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FACTORS CONTROLLING ESTROGEN RECEPTOR LEVELS IN NORMAL MOUSE MAMMARY TISSUE

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SUMMARY

Mouse mammary gland cytosol estrogen receptors have been investigated under conditions of differing endogenous hormonal environment. Between 2 and 4 weeks of age, corresponding to pubescence in the mouse, receptor levels increase from almost undetectable values to a full adult complement. This level remains high and is not depressed by ovariectomy. Administration of estrogen to immature animals, using either periodic low dosages or a single pharmacologic dose, does not result in induced enhancement of receptor levels. In the adult animal, however, mammary tissue responds to estrogen stimulation with the characteristic depletion and replenishment of cytosol receptors common to other estrogen-sensitive tissues. Receptor levels increase during early pregnancy, remain at this level through mid-pregnancy, rise dramatically again in late pregnancy, and fall following parturition. The sedimentation properties of the estrogen receptors change with pregnancy, from a 4–5 *s* form in the non-pregnant mouse to an 8 *s* form in the pregnant animal. Analysis of the pregnancy receptor in sucrose gradients containing 0.4 M KCl reveals a 5.3 *s* peak which is different from the non-pregnancy 4–5 *s* moiety. After parturition, the receptor form remains principally 8 *s* in lactating animals, but largely transforms to a 4–5 *s* form in animals which are prevented from lactating. The early phase of the depletion–replenishment response to estrogen in the adult animal is sensitive to inhibition by cycloheximide. The timing of this inhibition is not so critical as it is in other estrogen target tissues, suggesting that continuous protein synthesis might be an essential feature of replenishment in the mammary gland. The unusual features of hormonal regulation of mammary receptors found in this study provide a basis for precise definition of the factors involved and should find application to the study of loss of estrogen responsiveness during neoplastic transformation.

INTRODUCTION

Correlation between levels of cytoplasmic estrogen receptors in human breast tumor samples and subsequent responsiveness of tumor growth to endocrine treatment has provided a dramatic example of the direct clinical relevance of the measurement of these proteins [1]. However, the underlying basis for the loss of responsiveness of the tissue during the course of neoplastic transformation, and the relationship of this phenomenon to alterations in estrogen receptor activity, are almost totally unknown.

In the mouse mammary gland, it appears reasonable to state that tumorigenesis is accompanied by a period of initial absolute hormone dependence followed by a period of hormone independence. The central influence of estrogen upon tumor formation in this species has been undisputed since the early demonstration by Lacassagne [2] that estrogen administration induced mammary tumors in male mice. Discovery of specific uptake and retention of [^{3}H]-17β-estradiol by mammary tissue of ovariectomized mice [3] was followed by the finding that cells of mammary fat pads lack such retention capacity [4]. More recently, cytoplasmic and nuclear estrogen receptors have been described in the normal mammary gland of C3H [5] and BALB/c [6] mice. Shyamala [7] has reported that cytoplasmic, but not nuclear, estrogen receptors were detectable in certain estrogen-independent spontaneous mouse mammary tumors, implying a defect in the normal chain of hormone-induced events subsequent to specific uptake by the tissue.

In the present study, various aspects of the control of estrogen receptor populations in normal mouse mammary tissue have been investigated. It is expected that an analysis of the regulation of estrogen activity in the normal gland is a necessary prerequisite to, and will provide a basis for comparison with, studies designed to relate loss of receptor functionality during tumor formation with progressive diminution of tissue hormonal responsiveness.

