also indicate a fusion of viral envelope sequences to *sis* sequences is not a sole criterion for transformation by *sis* genes.

Although pSM-1 represents an incomplete cDNA transcript, it is likely that the sequence shown here encodes the entire precursor for PDGF chain A. This is suggested by the presence of an upstream TGA stop codon at position 1 which is in phase with the open reading frame of pSM-1. Thus, the ATG at position 118 is probably the actual initiator codon for the c-*sis* protein product. Sequences of a second c-*sis* cDNA clone, pSM-2, showed that it contained 11 base pairs 5′ to the sequences of pSM-1 with the TGA stop codon at the same position as in pSM-1 (*25*). Therefore, the TGA codon was not generated as a cloning artifact. The stop codon of pSM-1 is also in phase with the SV40 initiator between the 16*S* donor and acceptor splice sites, so that no read-through SV40-*sis* product is expected.

The ability of pSM-1 to transform 3T3 cells after transfection in contrast to the inability of PDGF to transform cells when applied externally is probably due to the intracellular overproduction of PDGF chain A as a consequence of transcriptional activation by the SV40 promoter. This overproduction could result in (i) differences in the posttranslational processing transport or compartmentalization of the c-*sis* gene product, (ii) differences in the subunit structure of the pSM-1 product and PDGF, that is, a homodimer instead of a heterodimer as in PDGF, or (iii) qualitative or quantitative differences in the interaction of PDGF chain A with cellular receptors.

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Independent Developmental Programs for Two Estrogen-Regulated Genes

Abstract. *Measurement of hepatic apolipoprotein II and vitellogenin II messenger RNA during chicken embryogenesis showed that these genes acquire estrogen responsiveness at different stages of development. Sensitive solution hybridization assays with excess complementary DNA showed that apolipoprotein II transcripts were induced to 500 molecules per cell at day 9, whereas induction of vitellogenin II messenger RNA was not found until day 11. Thus, two estrogen regulated genes of common function and coordinately regulated in the adult may be on independent developmental programs.*

Embryonic chick liver is a useful system to study the acquisition of responsiveness to the steroid hormone estradiol. As in the mature hen or hormone-treated rooster, estradiol induces the hepatic synthesis of egg yolk precursor proteins in the competent chick embryo. Proteins induced by estradiol include the very low density apolipoproteins B (apo-B) and II (apo-II) and the highly phosphorylated vitellogenins (VTG) (*1*). Synthesis of apo-II is inducible by estradiol

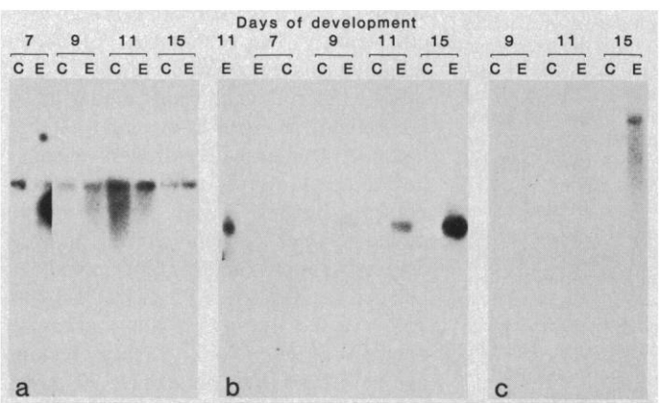

Fig. 1. Ontogeny of inducibility of apo-II and VTG-II mRNA's as assayed by Northern hybridization. For the indicated days of development, 5 μg of poly(A)$^+$-enriched RNA from the livers of estrogen-treated (E) or control (C) chick embryos were subjected to electrophoresis through 1.5 percent agarose gels containing 5 m*M* methylmercuric hydroxide. The RNA was transferred to nitrocellulose, and the blots were hybridized overnight with nick-translated recombinant plasmids (0.5×10^6 to 1.0×10^6 count/min) containing sequences complementary to chicken serum albumin mRNA (a), apo-II mRNA (b), or VTG-II mRNA (c). In each case the extent of migration corresponded to that expected for each mRNA in comparison to ribosomal RNA markers (*6*). Estrogen treatment consisted of injection of 1.25 mg of estradiol-17β in 50 μl of propylene glycol just beneath the air sac membrane 48 hours before the embryos were killed. Controls were given the vehicle alone.

