bonded phase, fused silica column). Single-ion monitoring was used to detect terpanes (mass-to-charge ratio m/z = 191) and steranes (m/z = 217). The use of trade names here and elsewhere in this report is for descriptive purposes only and does not constitute endorsement by the U.S. Geological Survey.
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Estrogen Memory Effect in Human Hepatocytes During Repeated Cell Division Without Hormone

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Transient stimulation of target tissues by sex steroids can cause long-lasting changes that may facilitate or alter responses to subsequent hormonal treatment. How these altered characteristics are propagated during cell division in the absence of the stimulating hormone is unknown. The human hepatocarcinoma cell line HepG2 was used as a model to examine the effects of estrogen on the synthesis of serum apolipoproteins in vitro. Treatment with low concentrations of estrogen for 24 to 48 hours resulted in long-lasting alterations in the kinetics with which the cells responded to subsequent stimulation with estrogen. Manifestation of this memory effect was correlated quantitatively with the induction and propagation of a moderate-affinity, nuclear, estrogen-binding protein with the characteristics of a type II estrogen receptor. The data indicate that transient exposure of these cells to estrogen can induce changes in their response characteristics and composition of nuclear proteins that are inherited by daughter cells grown in the absence of hormone for more than ten generations.

MANY EFFECTS OF SEX STEROIDS on gene expression in their target tissues are dependent on the continued presence of hormone. However, in some cases, transient stimulation is sufficient to induce long-lasting alterations in the ability of the tissue to respond to subsequent hormonal treatment. Persistence of these altered response characteristics for several months, even in very young animals, has raised the possibility that this so-called priming or memory effect may not simply be long lasting, but might be propagated during growth of the tissue in the absence of the stimulating hormone. How this is accomplished is unknown. A particularly well-characterized example of priming has been provided by studies of vitellogenesis in avian and amphibian liver. Short-term primary stimulation of adult male or immature animals with estrogen results in transient activation of genes specifying major yolk proteins, such as vitellogenin, together with long-lasting alterations in the kinetics with which they respond to secondary stimulation (*1–4*). To date, this long-term effect of estrogen on hepatic gene expression has been shown to occur only in egg-laying vertebrates. We now report that (i) an estrogen-memory effect can be reproduced entirely in vitro in an established line of human hepatoma cells and (ii) manifestation of this effect after repeated cell divisions in the absence of hormone correlates quantitatively with the induction and propagation of a moderate-affinity, nuclear, estrogen-binding protein with characteristics of the type II estrogen receptor.

The human hepatoma line HepG2, which was established in culture by Knowles *et al.* (*5*), has been shown to retain many of the differentiated characteristics of normal hepatocytes, including the ability to synthesize the major serum apolipoproteins of hepatic origin. Because of the importance of these proteins in determining atherosclerotic risk, we examined the possibility of using these cells as an in vitro model to study hormonal regulation of their synthesis. Our studies revealed that estrogen has two quite distinct dose-dependent effects on the synthesis of these proteins (*6*). At concentrations in the physiological range, it induces increases in the levels of messenger RNA's (mRNA's) specifying apolipoproteins apoAI and apoCII (*7, 8*), both of which are components of nascent high-density lipoprotein particles. At 25- to 50-fold higher concentrations, the hormone increases synthesis of two additional apolipoproteins, apoE and apoB, which are major components of nascent very low density lipoproteins (VLDL's), by a mechanism that also involves alterations in apolipoprotein mRNA levels (6).

In addition to requiring different concentrations of hormone, induction of these two pairs of apolipoproteins was found to occur with different kinetics. Induction of apoB and apoE, unlike that of apoAI and apoCII, was characterized by a lag of 6 to 8 hours (*6*). However, if the cells were preincubated for 24 to 48 hours in estrogen at concentrations well below the threshold required to induce apoB and apoE, the lag in induction of these two proteins on exposure to high concentrations of the hormone could be eliminated. This difference between the response characteristics of control and pretreated cultures is reminiscent of differences between the primary and secondary induction kinetics of avian and amphibian vitellogenin genes. The observation prompted us to examine whether these human cells also display a memory effect similar to that observed in the liver of egg-laying animals. Since established lines of amphibian and avian hepatocytes do not exist, this also provided for the first time the opportunity to examine the propagation of such an effect in a homogeneous population of dividing cells.