MATERIALS AND METHODS

Animals and sample preparation. Female mice of the C3H$^+$ strain were obtained from our own colony (Kirschbaum Memorial Mouse Laboratory) for use in these studies. Animals were sacrificed by cervical dislocation. In a caudal orientation, the second, third and fourth pairs of mammary glands were excised and placed in cold TD buffer (0.01 M Tris, 0.005 M dithiothreitol, pH 8.0). The tissue weight of six glands from a single animal ranged from 0.12 g in the 19-day-old mouse to 0.76 g in the adult. All subsequent manipulations were performed at 0–4°C. The tissues were homogenized (6 glands/ml) with a Tekmar tissue

grinder and centrifuged for 60 min at 105,000 *g*. The thick lipid layer was pierced with a needle and the underlying cytosol fraction was aspirated with a syringe. Analysis of the estrogen receptor content of the cytosol at this point proved unsatisfactory because of extensive interaction of the steroid with lipid particles remaining in suspension. Therefore, except for experiments involving sucrose gradient ultracentrifugation, the cytosol was subjected to fractionation by slow dropwise addition of a cold saturated solution of ammonium sulfate to 40% saturation of the cytosol. This resulted in complete precipitation of the receptor activity within 1 h, and separation was effected by centrifugation for 10 min at 2000 *g*. The pellet was then dissolved in TD buffer and the resulting solution was used as receptor source.

Steroids. [2,4,6,7-^{3}H]-17β-Estradiol (100 Ci/mmol) and [1,2,6,7-^{3}H]-progesterone (96 Ci/mmol) were obtained from Amersham Searle and subjected to descending paper chromatographic purification. Strips were analyzed on a Packard Radiochromatogram Scanner (Model 7201) and the steroids were identified by reference to migration of unlabeled standards. Radiochemical purity following this procedure was >98%. Unlabeled steroids were obtained from Mann and used without further purification.

Receptor assay. Aliquots (100 μl) of ammonium-sulfate-fractionated cytosol were added to plastic assay tubes containing either [^{3}H]-17β-estradiol alone or [^{3}H]-17β-estradiol plus a 100-fold molar excess of unlabeled 17β-estradiol. In experiments dealing with receptor ontogeny, 14 levels of labeled steroid were used, ranging from 10^{-9} to 10^{-12} M. In subsequent experiments, ten-point analyses were employed, in the range of 10^{-9}–10^{-11} M steroid. Samples were allowed to reach equilibrium (18 h, 4°C), after which receptor-bound steroid was precipitated by protamine sulfate (Sigma, Grade 1, 4 mg/ml) and separated by centrifugation at 2000 *g* for 10 min. Details of the protamine precipitation procedure standardized in this laboratory have been published previously [8]. The radioactive content of both supernatant and pellet were determined by liquid scintillation spectrometry (Beckman LS-230; efficiency for tritium: 50%). The scintillation mixture was composed of 5 g of Permablend II (Packard) dissolved in 1 liter of toluene. Disintegrations per min were determined by use of the external standards ratio method. Protein content of cytosol was routinely measured by the method of Lowry *et al.* [9]. Binding data were analyzed graphically by the method of Scatchard [10].

Receptor depletion and replenishment. Levels of cytoplasmic estrogen receptors at intervals following stimulation by 17β-estradiol administration [11–13] were determined as follows. At zero time, groups of animals were injected i.p. with 0.1 μg of 17β-estradiol in a vol. of 0.1 ml of 0.9% NaCl; control animals received vehicle only. At intervals of 1, 3, 5, 10 and 15 h, groups of animals were sacrificed and levels of receptor binding were determined by Scatchard analysis. Where applicable, cycloheximide (Sigma) was administered (0.1 mg in 0.1 ml of 0.9% NaCl) at designated time periods relative to the time of estrogen injection.

Sucrose density gradient centrifugation. Cytosol was incubated with the appropriate steroid for 4 h at 4°C and 300 μl samples were applied to preformed 5–20% sucrose gradients. The buffer used was TD, containing 0.4 M KCl where indicated. Free steroid was removed prior to sample application by adsorption onto dextran-coated charcoal [14]. Samples were centrifuged for 15.5 h at 225,000 *g* with bovine serum albumin as a reference marker. Sedimentation constants were approximated according to Martin and Ames [15]. When cytosol was to be used specifically for analysis on sucrose gradients, the ammonium sulfate fractionation step was eliminated from the sample preparation procedure.