by day 10 of embryonic development, but synthesis of VTG is not inducible until day 12 (*2*). Because the expression of the genes encoding these proteins is coordinated in the adult (*3*), the embryonic dissociation suggests that genes regulated by estrogen may acquire hormone responsiveness via independent transitions. However, measurements made at the level of protein synthesis are not sufficiently sensitive to warrant this conclusion. In addition, the failure to detect VTG-II synthesis before day 12 of development could reflect the absence of the complex biosynthetic pathway for this highly phosphorylated glycoprotein (*4*) as opposed to a lack of responsiveness at the level of the gene. We now report that the embryonic dissociation in apo-II and VTG-II responsiveness is due to events occurring at the level of transcription.

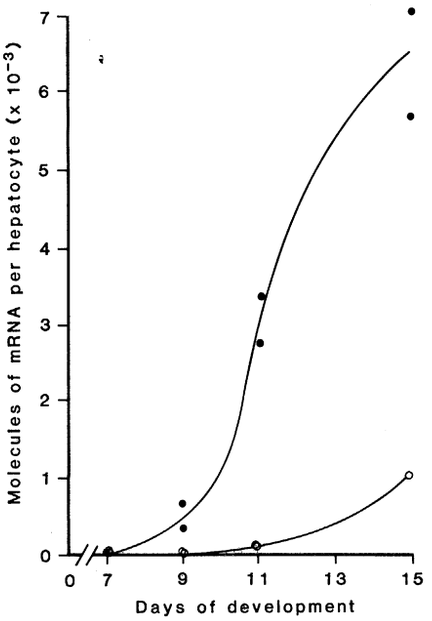

Fig. 2. Apo-II and VTG-II mRNA concentrations in liver RNA from estrogen-treated embryos as measured by solution hybridization with excess DNA. Single-stranded complementary DNA probes (100 pg) were hybridized with varying concentrations of total cellular RNA in 0.05 ml of 0.03*M* tris-HCl (*p*H 7), 0.3*M* NaCl, and denatured calf thymus DNA (100 μg/ml) at 68°C for 60 hours. The sample was digested with S1 nuclease (*6*), and hybrids were collected by acid precipitation. Values for mRNA are expressed as molecules per hepatocyte calculated from the hybridization value, the probe specific activity, an RNA per hepatocyte value of 10.9 pg, and rooster liver composition of 70 percent hepatocytes (*13*). With the exception of apo-II on day 7 and VTG-II on day 15, two separate RNA preparations from pooled livers of 7 to 8 dozen estrogen-treated and control (not shown) embryos were analyzed at each of the stages indicated for apo-II mRNA (●) and VTG-II mRNA (○). Single-stranded probes were prepared from complementary DNA clones for VTG-II and apo-II by replacement synthesis with T4 DNA polymerase (*14*). The VTG-II probe was the 580-base-pair Ava I–Hind III fragment (*6*), and the apo-II probe was the internal 240-base-pair Pst I fragment (*6*). Strand separation was achieved by hybridization with excess poly(A)$^+$ RNA from liver of estrogen-treated roosters and subsequent S1 nuclease digestion and alkaline hydrolysis. The resulting single-stranded probes were less than 1 percent resistant to S1 nuclease.

The Northern blotting procedure (*5*) was used to screen samples of polyadenylated [poly(A)$^+$] RNA from livers of estrogen-treated or control embryos at various stages of development. Hybridization with a probe specific for albumin messenger RNA (mRNA) (*6*) was used as a positive control in these experiments. The serum albumin gene was transcribed as early as day 7 of embryonic development and throughout the period under investigation in both estrogen-treated and control embryos (Fig. 1a). Hybridization of the RNA with apo-II or VTG-II probes did not reveal these mRNA's at any stage of development in control embryos (Fig. 1, b and c). In estrogen-treated embryos, apo-II mRNA was barely detected by day 9 and greatly increased by days 11 and 15 (Fig. 1b). In contrast, VTG-II mRNA was not detected by day 9, was barely detected by day 11, and greatly increased by day 15 (Fig. 1c).

