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This four year research program focused on the role of prolactin (PRL) in breast cancer and its interactions with estrogens. The first objective investigated PRL as a local mitogen in carcinogen-induced rat mammary tumors. Both PRL mRNA and immunoreactive PRL were detected in normal mammary glands and in mammary tumors. Addition of PRL antisera suppressed proliferation of mammary tumor cells, suggesting that locally produced PRL stimulates mammary tumor cell proliferation. The second objective examined the effects of xenoestrogens on breast cancer and on PRL release. The results indicated that the *in vivo* action of such compounds may be amplified by activating multiple targets, i.e., by having a direct effect on the breast and an indirect effect via increased PRL release. The third objective examined the presence of PRL in human breast tissue and the generation of a cleaved fragment of PRL that acts as an angiostatic factor. Immunoreactive PRL, but not mRNA transcripts, were present in normal breast tissue and carcinomas. PRL appears to be cleaved by thrombin, a proteolytic enzyme that is essential for endothelial cell biology. Additional research will undoubtedly uncover the full spectrum of PRL actions.

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INTRODUCTION:

The first phase of our studies was driven by the hypothesis that PRL is produced by rat mammary tumors and acts as a local mitogen that affects tumorigenesis. Growth factors are known as important mediators of carcinogenesis and have been implicated in malignant transformation, tumor cell proliferation and tumor progression. PRL shares several characteristics with growth factors, including multiple extrapituitary sites of synthesis, wide distribution of receptors, homology of the PRL receptor to those of the cytokine/ hematopoietic growth factor family, similarities in signal transduction pathways, and mitogenic and morphogenic actions (1). The demonstration that mammary tissue produces PRL (2), raised the possibility that PRL acts as a locally produced mitogen that exerts paracrine or autocrine effects independent of its circulating levels.

The second phase of our research examined the hypothesis that xenoestrogens promote mammary tumorigenesis via two interacting mechanisms: one, a direct effect on mammary cell proliferation and two, an indirect effect by increasing PRL synthesis and release. Xenoestrogens include non-steroidal compounds, both naturally occurring (phytoestrogens) and man-made such as pesticides, herbicides, surfactants and monomers of plastics (3). Xenoestrogens were reported to compete with estradiol in binding to uterine estrogen receptors, to induce progesterone receptors, and to increase proliferation of MCF-7 human breast cancer cells (4). Surprisingly, little is known about the *in vivo* effects of xenoestrogens on either mammary tumorigenesis or PRL secretion.

The third phase of our investigation examined the hypothesis that a cleaved fragment of PRL, named 16K PRL, is produced by breast tissue and functions as an angiostatic agent that suppresses neovascularization within growing tumors. Several lines of investigation have shown that cleavage of PRL generates a fragment that does not bind to classical PRL receptors (5) and exerts an anti-angiogenic function that is not shared by the parent compound (6). The shift in our hypothesis came after we were unable to demonstrate local PRL synthesis by human breast cancer cells. In spite of numerous trials, we detected no *de novo* synthesis of PRL by MCF-7 or T47D breast cancer cells. Additionally, although these cells express PRL receptors, proliferation of human breast cancer cells was unaffected by either exogenous PRL or PRL antiserum. Yet, PRL receptors are present in over 50% of breast tumor biopsy (7), and immunoreactive PRL is detectable in normal human breast and carcinomas, leaving the question of both the origin and functions of breast PRL unresolved.

BODY:

1. PRL as a local growth factor in rat mammary tumors

Experimental Model

For an *in vivo* model, we used rat mammary tumors induced by the carcinogen nitrosomethylurea (NMU). As an *in vitro* model, we used an NMU-derived rat mammary tumor cell line. The objectives were to: a) demonstrate local expression of both PRL and PRL receptor, and b) determine whether local PRL stimulates proliferation of mammary tumor cells.

Detection of PRL in mammary tissue

PRL transcript was detected in the lactating mammary gland, NMU-induced mammary tumors, and the NMU tumor cell line. Products of the expected size were seen and confirmed by Southern hybridization to a rPRL cDNA probe. The PRL PCR products from mammary and tumor tissues were sequenced and

found identical to pituitary PRL. An additional band of 210bp was seen in the tumor, but not in the lactating mammary tissue. Ovariectomy (OVEX) resulted in a 50% decrease in the tumor size but its effects on local PRL expression was neither large nor consistent. Immunoreactive PRL was detected in the mammary tumors, and tumor tissues contained an average of 0.3 ± 0.3 ng of immunoreactive PRL/mg protein.

Expression of the PRL receptor by mammary tissue

All mammary tissues expressed mRNA for the PRL receptor, including the long and short isoforms. The PCR products were of the expected sizes, and hybridized strongly to a cDNA probe common to the two isoforms. The long form was predominant in the lactating mammary tissue, whereas equal amounts of long and short forms were observed in tumors. The short form could not be detected in the NMU cell line. OVEX had no consistent effect on the expression of the PRL receptor, nor on the relative amounts of the two isoforms in the tumors. An additional, smaller PCR product was seen in the tumor, which also hybridized to the PRL receptor probe; this product was not seen in the lactating mammary tissue.

Suppression of NMU cell proliferation by PRL antisera

The addition of rPRL antiserum to cultured NMU tumor cells markedly inhibited their proliferation, compared to cells incubated with either NRS or rGH antiserum. This inhibitory effect was dependent on cell density, resulting in 65-70% suppression at the low initial cell density and 35-40% at the higher density. This inhibition was statistically significant at all initial cell densities. Exogenous rPRL did not reverse the effect of the PRL antiserum, and caused only a modest stimulatory effect (20-25%) on NMU cell growth in the absence of PRL antisera.

2. Effects of xenoestrogens on breast cancer and on PRL release

Experimental model

Two xenoestrgens were used. One, β-hexacholocyclohexane (β-HCH), and organochlorine pesticide which is both abundant and persistent in the environment and was reported to induce progesterone receptors in MCF-7 cells and to increase rat uterine weight. Two, bisphenol A (BPA), a monomer of polycarbonate plastic and epoxy resins that has been detected in autoclaved water, canned vegetables, and human saliva. Similar to β-HCH, BPA mimics the action of estrogens on cultured breast cancer cells.

Stimulation of MCF-7 cell proliferation by β-HCH in vitro

The direct growth-promoting effects of β-HCH were examined using the estrogen-sensitive MCF-7 cells and the estrogen-insensitive MDA-MB231 cells. Cells were plated in phenol red-free MEM supplemented with 3% charcoal-stripped FBS and incubated with β-HCH (10^{-10} to 10^{-5} M) in the presence or absence of the antiestrogen ICI164348 (10^{-6} M) for 8 days. β-HCH increased MCF-7 cell proliferation in a dose-dependent manner, and this stimulation was abolished by co-incubation with ICI. As expected, incubation of the estrogen-insensitive MDA-MB231 cells with either estradiol or β-HCH did not increase cell proliferation. These results suggest that β-HCH mimics estradiol in directly stimulating proliferation of human breast cancer cells albeit at a 1000 fold lower potency. These data also indicate that functional estrogen receptors are required for mediating the mitogenic action of xenoestrogens.

Increased growth of grafted MCF-7 cells by β-HCH in vivo

The efficacy of β-HCH as a tumor promoter *in vivo* was tested using a mouse xenograft model. MCF-7 cells were inoculated in athymic OVEX female nude mice that were implanted with silastic capsules

containing estradiol, β-HCH or cholesterol. At various days thereafter, tumor sizes were measured with calipers and their volume estimated. After 16 days, mice were sacrificed and both tumors and uteri were removed, weighed and frozen. β-HCH was nearly as effective as estradiol in stimulating tumor growth *in vivo*. The final weights of the tumors (1.9±0.3 g for β-HCH and 2.4±0.6 g for estradiol) were similar being 3-4 fold higher than in control mice. In contrast, the effect of β-HCH on uterine weight was significantly lower than that of estradiol. Assuming that estradiol and β-HCH diffuse equally from the capsules, these results suggest that unlike its lower potency *in vitro*, β-HCH has a similar biopotency to estradiol *in vivo*. However, its lower uterotropic effects indicate that the estrogen-mimicking activity of β-HCH is tissue-specific.

BPA stimulates PRL release in vitro

We first examined whether BPA mimics the actions of estradiol on PRL release *in vitro*, using primary anterior pituitary cells and GH_3 cells, a somatomammotroph cell line. Cells were plated under serum-free conditions and incubated with the test substances for various times. PRL release from primary anterior pituitary cells increased in a dose-dependent manner in response to estradiol. BPA stimulated PRL release up to 2.5 fold, but at a 1000-5000 lower potency than estradiol. GH_3 cells were incubated with estradiol (10 nM), BPA (1 μM) or testosterone (10 nM) for 7 days. Both estradiol and BPA increased PRL release in a time-dependent manner while testosterone was without effect. Collectively, these data suggest that similar to its effect on MCF-7 proliferation and progesterone receptor expression (8), BPA increases PRL, another estrogen-dependent gene.

Strain-dependent increase in PRL release in vivo by BPA

To determine whether BPA increases PRL release *in vivo*, we chose two strains of rats: Fischer 344 (F344) and Sprague Dawley (SD). F344 is an inbred rat strain with predisposition to estrogen-induced hyperprolactinemia, while other rat strains respond to estrogens with a moderate increase in PRL release (9). OVEX rats from both strains were implanted with silastic capsules filled with crystalline estradiol or BPA; controls receive empty capsules. Within three days, estradiol increased serum PRL levels in F344 rats 10 fold, but only 3 fold in SD rats. BPA increased serum PRL levels 7-8 fold in F344 rats but was without effect in SD rats. Uterine and pituitary weights in F344 rats increased 4 and 2.5 fold, respectively, by estradiol whereas BPA was significantly less effective. These results demonstrate that BPA mimics the *in vivo* effects of estradiol by inducing hyperprolactinemia in an estrogen-sensitive rat and can, indirectly, affects the development of mammary tumors.

3. Presence of PRL in human breast tissue and the generation of 16K PRL

Experimental model

16K PRL was reported to inhibit endothelial cell proliferation and to antagonize the stimulatory effects of basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) *in vitro* and in *vivo* (10). Local generation or involvement of 16K PRL in breast cancer have not been investigated. The objectives were threefold: a) to examine whether 16K PRL is detectable in the normal human breast or breast carcinomas, b) to determine if breast carcinomas and breast cancer cells can cleave PRL, and c) to identify the proteolytic enzyme responsible for such cleavage.

Immunoreactive PRL in normal human breast and in carcinomas

The presence of PRL in human breast tissues was examined by immunocytochemistry, using anti human PRL antiserum from the NIDDK. A strong PRL signal was detected in a lactating breast and in both grade

I and grade III ductal carcinomas. As expected, PRL was localized in the cytoplasm of the epithelial cells and also appeared to be localized in migratory cells such as macrophages or lymphocytes. Northern analysis and *in situ* hybridization failed to detect mRNA for PRL in such tissues, leading us to conclude PRL in breast tissue is taken up from the blood. The precise nature of intramammary PRL, i.e., whether it represents an intact or a cleaved molecule, could not be determined since the existing polyclonal antibodies do not differentiate between 23K PRL and 16K PRL and will require immunoaffinity purification.

Generation of 16K PRL by breast cancer homogenates

Lactating rat mammary glands have the highest PRL cleaving activity of all tissues examined (11). The cleaving enzyme was identified as Cathepsin D, an estrogen-inducible, acid protease that is highly expressed in breast cancer (12). We, therefore, tested the cleaving capacity of normal human breast and carcinomas. Tissues were homogenized in Tris buffer, centrifuged at 25,000xg and the pellets suspended in citrate-phosphate buffer pH 3.4 and incubated with purified rat PRL (rPRL) for 4h. Serum-free conditioned media from T47D breast cancer cells were also acidified to pH 3.4 and incubated with rPRL. As shown by SDS-PAGE, all samples cleaved rPRL with an apparent higher cleaving activity by tumors than normal breast tissue. Since pH 3.4 is optimal for cathepsin D, these data are consistent with the reports that cathepsin D is expressed in carcinomas and is secreted by breast cancer cells. As rat PRL is not the native substrate for human cells we next examined cleavage of human PRL. To our surprise, the acidified microsomal pellet from MCF-7 cells cleaved rat PRL but not the human hormone. Similarly, acidified microsomal preparations from breast carcinomas cleaved rat, but not human, PRL. Purified human cathepsin D, incubated with hPRL for up to 24h, also failed to cleave the hormone. These results lead us to conclude that cathepsin D is unlikely the natural enzyme that cleaves human PRL.

Cleavage of human PRL by thrombin and binding to heparin

The failure of cathepsin D to cleave hPRL prompted a search for other proteolytic enzymes. Among these, we tried thrombin, a liver-derived serine protease best known for its function in the coagulation cascade. Thrombin plays an essential role in endothelial cell biology and like plasmin, its concentration is enriched at the endothelial surface. As was recently reported, thrombin binds to anti-thrombin and vitronectin, and the inactive complex is immobilized via an heparin-binding domain in the extracellular matrix of endothelial cells (13). Our studies revealed, for the first time, that purified thrombin at pH 7.4 cleaved both rat and human PRL whereas cathepsin D at pH 3.4 cleaved only rat PRL. As judged by their similar mobility on SDS-PAGE, cathepsin D-cleaved rPRL and thrombin-cleaved hPRL have an apparent molecular weight of 16 kDa. Confirmation of the angiostatic activity of the thrombin-cleaved PRL and determination of the exact cleavage site and currently undergoing.

Another striking and unexpected finding is the binding of hPRL to heparin. Almost all angiogenic/ angiostatic factors bind to heparin/heparan sulfate chains of cell-associated proteoglycans. Therefore, we tested if hPRL binds to heparin. Upon fractionating hPRL on a heparin affinity column, the hormone was retained and was eluted with 0.5M NaCl. Such affinity is similar to that of FGF-1 (0.5M NaCl), slightly lower than that of $VEGF_{165}$ (0.9M NaCl) and significantly lower than that of FGF-2 (1.5M NaCl). Neither rat PRL nor human GH, both of which have structural and functional homology to hPRL, were retained on the column.

CONCLUSIONS:

Our studies with carcinogen-induced rat mammary carcinomas lead to the following conclusions: a) rat

mammary tissues, including mammary tumors, express PRL mRNA and contain immunoreactive PRL, b) these tissues express PRL receptor mRNA, including both the long and short isoforms, and c) the addition of PRL antiserum to cultures of an NMU cell line markedly inhibited cell proliferation. This effect was not seen with either NRS or GH antiserum. Collectively, these results suggests that at least in rodents, locally produced PRL functions in a paracrine/autocrine manner to induce proliferation of mammary epithelial cells.

Environmental estrogens represent an unsuspected source of estrogens that can alter the development and progression of breast cancer. Our studies with two synthetic estrogen mimetic chemicals that are abundant in the environment can be summarized as follows: a) they mimic estradiol by increasing growth of breast cancer cells and by stimulating PRL synthesis and release, b) the *in vivo* efficacy of these compounds appears to be significantly higher than their *in vitro* potency, c) the estradiol-like effect of these compounds is tissue-specific, and d) a genetic strain of rats with enhanced sensitivity to the effects of xenoestrogens has been identified. Collectively, the *in vivo* action of xenoestrogens may be amplified by activating more than one target, i.e. by having both a direct effect on the breast and an indirect effects via increased PRL secretion. There may be a human homolog to the estrogen-sensitive F344 rat in terms of predisposition of certain individuals to the effects of environmental estrogens.

Angiogenesis is an integral part of tumorigenesis. The mechanism that normally maintains the endothelium quiescent but initiates proliferation as tumors grow is enigmatic. The current view is that a fine balance exists between angiogenic and angiostatic factors and its disturbance causes neovascularization. A proteolytic fragment of PRL acts as an angiostatic agent, leading to our working hypothesis that 16K PRL may be important in controlling mammary angiogenesis. Our results can be summarized as follows: a) normal and carcinogenic human breast tissues contain significant amounts of immunoreactive PRL, most of which is not locally produced, b) human breast carcinomas and breast cancer cells under acidified conditions can cleaved rat, but not human, PRL, c) thrombin, an enzyme which is essential to endothelial cell biology, cleaves both rat and human PRL, and d) similar to most angiogenic/angiostatic factors, human PRL binds to heparin. Collectively, our results established a plausible mechanism by which an angiostatic agent is locally produced by breast cells and may act to suppress neovascularization.

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Prolactin Is a Local Growth Factor in Rat Mammary Tumors*

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ABSTRACT

PRL is a mitogenic hormone that shares many characteristics with growth factors. The recent demonstration that rat mammary tissue expresses PRL messenger RNA (mRNA) led us to hypothesize that PRL may act as an autocrine/paracrine growth factor in the mammary gland and may be a determinant in mammary carcinogenesis. To examine this, mammary tumors were induced in rats by injection of the carcinogen nitrosomethylurea (NMU). *In vitro* studies used a cell line derived from NMU-induced mammary tumors. Expression of PRL and PRL receptor was assessed by reverse transcriptase-polymerase chain reaction. The NMU-induced mammary tumors and the cell line express mRNA for both PRL and PRL receptor (the long and short isoforms); additional hybridizing polymerase chain reaction products were seen in the tumors, but not in lactating mammary tissue. Immunoreactive PRL was detected in the NMU-induced tumors. The effect of PRL on cell proliferation was assessed by culturing NMU cells with PRL antiserum. The PRL antiserum inhibited cell proliferation by up to 70% compared to the effect of normal rabbit serum or GH antiserum.

In summary, we showed that NMU-induced mammary tumors express mRNA for PRL and PRL receptor. Addition of PRL antiserum to cultured NMU cells significantly inhibited their growth. We propose that PRL may be acting as a local growth factor that stimulates the proliferation of mammary tumors. (*Endocrinology* **136:** 3619–3623, 1995)

PRL IS A pituitary polypeptide hormone whose classic target is the mammary gland. The mitogenic effects of PRL in the mammary gland (1) support a role for the hormone in mammary carcinogenesis. However, circulating PRL levels correlate poorly with the incidence and severity of breast cancer, and treatments that suppress pituitary PRL release have not improved outcome (2). Consequently, PRL has been largely disregarded as a significant determinant in breast cancer. The recent demonstration that mammary tissue produces PRL (3–6) raises the possibility that PRL acts as a locally produced growth factor, exerting paracrine or autocrine effects, independent of its circulating levels.

Growth factors are emerging as important mediators of carcinogenesis and have been implicated in malignant transformation, tumor cell proliferation, and tumor progression (7). PRL shares several characteristics with growth factors, including multiple extrapituitary sites of synthesis (8–10), wide distribution of receptors (11), homology of the PRL receptor to those of the cytokine/hematopoietic growth factor family (12), similarities in signal transduction pathways (13), and mitogenic and morphogenic actions (9, 14). Indeed, PRL has been shown to act as an autocrine growth factor in two cell types, the somatomammotroph (GH_3) cell line (15) and lymphocytes (16).

In this study, we examined the hypothesis that PRL is produced by mammary tumors and acts as a local mitogen involved in tumorigenesis. For an *in vivo* model, we used rat mammary tumors induced by the carcinogen nitrosomethylurea (NMU) (17). As an *in vitro* model, we used an NMU-derived rat mammary tumor cell line. The objectives were to 1) demonstrate local expression of both PRL and PRL receptor, and 2) determine whether local PRL affects the proliferation of mammary tumor cells.

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Materials and Methods

Animals

Forty-five-day-old female Fischer 344 rats, obtained from Harlan (Indianapolis, IN), were given two iv injections, 1 week apart, of NMU (Sigma Chemical Co., St. Louis, MO; at 5 mg/100 g BW). Mammary tumors developed within 80–100 days. Rats with large tumors (2–3 cm in diameter) were either killed or ovariectomized and killed 10 days later. Tumors were removed and stored at −70 C until use. Mammary tissue, kidney, heart, and cerebral cortex from lactating rats (1–2 days postpartum) served as controls. All experiments were approved by the University of Cincinnati institutional animal care and use committee.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Tissues were homogenized in guanidinium thiocyanate buffer, and total RNA was isolated by phenol/chloroform extraction. Reverse transcription of 1 μg RNA was performed using Superscript RT (Gibco, Grand Island, NY), primed with oligo(deoxythymidine). Ten percent of the reaction was subjected to PCR, using the following primers. For rat (r) PRL (18), the 5′-primer, located in the third exon, had the sequence 5′-GATCGTGAGTTTATTGCCAAGGCC-3′ (nucleotides 268–291), and the 3′-primer, located in the fifth exon, was 5′-CTTGCAGGGATGGGAGTTGTGACC-3′ (nucleotides 578–601), with a predicted product size of 334 base pairs (bp). For the rPRL receptor, 2 sets of primers were used to detect the long (19) or short (20) isoform. The upstream primer for both, 5′-ATGGATACTGGAGTAGATGGAGCC-3′, was derived

from a sequence in the region common to the 2 receptors (nucleotides 620–643). Using downstream primers specific to the long (5′-CCAGAGTCACTGTCGGGATCTAAG-3′; nucleotides 1011–1034) or short (5′-GAGGCTCCTATTTGAGTCTGCAGC-3′; nucleotides 935–958) forms yielded product sizes of 415 and 339 bp, respectively. After denaturation at 95 C for 5 min, the reaction was carried out as follows: 94 C for 30 sec, 65 C for 15 sec, and 72 C for 45 sec. The PCR products were visualized on ethidium bromide-stained agarose gels. To determine a nonsaturating number of cycles, one of the PCR primers was radiolabeled with ^{32}P using polynucleotide kinase (Gibco-BRL, Gaithersburg, MD). The PCR reaction was carried out under conditions identical to those described above, with the addition of 1 μl radiolabeled primer. A 10-μl aliquot was removed every 5 cycles from 25–45 cycles and electrophoresed on a 1% agarose gel. The bands were isolated, and incorporation of radiolabeled primer was measured by scintillation counting.

For Southern analysis, PCR products were transferred to nylon membranes (Schleicher and Schuell, Keene, NH) and hybridized to complementary DNA (cDNA) probes, which were random primer labeled with [^{32}P]deoxy-CTP (DuPont-New England Nuclear, Boston, MA). The rPRL cDNA probe was obtained from Dr. R. Maurer (Oregon Health University, Portland, OR). The probe for the PRL receptor was derived from a rat cDNA obtained from Dr. P. Kelly (INSERM Unit, Paris, France). It consisted of approximately 200 bp included in the region common to the two products, thus enabling a comparison of their relative band intensities. The blots were washed to a final stringency of 0.1 × SSPE-1% sodium dodecyl sulfate at 65 C. These reactions were performed on three different tissue samples in each group.