RESULTS

Ontogeny of the estrogen receptor activity. As an initial means of investigating hormonal control of mouse mammary estrogen receptor concentration, virgin mice were sacrificed at ages ranging from 2 weeks to 5 months and cytoplasmic estrogen receptor content was measured. The results, shown in Fig. 1, indicate that receptor activity increases 10-fold between 2 and 4 weeks of age, the time of puberty in these animals. With increasing age, the levels fall somewhat and appear to reach a plateau. The decline in values was thought to be the result of increasing

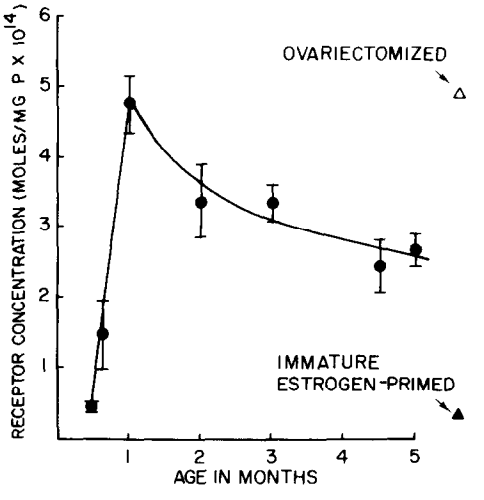

Fig. 1. Mammary tissue estrogen receptor concentration in virgin mice of different ages. Mice of the indicated ages were sacrificed in groups of 3–4 animals and assessed for levels of cytoplasmic estrogen receptors in the mammary gland (●). Each point represents the mean ± S.E. of at least 3 determinations from separate experiments. A group of 2-month-old mice, ovariectomized for 2 weeks, were also analyzed for receptor content (△). Immature mice (10 days old) were treated with 0.02 μg of 17β-estradiol i.p. daily for 5 days and sacrificed 2 days after the treatment period for determination of receptor level (▲).

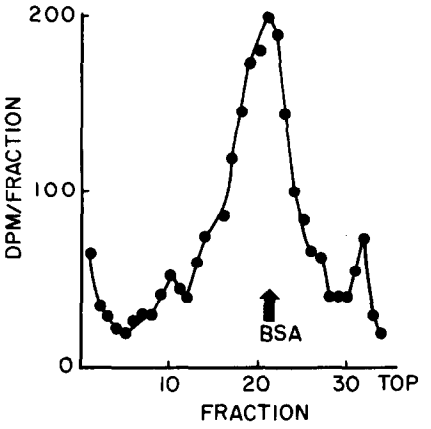

Fig. 2. Sucrose density gradient analysis of estrogen binding in mouse mammary cytosol. Cytosol was extracted from 4 virgin mice injected 24 h previously with 0.1 μg of 17β-estradiol and was incubated with [^{3}H]-17β-estradiol at a concentration of 5×10^{-11} M for 4 h at 4°C. Free steroid was removed by dextran-coated charcoal adsorption and a sample containing 2.55 mg of protein was layered onto a 5–20% sucrose gradient in TD buffer. Bovine serum albumin was used as internal standard. Fractions of 0.1 ml were collected for quantification of radioactivity.

titers of endogenous estrogen, and this presumption was borne out by the observation that receptor levels in 2-weeks' ovariectomized mice were equivalent to the highest values seen at pubescence. The latter finding also demonstrates that this receptor system does not degenerate following estrogen deprivation, consistent with estrogen receptor dynamics in other responsive tissues [16]. Estrogen priming of immature animals for 5 days was ineffective in inducing an increase in receptor levels. Thus, it appears that, unlike other estrogen-sensitive tissues, the receptor-synthesizing machinery of the mammary gland becomes activated at a relatively late age when the tissue has been prepared for stimulation by estrogen.