Northern analysis confirmed the temporal dissociation in apo-II and VTG-II responsiveness as observed at the level of protein synthesis (*2*). However, since this analysis was only semiquantitative and was carried out with poly(A)$^+$ RNA, we could not eliminate the possibility that VTG-II mRNA was induced by day 9 but was below the limit of detection or was not polyadenylated. A quantitative analysis of apo-II and VTG-II mRNA induction was obtained by a sensitive solution hybridization assay with excess single-stranded DNA probes of high specific activity. This procedure (Fig. 2) can reliably measure one molecule of VTG-II mRNA per cell when total cellular RNA is assayed. In liver from embryos that had been treated with estrogen 48 hours before assay, apo-II mRNA was barely detected by day 7, was present at 500 molecules per cell by day 9, and increased to approximately 6500 molecules per cell by day 15. VTG-II mRNA was not detected by day 7, was marginally detected at day 9 (one to ten molecules per cell), and was present at 70 molecules per cell by day 11 and 1000 molecules per cell by day 15. Thus the ratio of apo-II to VTG-II transcripts changed from about 50:1 by days 9 and 11 to 6:1 by day 15. In adult birds, the ratio of apo-II to VTG-II transcripts at all stages between 1 and 5 days after estrogen treatment is 3:1 or 4:1 (*3*). Thus we conclude that by day 9 little significant estrogenic induction of VTG-II mRNA occurs compared to apo-II mRNA, and even at day 11 VTG-II induction is considerably retarded.

Earlier studies on the embryonic development of the estrogen receptor system showed that nuclear receptor could be detected in 12-day-old embryos that had been injected with estradiol 2 days before assay (*1*). With an improved assay (*7*) we found a small amount of receptor in nuclear extracts prepared on day 9 from embryos injected with estradiol on day 7 (Fig. 3). Nuclear receptor was not detectable at earlier stages. After day 9, nuclear receptor concentrations increased in parallel with the induction of apo-II mRNA by estradiol.

The appearance of specific steroid receptors in development has been linked to the capacity for hormonal responsiveness in several systems (*8*). The parallel development of nuclear receptor and apo-II inducibility suggests that the apo-II gene acquires competence at or before the appearance of receptor and that the extent of induction is directly related to the concentration of nuclear receptor. However, for the VTG-II gene responsiveness is not proportional to the development of the receptor system. The dissociation in responsiveness of the apo-II and VTG-II genes might be explained by different requirements for or sensitivity to the limited quantity of nuclear recep-

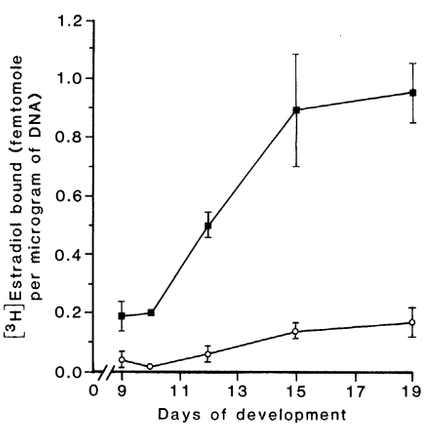

Fig. 3. Ontogeny of estrogen-induced, salt-soluble nuclear estrogen receptor activity. Embryonated eggs were injected with 1.25 mg of estradiol-17β in 50 μl of propylene glycol, and hepatic nuclear estrogen receptor concentrations were determined 48 hours later (*7*). The number of samples analyzed for each stage was as follows: day 9, 2; day 10, 1; day 12, 6; day 15, 6; day 19, 2. The results are expressed as the mean ± standard deviation except for days 9 and 19 where they are expressed as the mean ± range. Upper curve, estrogen-treated embryos; lower curve, controls given propylene glycol vehicle.

tor present at days 9 and 11. However, this seems unlikely since nuclear receptor increases in this interval whereas the ratio of apo-II to VTG-II responsiveness is the same at days 9 and 11. In addition, we have not detected significant differences in the sensitivity of the apo-II and VTG-II genes to low doses of exogenous estrogens in 2-week-old chicks (*9*).