PRL immunoreactivity

Tissues were homogenized in 1 N acetic acid and centrifuged at 10,000 × *g* for 10 min, and the supernatants were dialyzed overnight and lyophilized. Samples were reconstituted in RIA assay buffer and analyzed in serial 2-fold dilutions. Rat PRL RIA was performed using reagents obtained from the NIDDK Hormone Distribution Program, with an assay sensitivity of 50 pg/tube. Intra- and interassay coefficients of variation were 7% and 9%, respectively.

Cell culture and cell proliferation assay

NMU cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum. Before an experiment, cells were incubated for 24 h in DMEM-1% fetal bovine serum, detached by trypsinization, and plated at a density of 2–16 × 10^3 cells/well in 96-well plates in 200 μl DMEM. After confirming cell attachment, the cells were incubated with dialyzed antiserum to rPRL (NIDDK, IC4) or rGH (NIDDK, IC1), obtained from the National Hormone and Pituitary Program, or with dialyzed NRS at a dilution of 1:300. This dilution was found to significantly inhibit cell proliferation in a dilution series analysis (data not shown). Cells were visualized twice daily to monitor cell growth and attachment. After 48 h, relative cell density was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] assay (21). In this assay, cell number is proportional to optical density at 570 nm. This was validated by performing the MTT assay on cells plated at different cell densities, as determined by hemocytometer counting. All data are presented as the mean ± SEM; significant differences were calculated by two-way analysis of variance using Duncan's multiple range test. Each experiment was performed three times.

Results

Detection of PRL in mammary tissue

Using RT-PCR conditions identical to those used for the tissue samples, PRL product increased in a linear fashion through 40 cycles, without a further increase at 45 cycles (Fig. 1). PRL expression was readily detectable at 35 cycles, which was used for analysis of the mammary samples. PRL transcript was detected in the lactating mammary gland, NMU-induced mammary tumors, and the NMU cell line (Fig. 2, *upper panel*). Transcript was also seen in the kidney and, after 45 cycles, in the heart and cerebral cortex (data not shown). Products of the expected size were seen and were confirmed by Southern hybridization to a rPRL cDNA probe (Fig. 2, *lower panel*). The mammary and tumor PRL PCR products were sequenced and found to be identical to pituitary PRL.

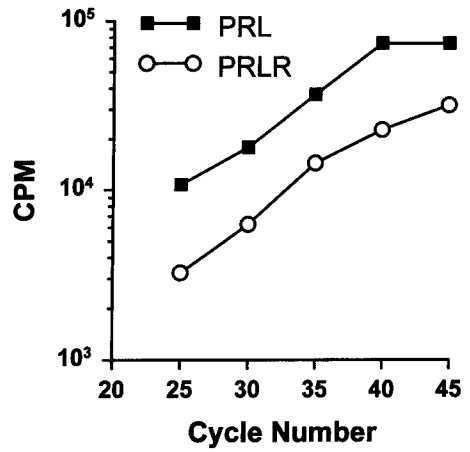

FIG. 1. A linear increase in product *vs.* cycle number in RT-PCR of mammary tissue for both PRL and the short form of the PRL receptor (PRLR). The long form of the PRL receptor showed similar results (data not shown). The PCR primer was end labeled with ^{32}P, and reactions were carried out under conditions identical to those used in the experiments. The PCR products were isolated and counted in a scintillation counter. Thirty-five cycles were chosen for subsequent experiments.

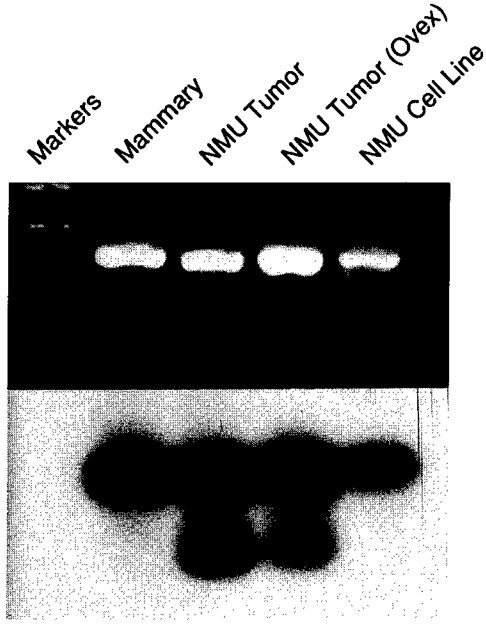

FIG. 2. PRL expression in mammary tissues, as detected by RT-PCR. *Upper panel*, PCR amplification of PRL mRNA in lactating mammary tissue, NMU-induced mammary tumors, and the NMU cell line. *Lower panel*, Southern blot analysis of the PCR products. The PCR products were transferred to nylon membranes and hybridized to the full-length ^{32}P-labeled rPRL cDNA probe. All samples demonstrated detectable levels of PRL transcript.

Interestingly, an additional band of approximately 210 bp was seen on the Southern blot in the tumor, but not in the lactating mammary tissue. On prolonged exposure, this band was also seen in the NMU cell line. OVEX resulted in a marked decrease (>50%) in the size of the tumors studied. However, the effect of OVEX on the expression of PRL was neither large nor consistent (Fig. 2).

Immunoreactive PRL was detected in the mammary tumors (Fig. 3). Homogenates of tumor tissues contained an average of 0.3 ± 0.3 ng/mg protein. The dilution curve of mammary tumor PRL was parallel to the standard curve. Immunoreactive PRL was also detected in the kidney, but not in the heart or cerebral cortex (data not shown).

Expression of PRL receptor by mammary tissue

Analysis of RT-PCR product *vs.* cycle number for the long and short forms of the PRL receptor revealed a linear increase through 40–45 cycles (Fig. 1). Thirty-five cycles were chosen for sample analysis. All mammary tissues expressed messenger RNA (mRNA) for the PRL receptor, including the long and short isoforms (Fig. 4, *upper panel*). The PCR products were of the expected sizes and hybridized strongly to a cDNA probe common to the two isoforms (Fig. 4, *lower panel*). A predominance of the long form was seen in the lactating mammary tissue, whereas equal amounts of the two isoforms were observed in the tumors. The short form could not be detected in the NMU cell line (Fig. 4); although a PCR product of greater than the expected size was seen, it did not hybridize on the Southern analysis and was considered an artifact. OVEX had no consistent effect on the expression of the PRL receptor or on the relative amounts of the 2 isoforms in the tumors. An additional smaller PCR product was seen in the reaction for the long form of the receptor in the tumor, which also hybridized to the PRL receptor probe; this product was not seen in the lactating mammary tissue.

Suppression of NMU cell proliferation by PRL antiserum

The addition of rPRL antiserum to NMU cells markedly inhibited their proliferation compared to that of cells incubated with either NRS or rGH antiserum (Fig. 5). This inhibitory effect was dependent on cell density, resulting in 65–70% suppression at the low initial cell density and 35–40% suppression at the higher density. This inhibition was statistically significant at all initial cell densities ($P < 0.05$). It is of interest that such a marked inhibitory effect was not seen with two other PRL antisera, *i.e.* IC5 from NIDDK or an antiserum produced by this laboratory (data not shown). The addition of exogenous rPRL (bioactive PRL, NIDDK) to the NMU cells did not reverse the effect of the PRL antiserum and caused only a modest stimulatory effect (20–25%) on cell growth in the absence of PRL antiserum (data not shown).

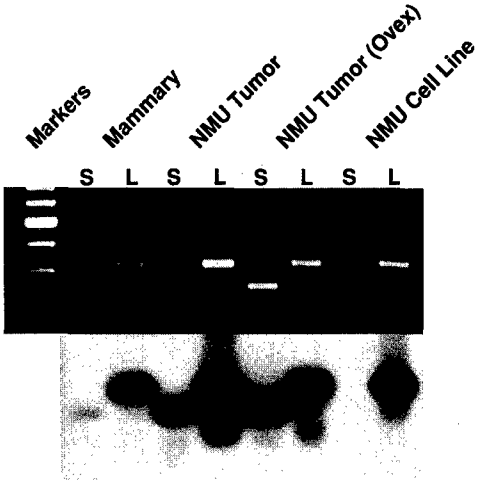

FIG. 4. PRL receptor expression in mammary tissues determined by RT-PCR. *Upper panel*, PCR amplification of PRL receptor mRNA in lactating mammary tissue, NMU-induced mammary tumors, and NMU cell lines. S and L denote long and short receptor isoforms, respectively. *Lower panel*, Southern blot analysis of the PCR products. After transfer to a nylon membrane, the PCR products were hybridized to a segment of the rPRL receptor common to the two isoforms, allowing comparison of the relative band intensities. All samples contain both PRL receptor isoforms, with the exception of the NMU cell line, which lacks the short isoform.

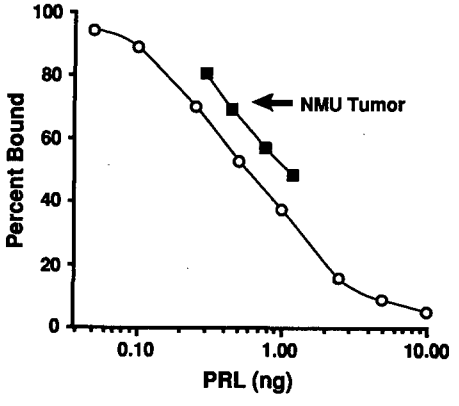

FIG. 3. Immunoreactive PRL in the NMU-induced mammary tumor. An NMU-induced mammary tumor was extracted with 1 N acetic acid, dialyzed, and lyophilized. Serial 2-fold dilutions were assayed for PRL. Displacements of tumor extract were parallel to the standard curve. This is a representative example of one of three extracted tumors.

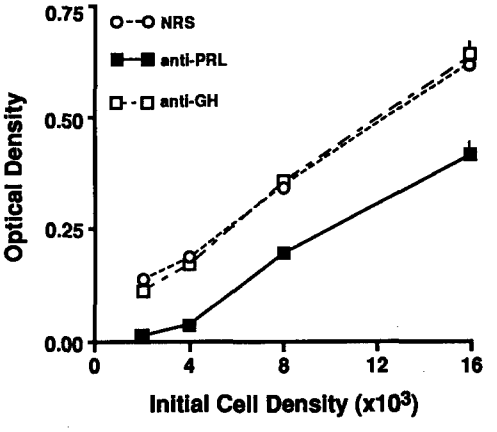

FIG. 5. Inhibition of NMU cell proliferation by 48-h incubation with PRL antiserum (anti-PRL) and lack of effect of anti-GH and normal rabbit serum (NRS), each at a dilution of 1:300. Optical density (570 nm), as determined by the MTT assay, is proportional to cell number. The inhibitory effect of PRL antiserum was more pronounced at low cell density.

Discussion

We demonstrated that rat mammary tissues, including mammary tumors, express PRL messenger RNA and contain immunoreactive PRL. Mammary tumors, but not lactating mammary tissue, appear to express a smaller PRL variant. In addition, these tissues express PRL receptor mRNA, including both the long and short isoforms. The NMU-induced tumors also may express PRL receptor variants smaller than the expected size. The addition of PRL antiserum to cultures of an NMU cell line markedly inhibited cell proliferation. This effect was not seen with either NRS or GH antiserum.

Expression of PRL mRNA in rat mammary tissues was recently demonstrated by Northern analysis (3, 6), RT-PCR (4, 6), and *in situ* hybridization (3). We extended these findings to both NMU-induced mammary tumors and a cell line derived from such tumors. The demonstration of detectable levels of immunoreactive PRL further indicate that the message is translated into protein. This is supported by the finding of PRL mRNA in the polysomal fraction of ovine mammary tissue (6). The concentration of immunoreactive PRL in the tumor (0.3 ng/mg protein), although significantly lower than that of pituitary PRL, is similar in magnitude to that reported in the brain (22) and could reach a significantly high concentration in the vicinity of responsive cells. It cannot be excluded that the PRL present in mammary tissues is derived in part from nonmammary cells, *e.g.* lymphocytes (23). However, the localization of PRL mRNA to the mammary epithelium by *in situ* hybridization (3) together with our finding of PRL expression by the NMU cell line argue against lymphocytes being the major source of mammary PRL.

Two hybridizing bands were seen in the Southern blots for PRL in the mammary tumors. As the smaller band hybridized to the PRL cDNA probe under the same high stringency conditions as the expected fragment, it is unlikely that it represents a PCR artifact. Instead, it may represent alternative splicing or a deletion mutation in the tumor tissues. Interestingly, Emanuele *et al.* (24) also detected a smaller PCR product in brain tissue and suggested that it represents an alternatively spliced PRL transcript skipping the fourth (180-bp) exon. In our case, a product size of 210 bp, indicating a deletion of approximately 120 bp, is not consistent with a deletion of the fourth exon, and this issue is currently being investigated.

The rat mammary tissue expresses both short and long forms of the PRL receptor. As the difference between the two isoforms lies in the intracellular domain of the receptor, it has been proposed that the two receptors are linked to different signal transduction pathways (12). Predominant expression of the long form of the receptor by normal mammary gland has been previously reported (25). Our results indicate that the NMU-induced tumors express approximately equal amounts of the two receptors. The physiological significance of an altered ratio of the two receptors in the tumor is yet unknown. Similarly, the presence of a PRL receptor variant in the tumor, as suggested by the additional hybridizing band in the PCR reaction for the long form of the receptor, may be important. Indeed, a mutation of the PRL receptor due to a large deletion in the intracellular domain appears to confer PRL-dependent growth on the NB_2 lymphoma cell line (26).

OVEX is known to result in a marked decrease in size of the majority of NMU-induced tumors (17), an effect also seen in the present studies. However, both PRL and PRL receptor transcripts remained easily detectable after OVEX, suggesting that estrogen is not required for their basal expression in the rat mammary gland. Two laboratories examined the effect of OVEX on PRL receptors in NMU-induced mammary tumors using ligand binding analysis (27, 28). Both found a modest (25–30%) decrease in binding in the tumors after OVEX, an effect that was significant in only one of the studies (28). In the ovine and caprine mammary gland, PRL expression appears to be transcribed from the same promoter as the pituitary (5), which contains an estrogen response element (29). Additional, more quantitative, experiments would be necessary to resolve this issue in NMU tumors.

To determine whether local PRL acts as a mitogen in the mammary tumor, we added PRL antiserum to NMU cells cultured under serum-free conditions. The marked inhibition of cell proliferation (>70% at the lower initial cell densities) suggests that endogenous PRL contributes to the maintenance of basal cell growth of this mammary tumor cell line. An autocrine or paracrine effect of PRL on cell proliferation has previously been demonstrated in pituitary cells (15) and lymphocytes (16). The addition of exogenous PRL did not reverse the inhibitory effect of the antibody in the NMU cell line. This was also seen in the GH_3 cells and was apparently due to the presence of different PRL isoforms in the reference preparation, with opposing effects on cell proliferation (15). Other growth factors, including transforming growth factor-β, are known to display opposing effects on cell proliferation under different conditions (7).

We are puzzled by the finding that the IC4 antiserum, but not two other PRL antisera, inhibits proliferation of NMU cells. Cell growth was inhibited by the IC4 antiserum after extensive dialysis and by the immunoglobulin G fraction after ammonium sulfate precipitation. It is of interest that variable effects of different PRL antisera on cell proliferation were also reported for lymphocytes (16, 30) and GH_3 cells (15). Together, these findings suggest that only certain PRL isoforms might act as mitogens. Given the large number of PRL isoforms and their diverse biological effects (31), it is possible that the NMU cells are producing a specific PRL isoform that is recognized by IC4 but not the other antisera. We speculate that the rPRL preparation from NIDDK either contains low levels of this isoform or possibly other isoforms that function as growth inhibitors.

Whereas the expression of PRL by mammary tissue is now well established, previous reports have not addressed the possible function(s) of local PRL. Preliminary evidence is provided here that locally produced PRL may act as a growth factor in the mammary gland. These autocrine/paracrine actions of PRL in mammary tissue could have important implications in the field of breast cancer. The presence of a locally produced hormone, acting as a growth factor in the mammary gland, raises the possibility that disturbances in its regulation, such as overexpression or mutation of PRL or its receptor, promote malignancy or enhance its progression. These effects are now well documented for other growth

factors in the breast, including epidermal growth factor, transforming growth factor-α, and c-*erb*B-2 (32).

Acknowledgement

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Note Added in Proof

Clevenger *et al.* (33) provided evidence of an autocrine/paracrine loop for prolactin in human breast cancer.

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Extrapituitary Prolactin: Distribution, Regulation, Functions, and Clinical Aspects*

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I. Introduction

PRL affects more physiological processes than all other pituitary hormones combined. Among these are the regulation of mammary gland development, initiation and maintenance of lactation, immune modulation, osmoregulation, and behavioral modification. At the cellular level, PRL exerts mitogenic, morphogenic, or secretory activities. This raises the question as to the mechanism by which a single hormone can modulate so many seemingly unrelated functions. This review addresses the concept of the dual function of PRL, as a circulating hormone and a cytokine. The diversity of PRL actions is derived from three components: structural polymorphism, local production and processing, and divergent intracellular signaling pathways and target genes. PRL can be easily classified as a cytokine based on its shared properties with hematopoietic growth factors. These include comparable structural motifs, multiple sites of synthesis, ubiquitous receptor distribution, homologous receptor structure, and similar signal transduction pathways.

A seminal report by Nagy and Berczi (1) drew attention to extrapituitary PRL. They reported that hypophysectomized female rats had 10–20% lactogenic activity in their serum as compared with controls. Within 2 months, the lactogenic activity gradually increased to 50% of controls. Immunoneutralization of PRL in these hypophysectomized rats markedly reduced this lactogenic activity and caused multiple immunological deficiencies and death. These findings elucidated several vital functions subserved by PRL and raised the possibility that extrapituitary PRL compensates, at least in part, for a deficiency in pituitary PRL.

The presence of PRL at any given site should not be considered evidence for local synthesis. Some tissues can transfer PRL from the circulation into another compartment by PRL-binding proteins acting as transporters. PRL can be delivered to other sites by infiltrating lymphocytes or migratory macrophages. Certain cells are capable of accumulating PRL by internalization. Consequently, PRL can be concentrated in some sites, accounting for a common discrepancy between PRL detection by antibody-based methods and those measuring gene expression.

The full spectrum of PRL functions in humans is not completely understood. The common view is that PRL is essential for lactation, is deleterious to reproduction when produced

in excess, but has no distinct functions in nonpregnant, nonlactating women or in normal men. Several important points should be considered, however. First, although the human fetus is exposed to unusually high levels of PRL, potential morphogenic or regulatory functions of PRL during fetal development are enigmatic because human fetuses are inaccessible for experimentation, and comparable animal models are not available. Second, unlike GH, dysfunctional mutations in either PRL or receptor have not been identified in humans, suggesting that PRL might subserve a vital function. Third, the consequences of PRL absence in adults are unclear because hypophysectomy or panhypopituitarism does not eliminate extrapituitary PRL. Fourth, GH, which has a substantial lactogenic activity and can bind to PRL receptors in humans, can compensate for some of PRL's functions in cases of severe PRL deficiency.

Using pituitary PRL as a model, we first briefly discuss PRL-producing cells, gene regulation, molecular heterogeneity, receptor structure, and mechanism of action. The main focus of this review is on local production and regulation of PRL in extrapituitary sites and its putative functions as an autocrine/paracrine factor. These sites are organized into four categories: reproductive organs, immune system, brain, and other organs. Clinical aspects are discussed in the final section. The overall concept of PRL as an endocrine/paracrine/autocrine factor is presented in Fig. 1.

II. Pituitary PRL as a Model

A. PRL-producing cells

Lactotrophs are the last anterior pituitary cells to differentiate and are preceded by GH and dual GH/PRL-producing cells (2). A functional POU homeodomain transcription factor, Pit-1, is required for the development of somatotrophs, lactotrophs, and thyrotrophs, and its inactivation results in virtual absence of these cell types (2). The factors that induce terminal differentiation of lactotrophs are unknown, but nerve growth factor (3), epidermal growth factor [EGF (4)], and basic fibroblast growth factor (5) may be involved since they promote lactotroph maturation in neonates. Lactotrophs are among the APUD (Amine Precursor Uptake and Decarboxylation) cells of neuroectodermal origin because of their neuronal-like properties (reviewed in Ref. 6). Although this classification is not universally accepted, there is evidence that some anterior pituitary cells originate from the neural primordium (7). The use of neuronal/glial stem cell markers could help in tracking neuronal-like cells that may populate the adenohypophysis during morphogenesis.

Lactotrophs comprise 20–50% of total anterior pituitary cells. Morphological criteria have distinguished two main cell types: densely granulated cells that function as storage or resting cells and sparsely granulated secretory cells that are particularly abundant in prolactinomas (8). Functionally, lactotrophs are heterogeneous with respect to basal hormone

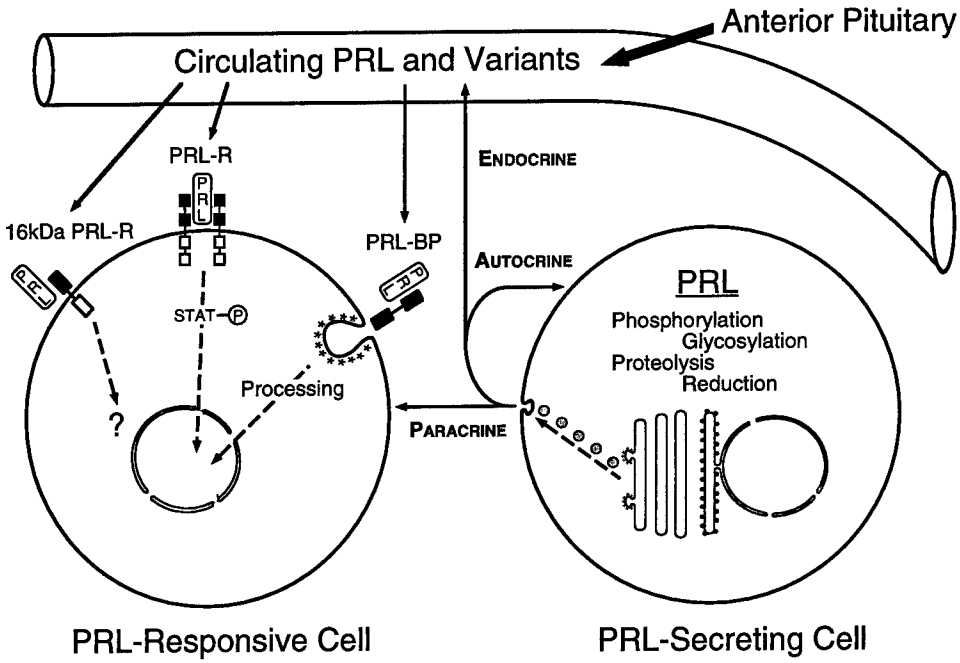

FIG. 1. Diagram demonstrating the pleiotropic actions of PRL. Circulating PRL and variants are derived primarily from the anterior pituitary with a lesser input from extrapituitary PRL-secreting cells (endocrine). Locally produced PRL can affect either adjacent cells (paracrine) or the PRL-secreting cell itself (autocrine). Processing of PRL can occur at both the production site and the target tissues. PRL-responsive cells have one or more receptor/binding proteins. The classic membrane receptor (PRL-R) dimerizes upon binding to PRL and activates the JAK/STAT pathway. After phosphorylation, STAT proteins translocate to the nucleus. Other signaling pathways might also be involved. The receptor that binds 16-kDa PRL (16 kDa PRL-R) differs from the classic receptor, but neither its structure nor signaling pathways are known. The PRL-binding protein (PRL-BP) is smaller than the classic receptor and could be either a truncated form of the PRL-R or the product of another gene. The PRL-BP may be involved in internalization of PRL into responsive cells and in transcellular transport of PRL.

release, electrical activity, and responsiveness to secretagogues (reviewed in Ref. 9). Lactotrophs located in the central or lateral areas of the pituitary have different responsiveness to releasing/inhibiting factors (10), indicating regional differences in exposure to hypothalamic or posterior pituitary factors (6).