Nature and specificity of estrogen binding in virgin mice. Upon sucrose density gradient analysis of estrogen binding in virgin mice administered 0.1 μg of 17β-estradiol 24 h previously, the major portion of the radioactivity sediments in the 4–5 *s* region, with a small amount of 8 *s* binding (Fig. 2). Animals which were not pretreated with estrogen showed similar patterns, but the levels of binding were lower and much greater concentrations of cytosol protein were required for quantitative evaluation on sucrose gradients. It was also found that the binding activity in glands from ovariectomized mice sedimented in the 4–5 *s* region.

Investigation of the steroidal specificity of the 4–5 *s* binding (Table 1) suggested the presence of two populations of estrogen-binding components. Neither progesterone nor testosterone, in high molar excess amounts, was capable of competing for a significant portion of the 4–5 *s* binding sites. Unlabeled 17β-estradiol, however, reduced the binding by half, indicating the presence of approximately equivalent amounts of low-capacity and high-capacity binding systems. From analogous results in other estrogen-sensitive tissues, it is likely that the non-displaceable component of the binding is albumin. It was of some interest that mammary tissue cytosol incubated with [^{3}H]-progesterone showed 35% of the binding observed with [^{3}H]-17β-estradiol; competition by either unlabeled progesterone or cortisol indicated that approximately half of the binding was of the receptor type.

Estrogen receptor levels during pregnancy. During the course of pregnancy in the mouse, dramatic changes occur in levels of mammary tissue estrogen receptors (Table 2). The results are expressed in terms of the total binding capacity of the mammary tissue from a single animal, rather than the specific binding per mg of cytosol protein. The reason for this departure from our usual form of presentation is that the cytosol protein content increases significantly throughout pregnancy, in accordance with the large changes in tissue weight, and expression of the values on the basis of this parameter become meaningless. In early pregnancy, the receptor content increases significantly over that of the non-pregnancy level, and remains constant through mid-pregnancy. A distinct

Table 1. Specificity of 4–5 *s* binding of 17β-estradiol to mammary tissue cytosol*

Steroid addition	% Retention of 4–5 *s* radioactivity
[^{3}H]-17β-Estradiol	100
[^{3}H]-17β-Estradiol plus:	
200-fold testosterone	91
200-fold progesterone	97
100-fold 17β-estradiol	45
[^{3}H]-Progesterone	35

* Cytosol was prepared from glands of virgin mice injected with 0.1 μg of 17β-estradiol 15 h prior to sacrifice. Aliquots of 300 μl were incubated for 5 h at 4°C with [^{3}H]-17β-estradiol alone (10^{-9} M) or supplemented with the indicated molar excess amounts of unlabeled steroid. One sample was incubated with 2×10^{-10} M [^{3}H]-progesterone. Samples were then subjected to sucrose density gradient centrifugation and radioactivity sedimenting in the 4–5 s region was quantified.

Table 2. Estrogen cytosol receptor levels in mammary tissue during pregnancy

Group	Receptor concentration* (mol × 10^{14})
Non-pregnant	3.10 ± 0.91
Early pregnancy (5–9 days)	5.05 ± 0.69
Mid-pregnancy (10–15 days)	5.68 ± 1.44
Late pregnancy (16–20 days)	11.01 ± 1.78
Postpartum, 3 days	9.07 ± 1.02
Postpartum, 6 days	6.94 ± 0.63

* Values are the mean ± S.E. for a minimum of 3 separate duplicate determinations. Receptor levels were determined using cytosol from groups of at least 4 animals each and are expressed as total specific binding of 17β-estradiol in the tissue from one animal.