A further consideration is that acquisition of hormone responsiveness may be associated with modifications in gene or chromatin structure that do not take place in coordinate fashion for all estrogen responsive genes. For example, the methylation status of regions surrounding the apo-II gene changes between days 7 and 9 of development (*10*). Such changes could reflect the transition of the apo-II gene to the responsive state. In contrast, the VTG-II gene remains fully methylated before the administration of hormone and shows only minor changes in methylation status even in the adult (*11, 12*). The VTG-II gene shows some evidence of developmental change as judged by tissue-specific nuclease hypersensitive sites present at day 9 (*12*). However, since these changes also occur in the oviduct, they are not sufficient for estrogen inducibility of the VTG-II gene. These results may indicate that hormone responsiveness for each gene is acquired in a series of independent events. The comparison of apo-II and VTG-II responsiveness (Fig. 2) also shows that genes that are of common function and coordinately regulated in the adult may be on independent developmental programs during embryogenesis.

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Calcium Source for Excitation-Contraction Coupling in Myocardium of Nonhibernating and Hibernating Chipmunks

Abstract. *The amplitude of the early plateau phase of the action potential and the slow action potential of cardiac muscle were much lower in hibernating chipmunks than in nonhibernating chipmunks. The frequency-dependent contraction was decreased in hibernating animals but increased in nonhibernating animals. Caffeine caused a negative inotropic effect in hibernating animals but a positive inotropic effect in nonhibernating animals. Ryanodine caused greater inhibition in hibernating animals than in nonhibernating animals. These results suggest that the respective roles of the sources of calcium for cardiac excitation-contraction coupling are changed during hibernation.*

The hearts of hibernating animals, in vivo and in vitro, continue to function under temperature conditions (below 5°C) at which the hearts of nonhibernating animals cease to function (*1*). Changes in the electrocardiogram and in myocardial performance that occur in animals during hibernation, such as prolongation of various waves and intervals or a marked reduction in heart rate, have been characterized (*2*). However, the physiological mechanism responsible for changes in myocardial performance during hibernation is not well defined. We used microelectrode techniques to investigate the electromechanical characteristics of isolated papillary muscle from hibernating and nonhibernating chipmunks. Our results suggest that hibernation causes marked changes in some fundamental characteristics of cardiac muscle function in this species.

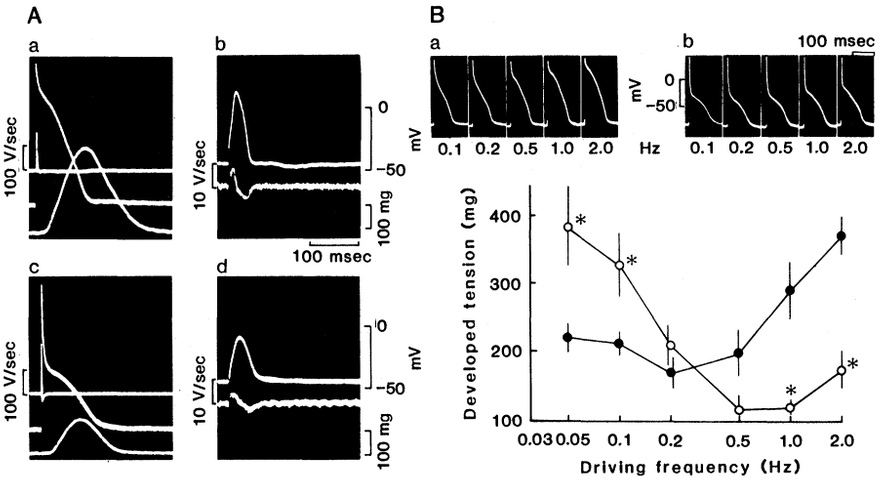

Fig. 1. (A) Transmembrane action potentials and slow action potentials in preparations from nonhibernating (a and b) and hibernating (c and d) animals. Both preparations in normal medium (a and c) and in the high K^+ medium (b and d) were driven at 1.0 Hz and 0.5 Hz, respectively. The first derivative of the action potential is shown in the top trace in (a) and (c) and in the lower trace in (b) and (d). A membrane action potential is shown by the middle trace in (a) and (c) and by the upper trace in (b) and (d). The bottom trace in (a) and (c) shows a developed tension. (B) Frequency-dependent changes in the membrane action potential (a and b) and contractile activity (graph) on preparations from nonhibernating [(a) and (●) on graph] and hibernating animals [(b) and (○) on graph]. The driving frequency is shown under each panel. Values plotted on the graph represent the mean ± standard error of the mean of six preparations. The asterisk indicates that the difference between the preparations from hibernating and nonhibernating animals is significant. The probabilities were adjusted by Bonferroni type statement.