Dual secreting cells, somatomammotrophs, can be interconverted to somatotrophs or lactotrophs, depending upon the stimulus. This process facilitates rapid recruitment of PRL-producing cells while bypassing the metabolically costly cell division (11). Unlike most pituitary cells, lactotrophs maintain a robust proliferative capacity during adulthood, accounting for the higher incidence of prolactinomas than other types of pituitary tumors (8). Some rat strains (*e.g.* Fischer 344) are exquisitely sensitive to induction of prolactinomas by estrogens (12), but a similar role for estrogens in the etiology of human prolactinomas has not been demonstrated (8). Taken together, lactotrophs constitute a dynamic and heterogeneous population of cells that display a remarkable adaptation to altered physiological conditions.

Two PRL-secreting cell lines, GH_3 and MMQ, have been especially valuable for PRL research. The GH cell line has many derivatives that express either PRL alone, GH alone, or both. These cells have functional receptors for several hypothalamic peptides, growth factors, neurotropins, and steroid hormones (13, 14). Although they lack high-affinity dopamine receptors, they respond to dopamine when transfected with dopamine receptor constructs (15), indicating the presence of functional postreceptor signaling mechanisms. Unlike GH_3 cells, MMQ cells are nonadherent, secrete PRL only, and express receptors for dopamine (16) and oxytocin (17).

B. Control of PRL gene expression

The PRL gene is present as a single copy on chromosomes 6 and 17 in humans and rats, respectively. PRL shares 40% homology with GH and placental lactogen, and the three hormones were derived by gene duplication from a common ancestral gene some 400 million years ago (18). The rat PRL gene is approximately 10 kb in size and is composed of five exons (19). The mature PRL mRNA is about 1 kb and encodes a 227-amino acid protein that includes a 28-amino acid signal peptide that is cleaved upon entering the endoplasmic reticulum.

A 2- to 2.5-kb sequence at the 5′-flanking region of the rat PRL gene controls tissue-specific and hormone-dependent gene expression (20). Functionally, two distinct domains are recognized: a proximal promoter (between +33 and −250 bp) and a distal enhancer (between −1300 and −1800), both of which are required for pituitary-specific expression (21, 22). Four *cis*-elements in the proximal promoter (sites 1P to 4P) and four in the distal enhancer (sites 1D to 4D) bind Pit-1 (23). Pit-1 has an N-terminal transactivating domain and a POU homeodomain that binds to a consensus DNA sequence. Pit-1 is not restricted to the lactotrophs and may require interactions with other transcription factors to confer lactotroph phenotype. A basal transcriptional element at −85 to −112 may bind a repressor molecule that restricts the expression of the PRL gene in nonpituitary cells (24).

Many hormones, neurotransmitters, and growth factors affect the PRL gene. Ligands that bind to G protein-linked receptors, *e.g.* TRH, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), and dopamine, activate protein kinase A, protein kinase C and/or calcium/calmodulin-dependent pathways (reviewed in Ref. 22). A degenerate cAMP response element in the proximal promoter partially mediates the response to cAMP. The PRL promoter, however, does not bind cAMP response element binding protein CREB (25). Since the rat Pit-1 promoter has two CREB sites, cAMP affects the PRL gene also indirectly, via increased production of Pit-1 (26). Response elements for calcium and TRH are located in both the proximal and distal promoter regions, and Pit-1 binding is necessary, but insufficient, for eliciting maximal stimulation by these compounds (27). Additional factors could be involved since the GH gene, which also binds Pit-1, is not activated by many substances that regulate the PRL gene.

Steroid hormone receptors act as transcription factors by binding to consensus sequences on target genes. An imperfect palindromic estrogen response element (ERE) is located in the distal enhancer, adjacent to the D1 Pit-1 site (28). To exert its actions, estrogen requires multiple Pit-1 binding sites (21). Binding of the estrogen receptor to ERE causes looping of the DNA, bringing the enhancer and promoter regions to juxtaposition via protein-protein interactions (29).

Among the growth factors, insulin (30) and EGF (31) stimulate, whereas transforming growth factor-β suppresses (32), the PRL gene. Growth factors often exert pleiotropic actions, as exemplified by EGF, which increases PRL gene transcription and hormone storage and alters the morphology of lactotrophs (4, 33). Many growth factors bind to transmembrane receptors with intrinsic tyrosine kinase activity. Binding triggers signal transduction via the ras/raf/mitogen-activated peptide kinase pathway and induces nuclear transcription factors such as c-*jun*, c-*myc*, and c-*ets*. A *ras*-signaling pathway, resulting in induction of *ets*, is functional in GH_3 cells (34), but the native ligand that activates this *ras* pathway is unknown.

The lack of a human PRL-producing cell line has impeded progress in understanding the regulation of human PRL gene expression. As illustrated in Fig. 2, the human gene differs from the rat gene in several respects. It is composed of six, rather than five, exons and is more than 15 kb long (35). The extra noncoding exon, exon 1a, has a transcriptional start site 5.8 kb upstream of the pituitary start site (36). In extrapituitary sites such as decidua, myometrium, and lymphoid cells, exon 1a is spliced to the first pituitary exon 1 (or 1b). This generates a RNA transcript that is approximately 150 bp larger than the pituitary counterpart (37), differing only in the 5′-untranslated region (Fig. 2). A super distal promoter region at −3500 to −5000 (38), which regulates PRL gene expression in extrapituitary sites, will be discussed in *Sections III and IV*. Compared with the rat gene, the pituitary-type proximal promoter of the human gene has three, rather than four, Pit-1-binding sites, and the distal enhancer has eight Pit-1-binding sites of which only two (D2 and D6) contain the exact consensus sequence. Site D5 appears to bind to a factor

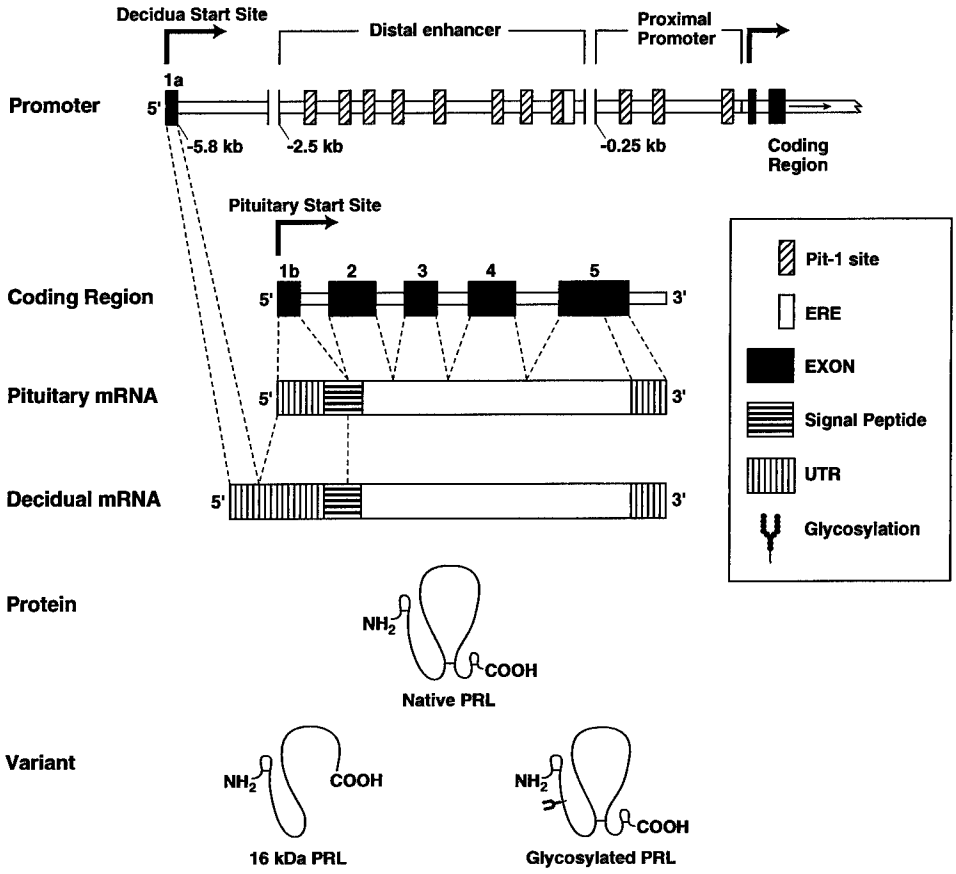

FIG. 2. Diagram of the human PRL gene, mRNA, native protein, and selected PRL variants. The PRL promoter has three regulatory domains: 1) a proximal region with three Pit-1-binding sites, 2) a distal region with eight Pit-1-binding sites, only two of which (designated with *) have an exact consensus sequence, and an estrogen response element (ERE) sequence, and 3) a superdistal region (from −3.5 to −6 kb). Extrapituitary sites (*e.g.* decidua) use an alternative promoter with the start site located 5.8 kb upstream of the pituitary start site. The decidual-type mRNA is about 150 bp longer in the 5′-untranslated region (UTR) than the pituitary-type mRNA. The native PRL protein is composed of 199 residues and has three intramolecular disulfide bonds. Two PRL variants, formed by posttranslational modification, are shown: the 16-kDa PRL variant formed by cleavage and reduction and glycosylated PRL with a single N-linked glycosylation site at position 31. [Derived from Refs. 35–39 and 60.]

belonging to the AP-1 (*jun*) family (39). Although it contains a degenerate ERE sequence, the human PRL promoter responds poorly to estrogen (40).

C. Regulation of PRL release

Multiple secretagogues affect PRL release. The older notion that secretagogues act rapidly while gene regulators act slowly has been questioned, given the very fast induction of genes such as c-*fos*. The two types of regulators may be better classified by their preferred utilization of cellular compartments, *i.e.* secretagogues act on the cell membrane and activate calcium-dependent exocytosis while gene regulators, directly or indirectly, utilize the nuclear compartment. These are not mutually exclusive since many (though not all) secretagogues also affect gene expression. The list of PRL secretagogues is rather long, but the physiological relevance of compounds that affect the lactotrophs *in vitro* only, in the absence of dopaminergic inhibition, is uncertain. Selected features of dopamine and TRH, representing an inhibitor and stimulator, respectively, will be presented. Comprehensive reviews on PRL release are found elsewhere (9, 14, 41, 42).

Dopamine, the primary inhibitor of PRL release, acts by binding to a seven- transmembrane, G protein-coupled (G_o/G_i) receptor. There are two D2 receptor subtypes, long ($D2_{444}$) and short ($D2_{415}$), which result from alternative splicing (43). These exist at different ratios in pituitary lactotrophs and may be coupled to different G proteins (44). The exact mechanism by which dopamine inhibits PRL release is uncertain. Within seconds, dopamine increases potassium conductance and inactivates voltage-sensitive calcium channels, resulting in membrane hyperpolarization and lowering of intracellular free calcium (45). Within minutes to hours, dopamine suppresses adenylate cyclase activity (46), inositol phosphate metabolism (47), arachidonic acid release (48), and PRL gene expression (49). Within days, dopamine suppresses cell proliferation (50). Paradoxically, very low levels of dopamine appear to stimulate PRL release (51). It is unclear whether these processes occur within the same cell or in different subpopulations and to what degree they proceed sequentially or in parallel. Until resolved, the multiple effects of dopamine on the lactotrophs remain poorly understood.

TRH induces a biphasic stimulation of PRL release: a rapid rise followed by a sustained lower release (52, 53). The first phase involves inositol triphosphate (IP_3)-mediated calcium

release from the endoplasmic reticulum. The second phase results from increased calcium influx through L-type, voltage-dependent calcium channels (52, 54). Like the dopamine receptor, the TRH receptor belongs to the superfamily of G protein-coupled receptors. Binding of TRH activates the two transduction pathways via different G proteins. Stimulation of phospholipase C and generation of IP_3 and diacylglycerol occur by receptor coupling to G_q/G_{i1}, while activation of calcium channels involves G_{i2} and G_{i3} and requires a concurrent protein kinase C action (55). The coupling of the receptor to different G proteins may indicate the presence of TRH receptor subtypes.

D. Structural heterogeneity

PRL is a single chain polypeptide of 23 kDa. It has three intramolecular disulfide bridges between residues 4–11, 58–174, and 191–199, N-linked (in some species, O-linked) glycosylation sites, and three phosphorylation sites (Ref. 56; see Fig. 2). A model of the three-dimensional structure of PRL, fashioned after x-ray crystallography of GH, predicts that it is composed of four α-helices arranged in an antiparallel bundle (reviewed in Ref. 57). The helix-bundle motif is shared with hematopoietic factors such as interferon, many interleukins, and ciliary neurotropic factor. The 60% to 100% sequence homology of PRL molecules in different mammalian species reflects their phylogenetic relationship. About 30 residues, clustered in four distinct regions, are highly conserved among species and may be important for binding to the receptor (58).

Many variants of PRL are known and could theoretically be formed by transcriptional or translational mechanisms. Since the PRL gene has several potential splice sites, variants could arise by differential splicing. An alternatively spliced PRL mRNA, presumably missing exon 4 and encoding a PRL molecule of 137 amino acids, has been reported in the brain (59). Retention of an intron, potentially resulting in a 28-kDa molecule, has also been reported (reviewed in Ref. 60). However, translation products of these mRNAs have not yet been identified. Instead, most if not all PRL variants are formed by posttranslational modifications and differ in size and functional groups. Larger forms of PRL are mostly formed by aggregation. In general, only some forms retain PRL-like activities while others have unique properties or no known functions. An excellent review by Sinha (60) covers this topic. Here, we highlight only aspects that are relevant to our topic.

Smaller molecular mass variants of PRL are produced by proteolytic cleavage, both at the sites of synthesis (61, 62) and at some target tissues (63, 64). Two variants, a 22-kDa and a 16-kDa isoform, are of particular interest. The 22-kDa form, PRL_{1-173}, is generated by kallikrein, a trypsin-like serine protease that is abundant in lactotrophs (62). It cleaves PRL at Arg^{174}-Arg^{175}, followed by the removal of the last Arg residue by carboxypeptidase. The production of this variant is sex-dependent and is stimulated by estrogen and suppressed by dopamine (65). Although it has low potency in the Nb2 lymphoma bioassay, the higher release levels in females than in males suggest a potential role in female reproduction.

The 16-kDa PRL is of interest because of its antiangiogenic activity. Its formation proceeds in two steps: cleavage by a cathepsin D-like protease around residues 145–149, which generates a two-chain molecule joined by a disulfide bond, followed by a reduction yielding 16-kDa (PRL_{1-143}) and 8-kDa fragments (see Fig. 2). The 16-kDa PRL has been detected in the hypothalamus (66), pituitary, and serum (67), accounting for about 1% of total secreted PRL. Early reports that it has a higher lactogenic and mitogenic potency in the mammary gland than intact PRL (68) have been challenged. In fact, 16-kDa PRL binds only weakly to PRL receptors (69). Since 16-kDa PRL lacks the putative fourth α-helix, it could assume a different configuration. The 16-kDa PRL inhibits basal and FGF-stimulated growth of capillary endothelial cells (70). This antiangiogenic activity, shown both *in vivo* and *in vitro*, is mediated by a high-affinity receptor distinct from the PRL receptor (71).

Glycosylated PRL is detected in pituitary and plasma, composing 1%, 15%, and 50% of total PRL in bovine, human, and porcine pituitaries, respectively (60). There is evidence for constitutive, rather than regulated, secretion of glycosylated PRL (72). The carbohydrate moieties vary among species, and their heterogeneity accounts for differences in bioactivity, immunoreactivity, receptor binding, and MCR (reviewed in Ref. 60). Glycosylation often decreases PRL bioactivity, although unique physiological functions of glycosylated PRL have not been identified. PRL can also be mono- or diphosphorylated on serine and/or threonine residues, a modification that results in charge variability (73). The ratio of phosphorylated/nonphosphorylated forms is altered during the estrous cycle and pregnancy (73). Since phosphorylated PRL inhibits the release of native PRL from GH_3 cells (74), it may have an autocrine/paracrine function.

E. Receptors and mechanism of action

The PRL receptor, originally cloned from rat liver, encodes a 291-amino acid protein that is considerably shorter than the homologous GH receptor. It was later discovered that the rat has a second, 'long' form that was cloned from the ovary and contains 591 amino acids (reviewed in Ref. 75). A third, 'intermediate' form, containing 391 amino acids, has been detected in the Nb2 transformed rat T cell line (76). It is a truncated form of the long receptor missing the last 198 amino acids due to a deletion in the last exon. The human PRL receptor is encoded by a single gene, located on chromosome 5 in close proximity to the gene of the GH receptor (77). There is evidence for more than one PRL receptor form in humans (78, 79), but to date only a single (long) form has been cloned.

The PRL receptor belongs to the hematopoietic receptor family that includes GH, many cytokines, and some growth factors. All are characterized by a single hydrophobic transmembrane domain that divides the receptor into an extracellular ligand binding domain and an intracellular domain (75). Features common to the extracellular domain include four paired cysteine residues and a wzxwz motif (tryptophan-serine-any amino acid-tryptophan-serine), both of which are necessary for forming the ligand-binding pocket. The cytoplasmic domains of these receptors differ in size and structure. A hydrophobic proline-rich motif (homology box 1), located near the transmembrane region, is essential for

signal transduction for all ligands studied (57, 80). The PRL receptor and several other hematopoietic receptors also contain a less conserved cytoplasmic region, denoted box 2 (81, 82).

The 'long' and 'short' PRL receptor isoforms in the rat have identical extracellular domains but differ in the length and sequence of the intracellular domain. These differences appear to be the result of alternative splicing of a single PRL receptor gene. Both isoforms of the PRL receptor exist in most rat tissues, and their ratio is altered under some conditions (83, 84). When transfected into COS cells, the short PRL receptor does not stimulate the milk protein gene promoter (81). Although the exact function served by each isoform is unknown, one might predict that they are coupled to different signal transduction pathways and account for some of the pleiotropic actions of PRL.

PRL receptors are present in nearly all tissues in the rat; the highest concentrations are in the liver, choroid plexus, ovary, and mammary gland (83). It is likely that signal transduction by PRL requires binding of one PRL molecule to two receptor molecules. The dimerization model, fashioned after x-ray crystallography for GH (85), assumes that each PRL molecule has two binding sites that interact sequentially with the receptor (86, 87). At high concentrations, PRL can saturate the receptor and hinder further receptor dimerization, which explains the often observed bell-shaped dose-dependent curves. In some lymphocytes, PRL is also rapidly internalized and is translocated to the nucleus (88). Intranuclear accumulation of PRL is stimulated by interleukin-2 (IL-2), is maximal within 6 h of treatment, and is inhibited by extracellular anti-PRL antibodies (89). However, the specific function of intranuclear PRL remains to be elucidated. There is evidence that in the choroid plexus (90), amniochorion (79, 91) and mammary epithelial cells (92, 93), a PRL receptor/binding protein, possibly of a different structure, acts as a transporter that translocates PRL from blood to their respective fluid compartments, *i.e.* cerebrospinal fluid (CSF), amniotic fluid, and milk (see Fig. 1).

PRL signaling is mediated through a cytoplasmic tyrosine kinase pathway, a mechanism originally described for the interferon receptor (94). The JAK (Janus kinase) tyrosine kinases mediate signal transduction for most members of this superfamily. One of these, JAK2, is constitutively associated with the PRL receptor. Binding of PRL to its receptor causes phosphorylation of JAK2 and autophosphorylation of the distal intracellular domain of the receptor (81, 95). The activated JAK2 phosphorylates other associated proteins, most notably the STAT proteins (Signal Transducer and Activator of Transcription). Of the STAT proteins presently known, STAT 1 and STAT 5 are activated by PRL (96). The phosphorylated STAT proteins translocate to the nucleus where, probably in association with other proteins, they bind to specific response elements, called GAS ([Gamma]-interferon activated site), in the promoters of target genes. In T cells, there is evidence for an association between the PRL receptor and a serine threonine kinase RAF-1, although this kinase is not tyrosine phosphorylated by PRL as it is by other members of this superfamily (97).

III. PRL in Reproductive Organs

A. PRL production by the human decidua

During pregnancy, both the maternal and fetal components of the placenta produce PRL-like substances. These include placental lactogens that resemble either GH or PRL, compounds with unknown functions that share sequence homology with PRL, and PRL itself. These proteins differ in structure and function among species and are expressed in a cell- and time-specific manner during pregnancy. Discussion of the PRL-like proteins of pregnancy is beyond the scope of this review and here we focus on decidual PRL.

The human decidua is a specialized endometrial stromal tissue that differentiates in the late luteal phase of the menstrual cycle in preparation for blastocyst implantation. Under the influence of progesterone, histological signs of decidualization, *e.g.* enlargement and rounding of the spindle-shaped stromal cells, are recognized on about day 22 of the cycle. If pregnancy occurs, decidual cells proliferate and undergo further morphological and physiological changes. Eventually, the decidua forms the maternal boundary to the fetal chorion and amnion. PRL synthesis coincides with the initiation of decidualization, increases after implantation, reaches a peak at 20–25 weeks of gestation, and declines toward term (98, 99).