Newly confluent cells were maintained for 48 hours in medium containing 17β-estradiol at an initial concentration of 20 n*M*. This treatment maximally induces apoAI and apoCII but does not alter the production of

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apoB and apoE (*6*). Cells were then replated and allowed to divide six times in the absence of estrogen. When these pretreated cells were subsequently exposed to high concentrations of 17β-estradiol, enhanced synthesis of apoB and apoE occurred with no appreciable lag (Fig. 1A). Similar experiments were performed with cells after 10 and 16 generations. The rates of synthesis of apoB and apoE 6 hours after reexposure of cultures that had undergone six or ten cell divisions after pretreatment were approximately five and four times as great, respectively, as those in cells not previously exposed to estrogen. By comparison, pretreated cells that were shifted directly to a high concentration of estrogen without intervening growth in the absence of hormone had rates of synthesis of apoB and apoE approximately eight times those of controls (*6*). Thus, on a per cell basis, the magnitude of the memory effect after ten generations was still 50% of that observed in a fully primed culture, even though the cell population had increased more than 1000-fold during growth in the absence of hormone. It was not until the cells had divided 15 or 16 times in the absence of estrogen that the response kinetics became indistinguishable from those of control cells.

Studies of vitellogenesis in avian liver have drawn attention to relatively long-lasting, hormonally induced alterations in patterns of methylation and nuclease hypersensitivity of genes specifying the major yolk apolipoproteins, vitellogenin and apoVLDL II (*9–12*). It has been suggested that these modifications could reflect changes in chromatin structure that are involved in the persistence and possibly the propagation of the memory effect. However, in at least some cases, it appears unlikely that the modifications are maintained long enough to be directly involved in the memory response (*12–13*). Alterations in the spectrum of nuclear proteins that could be required to maintain such modifications, or to facilitate activation of the genes by some other mechanism, have not yet been identified. Studies detailed below suggest the lower affinity type II estrogen-binding protein described by Clark *et al.* (*14*) as a candidate for such a function.

Exposure of HepG2 cells to low concentrations of estrogen induces a 14- to 15-fold increase in high affinity type I estrogen receptors and, after a lag of several hours, a comparable increase in lower affinity nuclear binding sites that display the extreme sensitivity to reducing agents that is characteristic of type II estrogen-binding proteins (*8*). These lower affinity, matrix-associated sites have been identified in nuclei from a number of estrogen target tissues, and it has been suggested that they could play an as yet undefined role in cellular proliferation (*14–16*). However, the liver does not display a typical mitotic response to estrogen, nor are the growth characteristics of HepG2 cells altered by exposure to the hormone. We have proposed—on the basis of their induction kinetics, their lower affinity for estrogen, and the ability to induce them with low concentrations of hormone—that in HepG2 cells type II sites could be involved in mediating the enhanced synthesis of apoB and apoE in response to high levels of estrogen (*6*). This suggestion is not inconsistent with the proposed role of type II sites in other target organs since high levels of apoB and apoE may serve to meet the increased demand for cholesterol during estrogen-induced proliferation of nonhepatic tissues. If type II sites do fulfill such a function in liver, the memory effect observed after priming of the cells with estrogen requires that, once induced, the type II sites should be propagated in daughter cells in the absence of hormone. Experiments designed to test this possibility are described in Fig. 1B.

Type I and type II sites can be induced in HepG2 cells during either the log phase of cell growth or in confluent cultures, with no detectable difference in the extent or kinetics of induction. In the experiments described here, newly confluent cultures were treated with low concentrations of estrogen for 48 hours to fully induce type I and type II binding sites. During this period there was no significant change in cell number. When the hormone was removed, nuclear type I receptors declined to preinduction levels within 24 hours (Fig. 1, inset). To some extent, the extremely rapid decrease observed during the first 3 to 6 hours could be accounted for by an increase in the proportion of receptors recovered in cytoplasmic fractions. However, within 40 hours the cytoplasmic levels of receptor also declined to control values. The loss of type I receptors was even more rapid when the cells were harvested and replated after removal of the hormone. Thus basal levels were reestablished within approximately one doubling time.