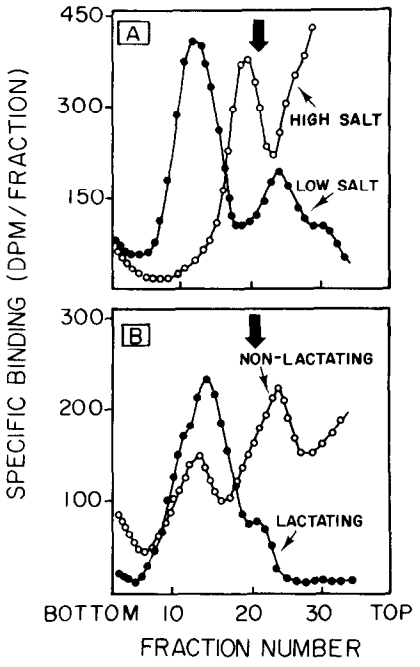

Fig. 3. Sucrose density gradient profiles of specific estrogen binding during pregnancy, and in postpartum lactating and non-lactating tissue. (A). Mid-pregnant (10–15 days) mice were sacrificed and cytosol was prepared in TD buffer. Samples were incubated with [^{3}H]-17β-estradiol for 4 h and applied to a sucrose gradient (●). Identical cytosol samples were extracted with TD- 0.4 M KCl buffer, and the extracts were applied to the gradient (○). Bovine serum albumin was used as standard marker (arrow). In each case, the contribution of nonspecific binding has been determined by simultaneous centrifugation of samples containing excess unlabeled 17β-estradiol, and this amount has been subtracted from the total radioactivity in each fraction. (B). Sucrose gradient patterns of cytosol in TD buffer from 7-day postpartum lactating (●) or 24-day postpartum non-lactating involuted (○) mammary glands. Conditions as in A.

second elevation is then observed in late pregnancy, and, following parturition, the levels fall in lactating mammary tissue.

Nature of estrogen binding during pregnancy. In pregnant mice, the major portion of mammary tissue cytoplasmic receptors sediment in the 8 *s* region of a sucrose gradient (Fig. 3, panel A), in contrast with the virtually complete appearance as a 4–5 *s* component in virgin mice (Fig. 2). Samples of cytosol have been compared from animals in early (5–9 days) and late (16–20 days) pregnancy, and the sucrose gradient patterns are identical. When examined in the presence of 0.4 M KCl, pregnancy cytosol receptors sediment principally as a 5.3 *s* peak; it has been clearly shown that this component is distinct from the major peak found in non-pregnant animals.

Following parturition, the sedimentation characteristics of the receptors are different between animals which are permitted to lactate and those which are removed from their young at birth, allowing the glands to involute (Fig. 3, panel B). In the lactating animals, the receptor remains essentially in the same form as found throughout pregnancy. In the non-lactating mothers, however, both the 8 *s* and the 4–5 *s* components are observed, with the latter form being predominant. It is emphasized that the patterns shown in Fig. 3 represent specific estrogen binding, assessed in each fraction as the difference between samples containing [^{3}H]-17β-estradiol alone and those containing [^{3}H]-17β-estradiol plus a 100-fold molar excess of unlabeled 17β-estradiol.

Receptor turnover as influenced by 17β-estradiol. The initial response of cytoplasmic receptors to a sudden injection of 17β-estradiol has been characterized in several responsive tissues of the rat [11, 12, 17] as consisting of rapid depletion by translocation to the nucleus followed by replenishment to levels equal to or greater than initial values by 15 h. In mammary gland cytosol of mature mice (Fig. 4), this depletion–replenishment process is similar to that observed in these other studies. This response to estrogen is manifested in identical fashion in the 21-day-old mouse, but not in animals of a younger age. This reinforces our finding that induction of receptor binding cannot be invoked in the immature gland, even as an acute response. Simultaneous administration of 17β-estradiol and cycloheximide at a level which inhibits overall protein synthesis by >96% (data not shown) results in a markedly diminished, though clearly not obliterated, replenishment response. It should be