PRL, produced *de novo* by decidual cells (100, 101), is indistinguishable from pituitary PRL by chemical, immunological, and biological criteria (reviewed in Ref. 102). Sequence analysis of decidual PRL cDNA (103) has established its structural identity with pituitary PRL. However, it was later discovered that the decidual RNA transcript is larger than that in the pituitary, differing only in the 5'-untranslated region (37). The use of the alternative promoter accounts for the tissue-specific regulation of PRL gene expression. Indeed, decidual PRL is regulated by autocrine and paracrine factors from the feto-placental unit, while classic regulators of pituitary PRL such as dopamine, TRH, and VIP are ineffective (Refs. 102 and 104; see Table 1). Moreover, Pit-1, which is mandatory for PRL expression in the pituitary, is not produced in decidual tissue (2). Although the alternative promoter contains two consensus Pit-1-binding sites, Pit-1 does not affect PRL transcription in decidual cells (105).

Progesterone is the factor that controls decidual PRL most effectively. In its absence, endometrial stromal tissue from the proliferative phase does not secrete PRL, and secretory phase endometrium ceases production within 2–3 days in culture. When progesterone is present, the decidualized endometrium produces PRL at increasing levels (106–108). Evidence suggests, however, that progesterone is important for inducing and maintaining decidualization rather than as a direct stimulator of PRL gene expression. For instance, an activated progesterone receptor does not increase transcription of the decidual PRL promoter even though the promoter contains seven half-sites for glucocorticoid/progesterone receptors (105). Also, progesterone receptors do not colocalize in PRL-producing cells in decidualized tissue (109). The decidua contains heterogeneous cell populations, and progesterone acts as a differentiating agent that induces fibroblasts from term decidua to express PRL (110). This suggests the

TABLE 1. Differential regulation of PRL synthesis and release by selected substances in the decidua, myometrium, and pituitary[a]

Substance	Decidua	Myometrium	Pituitary
Neuropeptides:			
TRH	⇋ (104)[b]	nd	↑ ↑ ↑ (52)
VIP	nd	⇋ (154)	↑ ↑ (156)
Neurotransmitters:			
Dopamine	⇋ (104, 131)	nd	↓ ↓ ↓ (157)
Steroids:			
Estrogen	⇋ (107)	↑ (150)	↑ ↑ (158)
Progesterone	↑ ↑ ↑ (107, 108)	↓ ↓ ↓ (150, 152)	⇋ (158)
Growth factors:			
EGF	nd	↓ (154)	↑ (31)
IGF-1	↑ ↑ (114)	nd	↑ ↑ (159)
PDGF	↓ (118)	nd	nd
Hematopoietic factors:			
IL-1α and β	↓ ↓ (118, 119)	nd	↓ (160)
IL-4	nd	↓ ↓ (154)	nd
TNFα	↓ ↓ (118, 119)	nd	nd
Protein hormones:			
Endothelin-1	↓ ↓ (116)	nd	↓ ↓ (161)
hCG	nd	↑ ↑ (163, 164)	nd
Insulin	↑ ↑ (113)	nd	↑ (30)
Lipocortin-1	↓ ↓ ↓ (115)	nd	nd
Relaxin	↑ ↑ (107)	nd	↑ (162)

Symbols are as follows: ↑, small increase; ↑ ↑, moderate increase; ↑ ↑ ↑, strong increase; ⇋, no effect; ↓, small decrease; ↓ ↓, moderate decrease; ↓ ↓ ↓, strong decrease; nd, not determined.

[a] Human decidua and myometrium, rat pituitary

[b] References in parentheses

presence of a population of stem cells capable of proliferation or terminal differentiation, depending upon the hormonal/extracellular matrix environment.

Unlike pituitary lactotrophs, which possess dense secretory granules, decidual cells resemble the transformed GH_3 cells by having little storage capacity (reviewed in Ref. 111). Decidual PRL is rapidly released after synthesis and is not affected by increased extracellular calcium (112). This is unusual for cells that secrete protein hormones and is more typical of steroid-producing cells. Among the stimulators of decidual PRL are insulin-like growth factor-I, insulin, and relaxin, each acting through a distinct plasma membrane receptor (107, 111, 113, 114). Lipocortin-1, a calcium- and phospholipid-binding protein (115), and endothelin-1 (116) cause inhibition. Intracellular second messengers mediating these effects are unknown, although cAMP, diacylglycerol, phospholipase A2, and arachidonic acid, have all been implicated (117). As presented in Table 1, hematopoietic factors, *e.g.* tumor necrosis factor, IL-1, and platelet-derived growth factor (Refs. 118 and 119), also inhibit decidual PRL release. These are produced by macrophages and large granular lymphocytes, which populate the decidua.

Local proteins that stimulate and inhibit decidual PRL have also been reported (120). A releasing factor (PRL-RF), a partially purified 23-kDa protein (121) stimulates decidual PRL release in a biphasic manner, with only the second phase inhibited by cycloheximide (122). An inhibitory factor (PRL-IF), a 35- to 40-kDa protein present in decidual conditioned media, decreases basal decidual PRL release and reverses the stimulatory effect of PRL-RF (117). Neither of these factors affects pituitary PRL release, and their structure remains to be elucidated.

B. Amniotic fluid PRL and putative functions

PRL-like activity was detected in the amniotic fluid in the early 1970s and by all criteria was found to be identical to pituitary PRL (123–126). In addition to the 23-kDa native PRL, several PRL variants are present in the amniotic fluid, including large aggregates, small molecular mass forms, and cleaved PRL (91, 127). The amniotic fluid also contains significant amounts of glycosylated PRL, which increases in concentration with the length of gestation (128). The very high concentrations of PRL in the amniotic fluid, reaching 50- to 100-fold over maternal or fetal blood PRL levels, are affected by two factors: the massive size of the decidua (production rate estimated as 1 μg/g of decidua) and the long half-life of PRL in this compartment (4.5 h as compared with 15–20 min in the blood; reviewed in Ref. 129).

Although the source of PRL in the amniotic fluid was not immediately apparent, there is now compelling evidence that it originates from the decidua (reviewed in Ref. 130). For example, the dynamics of PRL concentrations in amniotic fluid and maternal and fetal blood throughout gestation are very different. Whereas peak PRL concentration in the amniotic fluid occurs at 20–24 weeks, PRL in either the maternal or fetal blood reaches its highest levels at term (Refs. 131–133; see Fig. 3). Moreover, suppression of maternal serum PRL levels by hypophysectomy (134) or bromocriptine therapy (135) does not reduce amniotic fluid PRL. Also, amniotic fluid PRL concentrations parallel the changes in decidual PRL mRNA levels (99) and release capacity (136) during most of gestation. The decline toward term, however, cannot be readily explained by either a reduction in decidual synthetic capacity or a dilutional effect of increased amniotic fluid volume.

From its decidual site of production, PRL is transported to

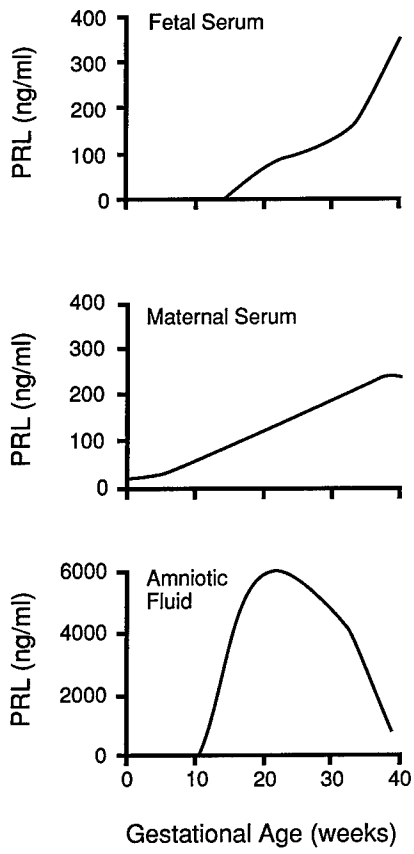

FIG. 3. Profile and relative levels of PRL in three fluid compartments during human pregnancy. PRL in the amniotic fluid peaks at about 20–24 weeks of gestation, whereas that in the maternal circulation gradually increases until delivery. PRL in fetal blood becomes detectable at about 15 weeks and markedly increases from the 34th-35th week until delivery. Note that PRL concentrations in the amniotic fluid PRL are 10- to 50-fold higher than either the maternal or fetal blood. [Derived from Refs. 102, 128, 131–133, 347, and 348.]

the amniotic fluid by the chorioamnion membranes, tissues of fetal origin. Under *in vitro* conditions, PRL crosses from the maternal to the fetal side only if tissue integrity is maintained, *i.e.* the decidua remains adherent to the fetal membranes (91). The process by which PRL unidirectionally traverses several layers of fetal membranes but is prevented from entering the maternal side presumably requires cellular polarity and specific transporter/binding proteins, as illustrated in Fig. 4. Such binding proteins account for the presence of PRL in the cytoplasm of amnionic and chroionic cells (137, 138), which themselves do not produce PRL. Indeed, PRL-binding sites in these tissues become detectable only after 'stripping' endogenously bound PRL (139, 140). Using autoradiography, only a small subset of amniotic epithelial cells were capable of binding PRL (141). A recent study found multiple molecular species of the PRL receptor (79). Two of these, 30 and 63 kDa, were detected in the cytosol of amnion and chorion cells as well as in the amniotic fluid, suggesting that they function as transporter/binding proteins.

In spite of its prominence throughout human gestation, the functions of amniotic fluid PRL are speculative. Since PRL regulates water balance and electrolytes in lower vertebrates, a similar osmoregulatory function during the aquatic phase of human embryogenesis has been proposed (129). Introduction of ovine PRL into the amniotic fluid of pregnant rhesus monkeys decreased amniotic fluid volume due to transfer of water to the maternal compartment (142). This was supported by *in vitro* studies in which PRL reduced the permeability of the human amnion and increased net movement of ^{3}H-labeled water from the amniotic to the maternal side (143). Additionally, excessive production of amniotic fluid, or polyhydramnios, is associated with abnormally low levels of PRL in the amniotic fluid, possibly due to a reduced number of lactogenic hormone receptors in the chorion (144). Yet, the production of PRL itself by decidual cells is unaffected by the osmolality of the incubation medium (102, 112).

Fetuses swallow and ingest up to 500 ml/day of amniotic fluid, and their immature gastrointestinal tract can transport intact proteins (145, 146). Presumably, PRL from the amniotic fluid can gain access to the fetal circulation via this route and regulate fetal functions. An early report implicated PRL in lung maturation by enhancing surfactant production in rabbit fetuses (147), but these results have not been reproduced in other species. There are correlations, however, between amniotic fluid PRL concentrations and fetal lung maturation under certain conditions (148). Another intriguing speculation is that amniotic fluid PRL may affect the maturation of the fetal immune system. If so, the amniotic fluid could provide a ready supply of PRL to the developing fetus at a time when PRL synthesis by its own pituitary is low (132). Based on circumstantial evidence only, functions for decidual PRL outside of the amniotic fluid compartment have also been proposed. These include effects on implantation, prevention of immunological rejection of the blastocyst, and inhibition of uterine contractility before labor (111). An association of PRL with abnormalities of pregnancy is discussed in *Section VII.*

C. Myometrial PRL

The myometrium, or muscle layer of the uterus, is another source of PRL production. The smooth muscle cells of the myometrium share a common mesenchymal embryonic origin with the stromal cells of the endometrium. Initial reports demonstrated that explants of normal myometrium as well as proliferative leiomyomas (fibroids) secreted immunoreactive PRL into the culture medium (149, 150). Subsequent investigation established that myometrial PRL is identical to pituitary PRL, based on physicochemical and biological criteria (151, 152). Like the decidua, the human myometrium utilizes the alternative promoter, and its RNA transcript is 150 bp larger in the 5′-untranslated region (152). In addition to the primary 23-kDa PRL, myometrial explants synthesize a glycosylated PRL variant. The exact cell type that secretes PRL is unknown, with conflicting reports as to whether monolayer cultures derived from myometrial explants continue to release PRL (152, 153).

Of considerable interest is the finding that the regulation of myometrial PRL secretion differs significantly from that of the decidua. The observation that myometrial PRL release increases with time in culture suggested that PRL is under inhibitory control *in vivo*. Indeed, progesterone, the most important stimulator of decidual PRL, inhibits myometrial

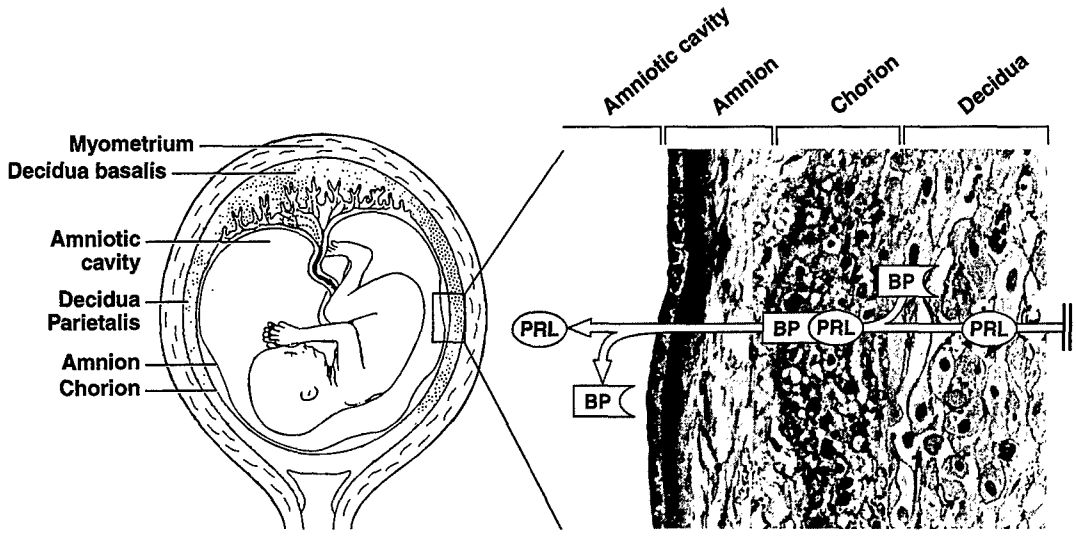

FIG. 4. Postulated mechanism for the production and transport of PRL from the decidua to the amniotic fluid. The drawing of the pregnant uterus (*left*) shows structural relationship between the maternal components (myometrium and decidua) and fetal membranes (chorion and amnion). The fetus is shown within the amniotic cavity, which is filled with amniotic fluid. *Inset on the right* shows cellular details of the decidua, chorion, and amnion. PRL is produced by decidual cells and is transported across the chorion and amnion into the amniotic cavity but is not transferred into the myometrium or the maternal circulation. The model postulates that PRL transport requires a PRL-binding protein (BP) and cellular polarity. [Derived from Refs. 79, 91, 100, 139, 140, and 141; Figure on the *left* is adapted from MB Renfree: Implantation and placentation. In: Austin CR, Short RV (eds) Embryonic and Fetal Development. Cambridge University Press, Cambridge, England, 1982, pp 66. Reprinted with the permission of Cambridge University Press.]

PRL expression at concentrations as low as 10^{-10} M (152); this has not been observed using uterine fibroids (153). Thus, the PRL gene in two adjacent tissues might be regulated in opposite directions by progesterone, representing an unusual situation. Other substances could also be involved, since PRL expression in myometrial explants was strongly inhibited by placental conditioned media from which progesterone had been removed (154). Among the growth factors and cytokines tested, IL-4 was the most potent inhibitor of myometrial PRL expression (see Table 1).

The function of myometrial PRL is unknown. If progesterone is inhibitory, then the myometrium may not express much PRL during the luteal phase of the cycle or during pregnancy, when circulating progesterone levels are elevated. On the other hand, if PRL expression increases during the proliferative phase, when the endometrium does not produce PRL, this may be the source of the higher levels of PRL found in the uterine luminal fluid than in circulating levels (155). The potential role of PRL in the pathogenesis of leiomyomas is discussed in *Section VII.*

D. Mammary and milk PRL

PRL has been detected by immunoassays and bioassays within the epithelial cells of the lactating mammary gland (165, 166) and in milk (reviewed in Ref. 167). Sequestration of PRL from the circulation constitutes one mechanism by which PRL enters the milk compartment. This was supported by tracing radiolabeled PRL from blood to milk (168, 169) and by demonstrating transit of PRL through mammary epithelial cells *in vitro* (170, 171). For intercellular transit of PRL to occur, one must postulate a multistep process involving endocytosis, sorting, and a directional transport toward the alveolar lumen, as depicted in Fig. 5. This could involve either PRL alone or PRL bound to a receptor/binding protein (170). High-affinity PRL-binding proteins were identified in rabbit (92, 172) and human (93) milk, with an estimated molecular mass of 33 kDa, but the details of their structure and function remain to be defined.

Local synthesis is another source for milk PRL. This was initially inferred from indirect observations. For example, suppression of plasma PRL in lactating rats (167) and dairy cows (174) by bromocriptine did not lower immunoreactive PRL in milk. Similarly, treatment of lactating goats with bromocriptine did not impair lactational performance (175). Although it has been argued that PRL is not critical for supporting established lactation in ruminants, the alternative interpretation is that locally produced PRL compensates for reduced pituitary PRL release. Further, milk contains disproportionally high levels of PRL variants (176), suggesting that the mammary gland either modifies sequestered PRL and/or synthesizes its own PRL.

Emerging evidence indicates that mammary tissue from rodents (177–179), ruminants (173), and human breast carcinomas (78, 180) expresses PRL. PRL mRNA in mammary glands from pregnant and lactating rats has been detected by Northern analysis (177), RT-PCR (178, 179), and *in situ* hybridization (177, 178). The PRL transcript is unevenly expressed in the alveolar and ductal epithelium, suggesting functional heterogeneity among the secretory cells. Immunoreactive PRL, albeit at very low concentrations, was measured in mammary tissue extracts from lactating rats and in mammary tumors from carcinogen-injected cycling rats (179). Within 24–48 h of incubating mammary gland explants, both PRL mRNA and PRL released into the media declined to undetectable levels (178). The decline in PRL production by the explants suggests the absence of a critical stimulatory factor(s) *in vitro.*

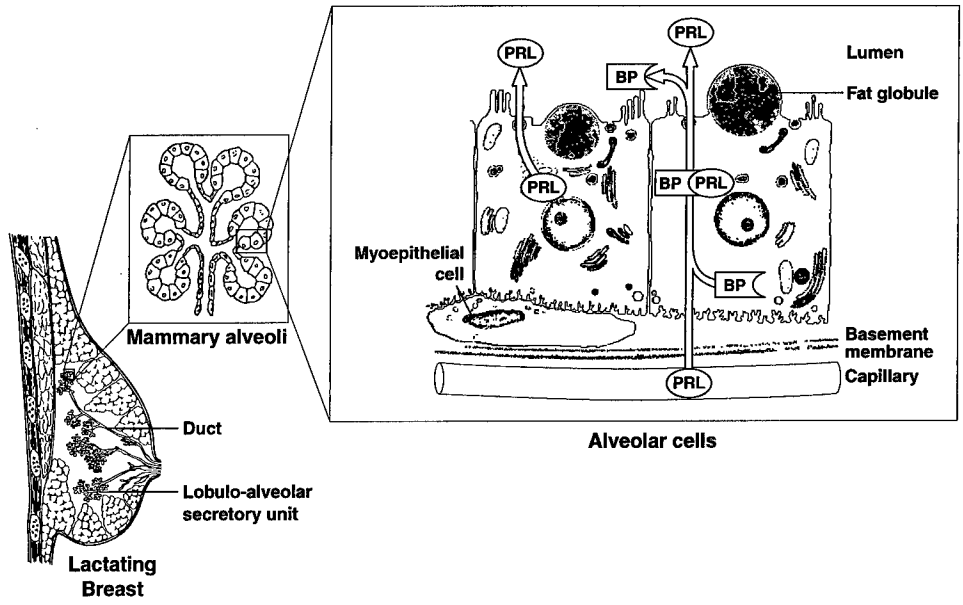

FIG. 5. Postulated mechanism for PRL accumulation in milk. The lactating breast (*left*) shows lobuloalveolar secretory units. *Inset in the middle* shows details of the round alveoli surrounding the lumen which contains milk. *Inset on the right* shows a schematic diagram of two alveolar epithelial cells interposed between a capillary and the lumen. PRL in milk can originate from two sources: 1) local synthesis (cell on the *left*), and 2) transport from the blood across the basement membrane and through the alveolar cell (cell on the *right*). For transport to occur, the model postulates the presence of a PRL-binding protein (BP) and cellular polarity. The relative contribution of the two sources to milk PRL is unknown. [Derived from Refs. 92, 93, 165–174, and 176–180; *Figure on the right* is adapted from AT Cowie: Lactation. In: Austin CR, Short RV (eds) Hormonal Control of Reproduction. Cambridge University Press, Cambridge, England, 1984, pp 205. Reprinted with the permission of Cambridge University Press.]

PRL was detected by Northern analysis and RT-PCR in mammary glands from pregnant and lactating sheep and goats (173). Both the transcript size and the sequence of the entire coding region are identical to those in the pituitary. Primer extension analysis revealed a 5′-untranslated region of 56 nucleotides that was similar to that in the pituitary, suggesting that both tissues utilize the same promoter. The finding of PRL mRNA in the polysomal fraction implied that ovine and caprine mammary tissue translate the message into protein (173). Future studies should examine whether human breast tissue that expresses PRL (78) utilizes the pituitary- or extrapituitary-type PRL promoter.

PRL is also processed by the mammary gland. Incubation of PRL with mammary slices resulted in the formation of cleaved products of 11- and 14 kDa (63); the 14-kDa form is similar to the 16-kDa PRL previously reported (181). PRL processing was higher in mammary glands from pregnant than from lactating rats and differed, qualitatively and quantitatively, from that occurring in other tissues. Cleavage was done by an acid protease and required internalization of PRL into an acidifiable compartment in the mammary cells. Milk contains more PRL variants, PRL aggregates, and a higher percentage of glycosylated and phosphorylated PRL than blood (167, 176). These could result from a preferential transport of PRL variants from blood to milk, an intracellular modification of PRL during transport, or a local synthesis of certain PRL variants.