In contrast, type II sites were maintained during both replating and subsequent cell division. The number of sites per cell declined by less than 10% at each division, so that, for example, after six divisions in the absence of hormone the levels were still 60% to 65% of maximum (Fig. 1B). A theoretical dilution curve, based on the assumption that type II sites are infinitely stable after

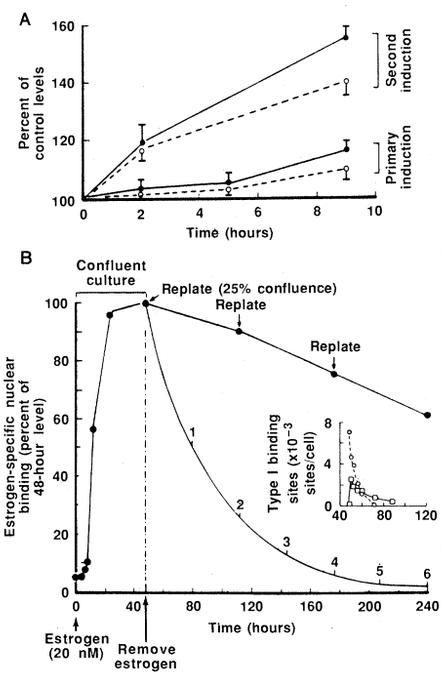

Fig. 1. (A) ApoB and apoE induction in HepG2 cells treated with estrogen and propagated in the absence of hormone. Newly confluent cultures were maintained for 48 hours in medium that was adjusted to 20 n*M* in 17β-estradiol every 12 hours. The cells were then washed three times, replated, and propagated for six divisions in the absence of hormone for 10 days. Confluent cultures were reexposed to estradiol (1.0 μ*M*), and the levels of newly secreted apoB and apoE were determined by radioimmunoassay (*19*). Similar analyses were carried out on cultures treated with estrogen for the first time. Results are expressed as percentages of the levels of each apolipoprotein in cultures of pretreated cells that were not reexposed to hormone. Primary induction: apoB (●) and apoE (○); secondary induction: apoB (●) and apoE (○). Results shown are the means of three experiments with error bars indicating one SEM. (B) Propagation of type II nuclear, estrogen-specific binding sites in HepG2 cells after hormone withdrawal. The numbers of type II sites were monitored in cells treated with estrogen and subsequently propagated in the absence of hormone as described in (A). The line without data points represents a theoretical dilution curve of the number of type II sites per cell, assuming that no synthesis or turnover occurs after hormonal withdrawal. Numbers above the line indicate the number of cell divisions that have taken place since the removal of estrogen. Inset: Kinetics of disappearance type I sites from nuclei and cytosol of HepG2 cells after estrogen withdrawal. Newly confluent cells were maintained in medium containing 20 n*M* 17β-estradiol for 48 hours. Cells were then washed three times and cultured in control medium; the number of type I sites in nuclear (○) and cytosolic (□) fractions were plotted as a function of time after hormonal withdrawal. Data shown are the means of two independent experiments. For all data points independent results were within 10% of the mean. Procedures for cell growth and maintenance and for radioimmunoassay of apolipoproteins have been described in detail (*19*). Specific binding of 17β-[^{3}H]estradiol to nuclear type I and type II binding sites was carried out by modification of the method of Lazier and Jordan (*20*).

hormone withdrawal but are not produced in daughter cells, is also illustrated (Fig. 1B). Under these circumstances, the predicted number of sites per cell after six divisions would be only 2% to 3% of that observed. Analyses at later times indicated that it took more than 15 generations for the levels of type II sites to become indistinguishable from those in control cells. It is now possible to distinguish between a uniform decrease in the levels of type II sites in all cells and a propagation mechanism that results in a small percentage of cells at each division (7% to 8%) that do not express these sites. However, there is clearly an excellent quantitative correlation between the levels of type II sites and the magnitude of the memory effect, as revealed by comparison with data on the kinetics with which apoB and apoE synthesis increases after restimulation of pretreated cells (Fig. 2).

Since induction of type II sites lags behind that of type I sites by 5 to 6 hours, we examined the possibility that brief exposure to estrogen (5 to 6 hours) might trigger a sequence of events that would result in subsequent appearance of type II sites. Cells treated in this fashion were also examined to determine whether they displayed any evidence of a memory effect 48 hours after removal of estrogen. After 6 hours of treatment with 20 n*M* 17β-estradiol, type I sites reached 80% of maximum whereas type II sites increased by less than 10%. Removal of hormone at this time resulted in loss of type I receptors within 24 hours and no subsequent increase in type II sites. Thus, the presence of estrogen or type I receptors (or both), although not necessary for the propagation of type II sites, is required during at least part of their initial accumulation phase. Consistent with the suggestion that the type II sites are involved in the memory response, the cells displayed apoB and apoE induction kinetics that were indistinguishable from those of control cultures.