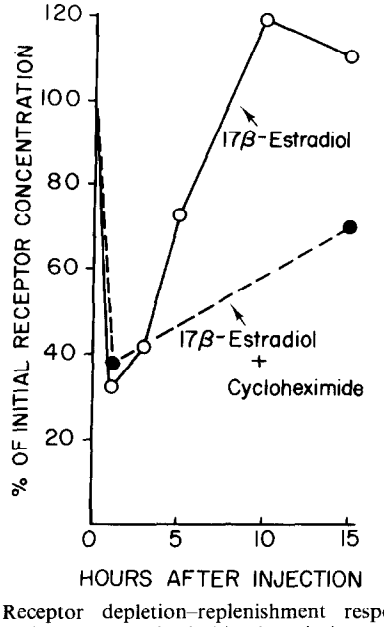

Fig. 4. Receptor depletion–replenishment response to 17β-estradiol and cycloheximide in virgin mammary glands. 0.1 μg of 17β-estradiol was injected i.p. at time zero. Groups of animals (4 mice/group) were killed at intervals following the injection and the concentration of cytosol receptor binding sites was determined (○). Alternatively, 0.1 μg of 17β-estradiol and 0.1 mg of cycloheximide were injected simultaneously at time zero. Groups of 4 mice were sacrificed at different intervals and receptor content was measured (●). Values were calculated on the basis of specific binding and expressed as the % of specific binding observed in control vehicle-injected groups at each interval.

Table 3. Effect of timing of cycloheximide administration on 5-h estrogen receptor replenishment levels*

Time of administration (h)		% of saline-injected 5-h receptor level
17β-Estradiol	Cycloheximide	
—	zero	98.0 ± 1.2
zero	—	72.5 ± 5.5
zero	+0.5	50.8 ± 2.8
zero	+1.0	54.7 ± 5.7
zero	+3.0	54.7 ± 5.8

* Animals were injected with 0.1 μg of 17β-estradiol at time zero, followed by 0.1 mg of cycloheximide at times indicated. All animals were killed at 5 h and cytosol estrogen receptor content was measured. Values were calculated as the mean ± S.E. of the moles of 17β-estradiol bound per mg of cytosol protein in 3 individual determinations and are expressed as percentages relative to the replenishment level in a vehicle-injected group.

pointed out that the depletion–replenishment response measured in these experiments is not intended as a quantitative assessment of cytoplasmic receptor turnover (since occupation of some sites by injected unlabeled steroid would clearly lead to underestimation of the receptor level); rather, this analysis is most valuable as an index of the relative responsiveness of the cells to estrogen stimulation under different conditions.

A more detailed analysis of the cycloheximide effect was made by comparing the 5-h receptor replenishment levels obtained when cycloheximide was administered at different times relative to 17β-estradiol injection at time zero. The 5-h level was chosen because it represents the period during which replenishment is occurring at a maximal rate. The results (Table 3) demonstrate that cycloheximide administration is equally effective in inhibiting early replenishment whether given as long as 3 h after estrogen or given earlier or even concomitantly with estrogen (Fig. 4). Control experiments showed that the drug itself did not alter the 5 h receptor levels from those of vehicle-injected animals.

DISCUSSION

Several aspects of the hormonal influences on mammary gland cytoplasmic estrogen receptor population render this system unique from that of any other estrogen-responsive tissue studied to date. The ontogeny of estrogen receptors has been analyzed in the rat uterus [18, 19]. The concentration of cytoplasmic receptors in this tissue peaks at 10 days of age, at which time 17β-estradiol can also induce receptor synthesis. In the rat hypothalamus, the major increase in estrogen receptor levels occurs between 14 and 21 days of age [20, 21]. All these events are occurring well before the onset of puberty in this species. In the present study, we have shown that rising levels of mammary tissue receptor are observed at an age when estrogen levels are also rising and, furthermore, that 17β-estradiol cannot effect an increase in receptor content at an earlier age. Thus, the proposal made for other tissues, that receptor synthesis is a constitutive property of cells and is independent of estrogen [18], cannot be extrapolated to the situation in the mammary gland. In addition, it appears likely that glandular differentiation under the control of other hormones may be required before maturation of a functional estrogen receptor system occurs.