As indicated, locally produced PRL serves as an accessory source for milk PRL. In addition to its essential nutritional value, milk contains many biologically active substances such as hormones, growth factors, enzymes, and immunoglobulins. With the use of several bioassays and immunoassays, PRL has been detected in milk from cows, goats, sheep, pigs, rats, monkeys, and humans (reviewed in Refs. 167 and 182). The highest PRL concentrations are found in colostrum, followed by a decline during the course of lactation. Milk from early lactation contains a higher ratio of bioactive *vs.* immunoreactive PRL (B/I ratio) than that from late lactation. The early milk from rats has a B/I ratio of 5, its PRL concentration exceeds that in blood, and most of the PRL is glycosylated or phosphorylated (176); similar findings were reported for human milk (183).

PRL provided via the milk may be especially important for species such as rodents, which are born immature. The gastrointestinal (GI) tract of neonatal rats allows absorption of intact proteins for a short time after birth. This is a valuable mechanism for conferring passive immunity to the offspring via milk-borne immunoglobulins (reviewed in Ref. 184). About 15–20% of milk PRL ingested by rat pups passes into their circulation (185). Perhaps the heavily glycosylated milk PRL is better protected from proteolysis and degradation in the neonatal GI tract. At this early age, the neonatal pituitary produces very little PRL (186). Several studies have established that PRL, provided by the milk during postnatal days 2–5, is necessary for the proper development and subsequent function of the neonatal neuroendocrine (187, 188) and immune (189–191) systems. In contrast, ruminants and primates, where fetal maturation is completed *in utero*, may utilize amniotic fluid PRL for similar purposes. If so, the benefits of milk PRL to human infants remains obscure.

Whether locally produced PRL plays a role within the mammary cells remains to be determined. Similar to pituitary-derived PRL, mammary PRL could promote production of milk constituents or functions as a mitogen/mor-

phogen that affects mammary cell proliferation and differentiation. The potential involvement of PRL in carcinogenesis of the breast is discussed in *Section VII.*

IV. PRL and the Immune System

A. Production of PRL by immune cells

The immune system is composed of many classes of cells, all of which are derived from a small number of progenitors in the bone marrow and thymus. Maturation and differentiation of lymphocytes occur at different sites, including bone marrow, thymus, spleen, and lymph nodes. The recognition of PRL as an immune cytokine evolved slowly because its synthesis by immune cells is very low, the cells are highly heterogeneous, and resting cells normally require activation to express PRL. Initial evidence was based on increased proliferation of Nb2 cells by conditioned media from Con A-stimulated splenocytes (192). The mitogenic effect was blocked by PRL antisera and partially reversed by exogenous PRL. Similarly, PRL antibodies blocked mitogen-induced proliferation of rat T and B lymphocytes and human peripheral blood mononuclear cells (193). Since this occurred in serum-free media, the authors suggested that a PRL-like molecule is produced by the lymphocytes and affects cell cycle progression. The failure of exogenous PRL to promote lymphocyte proliferation was attributed to the occupation of the PRL receptors by locally produced PRL (193).

The evidence for PRL production by immune cells is now compelling. PRL mRNA in rat and human lymphocytes has been detected by Northern analysis (194–196), RT-PCR (195, 197, 198), and *in situ* hybridization (199, 200). The PRL transcript in human lymphocytes is identical to the pituitary-derived PRL except for the longer 5′-untranslated region (195, 197); the PRL transcript from rat lymphocytes has not been characterized. Using Western blot analysis and metabolic labeling, a 22-kDa immunoreactive PRL was detected in murine thymocytes, and after mitogen activation, in their culture media (201); similar findings were reported for human immune cells (194, 197, 200). PRL variants with molecular sizes ranging from 11 to 60 kDa were detected in human lymphocytes (194, 197, 198), whereas variants ranging from 35 to 46 kDa were found in the mouse (202). A glycosylated 25-kDa form was also detected in human lymphoblastoid cells (203, 204). Although some forms may result from methodological artifacts, the presence of PRL variants could explain some inconsistent efficacy of PRL antisera in blocking lymphocyte proliferation (193, 198, 205, 207).

Flow cytometry and *in situ* hybridization were used to classify PRL-producing immune cells. Data to date indicate that human thymocytes and T cells, and to a lesser extent B cells, express PRL (197). As presented in Table 2, PRL expression in human lymphoid tissues was detected in the periarterial lymphatic sheath and marginal zone of the spleen, the subcapsular cortex of the thymus, and the paracortex of lymph nodes and tonsils (200). Vascular endothelium and epithelial cells, neither of which is of immune lineage, also have detectable signals. PRL was also detected

TABLE 2. Comparison of the distribution of PRL and PRL receptors in the human immune system and lymphoid cell lines

Tissue/cell	PRL		PRL receptor	
	mRNA[a]	Protein	mRNA	Protein
Immune cells:				
B lymphocytes	1 P (197)		3 (197)	3 F (213, 216)
Macrophages				2 F(213)
Mononuclear cells	2 P (197, 198)	2 E,W (194, 197, 198)	2 (197)	2 Bin,F (213, 215)
Natural killer		1 W (217)		2 Bin (217)
Thymocytes	3 N,P (194, 195, 197)	3 B,W (194)		
T lymphocytes	2 P (197)		2 (197)	2 F (213, 216)
Tissues:				
Lymph nodes	1 P,S (200)			
Spleen	2 P,S (200)			
Thymus	2 P,S (200)			
Tonsils	1 P,S (200)			
Tumors:				
Lymphoma	2 P,S (200)			
Thymoma	2 P,S (200)			
Myeloid leukemia		2 W (206)		
Cell lines:				
B cell origin				
DAUDI Burkitt		1 W,B (198)		
IM9-P-3	2 N,P (152, 204, 218)	2 W (204, 218)		
Raji				3 F (213)
Natural killer				
YT	3 P (197)			1 F (213)
T cell origin				
CEM				2 F (213)
HSB2				3 F (213)
Hut 78	1 P (197)			
Jurkat	2 P (195, 197)	1 W (197)		1 F (213)

Abbreviations: B, bioassay; Bin, binding; E, enzyme-linked immunoassay; F, flow cytometry/Ab; N, Northern analysis; P, RT-PCR; S, *in situ* hybridization; W, Western blot. Levels of detection: 0, non detectable; 1, weak; 2, moderate; 3, strong.

[a] Numbers designate levels of detection; letters designate methods of detection; references are in parentheses.

by immunoblotting in myeloblasts from patients with acute myeloid leukemia (206). In the rat, a few bone marrow cells, some cells in the red pulp and marginal zone of the spleen, and occasional cells in the thymus were positive for PRL (199, 208). Interestingly, a combined *in situ* hybridization and immunocytochemistry revealed significantly more cells containing the PRL protein than those expressing its mRNA, suggesting PRL accumulation by endocytosis (208).

Some transformed human lymphoid cell lines also produce PRL (Table 2). The B lymphoblastoid-derived subclone IM-9-P3 constitutively expresses the PRL message and hormone (204). As reported by Pellegrini *et al.* (197), PRL transcripts were also detected in two T lymphocyte cell lines (Jurkat and Hut), lymphoma cells (U937), and a natural killer cell line (YT). There is evidence for PRL release only from the Burkitt lymphoma-derived cell line IM-9-P3 (204). Additional research is necessary before the complete picture of PRL production by different classes of immune cells will emerge.

B. PRL receptors in immune cells

To validate PRL as an autocrine/paracrine factor, both the hormone and its receptors should be present in immune cells. Binding of PRL to lymphocytes was first demonstrated by Russell and co-workers (209). Subsequent studies detected PRL receptors on approximately 20% of primary rat splenocytes, using PRL receptor antibodies. However, as compared with Nb2 cells with about 12,000 receptors per cell, primary lymphocytes have only 1000–2000 receptors per cell (210). A much higher percentage of lymphoid cells that express the receptor, including all B cells and about 50–60% of T cells, was reported by another group (211). Both long and short forms of the receptor are expressed by rat lymphoid organs, with a slightly higher prevalence of the long form (83). Notably, bone marrow cells, which include all hematopoietic precursors, as well as peritoneal macrophages, express high levels of the receptor (211, 212).

Among human peripheral blood lymphocytes, B cells express a high density of the receptor whereas most resting T cells have a low density. However, upon mitogen activation, PRL receptor expression by T cells rapidly increases (211, 213). The PRL receptor was also detected in many human lymphoid cell lines (Table 2). A more systematic investigation, comparing the extent of receptor expression *vs.* its translation product, is necessary.

Maturation of lymphoid organs and their cell constituents is slow and complex. In rodents, this process begins *in utero* and is completed after birth. PRL receptor expression was detected in the thymus, but not spleen, at embryonic day 20 in the rat (214). At birth, the thymus already expressed adult levels of the receptor while the spleen showed an age-dependent increase (190). Neonatal rat splenocytes have a higher ratio of long/short forms of the receptor, the significance of which is unclear. PRL receptor expression in subclasses of human thymocytes may also parallel their maturational process. This is suggested by the low number of CD4− CD8− cells (the most immature thymocytes) that express PRL receptor, compared with the majority of the CD4+ CD8+ cells that express the receptor (213).

A relevant question is whether PRL receptor expression in immune cells is altered under various physiological conditions. The evidence thus far is inconclusive. Using quantitative PCR, Di Carlo *et al.* (84) reported that hyperprolactinemia slightly decreased, whereas bromocriptine administration increased, the expression of the long PRL receptor isoform in rat peripheral lymphocytes. Some changes in PRL receptor expression in thymocytes and splenocytes in response to milk-borne PRL were observed in neonatal rats (189). PRL receptor levels in peripheral lymphocytes in women did not show a clear correlation with either serum PRL levels or the stage of the menstrual cycle (215). Similarly, no significant changes in PRL receptor expression by lymphocytes were observed in hyperprolactinemic patients, with or without bromocriptine treatment (216). Collectively, these data suggest that factors other than pituitary PRL may regulate the expression of the PRL receptor on immune cells.

C. Immunoregulatory functions of PRL

An association between the pituitary and the immune system was first proposed based on the atrophy of the thymus after hypophysectomy (219). Later studies revealed that dwarf mice that are deficient in PRL and GH have atrophy of the thymus and peripheral lymphoid organs and abnormal immune responses (220, 221). Subsequent studies implicated PRL and GH in antibody production, graft survival, mitogen-induced lymphocyte proliferation, and alteration of splenocytes and thymocytes (reviewed in Refs. 208 and 222). Both hormones were also reported to reverse age-related decline in immune functions (223), antagonize the immunosuppressive effects of cyclosporine (196), and promote macrophage activation and T lymphocyte function (224).

A wide range of immunological perturbations, caused by PRL deficiency, was described by Berczi (225) and Berczi and Nagy (226). Humoral and cellular immune deficiencies in hypophysectomized rats were restored by anterior pituitary transplants or exogenous PRL (227–229). While GH produced similar responses to PRL, other pituitary hormones were ineffective. Most important, a combination of hypophysectomy and PRL immunoneutralization resulted in anemia, reduced delayed type hypersensitivity, and death (1), whereas hypophysectomy alone caused less severe perturbations and enabled survival. The difference between the two conditions can be attributed to extrapituitary PRL and underscores the essential role of local PRL production.

PRL is mitogenic for lymphocytes. The mechanism by which it promotes lymphocyte proliferation has been extensively studied using Nb2 cells, a rat cell line derived from immature T lymphocytes (230, 231). Nb2 cells are exquisitely sensitive and growth-dependent on PRL, which may be related to the presence of a truncated PRL receptor (76). Addition of PRL to growth-arrested Nb2 cells synchronizes the cells to enter the cell cycle by inducing transcription of growth-related genes. Within 1–2 h, PRL increases the transcription of *c-myc*, *c-fos*, β-actin (232), and interferon-regulatory factor-1 (IRF-1; Ref. 233), followed by a delayed induction of heat-shock protein 70, ornithine decarboxylase, and cyclins (reviewed in Ref. 57). In particular, IRF-1 (234)

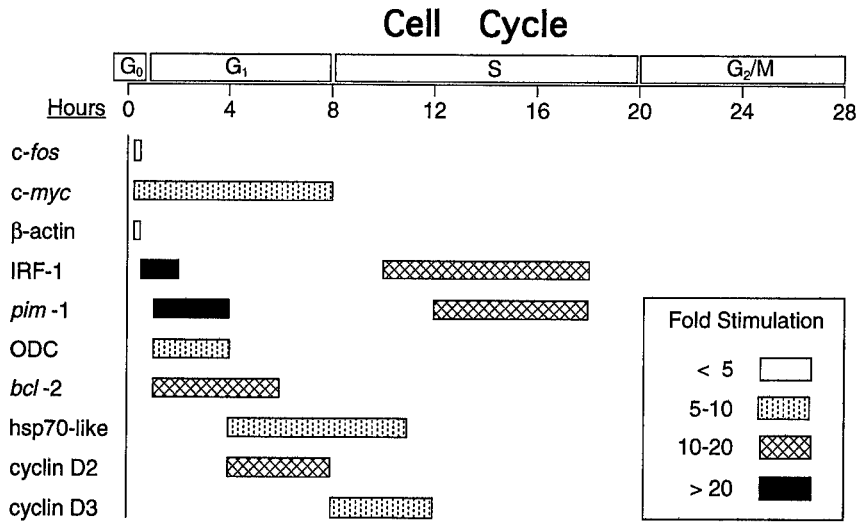

FIG. 6. Expression of various genes during PRL-initiated mitogenesis in Nb2 lymphoma cells. Cells were serum-starved before exposure to PRL at time 0. The relative increase of each gene is designated by the fold stimulation, and the duration of gene activation is designated by the length of the *bars*. Note the biphasic increase in at least two genes, IRF-1 and *pim*-1. [Derived from Refs. 232–235.]

and *pim-1* (235) are induced by PRL in a biphasic manner and may participate in cell transition into the G1 phase and then its entry into the S phase of the cell cycle. PRL also blocks glucocorticoid-induced apoptosis in Nb2 cells (235, 236), but it is unclear how the two pathways, *i.e.* stimulation of proliferation and inhibition of apoptosis, interact. The temporal induction of the various genes by PRL in Nb2 cells is illustrated in Fig. 6.

PRL often interacts with cytokines and functions as a coactivator. In the murine T-helper L2 cells, PRL alone induces the expression of IRF-1 but requires coincubation with IL-2 for inducing c-*myc*, proliferating cell nuclear antigen, thymidine kinase, cyclin B, and histones (237). PRL also induces IL-2 receptors, promotes IL-2 release and DNA synthesis in primary rat lymphocytes (238), augments IL-2-induced proliferation of human natural killer cells (239), and enhances mitogen-stimulated production of γ-interferon by mononuclear cells (240). There is also evidence for reciprocal interaction with IL-2 since treatment of L2 T cells with IL-2 causes a marked increase in PRL receptor expression during cell cycle progression (241).

Difficulties encountered in demonstrating the mitogenic effects of PRL on immune cells are partially attributed to the endocrine background of the donor animals, especially due to gonadal (238) and adrenal (242) steroids. Availability of PRL itself can also complicate the outcome. For instance, when thymic and spleen cells were harvested from hypophysectomized rats and then incubated in autologous serum, which is low in PRL, exogenous PRL (as low as 2.5 ng/ml) increased cell proliferation (243). This suggests that circulating PRL may saturate or down-regulate the receptors in immune cells, rendering them unresponsive to exogenous PRL *in vitro*. Moreover, since cells are usually cultured in sera containing lactogenic hormones, the effect of released PRL that was previously internalized, as was demonstrated for both lymphocytes (89) and lactotrophs (244), should not be overlooked.

Other functions of immune cells are also affected by PRL. The production of the thymic hormone thymulin is stimulated by PRL (245). PRL increases antibody production in both mouse (246) and human (247) lymphocytes, possibly by synergizing with IL-2. Transplantation of syngeneic pituitaries in mice, which results in hyperprolatinemia, increases circulating immunoglobulins (248). PRL may also provide protection against lethal effects of salmonella by increasing the phagocytic capacity of peritoneal macrophages (249). In natural killer cells, which defend against infection and tumor growth, PRL increases the expression of IL-2 receptors, enhances cellular responsiveness to IL-2, and stimulates colony formation and cytotoxicity (217). As emphasized earlier, it is presently impossible to dissect out the contributions of locally produced *vs.* pituitary-derived PRL to any of its immune functions. Transgenic animals with a site-specific PRL knock-out may provide the best model by which to test this notion.

In summary, the full spectrum of immune functions subserved by PRL is not completely understood for the following reasons. One, *in vivo* results can be prone to misinterpretations because of hormonal and cellular specificity, direct *vs.* indirect effects, and pharmacological as opposed to physiological, effects. Two, transformed cells such as Nb2, which are used as the 'gold' standard for PRL bioactivity, do not necessarily mimic primary immune cells. Three, there are no established assays for nonmitogenic actions of PRL. Four, like most cytokines, PRL does not act alone but requires costimulators. In spite of these caveats, the general consensus is that PRL is involved, directly or indirectly, with the development and maturation of immune cells in the thymus and peripheral lymphoid organs and may also play a role in the migration of immature lymphocytes to the periphery and in selected T- and B-dependent cellular immune responses (1, 210, 223, 224, 227, 228, 250–252). An association between PRL and autoimmune diseases is discussed in *Section VII.*

V. Brain PRL

A. Distribution of PRL within the brain

Fuxe and colleagues (253) first detected PRL in the hypothalamus, and their findings have since been replicated and extended. However, the exact mapping of PRL-producing neurons has been difficult because of the hierarchical orga-

TABLE 3. Distribution of PRL and PRL receptor in the rat brain

Site	PRL						PRL Receptor				
	mRNA[a]			Protein			mRNA			Protein[b]	
Telencephalon:											
Cerebral cortex	0			0–1	B,I,R	(255,257,265,268,270)	1	P,S	(83,273[c])	0–1	(274,275)
Hippocampus				1–2	B,I,R,W	(255,257,270,276,277)				0–1	(275)
Amygdala		1P	(59)	1–2	I,R	(255,265,268)					
Septum				0–1	I,R	(255,265,270)					
Caudate putamen	1	P	(59)	0–1	B,I	(257,265)				0–1	(275,278)
Diencephalon:											
Thalamus	1	P	(59)	0–1	B,I,R	(255,257,270)					
Hypothalamus[d]	3	N,P,S		3	B,I,R,W		2	P,S		1–2	
Brain stem:	2	P	(59)	0–1	I	(265,270)					
Midbrain				0–1	I	(265,266,270)					
Pons-Medulla	2	N	(260)	1–2	B,I,R,W	(255,257,268,270,276,277)					
Cerebellum	1	P	(59)	0–1	B,R	(255,257)				0–1	(278)
Spinal cord				1–2	B,I,R	(265,268,279)				0–1	(278)

Abbreviations: B, bioassay; I, immunocytochemistry; N, Northern analysis; P, RT-PCR; R, radioimmunoassay; W, Western blot. Levels of detection: 0, nondetectable; 1, low; 2, moderate; 3, strong.

[a] Numbers designate levels of detection; letters designate methods of detection; references are in parentheses.

[b] By receptor binding only.

[c] In the fetus only (primarily in the olfactory bulbs).

[d] Details in Table 4.

nization of the brain, the unique structure of neurons, and methodological limitations. Consider, for example, the fact that less than 5–10% of brain cells are neurons and of these, polypeptide-producing neurons constitute a minute minority. Because neurons have small perikarya, long and slender projections, and extensive arborization, the transcriptional-translational apparatus is often located at a great distance from sites of storage and release. Such features hamper determination of PRL gene expression by methods with insufficient sensitivity (*i.e.* Northern analysis) or poor resolution (*i.e.* RT-PCR). Immunological methods, on the other hand, depend on antibody selectivity, cannot discriminate between sequestered and locally produced PRL, and do not distinguish PRL, its variants, or molecules with shared epitopes.

Table 3 summarizes the distribution of PRL in the brain with more detailed information on the hypothalamus presented in Table 4. PRL-containing cell bodies appear to be located in the hypothalamus but not elsewhere in the brain. Colchicine-enhanced PRL accumulation (254, 255) and sustained PRL levels after hypophysectomy (255–258) served as the initial evidence for a pituitary-independent PRL network. More recently, PRL mRNA was detected by Northern analysis (259) and RT-PCR (59, 260–262) but not by *in situ* hybridization. PRL-positive neurons have been visualized in the arcuate, supraoptic, paraventricular, dorsomedial, and ventromedial nuclei (253, 254, 263–266). Projections from the magnocellular neurons reach the external layer of the median eminence, in close proximity to blood vessels and ependymal cells, and the neural lobe of the pituitary (264, 267, 268). It is unknown whether PRL in the magnocellular neurons is colocalized with vasopressin and oxytocin or is confined to a subset of cells.

PRL-containing fibers are not restricted to the hypothalamus. Many brain regions, including the bed nucleus of the stria terminalis, central nucleus of the amygdala, organum vasculosum of the lamina terminalis, and subfornical organ, contain PRL-positive neurons (268, 269). Caudal projections are found as far as the raphe nucleus, locus ceruleus (254, 270), midbrain central gray, lower brain stem, and spinal cord (265). Because of cross-reactivity of some antibodies with POMC- and dynorphin-derived peptides (263, 265, 271), many of these fibers may contain other molecules that share epitopes with PRL. When extracts of brain regions were analyzed by RIA, PRL was found mainly in the hypothalamus, with intermediate levels in the pons-medulla and low to undetectable levels in other regions (260).

At the subcellular level, immunoreactive PRL was detected by electron microscopy within granules (90–120 nm in diameter) concentrated in the soma and sparsely distributed in axons, dendrites, and synapses (266–268). Sucrose density centrifugation showed that most hypothalamic PRL is localized in membrane-bound, synaptosome-rich fractions (272). Equilibrium-density centrifugation of hypothalamic and pituitary extracts detected two fractions containing hypothalamic-derived PRL, but only one containing pituitary-derived PRL, suggesting tissue-specific differences in the size of PRL-containing granules (258). A question that should be addressed is whether membrane-bound PRL in the neurons is localized in exocytotic vesicles destined to be released or in endocytotic vesicles en route for internalization.

PRL at low to moderate levels is also present in the CSF, and like vasopressin, it exhibits a circhoral pattern of release (280–283). PRL in the CSF is derived from both internal and external sources; the relative contributions of each is unclear. On the one hand, PRL is clearly transported from the pituitary by retrograde blood flow (284) and is also taken up from the circulation by the choroid plexus (90, 285). On the other hand, the lack of tight correlation between PRL in blood and CSF during hyperprolactinemia (91, 283) and the sustained levels of PRL in the CSF after hypophysectomy (255) support a brain origin.

