate that estradiol induces the transcription of new organ-specific nuclear RNA species in the rabbit uterus and that some of these new species, but not all, also appear in the cytoplasm.[26] Therefore a comparison of nuclear and cytoplasmic RNA may reveal differences in hormonal response of given target organs that are "masked" when total RNA is examined. It is likely that when detailed studies on nuclear and cytoplasmic RNA are done, hormonal regulation (direct or indirect?) at the level of transport and translation of genetic information will be substantiated. A comparison of various organs in terms of their nuclear and cytoplasmic RNA species indicates that greater differences are found when cytoplasmic RNA is examined.[27] Since selective transport of RNA from the nucleus to the cytoplasm seems to be one of the mechanisms involved in cellular differentiation,[28, 29] it may also be a means whereby a differential response to a hormone, which is organ- or cell-specific, is achieved.

Changes in RNA synthesis induced by estrone, as shown in our experiments, are not necessarily part of the primary action of this hormone. They do, however, further substantiate that hormone action is mediated, at least in part, by the nucleic acid system and that some of the specificity of hormone action can be accounted for by the induced synthesis of specific RNA molecules.

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