The responsiveness of cytoplasmic mammary tissue receptor turnover to estrogen stimulation is generally similar to that observed in other tissues [11, 12], but differs significantly with respect to sensitivity to cycloheximide. The results indicate a requirement for prolonged protein synthesis during early replenishment, in contrast to the situation in uterus and anterior pituitary, where inhibition of early replenishment is manifested only when cycloheximide is administered within 1 h of estrogen injection [22]. In these latter tissues, the pattern is consistent with rapid synthesis of a receptor-activating species, whereas such a mechanism would not provide a satisfactory explanation for the mammary gland data. The anterior pituitary and uterus respond to estrogen acutely with the occurrence of very rapid changes in tissue function and morphology; the mammary gland response, on the other hand, appears to be more gradual, involving a prolonged process of tissue differentiation. It is teleologically satisfying then to consider that the initial phase of responsiveness to estrogen (i.e. receptor interactions) might be more rapid in pituitary and uterine tissue (consisting of a receptor activation process) than in mammary tissue (requiring continued synthesis of receptor).

The pattern of changes in receptor content throughout the course of pregnancy affords some clues as to the nature of the causative factors involved. The increased level of receptor during early pregnancy would result from rising levels of estrogen during this period, with a possible secondary contribution by progesterone, the titer of which is also increasing. The combination of these two hormones can invoke changes in virgin mouse mammary tissue which are similar to those encountered in the prelactating gland of the pregnant mouse [23, 24]. The sudden rise in receptor levels during late pregnancy may be attributed to high levels of prolactin at this time in these lactating animals, confirming the observation that prolactin potentiates estrogen binding in mammary explants [25]. At parturition, both estrogen and progesterone levels fall sharply and remain low during subsequent lactation [26], but prolactin remains high if lactation proceeds [27]. Thus, it might be expected that submaximally-stimulated levels of receptor would be present during this period, and our results indicate that this is the case (Table 2).

The changes which we observe in sedimentation characteristics of the estrogen receptors during pregnancy and lactation are of special interest in view of the provocative suggestion [28] that differentiation between 4 *s* and 8 *s* forms of human breast tumor

estrogen receptors might be instrumental in predicting the responsiveness of the tissue to estrogen therapy. Our results indicate that, under conditions where estrogen, progesterone or prolactin are present in stimulatory amounts (pregnancy and lactation), the preferred form of the receptor is 8 *s*. Under quiescent tissue conditions (virgin and involuted glands, and tissue from castrate animals), the 4 *s* form is predominant. It should be noted that the 8 *s* form has been described previously in the lactating gland of the mouse [29]. Our data do not yet permit distinction among the individual influences of the hormones on receptor nature, but these studies are in progress.

Mammary tumorigenesis in the mouse has been likened to an uncontrolled pregnancy-type stimulation, in that the ability of the tissue to halt its growth in the presence of excessive levels of hormones is lost [30]. While this approach may be somewhat simplistic, it is strongly supported by the fact that pregnancy, and specifically the hormonal changes accompanying this state, is a definite causative determinant in the etiology of mammary tumorigenesis in this species [31]. The precise roles of estrogen, progesterone and prolactin in tumorigenesis remain unclear, but the involvement of estrogen receptors may well be a central control point. While prolactin is thought to be of prime importance [32], this hormone is not mitogenic [33] and may only be acting to sensitize the tissue (by altering receptor levels) to the action of the mitogenic ovarian hormones [34]. Ultimately, then, estrogen may still be the primary factor since it enhances prolactin secretion and regulates the peripheral activity of prolactin directly at the mammary gland [35]. Further analysis of the control of estrogen receptor content, nature and physiological activity therefore appears warranted as a means of gaining insight into both the normal and pathological functioning of this gland.

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