Upon reviewing the literature, it soon becomes apparent that there is a major discrepancy between the relative abundance of PRL detected by immunocytochemistry and the scarcity of *bona fide* PRL-synthesizing neurons. As indicated by some investigators (265), this could be due, in part, to the

TABLE 4. Distribution of PRL and PRL receptor within the rat hypothalamus, choroid plexus, and circumventricular organs

Site	PRL		PRL Receptor	
	mRNA[a]	Protein	mRNA	Protein
Whole hypothalamus	2–3 N, P, S (59, 259, 260, 262)	1–2 B, R (256)		1–2 Bin, W (275, 278)
Hypothalamic areas:				
Anterior hypothalamus	1–2 P (261)			
Dorsal hypothalamus		1 I, R (255, 260, 264)		
Lateral hypothalamus		1–2 E, I (271, 286)		
Medial basal hypothalamus		1–2 E, I (265, 266, 286)		
Median eminence		3 E, I, R (254, 255, 260, 261[b], 264, 267–269)		1–2 Bin, I (274, 287, 288)
Perifornical area		0–1 I (268, 271)		
Preoptic area		1–2 E, I (253, 264, 286)	1–2 S (289)	0–1 Bin, I (288)
Ventral hypothalamus		1–2 I, R, W (255, 260, 264, 276)		
Hypothalamic nuclei:				
Arcuate		1–2 I (253, 254, 263, 265)	1–2 S (289)	1–2 Bin, I (274,278)
Dorsomedial		0–1 I (253, 263)		
Paraventricular	1–2 P (261)	2 I (261[b], 263, 264)		0–1 Bin, I (288)
Supraoptic		2 I (254, 261[b], 264)	1–2 S (289)	0–1 Bin, I (263, 288)
Ventromedial		0–1 I (254, 263, 265)		0–1 Bin, I (288)
Circumventricular organs:				
Area postrema		2–3 I (269)		
Organum vasculosum of the stria terminalis		2–3 I (268, 269)		
Subcommisural organ		2–3 I (269)		
Subfornical organ		1–2 I (268, 269)		0–1 Bin (274)
Choroid Plexus		2–3 I (269)	2–3 P,S (83, 289–292)	3 Bin, I, W (90, 274, 275, 278, 285, 297)

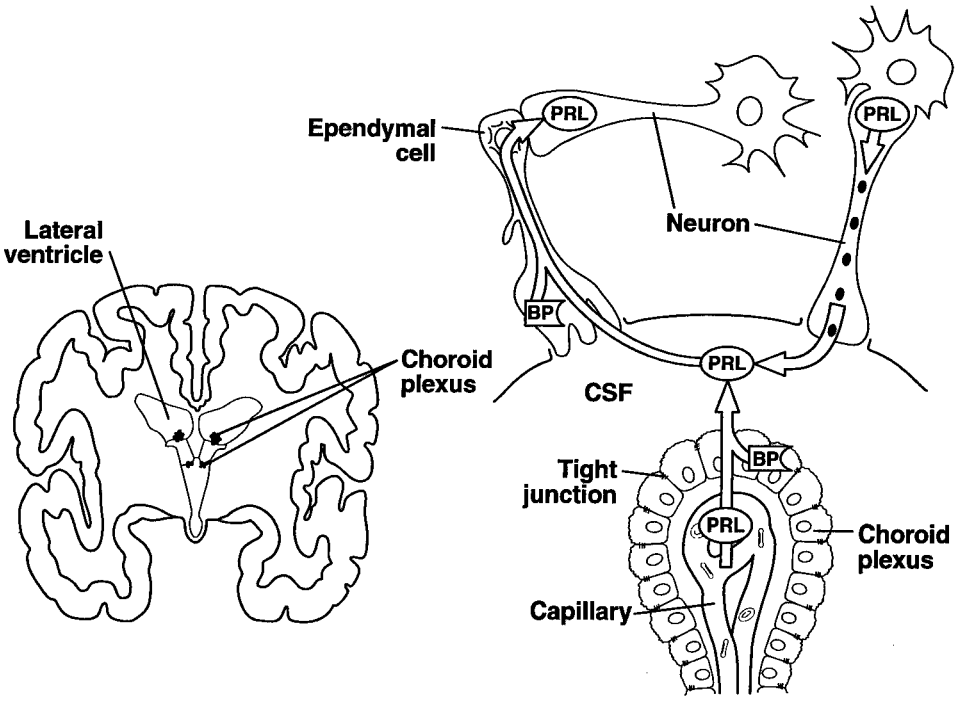

FIG. 7. Postulated mechanism for the production of PRL in the brain and transport into and out of the CSF. A coronal section of the human brain (*left*) shows the lateral ventricle and locations of the choroid plexus. The diagram on the *right* shows three structures in close proximity to the ventricles: the choroid plexus, neurons, and ependymal cells. PRL in the CSF originates either from PRL-producing neurons or is transported from the blood by the choroid plexus. PRL can be distributed by the CSF to distant brain locations and be taken up by ependymal cells and/or neurons. For these processes to occur, the model postulates the existence of PRL-binding proteins (BP). [Based on data from Refs. 90, 255, 269, 274, 281, 283, 285, 291, and 297; the figure was adapted from R Spector and CE Johanson: The mammalian choroid plexus. *Sci Am*, 261:68–74, ©1989 by Scientific American, Inc. All rights reserved.]

use of nonspecific antibodies. Yet, PRL can also be taken up from the CSF and retained by neurons, glia, or other cell types. This is supported by the detection of immunoreactive PRL in ependymal cells resembling tanycytes located at the ventricular lining of practically all circumventricular organs (269). Each of these is a potential site of PRL transport into, or from, the CSF, as illustrated in Fig. 7. A systematic mapping of prolactinergic neurons, using a combination of retrograde tracing, immunocytochemistry with well characterized antibodies, and *in situ* hybridization, is needed before their complete distribution within the brain is fully appreciated.

B. Characterization and regulation of brain PRL

The low abundance of PRL in the brain, together with the lack of neuronal cell lines, limits the extent of physicochemical characterization. Initially, gel filtration fractionation revealed that the bulk of hypothalamic (256) and extrahypothalamic (257) PRL eluted in larger molecular size fractions than pituitary PRL. Displacement curves of hypothalamic and pituitary PRL were not parallel, and the ratio of bioactivity to immunoreactivity was lower for brain PRL. Later studies suggested, however, that such dissimilarities are due to artifacts of the extraction procedure, which promoted PRL aggregation (255).

Three bands of immunoreactive (ir)-PRL, a major 24-kDa and two minor 16- and 8-kDa forms (260, 276), were detected in the hypothalamus by Western blotting. The same forms were present in the hippocampus and pons-medulla, but after metabolic labeling, PRL proteins were seen only in the hypothalamus and pons-medulla (260). Structural homology of brain and pituitary PRL was first suggested by the identity of their peptide maps (277). Sequencing of a PCR product from hypophysectomized rats established that hypothalamic and pituitary PRL are identical (293). A report on alternatively spliced PRL mRNA (presumably lacking exon 4; Ref. 59) was not confirmed by other groups using more stringent PCR conditions (260, 262). A single report utilizing RT-PCR detected PRL gene expression in the human hypothalamus (180). If substantiated, it raises the question of whether a pituitary- or extrapituitary-type PRL promoter is used in the human brain. More information is also needed on the characteristics of PRL present within the CSF in terms of variants and posttranslational modifications.

The rat brain can generate 16-kDa PRL by proteolytic processing. Proteolysis is done by an acid protease that might be colocalized within PRL-containing granules (64). A 14-kDa PRL, whose relationship to 16-kDa PRL is unclear, was recently detected in the magnocellular neurons and neurohypophysis, but not adenohypophysis, using antisera against 16-kDa PRL (66). The 14-kDa variant was found in the circulation and was released from neurohypophysial explants in response to elevated potassium. Conditioned media from the explants exhibit antiangiogenic activity, as was reported for 16-kDa PRL (261). There is some resemblance between the production, processing, transport, storage, and release of 14-kDa PRL and the better known magnocellular products, oxytocin and vasopressin. Several questions, *e.g.* the exact function of brain-derived 14/16 kDa PRL and the identification of its target tissues, remain to be determined.

Estradiol is recognized as a major regulator of brain PRL. This was proposed after observing that hypothalamic PRL levels, which are higher in female than male rats, are reduced after hypophysectomy in females only, presumably due to low estrogen levels (255). Treatment of hypophysectomized females with estradiol increased hypothalamic PRL gene expression, the releasable pool of PRL and PRL concentration in the CSF (260, 294). About one third of PRL-containing neurons in the medial basal hypothalamus accumulate [^{3}H]estradiol (295), indicating the presence of intracellular

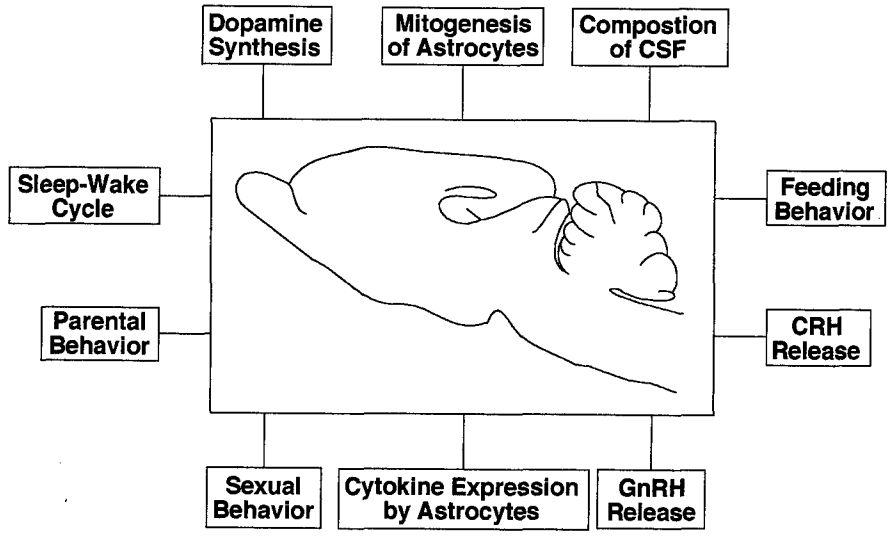

FIG. 8. Postulated functions of PRL within the brain. The diagram does not assign these functions to specific brain sites and does not emphasize more established *vs.* putative functions.

estrogen receptors. The sexual dimorphism in the expression of hypothalamic PRL is initiated before embryonic day 14, as shown by the higher number of prolactinergic neurons in cultures obtained from female compared with male embryos (296). This time precedes the critical period of steroid-dependent sexual differentiation and may involve steroid-independent genetic programming.

Information on substances that alter hypothalamic PRL synthesis and release is limited by the instability, high basal PRL release, and eventual loss of integrity of incubated hypothalamic fragments. PRL release was stimulated by depolarizing agents and angiotensin II but not by TRH (294). Other agents known to affect pituitary PRL release were either not tested or were ineffective. Increases in hypothalamic PRL mRNA levels in response to intraventricular injections of VIP and PACAP were recently reported (262), leading the authors to suggest that PRL participates in the sleep-wake cycle. Obviously, much more information is needed on the regulation of brain PRL.

C. Functions of brain PRL

As illustrated in Fig. 8, numerous putative functions have been attributed to PRL in the brain, including modulation of CSF composition, mitogenic effects on astrocytes, control of releasing/inhibiting factors, regulation of sleep-wake cycle, and modification of reproductive, parenting, and feeding behavior. Available information on the distribution of PRL receptors in the brain has not been particularly useful for identifying PRL target sites. In fact, except for the choroid plexus, there is a surprisingly low expression of PRL receptors in the brain (Table 3). Binding sites and PRL receptor mRNA are present at low levels in few hypothalamic nuclei, the medial preoptic area, and the median eminence (274, 278, 287–289). Similar to its effect on PRL, estradiol up-regulates the number of PRL-binding sites (278). The receptor density, if detectable, in other parts of the brain is 10- to 100-fold lower. Specific, high-affinity and low-capacity PRL-binding sites were also seen in the human choroid plexus, hypothalamus, and hippocampus (297, 299), with twice as many binding sites in the hypothalami of women as of men (297). The distribution of PRL receptors in the dove brain is similar to that in mammals (298).

The choroid plexus is a highly vascularized epithelial tissue that lines the brain ventricles. Its main functions are the production of CSF, maintenance of its composition, and active transport of molecules otherwise excluded from the brain by the blood-brain barrier. The density of PRL receptors in the choroid plexus is among the highest of all tissues, as shown by autoradiography (90, 274, 285), binding assays (275, 278, 297), RT-PCR (83, 290, 291), ribonuclease (RNAse) protection assay (290), and *in situ* hybridization (Refs. 289 and 291; see Table 4). Both acute and chronic elevations in circulating PRL markedly increase receptor expression (290) and binding capacity (285) in the choroid plexus and stimulate PRL transport from blood to CSF. Interestingly, cross-linking of labeled PRL to choroid plexus receptors from humans (299) and rats (275) revealed a major hormone-binding form of 44–45 kDa. This is much smaller than the classic membrane-bound PRL receptor, resembling in size the PRL-binding proteins present in the amniotic fluid (79) and milk (93). Again, the presence of such receptors supports the notion of a transporter/binding protein.

A working model that incorporates these data is depicted in Fig. 7. The model presumes that the PRL receptor and/or binding protein in the choroid plexus functions as a transporter, enabling circulating PRL to gain access to various brain regions. PRL, in line with its established osmoregulatory role in other systems, has been proposed to participate in the regulation of the composition of CSF, but this remains to be established. The CSF may be used as a route by which PRL reaches remote brain sites where it can be taken up by neurons, glia, or ependymal cells and exerts a variety of modulatory functions. It has been suggested that the capacity to transport PRL from the circulation to the brain proper can be used in the design of drugs, covalently bound to PRL, that circumvent the blood brain barrier (285).

Astrocytes are the most numerous cells in the brain. Unlike neurons, they are capable of proliferation in adulthood. Astrocytes often function as immunocompetent cells and react to brain injury by increased proliferation, cytokine release,

and antigen presentation. DeVito and co-workers (300) reported that PRL, but not GH, induced proliferation of growth-arrested astrocytes. Such mitogenic effects required serum-derived factors, suggesting that PRL functions as a coactivator. Indeed, PRL temporally increased the expression of tumor necrosis factor-α, IL-1α, and transforming growth factor-α in astrocytes, which in turn could synergize with PRL in promoting astrocyte proliferation and secretory activity (301). The effects of PRL may be mediated by protein kinase C, which rapidly translocates from the cytosol to the membrane in response to PRL (302). However, neither PRL receptors nor intracellular mediators, such as JAK2, have been demonstrated in astrocytes. The role of PRL in mediating brain response to injury is supported by the report that infliction of hypothalamic wounds increased local PRL content and mRNA levels (303). Direct injection of PRL to the wound site increased the expression of glial fibrillary acidic protein and tumor necrosis factor-α, both markers of astroglyosis.

PRL in the brain participates in the regulation of several neurotransmitters and neuropeptides. One of its best established targets are the tuberoinfundibular dopaminergic neurons in the arcuate nucleus (reviewed in Ref. 157). Hyperprolactinemia increases, while hypoprolactinemia decreases, dopamine synthesis and release into hypophysial portal blood (304–306). The reciprocal interactions between PRL and dopamine are classified as a short loop negative feedback mechanism. It enables pituitary-derived PRL, reaching the arcuate nucleus by retrograde blood flow, to regulate its own release by altering dopamine, its primary inhibitor. PRL receptor mRNA (289) and binding sites (288) have been detected in the arcuate nucleus. The release of two other putative PRL secretagogues, oxytocin and VIP, is also affected by hyperprolactinemia (307); PRL-binding sites were detected in the paraventricular and supraoptic nuclei that produce these peptides (288, 289). In addition, hyperprolactinemia is associated with activation of the pituitary-adrenal axis, and this may be mediated by a direct stimulatory effect of PRL on hypothalamic CRH release (308).

Another well documented action of PRL is on GnRH neurons. Hyperprolactinemia results in reproductive dysfunction and anovulation in humans and other species. Excess PRL exerts its antigonadal effects by acting on the brain, pituitary and/or gonads. A central action has been suggested by the decrease of GnRH levels in hypophysial portal blood (309, 310) and the reduced pulsatility and frequency of LH release (311) in response to hyperprolactinemia. PRL directly suppresses GnRH release from the GnRH-producing GT1 cell line. These cells express the PRL receptor (primarily the short form) and respond to PRL by decreasing GnRH gene expression and release (312). Under normoprolactinemic conditions, however, local PRL may stimulate GnRH release as suggested by the suppression of GnRH release from hypothalamic fragments by PRL antiserum (313). Interestingly, PRL receptors are highly expressed in fetal, but not adult, olfactory bulbs (314). Since the olfactory placode gives rise to GnRH neurons (315), PRL may play a role in the migration or differentiation of GnRH neurons during fetal development. This and the potential role of PRL in the development of the fetal olfactory system remain to be determined.

A sleep-associated rise in plasma PRL levels in humans has been reported but its strict correlation to sleep cycles is controversial (316, 317). Recent evidence indicates that together with VIP, hypothalamic PRL may be involved in regulating sleep-wake cycles in experimental animals (262, 318–320). Based on intracerebroventricular injection of PRL and administration of PRL antisera, these authors suggested that hypothalamic PRL promotes rapid eye movement sleep during the light cycle and suppresses rapid eye movement sleep during the dark cycle. This raises the possibility of a functional association between hypothalamic PRL and the suprachiasmatic nucleus, the circadian pacemaker.

PRL is also involved in a wide array of reproductive and nonreproductive behavior (reviewed in Refs. 321, 322). In birds, PRL facilitates behavioral responses to reproduction and alters behavior associated with nesting (322). In ovariectomized, estrogen-primed rats, infusion of PRL to the midbrain central gray increased lordosis behavior or sexual receptivity (323); this PRL effect was not mimicked by GH and several neuropeptides. Conversely, infusion of PRL antiserum decreased lordosis behavior, indicating the specificity of action of PRL. The latter is supported by the identification of PRL-sensitive neurons in the ventromedial hypothalamus that increased electrical activity in response to iontophoretically applied PRL (324). Copulatory behavior in male rats is also inhibited by long-term exposure to PRL (325). Pup retrieval, used as a paradigm for assessing maternal behavior, is also stimulated by intracerebroventricular administration of PRL (326). The medial preoptic area was proposed as the key neural site for PRL regulation of maternal behavior.

Reproduction in seasonally breeding female mammals is also influenced by PRL (reviewed in Ref. 338). Depending upon the species, melatonin, catecholamines, GnRH, and feedback by gonadal steroids are involved, but the exact mechanisms governing such interactions remain poorly understood. Hyperprolactinemia in rats also results in increased food intake, but whether this was due to direct or indirect effects of PRL has not been determined (327); direct intracerebroventricular administration of PRL in birds significantly increased their feeding behavior (328).

VI. Other Organs

A. PRL in skin and associated structures

There is emerging evidence for local production of PRL by the skin and associated exocrine glands. A PRL-like substance was detected by immunocytochemistry in human sweat glands dissected from skin biopsies (329) and in human skin grafted into immunodeficient athymic mice (330). The PRL-like material was localized in the clear cells of the secretory coil whose main function is fluid secretion, and to a lesser extent in the basal layer of the luminal ducts. Treatment of mice bearing human skin grafts with antiserum against human PRL increased the concentration of chloride in sweat (331). Based on these and other indirect evidence, the authors proposed that PRL participates in the regulation of sweat composition and may be a determinant in sweat gland defects associated with cystic fibrosis (332).

PRL in skin and sweat glands can be derived from three

potential sources: uptake from the circulation, PRL-producing migratory lymphocytes, and local synthesis by epidermal keratinocytes or connective tissue fibroblasts. Fibroblasts were first considered as a source of PRL after noting an unusually prolonged release of immunoreactive PRL from connective tissue explants (333). Production of PRL required the presence of serum factors and did not respond to ovarian steroids. Two surges of PRL, peaking after 30 and 130 days in culture, were apparent, suggesting the presence of two cell populations that produce PRL. In a recent study, primary cultures of human dermal fibroblasts spontaneously released PRL for up to 30 days (334). PRL release was stimulated by a combination of estrogen, progesterone, and prostaglandin E_2. The PRL transcript in fibroblasts was larger than that in the pituitary, resembling the decidual transcript whereas the released PRL was similar in size to pituitary PRL.

The lacrimal gland is an exocrine gland that produces the lacrimal fluid or tears. An adequate production of lacrimal fluid is essential for maintaining the integrity of the ocular surface. Immunoreactive PRL has been detected in human tears and lacrimal glands (335). Both PRL mRNA and PRL-binding sites were detected in lacrimal glands from rats, with the immunoreactive PRL localized in secretory vesicles of the acinar cells (336). A larger fraction of the PRL-binding sites were occupied by PRL in females than in males, suggesting that in addition to local production, an uptake and internalization mechanism for PRL is operative. Interestingly, there is significant sexual dimorphism in lacrimal gland morphology, function, and susceptibility to disease (336), but its relationship to PRL remains to be determined.

In search of a function for PRL in skin biology and pathology, several putative functions have been proposed (332, 337). Among these are the regulation of salt concentration in sweat and constituents of the lacrimal fluid, stimulation of epithelial cell proliferation in skin, and support of hair growth. In some species, *e.g.* blue fox, red deer, and hamsters, seasonal changes in PRL comprise a component of the endocrine signals that induce moult from winter to summer coat (reviewed in Ref. 338). In sheep, however, selection of breeds with a continuously growing fleece may have resulted in unresponsiveness of hair follicles to seasonal changes in PRL. Nonetheless, PRL-binding sites were localized in the dermal papillae of wool follicles and in apocrine sweat glands in New Zealand Wiltshire ewes (339).

B. PRL in other tissues

Immunoreactive PRL has been detected in numerous other organs. As reviewed by Robertson (332), these include nonreproductive tissues, *e.g.* the GI tract, lung, adrenals, and Islets of Langerhans, as well as reproductive organs, *e.g.* ovary, ventral prostate, urogenital tract, and testes. In most cases, however, the presence of immunoreactive PRL has not been supported by demonstration of local synthesis. Because PRL cannot be strictly classified as a local hormone in these sites, they will not be further discussed. Nonetheless, future studies should examine why PRL is concentrated in these tissues and what its local functions might be.