The ability to induce type II sites with comparable efficiency in dividing and confluent cells strongly suggests that cell division is not required. However, the lag of several hours that precedes induction of the type II sites does raise the possibility that limited DNA synthesis may be necessary. We have tested this possibility by carrying out experiments identical to those described in Fig. 1, except that the cells were treated with hydroxyurea (0.5 m*M*) for 6 hours before addition of estrogen and were maintained in the presence of the inhibitor for the entire 48-hour induction period. This concentration of hydroxyurea is adequate to reduce DNA synthesis, as determined by incorporation of [^{3}H]thymidine, by more than 90%. Despite the inhibition of DNA

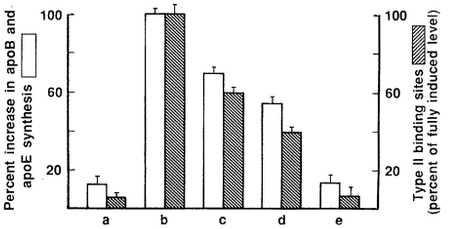

Fig. 2. Correlation of the levels of type II receptors and the kinetics of induction of apoB and apoE in HepG2 cells pretreated with estrogen and propagated in the absence of hormone. Cells were pretreated with 17β-estradiol (20 n*M*) and subsequently grown in the absence of hormone as described in Fig. 1A. After 6, 10, or 16 divisions the levels of type II sites were determined and the cells then exposed to a high concentration of the hormone (1 μ*M*). The increase in apoB and apoE synthesis, relative to control cells, was determined 6 hours later. The number of type II sites per cell is expressed as a percentage of the maximum level detected in cells maintained in 20 n*M* 17β-estradiol for 48 hours. Increases in the synthesis of apoB and apoE are expressed relative to those obtained when cells that had been pretreated with 20 n*M* estrogen for 48 hours were shifted directly to medium containing the hormone at a concentration of 1 μ*M*. Data shown were obtained from (a) untreated cells; (b) cells treated with 20 n*M* 17β-estradiol for 48 hours and shifted directly to medium containing the hormone at a concentration of 1 μ*M*; and (c to e) cells that had completed 6, 10, or 16 cell divisions in the absence of hormone, respectively, before reexposure to 1 μ*M* 17β-estradiol. Results shown are the means and standard errors of six independent experiments.

synthesis, hydroxyurea had no effect on either the kinetics of type II induction or the number of sites per cell. The sites were also propagated as efficiently as in untreated cells if the hydroxyurea was removed and the cells were allowed to divide.

In experiments that will be described in detail elsewhere, we examined the possibility that type II sites also play a role in the vitellogenic memory response observed in liver of egg-laying animals. To determine whether these sites could be propagated in vivo, we treated chicken embryos with a single injection of 17β-estradiol at day 13 of gestation. The numbers of type II sites and the rapidity of the vitellogenic response were then examined 17 days after hatching. Hepatic type II sites were present in chicks treated in ovo with amounts approximately 75% of those in laying hens and 35 to 40 times those in control chicks. Even 10 weeks after a priming injection, the numbers of type II sites were still 10 to 12 times those in control chicks. Analysis of vitellogenin mRNA levels also indicated that the pretreated chicks displayed an enhanced response to secondary stimulation. Since the DNA content of the liver increases 25- to 50-fold between day 13 of embryogenesis and 17 days after hatching, the data strongly suggest that type II sites and the memory effect can also be propagated in vivo in the absence of estrogen (*13*).

The ability of type II sites to be propagated in the absence of the original induction stimulus and the fact that their persistence correlates with manifestation of the estrogen memory effect suggests parallels with the predicted properties of factors proposed to be involved in maintenance of the determined state. It is possible that their expression could be the consequence of hormonally induced, heritable alterations in chromatin or DNA structure (*9, 10, 17*). These could be reflected by changes in nuclease hypersensitivity or specific demethylation events that take place during the lag period preceding induction of the sites. Both types of modification have been shown to be induced by estrogen, and similar alterations induced by other effectors have been shown to be capable of propagation. However, if this type of mechanism is involved, data both in vivo and in vitro indicate that, in this case, establishment of these alterations does not require cell division. Thus if demethylation plays a role, it cannot be accomplished simply by prevention of maintenance methylation. Since exposure of HepG2 cells to estrogen for the duration of the lag period does not result in the subsequent production of type II sites, it is also apparent that such modifications, if they do occur, require the continued presence of estrogen or type I receptors to elicit at least initial induction of the type II sites. While this requirement can be rationalized by a number of possible mechanisms, one possibility that is consistent with all of the existing data and that has been shown to be involved in "irreversible" alterations in gene expression in prokaryotes is that type II sites positively regulate their own synthesis (*18*). Initial induction would then require the presence of type I receptor-estrogen complexes until the type II sites reached a critical concentration beyond which their production would be expected to be self-sustaining. The availability of an established cell line that displays a defined memory effect will allow various predictions of these and other possible mechanisms to be tested experimentally.