The presence of PRL in human follicular fluid deserves attention. An early report compared the concentrations of pituitary hormones in follicular fluid collected from growing follicles during the menstrual cycle. Whereas LH and FSH levels increased toward midcycle, PRL concentrations decreased as the follicles grew in size (340). PRL in human follicular fluid is biologically active in the Nb2 assay (341). Although its origin has not been determined, transfer of circulating PRL into the follicular antrum is the most likely source. Since hyperprolactinemia is associated with anovulation and luteal phase dysfunction, a relevant question is whether this results from inappropriately elevated PRL in the follicular microenvironment. Indeed, large doses of PRL suppressed estradiol release by luteinized human granulosa cells (342) and inhibited LH binding to FSH-primed rat granulosa cells (343), supporting an antigonadal effect of elevated PRL concentrations at the level of the follicle. Follicular fluid PRL as a possible predictor of oocyte maturation during *in vitro* fertilization is discussed below.

VII. Clinical Aspects of PRL

A. General considerations

As emphasized in this review, PRL is produced, and has effects, in a wide variety of tissues. It should not be too surprising that abnormalities in the secretion or action of extrapituitary PRL might be associated with certain disease states. Amenorrhea, galactorrhea, and impotence are the best documented dysfunctions resulting from hyperprolactinemia (344, 345) but will not be discussed here. In addition, several other disorders appear to be associated with abnormal PRL physiology. In some cases, this involves aberrant local production of PRL. In other cases, PRL is elevated but its precise origin is unknown. In fact, in some cases of galactorrhea or amenorrhea, PRL levels are moderately elevated, but clinically recognized causes of hyperprolactinemia such as pituitary tumors, hypothalamic disease, medications, hypothyroidism, renal failure, or psychological stress are not found. This raises the possibility of an unidentified extrapituitary source of PRL.

The following discussion will focus on clinical disorders that involve PRL other than traditional syndromes of hyperprolactinemia. As evident, a clear 'cause and effect' for PRL is uncertain for most of these disorders. Rather, we intended to underscore suggestive, intriguing, or unexplored observations that may inspire further consideration and novel avenues of clinical investigation.

B. Disorders associated with reproduction

Since the discovery that amniotic fluid contained extremely large amounts of PRL, consideration has been given to its role in abnormal pregnancies. Most attention has focused not on PRL as a cause of disease but as a marker of certain conditions. Several disorders have been examined with respect to PRL levels in the amniotic fluid. For example, amniotic fluid PRL, both immunoreactive and bioactive, is elevated in pregnancy-induced hypertension (346), although this was not confirmed by another study (347). Since PRL is known to be a stress hormone, these elevations may represent physiological responses to fetal distress. The finding of

elevations in amniotic fluid PRL, which correlate with fetal lung maturity in rhesus monkeys, is intriguing (148). If confirmed in humans, it raises the possibility of using amniotic fluid PRL levels for assessing fetal lung maturation or even as a therapeutic modality.

Polyhydramnios, or excessive volume of amniotic fluid, is a common obstetrical condition leading to increased fetal morbidity. Polyhydramnios is often associated with diabetes mellitus, multiple pregnancies, or fetal malformation and is characterized by decreased levels of amniotic fluid PRL. Apparently, this does not simply represent a dilutional effect, as levels of other amniotic fluid hormones are not reduced (133, 348). Of interest is the finding of reduced number of PRL receptors within the chorion laeve of patients with chronic polyhydramnios (144). Consistent with the postulated osmoregulatory role of PRL during fetal life, loss of its receptors may impair water transport and be the cause of increased volume of the amniotic fluid.

Leiomyomas are benign fibromuscular tumors of the myometrium that occur frequently and represent a common indication for hysterectomy. PRL was produced by fibroid tissue in larger amounts than by normal myometrial explants, and its rate of release increased during culture, indicating removal from inhibition (153, 349). The control of PRL release from leiomyomas differs from that in the pituitary, decidua, or even myometrium. Estrogen, progesterone, and a GnRH agonist did not affect fibroid PRL release *in vitro* (349), while LH and TSH were stimulatory (164). Since PRL is mitogenic in other tissues, locally produced PRL may promote fibroid growth. A better understanding of the regulation of PRL in leiomyomas could suggest treatment regimens to inhibit tumor growth.

Postmenopausal myometrium, usually considered metabolically inactive, was significantly more responsive to stimulation of PRL release by human CG (hCG) than premenopausal myometrium (163). This is of interest since serum gonadotropin levels are elevated after menopause, as the negative feedback by gonadal steroids is reduced. This raises the possibility of PRL involvement with the pathogenesis of uterine sarcomas, a lesion almost entirely confined to the postmenopausal years.

Many women with disorders of reproduction can now achieve pregnancies by *in vitro* fertilization. A critical problem of *in vitro* fertilization, a procedure with limited success, is how to assess the maturity and fertilizability of oocytes. Neither parameter can be predicted by the morphology of recovered oocytes, which has led to a search for hormonal markers within the easily accessible follicular fluid. PRL has been proposed as a potential predictor of fertilization capacity of oocytes, although this issue remains highly controversial. Some reports found that PRL concentrations in the follicular fluid correlate positively with oocyte maturation and successful fertilization (350, 351) while others (341, 352) found no such correlations. This disagreement may be due to the different hormonal regimens used to induce ovulation in these patients, and perhaps the whole issue requires reexamination.

The involvement of PRL in the etiology of breast cancer has long been suspected, given the essential role of the hormone in the development and functioning of the normal gland. However, PRL has been largely disregarded as a determinant in breast cancer, based on insufficient correlation between circulating PRL levels and the incidence and severity of the disease and the ineffectiveness of treatments that suppress pituitary PRL release to improve outcome (353). However, if PRL is locally produced by breast tissue and acts as an autocrine/paracrine agent, then its actions may be independent of its circulating levels. There is emerging evidence supporting a role for local PRL in the development of mammary tumors in rodents (179) with preliminary data pertaining to human breast cancer.

Expression of PRL by human breast tissues has been reported by several investigators, relying primarily on RT-PCR (78, 180, 354). In one study, conditioned media from breast cancer cells, T47Dco (an estrogen receptor-negative subclone), contained a PRL-like substance that stimulated Nb2 cell proliferation and was abolished by absorbance with a monoclonal antibody against PRL (354). These authors also reported that anti-PRL antibodies inhibited proliferation of both T47Dco and MCF-7 breast cancer cells. Another group provided evidence that PRL is expressed by breast tissue (78). PRL expression in the epithelium of benign and malignant breast tissue was detected by both RT-PCR and *in situ* hybridization, and immunoreactive PRL was detected by Western analysis in several breast carcinoma cell lines. The presence of PRL receptors in these tissues, as well as the identification of PRL receptor isoforms (78), raises the intriguing possibility of an autocrine/paracrine role for PRL within human breast tissues.

C. Disorders of the immune system

Evidence derived from animal models and supported by few clinical observations suggests that PRL influences the course of some autoimmune diseases (reviewed in Refs. 355–357). Systemic lupus erythematosus (SLE) is a chronic inflammatory disease that is attributed, in part, to autoantibodies reactive with nuclear antigens. Deposition of immune complexes in target organs causes inflammation and extensive tissue damage. The much higher incidence of SLE in women than men has focused attention on estrogens while the potential role of PRL has been overlooked. Elevated PRL levels were found in subsets of SLE patients (358), with the disease being more active in patients with hyperprolactinemia without any obvious cause, compared with patients with known causes for hyperprolactinemia (359), raising the possibility of an extrapituitary source of PRL. In a clinical trial involving a small number of SLE patients, treatment with bromocriptine improved disease outcome (360). The role of PRL and its receptors in the progression of SLE-like disease in the NZB/W F_1 mouse has been well characterized (211, 248, 361).

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by infiltration of the joint synovium by macrophages and T lymphocytes. Like SLE, it is also more prevalent in women than men. Evidence for PRL involvement in the pathogenesis of RA is based on a report that PRL is elevated in girls with juvenile RA with antinuclear seropositivity compared with age-matched controls (362). Another study found abnormal diurnal rhythm of PRL in RA

patients (363). The possibility of increased PRL glycosylation, thereby altering its bioactivity, should be considered since immunoglobulins are abnormally glycosylated in RA patients. In addition, it would be of interest to test for the presence of PRL in the synovial fluid. PRL involvement in arthritis is supported by the induction of transient arthritis in rats with complete Freund's adjuvant. In this case, arthritis does not develop in hypophysectomized rats unless they are given PRL or GH (364).

Elevated PRL levels in patients with other diseases involving the immune system, such as thyroiditis, uveitis, and multiple sclerosis, have been sporadically reported, but the evidence is generally inconclusive. It is of interest that in one study, 60% of patients with acute myeloid leukemia had elevated serum PRL levels while all other measured hormone levels were normal. Ectopic production of PRL was detected by immunoblotting in leukemic myoblasts from one patient (206).

D. Other disorders

Cystic fibrosis (CF) is an autosomal recessive, genetic disorder characterized by pulmonary dysfunction and pancreatic insufficiency. A wide spectrum of abnormalities, both in terms of severity and site of the disease, has been described. The nature of the disease does not always correlate well with the recently identified genetic defect in the chloride channel, known as the CFTR. To explain this variability, one author has suggested that other factors are involved, including PRL (332). The putative role of PRL in CF stems largely from its demonstrated effects on chloride transport in lower vertebrates. While PRL levels in CF patients are not significantly different from controls, its pattern of secretion, *i.e.* diurnal rhythm, is greatly disturbed. Involvement of extrapituitary PRL is suggested by the detection of PRL in the sweat glands, a major site that is affected in CF (329, 330). Administration of antibody against hPRL to athymic mice carrying human skin grafts resulted in a significant increase in sweat chloride levels (331), in this respect mimicking CF.

An association between PRL and colorectal cancer has recently been reported by two groups (365–367). Their data suggested that plasma PRL levels correlated well with disease progression, were useful in identifying low- and high-risk patients, and served as a good predictor of overall survival. Patients with elevated PRL levels had more advanced disease than those with lower levels. PRL levels fell after tumor resection and in those patients who had cancer recurrence, PRL levels were a better predictor than carcinoembryonic antigen, a widely used tumor marker. Interestingly, 50% of colorectal tumors were positive for PRL receptors (365), and a subset of tumors also contain immunoreactive PRL (366). PRL has also been associated with other cancers, including tongue and cervix. Perhaps the association of PRL and cancer will develop into an area of clinical importance.

VIII. Summary and Perspectives

The widespread distribution of tissues capable of PRL synthesis is illustrated in Fig. 9. The most established extrapituitary sites are the decidua, immune system, brain, and myometrium, with emerging evidence for PRL synthesis by the skin and exocrine glands, including mammary, sweat, and lacrimal. PRL is produced by a wide variety of cells of different embryonic origin, morphology, and physiological functions. Some, *e.g.* lymphocytes and epithelia, are less differentiated and have a high proliferative capacity while others, *e.g.* neurons, are postmitotic and terminally differentiated. The heterogenous nature of PRL-producing cells, together with the expression of PRL receptors by almost every tissue in the body, supports the role of PRL as a cytokine and underlie its pleiotropic functions.

Another remarkable feature of PRL is its presence in most fluid compartments of the body. Although all hormones are present in serum and most are excreted into urine, PRL is found also in CSF and amniotic fluid and is secreted into milk, tears, and follicular fluid. Whereas PRL in the amniotic fluid originates from a local source (decidua), that in milk and CSF is derived from both locally produced and circulating PRL. Significant cellular resources must be spent in transporting PRL into these compartments, and yet little is presently known about the functions subserved by PRL in these sites.

Five areas in which future research could significantly advance the PRL field have emerged: 1) tissue-specific regulation of PRL gene expression; 2) biological functions of PRL variants; 3) characterization of PRL transporter/binding proteins; 4) PRL physiology in subsets of cells and within specific fluid compartments; and 5) extrapituitary PRL as a factor in pathophysiological processes.

Extensive research has characterized the promoter/enhancer region of pituitary PRL and identified *cis*- and *trans*-acting elements that regulate PRL gene transcription. In contrast, little is known about the control of the PRL gene in extrapituitary sites. With the identification of the alternative promoter in the human decidua and lymphoid tissues, a better understanding of tissue-specific regulatory factors should be forthcoming. It remains to be determined whether an alternative PRL promoter is also used in extrapituitary sites in other species or is unique to humans. Additionally, development of more sensitive methodology will be required to characterize PRL and its regulation in cells such as neurons and lymphocytes that express very low levels of PRL.

A large number of PRL variants result from posttranslational processing of native PRL. Their function, in general, is unknown. The commonly used assays, RIA and Nb2 lymphoma assay, are inadequate for determining their concentration or bioactivity. To fully recognize the function of PRL variants, alternative assays, which do not depend on routine antibody assays or on mitogenic activity, are needed. A case in point is 16-kDa PRL, an inhibitor of angiogenesis, which is not recognized by routine RIAs, does not bind to classic PRL receptors, and is not active in the Nb2 bioassay.

Since the cloning of the membrane PRL receptor, efforts have focused on studying its structure, distribution, and function. Evidence exists, however, for the presence of more than one type of PRL receptor. For example, PRL-binding proteins of varying sizes have been detected in several tissues and body fluids. They may have an active role in cellular internalization of PRL as well as its transport across cells and

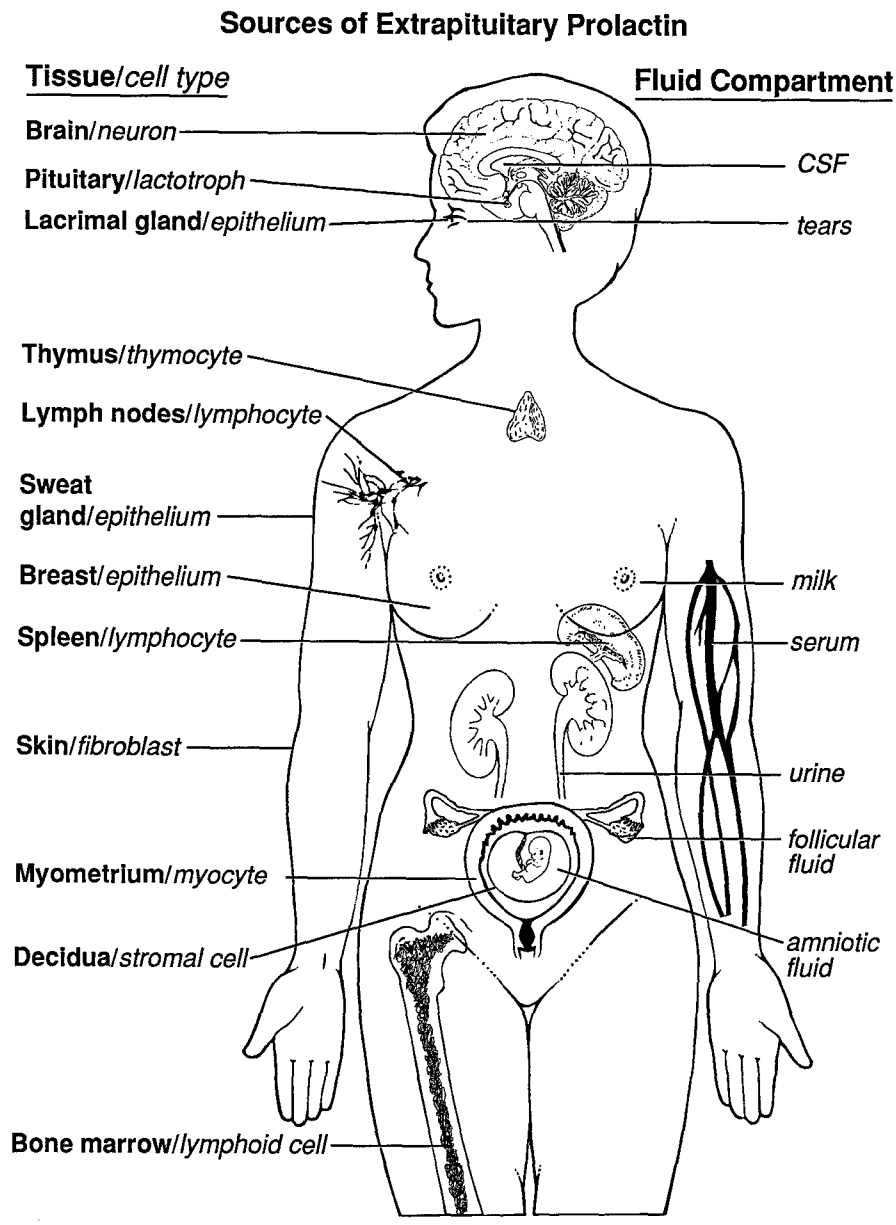

FIG. 9. Distribution of extrapituitary PRL throughout the human body. The structures listed on the *left* show tissues and cells that produce PRL. Those on the *right* show fluid compartments that contain PRL. Note the heterogeneity of PRL-producing cells (*italics*) in terms of different embryonic origin, morphology, and function.

thus be considered as "transporters." It is also puzzling that the mammary gland, the best recognized target for PRL, expresses significantly fewer membrane receptors than the liver or adrenal. Further, the brain, where PRL exerts a dozen different functions, has practically undetectable levels of the classic membrane receptor. The presence of another receptor is not unprecedented, as exemplified by the recent cloning of a second estrogen receptor (368). Information is now needed on the structure of the transporter/binding proteins and whether they represent a truncated form of the membrane receptor or are the product of another gene. If there is another PRL receptor, data generated by PRL receptor mutant transgenic animals should be interpreted with caution.

There is a discrepancy between cells producing and cells containing PRL in several tissues. This is perplexing not only for the identification of PRL-producing sites but also for assessing authentic paracrine interactions, especially when diffuse systems such as the brain and immune system are concerned. New techniques may help to delineate the contributions of circulating *vs.* extrapituitary PRL to local functions. For example, direct administration of PRL antisense oligonucleotides into the brain could help in sorting out the source of PRL in neurons, glia, or the CSF. The newly developed technique of site-specific gene 'knockout' is ideal for targeting pituitary PRL. If successful, it could mimic the effects of hypophysectomy but with much more selective and significantly less morbid consequences. This technology could also be used for specifically targeting mammary cells, lymphocytes, or neurons, enabling a closer look at paracrine/autocrine functions of PRL. A better understanding of PRL physiology in the mouse, the only species for which transgenic technology has been developed, is a prerequisite for these manipulations.

PRL has been implicated in a variety of diseases. The lack

of a strong correlation between circulating PRL levels and progression or outcome of certain diseases should not discourage further investigation for the following reasons. First, local rather than circulating PRL may be involved, as suggested by the recent demonstration of PRL expression by human breast cancer, lymphomas, and colorectal cancer. Second, mutations or deletions in PRL receptors could affect the actions of PRL in unexpected ways. Analogy can be made to the Nb2 cell line, which is dependent on PRL for growth possibly as a consequence of a mutated PRL receptor. Third, PRL variants may be involved. For example, it would be of interest to examine whether 16-kDa PRL, with its antiangiogenic activity, is produced by immune cells, *e.g.* tumor-infiltrating lymphocytes or macrophages, that are involved with tumorigenesis and tissue repair.

Finally, the field of extrapituitary PRL, still in its infancy, is now positioned for growth and expansion. The prospect of new and unexplored functions, the search for site-specific regulation, and the potential involvement of PRL in pathological processes all provide exciting research opportunities.

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Novel Estrogenic Action of the Pesticide Residue β-Hexachlorocyclohexane in Human Breast Cancer Cells[1]

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ABSTRACT

The estrogenic action of some persistent organochlorine pesticide residues may play a role in the progression of hormonally responsive tumors of the breast and uterus. The prototypical xenoestrogen *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT) acts by binding and activating the estrogen receptor (ER). The present study focuses attention on the mechanisms through which another organochlorine compound, β-hexachlorocyclohexane (β-HCH), exerts estrogen-like effects in human breast cancer cells. Both *o,p'*DDT and β-HCH stimulated proliferation in a dose-dependent manner in the ER-positive cell lines MCF-7 and T47D but not in the ER-negative lines MDA-MB231, MDA-MB468, and HS578T. Both compounds produced an increase in the steady state level of pS2 mRNA in MCF-7 cells. These responses were equal in magnitude to the maximal effect of estradiol, and they were inhibited by inclusion of the antiestrogen ICI164384. On the other hand, when tested in a competitive binding assay, β-HCH did not displace 17β-[^{3}H]estradiol from the ER even at a concentration that was 40,000-fold higher than the tracer steroid. Furthermore, nuclear retention of the ER during homogenization procedures was induced by a 2- or 24-h treatment of MCF-7 cells with *o,p'*-DDT and 17β-estradiol but not by treatment with β-HCH; this indicates that β-HCH nether activates the ER, nor is it converted intracellularly to an ER ligand.

Transcriptional activation by β-HCH occurs in estrogen-responsive GH3 rat pituitary tumor cells transfected with a luciferase reporter construct driven by a complex 2500-bp portion of the *PRL* gene promoter; this *trans*-activation response is inhibited by inclusion of ICI164384. However, β-HCH is ineffective in stimulating a reporter construct driven only by a consensus estrogen response element and a minimal promoter derived from the herpes simplex virus thymidine kinase gene. Thus, β-HCH cannot act on a simple, single estrogen response element; rather, it requires the combinatorial regulation found in a complex promoter.

These data are consistent with the notion that β-HCH stimulation of cell proliferation and gene expression is ER dependent, but its action is not through the classic pathway of binding and activating the ER. β-HCH may represent a new class of xenobiotic that produces estrogen-like effects through nonclassic mechanisms and, therefore, may be of concern with regard to breast and uterine cancer risk.

INTRODUCTION

Many nonsteroidal compounds, both naturally occurring and man made, possess estrogenic activity. The list of these xenoestrogens includes plant and fungal hormones (phytoestrogens and mycoestrogens; Ref. 1) pesticides and herbicides (2), nonionic surfactants (3), and monomers of certain plastics (4). Although xenoestrogens are generally less potent than endogenous estrogens when tested in bioassays, they are cause for concern due to their persistence in the environment, resistance to chemical or enzymatic degradation, altered excretion by the body, and sequestration and storage by adipose tissue (5, 6). In fact, the levels of organochlorines in fat are 200–300 times greater than those measured in serum (7). Additionally, these estrogen mimetics pollute the soil and ground water supply and contaminate the human food chain (5, 8).

The best known and most extensively studied xenoestrogen is the organochlorine pesticide *o,p'*-DDT.[3] The intracellular mechanism of action of *o,p'*-DDT has been shown to be mediated through the classic ER pathway (9–11). Specifically, *o,p'*-DDT binds and activates the ER; this complex then interacts with specific DNA sequences (ERE) in estrogen target genes and functions as a transcription factor to enhance (or inhibit) gene expression. The actions of *o,p'*-DDT include synthesis of progesterone receptors (12) and specific enzymes (13) and stimulation of uterine DNA synthesis (14).