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Neuronal Properties of Clonal Hybrid Cell Lines Derived from Central Cholinergic Neurons

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Clonal cell lines derived from specific types of central neurons can be used to identify and characterize properties specific to those neurons. With somatic cell fusion techniques, nine clonal hybrid cell lines have been developed from the septal region of the mouse basal forebrain. Two lines express characteristics typical of cholinergic neurons—choline acetyltransferase activity and immunoreactivity, neurite formation with neurofilament protein immunoreactivity, and aggregation in rotation-mediated cell culture. These cell lines may be useful for studying the trophic interactions that support the development and maintenance of central cholinergic connections.

CHOLINERGIC NEURONS OF THE mammalian basal forebrain play an important role in learning and memory and degenerate in Alzheimer's disease (*1*). This degeneration may result from a dysfunction of the trophic interactions that support cholinergic connections (*2*). Recent studies on mediators of central cholinergic trophic processes have focused on the possible roles of nerve growth factor (NGF) and other substances (*3, 4*). Much of this work has used primary cell culture techniques, which should theoretically permit analysis at the cellular and molecular level. However, the use of such techniques to study specific trophic mechanisms is limited because even distinct areas of the mammalian central nervous system contain neurons with various neurotransmitter phenotypes and different synaptic connections. To study cholinergic trophic mechanisms with greater resolution, we developed clonal hybrid cell lines from the mouse septal region. Of nine lines generated, two manifested stable and constitutive properties typical of septal cholinergic neurons. The availability of permanent cell lines such as these could facilitate investigations of cholinergic neuronotrophic processes.

Embryonic murine septal cells were fused with cells of a murine neuroblastoma line, N18TG2, that are deficient in hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8) (*5*). Neuroblastoma cells are embryologically related to neurons (*5*), and thus are likely to permit the expression of neuron-specific traits (*6*). In addition, the murine origin of both parental cell types minimizes the chromosomal loss that often occurs in xenogeneic fusions (*7*). Finally, this neuroblastoma line is deficient in the specific cholinergic marker choline acetyltransferase (ChAT), the enzyme that catalyzes the synthesis of acetylcholine (*8*).

The septal region was dissected from C57BL/6 mouse embryos at day 14 of embryogenesis (*9*), dissociated, and resuspended in phytohemagglutinin-containing, serum-free, modified Eagle's medium (*10*). The septal cell suspension was then added to a monolayer culture of neuroblastoma cells. Phytohemagglutinin facilitated the adherence of septal cells to the N18TG2 monolayer. After a 15-minute incubation, the medium was aspirated and the cells were fused with a 50% (by volume) polyethylene glycol (PEG 1000, Koch-Light) solution (*11*). The fusion products were plated at 0.05×10^6 cells per 35-mm plate in medium containing 10% (by volume) fetal calf serum, $100 \times 10^{-6}M$ hypoxanthine, $0.4 \times 10^{-6}M$ aminopterin, and $16 \times 10^{-6}M$ thymidine (HAT medium), which selects against HPRT-deficient cells. Individual colonies were isolated with cloning cylinders and expanded in medium without aminopterin. Primary septal cells or N18TG2 cells alone, treated similarly, produced no viable colonies.

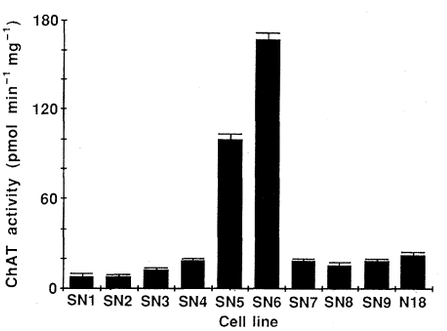

Fig. 1. ChAT activity (picomoles of ACh formed per minute per milligram of protein) of nine cell lines generated by fusion of septal and N18TG2 cells, and of N18TG2 (N18) cells (*26*). The values shown are means ± SEM ($n = 3$).

Nine cell lines from the fusion of septal cells and N18TG2 cells were isolated and screened for ChAT activity with a radiochemical assay (Fig. 1). Most of the cell lines showed ChAT activity similar to that of the N18TG2 parent cell type. Two lines, SN5 and SN6, showed marked ChAT activity and have been further evaluated.

The hybrid nature of the SN5 and SN6 lines is supported by growth in HAT medium and by chromosomal and isoenzyme

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