Lindane (γ-HCH), another organochlorine compound, is a mixture of several isomers, including the stable β isomer (β-HCH). β-HCH has negligible insecticidal activity but measurable estrogenic activity and, similar to a weak estrogen, it increases progesterone receptor content in MCF-7 cells, a human breast cancer cell line (15), and slightly augments rat uterine weights in a dose-dependent manner (16). Unlike estrogen, β-HCH does not compete with estradiol for binding to the ER (15), suggesting that although β-HCH induces classic estrogen-like responses in target tissues, the intracellular pathway mediating these actions may be different than that used by endogenous estrogen.

Xenoestrogens have been shown to promote tumorigenesis in rodents (10, 17), yet their role in the etiology of human cancers is less well documented and somewhat controversial. It is argued that due to their low potency and the small amounts that humans are exposed to, these compounds pose a negligible health risk. However, it is possible that through synergism with other xenoestrogens or endogenous weak estrogens they produce significant estrogenic effects (18). Additionally, there is steadily accruing evidence that these environmental estrogens are readily detected in humans and may contribute to carcinogenesis. β-HCH and *o,p'*-DDT residues have been measured in human maternal and cord blood, milk, and adipose tissue (19–21). Moreover, there is a strong association between the incidence of breast cancer in women and levels of β-HCH (22) and *o,p'*-DDT (23) in breast adipose tissue.

In the following studies, we examined the ability of β-HCH to promote MCF-7 cell proliferation in culture and in a xenograft tumor model. Using ER-binding studies, we confirmed previous observations concerning the lack of affinity of β-HCH for the ER. ER activation was monitored in MCF-7 cells following exposure to β-HCH, estradiol, or *o,p'*-DDT. Additionally, we studied the effect of β-HCH on mRNA levels for the estrogen-sensitive *pS2* gene and,

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[3] The abbreviations used are: *o,p'*-DDT, *o,p'*-dichlorodiphenyltrichloroethane; ER, estrogen receptor; ERc, cytosolic ER; ERn, nuclear ER; ERE, estrogen response element; HCH, hexachlorocyclohexane; FBS, fetal bovine serum; MEM/IF, MEM with insulin plus cortisol; cs, charcoal-stripped; PRF, phenol red-free; cmf, calcium- and magnesium-free; DCC, dextran-coated charcoal; DES, diethylstilbestrol; PRL, prolactin; luc, luciferase; wtc, wild-type consensus; AP-1, activating protein 1.

using gene transfer analysis, determined whether β-HCH induces the transcription of estrogen-responsive reporter gene constructs. Results reveal that β-HCH is a tumor promoter in breast cells and may represent a novel class of xenoestrogen, in that its intracellular mechanism of action differs from that of estradiol.

MATERIALS AND METHODS

Cell Culture and Growth Responses. The following human breast cancer cell lines were grown in culture: MCF-7 (obtained from Dr. George Sledge, Indiana University School of Medicine), T47D (obtained from Dr. Kathryn Horwitz, University of Colorado, Denver, CO), HS578T, MDA-MB231, and MDA-MB468 (all from the American Type Culture Collection, Rockville, MD). The former two of these lines express ER and are growth responsive to estrogens; the other three lines are estrogen insensitive. The maintenance medium for all cells consisted of MEM supplemented with 6 ng/ml insulin, 3.75 ng/ml cortisol, and 1% antibiotics (MEM/IF; all components from Sigma Chemical Co., St. Louis, MO) plus 5% FBS (Life Technologies, Inc., Grand Island, NY). Experimental medium consisted of PRF MEM/IF supplemented with 3% cs FBS. The serum was stripped of steroid by the addition of dextran-coated Norit A charcoal (1 mg of dextran/100 mg of charcoal) at 20 mg/ml, followed by centrifugation and filtration to clarify the stripped serum.

To assess growth effects of various estrogenic compounds, the cell number was estimated at the end of an 8-day treatment period. Cells were seeded at 7,500 or 15,000 cells/well in 24-well tissue culture plates depending on the growth characteristics of the individual cell line. Cells were plated in MEM/IF plus 5% FBS; the following day, medium was changed to PRF MEM/IF plus 3% cs FBS. Forty-eight h later, treatments were begun with a change of medium and consisted of vehicle (ethanol at 0.01%), 17β-estradiol (1 nM), β-HCH, or *o,p'*-DDT (both from Chem Service, West Chester, PA) at the indicated concentrations or the antiestrogen ICI164384 (1 μM; supplied gratis by A. Wakeling, Zeneca Pharmaceuticals, Cheshire, United Kingdom). The medium with treatments was changed every other day. At the end of 8 days of treatment, the cell number was estimated by one of the following techniques: (*a*) cells were dissolved in 0.5 N NaOH, the pH was neutralized, and the DNA content of the solution was analyzed by the Hoechst dye-binding assay of Labarca and Paigen (24); or (*b*) cells were incubated with the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), and the amount of blue chromogen that developed by mitochondrial reduction was determined spectrophotometrically in the cell lysate (25).

MCF-7 Tumors in Athymic Mice. All procedures involving animals were approved by the institutional animal care and use committee. To produce a transplantable tumor to be used in subsequent studies, MCF-7 cells (10^5) were injected s.c. on each flank of three athymic male mice. At the time of cell inoculation, animals were castrated, and a Silastic implant (0.25 inches long with a 0.062-inch inside diameter) containing crystalline estradiol was placed s.c. in the suprascapular region. After several weeks, tumors had grown to approximately 1.5 cm in diameter. These animals served as a source of transplantable tumors.

To perform the experiment, two of the tumors from a male host were excised, minced with scissors, and passed through a 20 mesh stainless steel sieve (Cellector; Bellco Glass, Vineland, NJ). The slurry was washed by settling through 50 ml of HBSS (Life Technologies). The resultant concentrated slurry was drawn into 1-ml tuberculin syringes, and 50 μl were injected s.c. into the flanks of nine adult, intact female hosts. The tumor grafts were allowed to establish for 10 days, at which time the hosts were ovariectomized and implanted with a Silastic capsule containing 20 mg of 17β-estradiol, β-HCH, or cholesterol (control). At the time of capsule insertion and at various days thereafter, the size of each tumor was measured with calipers, and the volume was estimated. At the end of 16 days of treatment, the hosts were sacrificed by cervical dislocation, and the tumors were excised and weighed. The host uterus was also excised, weighed, dried, and reweighed.

Steady State pS2 mRNA Levels. MCF-7 cells (2×10^5 cells/25-cm^2 flasks) were initially incubated in MEM/IF plus 5% FBS for 1 week, followed by 1 week in MEM/IF plus 3% cs FBS and 1 week in PRF MEM/IF plus 3% cs FBS; throughout, fresh medium was added on alternate days. Twenty-four h after the last medium change, flasks received one of the following treatments: vehicle (0.01% ethanol), 17β-estradiol (1 nM), or *o,p'*-DDT or β-HCH (1 μM each) either alone or in combination with 1 μM ICI164384. Twenty-four h after addition of treatments, media were removed, and total RNA was extracted with 1.5 ml of Tri-Reagent-LS (Molecular Research, Cincinnati, OH) using the manufacturer's protocol with the exception that following the homogenization and phase separation steps, the aqueous phase was re-extracted with 1.5 ml of Tri-Reagent. Twenty μg of each RNA sample were loaded on an agarose-formaldehyde gel for Northern blot analysis. The lane-to-lane loading of RNA was checked by estimating the amount of 18S rRNA staining with ethidium bromide. The nylon membranes were hybridized with ^{32}P-labeled cDNA for pS2 (gratis from Dr. P. Chambon, INSERM, Strasbourg, France), washed with 1, 0.5, and 0.1× SSC [1× SSC = 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0)], and autoradiographed (Kodak X-Omat AR; Sigma). The band representing pS2 mRNA in each lane was analyzed on a Bio-Rad imaging densitometer using the Molecular Analyst program (Bio-Rad, Melville, NY).

ER-binding Assays. MCF-7 cells were grown in a steroid-depleted environment for 2 weeks prior to harvest as described above for pS2 expression studies. Cells were washed four times in cmf PBS, and 10 mM EDTA in cmf PBS was added; after 10 min at 37°C, the cells were shaken free from the flask and collected. After one wash with cmf PBS at 4°C, the cells were homogenized in Tris-EDTA buffer (3.2 mM Tris buffer base, 1.9 mM Tris buffer-HCl, 1 mM EDTA, 0.5 mM DTT, 25 mM sucrose, and 0.2% NaN_3) using a Polytron dismembranator. The homogenate was centrifuged at 800 × *g* to produce the crude nuclear pellet; supernatant was subjected to an additional centrifugation at 100,000 × *g* to prepare cytosol.

To estimate the ERc, the standard DCC method with Scatchard analysis was used (26). After determination of the protein content of the cytosolic preparation, aliquots (0.5 mg protein/ml) were incubated with increasing concentrations (0.05–1.5 nM) of 17β-[^{3}H]estradiol (110 Ci/mmol; Amersham Corp., Arlington Heights, IL) in the absence or presence of a 100-fold molar concentration of DES (Sigma). After incubation at 4°C for 18 h, the free and bound hormone were separated by addition of DCC followed by centrifugation. An aliquot of the supernatant was counted in a liquid scintillation counter.

Two types of assay were used to determine the ERn content. In a single-point assay, the crude nuclear pellet was gently resuspended in a Dounce homogenizer, and aliquots of the suspension were incubated with 5 nM 17β-[^{3}H]estradiol in the absence or presence of DES. At the end of a 30-min incubation at 30°C, the nuclei were washed three times with cold (4°C) PBS, and the final pellet was extracted with 95% ethanol. The amount of radiolabeled steroid in an aliquot of the ethanol extract was determined. The specific uptake was determined by subtraction of the average radioactivity in extracts from the incubation tubes that included the excess DES. The total amount of ERn was calculated for each preparation. ERn was also expressed as a percentage of the total ER (ERc + ERn), which allows for estimation of the degree of receptor activation in the face of ligand-induced changes in receptor concentration.

In some experiments, the nuclear proteins were extracted from the crude nuclear pellet by addition of KCl to give a final concentration of 0.6 M. The protein extract was diluted (0.5 mg protein/ml) in Tris-EDTA buffer and used in the DCC assay with Scatchard analysis described earlier for ERc determination with the modification that assay tubes were incubated for an additional 30 min at 30°C to allow for exchange between endogenously bound ligand and tracer steroid. In this case, ERn was expressed both as a concentration (fmol/mg protein) and as the percentage of total ER.

The ability of β-HCH to compete with estradiol for binding to ER was determined by incubation of cytosolic protein with 2.5 nM 17β-[^{3}H]estradiol and increasing amounts of radioinert estradiol or β-HCH. In preliminary studies in which ethanol solutions were used, it was found that β-HCH, at concentrations of 10^{-5} M, did not remain soluble, and a precipitate was observed. Therefore, competitor compounds were dissolved in a mixture of propylene glycol:ethanol (4:1), and 50 μl of each were then added to assay tubes containing 200 μl of the cytosol preparations. There was no visible evidence of a loss of solubility even at a final concentration of 10^{-4} M β-HCH. Bound and free radiolabel were separated by the DCC method as above, and the amount of specifically bound tracer was expressed as a percentage of the maximum specific binding.

GH_3 Cell Cultures and Transient Transfection. GH_3 cells (American Type Culture Collection, Rockville, MD) were used in the transfection experiments. These cells were maintained in Ham's F-10 media supplemented with 15% horse serum and 2.5% FBS (Life Technologies) and allowed to grow to

70% confluency. Twenty-four h before transfection, the cells were subcultured in PRF serum-free media composed of DMEM and Ham's F-10 media (50 50, v/v), and supplemented with ITS+ premix (Collaborative Research Inc., Bedford, MA) and penicillin and streptomycin.

The PRL/luc plasmid (a gift from Dr. R. A. Maurer, Oregon Health Sciences, Portland, OR) consists of 2500 bp of the 5′ region of the rat *PRL* gene, including the promoter and enhancer regions, ligated to the firefly *luc* gene (27). The EREwtc/luc plasmid (a gift from Dr. E. Holler, Universität Regensburg, Regensburg, Germany) is composed of a single *Xenopus* vitellogenin A2 (wtc) ERE sequence (GGTCACAGTGACC) ligated 5′ to a minimal herpes simplex virus thymidine kinase promoter (−109 to +52 bp) and driving the expression of the *luc* gene (28).

GH_3 cell transfections and the luc assay were as reported previously (29). Briefly, 2–5 × 10^6 cells were cotransfected with 2–5 μg of PRL/luc or EREwtc/luc DNA plus 0.5 μg of cytomegalovirus β-galactosidase plasmid DNA (a gift from Dr. Arthur Gutierrez-Hartmann, University of Colorado Health Sciences Center) as an internal control. Electroporation parameters consisted of 220 V with 500 microfarads of capacitance. Following electroporation, GH_3 cells were resuspended in PRF serum-free media and plated (5 × 10^4 cells/well) in 96-well tissue culture plates that had been pretreated with protamine (Sigma) and NuSerum (Collaborative Research; Ref. 30). Treatments were added immediately as 100× solutions and consisted of vehicle, 17β-estradiol (10 nM), or β-HCH or *o,p′*-DDT (1 μM) alone or in combination with ICI164384 (1 μM). Cultures were incubated for 24 h at 37°C, at which time the media were removed, cells were lysed, and the luc activity was determined using the Promega (Madison, WI) Luciferase Assay System. luc results were normalized to β-galactosidase activity using the Galacto-Light chemiluminescent reporter assay (Tropix, Inc., Bedford, MA).

Statistical Analyses. ANOVA and the Scheffe F test were used to make comparisons between means. These analyses were carried out using the program Statview 4.02 (Abacus Concepts, Berkeley, CA).

RESULTS

MCF-7 Cell Proliferation. The growth-promoting properties of β-HCH and *o,p′*-DDT were compared using cultured MCF-7 cells. Both β-HCH and *o,p′*-DDT increased cell number in a dose-dependent manner, and the growth effects of both were inhibited by addition of the antiestrogen ICI164384 (Fig. 1). The stimulation of cell proliferation observed with *o,p′*-DDT or β-HCH (3–4-fold each) was comparable to the stimulation that was routinely observed with 17β-estradiol (data not shown). It should be noted that although both *o,p′*-DDT and β-HCH produced significant increases in cell number at a concentration of 10^{-8} M, maximal responses were achieved at 10^{-6} M *o,p′*-DDT or 10^{-5} M β-HCH. Similar results were observed with T47D cells, another ER-positive breast cancer cell line (data not shown). Conversely, when three ER-negative breast cancer cell lines, MDA-MB231, MDA-MB468, and HS578T, were incubated with β-HCH (1 μM) or 17β-estradiol (1 nM), no increase in cell number was observed (data not shown), indicating that functional ERs are required for β-HCH to be mitogenic.

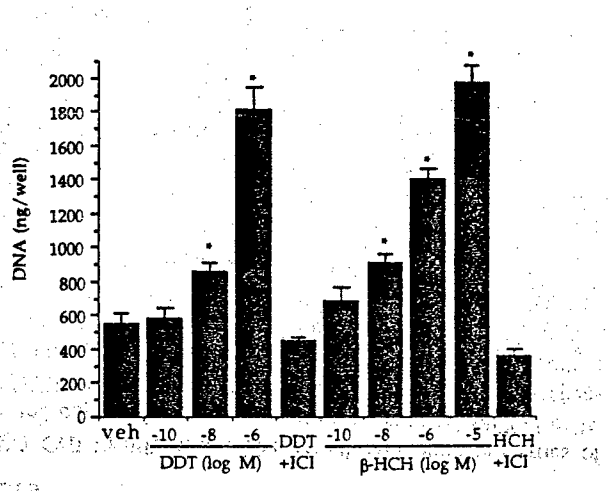

Fig. 1. Effects of *o,p′*-DDT and β-HCH on MCF-7 cell growth. MCF-7 cells were seeded into a 24-well plate at 15,000 cells/well in culture medium containing 5% FBS. The next day, the medium was changed to PRF MEM supplemented with 3% cs FBS. Two days later, the medium was replaced with medium containing one of the following treatments: vehicle (*veh*); *o,p′*-DDT (*DDT*) or β-HCH at the indicated concentrations; or *o,p′*-DDT or β-HCH at 1 μM plus 1 μM ICI164384 (*DDT + ICI* and *HCH + ICI*, respectively). The medium preparations were changed every other day up to 8 days after the start of treatment. The amount of DNA present in each well was assayed; the means of four wells are presented. Bars, *SD*; *, the mean differs from that of vehicle-treated values at $P < 0.05$.

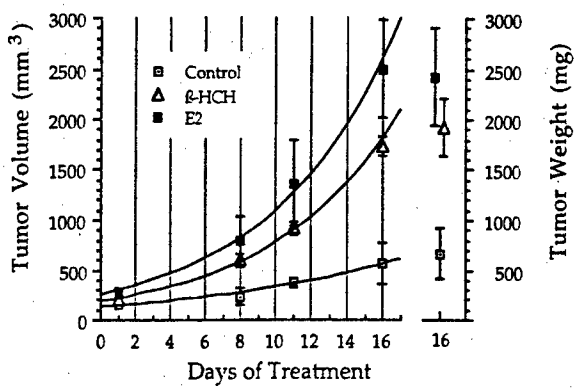

Fig. 2. Effects β-HCH or 17β-Estradiol (*E2*) on MCF-7 tumors grown in athymic mice. A tumor growing in an athymic mouse was harvested, minced, and injected, s.c. into nine intact female mice. Ten days later, the mice were ovariectomized, and a Silastic capsule was implanted s.c. (treatment day 1). Each capsule contained approximately 20 mg of either 17β-estradiol (*E2*) or β-HCH or cholesterol (*control*). The size of the tumor was estimated by measuring its dimensions through the skin. After 16 days, the tumors were excised and weighed. Values represent the means of tumors from three hosts. *Bars*, SD.

The efficacy of β-HCH as a tumor promoter *in vivo* was tested using a mouse xenograft model. β-HCH was nearly as effective as 17β-estradiol in stimulating the growth of MCF-7 cell tumors *in vivo* (Fig. 2). Tumors from the β-HCH- and 17β-estradiol-treated animals were 3–4-fold larger than tumors from the vehicle-treated animals. β-HCH stimulation of tumor growth paralleled that of 17β-estradiol at all time points, and the final weights of the tumors were not different in the two treatment groups. In contrast, the effect of β-HCH on the uteri of the host animals was less than one-fourth of that produced by 17β-estradiol. The dry weight of uterine horns in the 17β-estradiol-treated animals was 10.4 ± 0.52 mg compared with the control weight of 1.7 ± 0.03 mg, whereas β-HCH produced an intermediate weight of 3.8 ± 0.84 mg ($P < 0.05$ *versus* control).

Stimulation of *pS2* Gene Expression. The *pS2* gene product is a polypeptide that is expressed by one-half of all breast tumors, and although its function has not been determined, its presence identifies tumors that are sensitive to antihormonal therapy (31). The mRNA for pS2 is expressed by MCF-7 cells, and its levels have been shown to be increased by estrogen (31, 32). As shown, β-HCH, *o,p′*-DDT, and 17β-estradiol increased the levels of pS2 mRNA to a comparable extent, and the activity of all three compounds was blocked by antiestrogen treatment (Fig. 3).

ER Binding and Activation. Coosen and van Velsen (15) reported that β-HCH has no ER-binding affinity. Several experiments were performed to confirm and extend these previous observations. In a competitive binding assay, β-HCH, at concentrations as high as 10^{-4} M, did not compete with 17β-[^{3}H]estradiol for the ER (Fig. 4). Although it is possible that at this high concentration there is a loss of solubility of competitor compound, no visible precipitation was observed when β-HCH, in propylene glycol:ethanol, was added to the cytosol preparation. Moreover, the cell growth response seen with a 10^{-5} M dose of β-HCH was greater than that measured with a 10^{-6} M dose (Fig. 1), indicating that the former concentration represents a

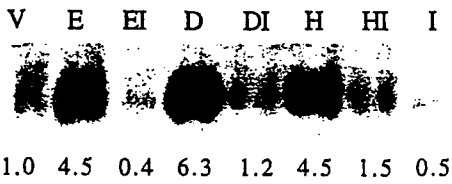

Fig. 3. Effects on steady state levels of pS2 mRNA in cultured MCF-7 cells. MCF-7 cells were cultured in for 1 week in MEM with 3% cs FBS and then 1 week in PRF MEM with 3% cs FBS as described in "Materials and Methods." Twenty-four h after the last change of medium, treatments were added to culture wells as follows: vehicle (*V*); 10 nM 17β-estradiol (*E*); 10 nM 17β-estradiol plus 1 μM ICI164384 (*EI*); 1 μM *o,p'*-DDT (*D*); *o,p'*-DDT plus ICI164384 both at 1 μM (*DI*); 1 μM β-HCH (*H*); β-HCH plus ICI164384 both at 1 μM (*HI*); and 1 μM ICI164384 (*I*). After an additional 24-h incubation, the medium was removed from the culture wells, and the cellular RNA was harvested as described. A Northern blot was prepared with 20 μg of total RNA/sample, and this was probed with labeled cDNA for pS2. Below each lane is the absorbance of each band relative to that of the vehicle control band.

reliably high standard. Thus, at concentrations of greater than 4000-fold (10^{-5} M) that of the radiolabeled tracer, β-HCH does not compete with 17β-estradiol for receptor binding.

It is generally believed that the ER normally resides within the nucleus; however, on cell homogenization, unliganded receptor leaks out into the cytosolic preparation, whereas ligand-activated receptors are retained in the nuclear fraction (33). We have used this phenomenon to compare the ability of 17β-estradiol, β-HCH, and *o,p'*-DDT to activate ER: the distribution of ER in MCF-7 ERc and ERn preparations was determined following incubation of the cells with the above compounds for 2 or 24 h. After 2 h, the concentration of ERc in cells incubated with β-HCH did not significantly differ from that of vehicle-treated controls, whereas the ERc concentration of *o,p'*-DDT-treated cells was significantly less (Fig. 5*A*). The decrease of ERc in *o,p'*-DDT-treated cells was accompanied by an increase in ERn levels; β-HCH treatment had no significant effect on the levels of either ERc or ERn (Fig. 5, *A* and *B*). Similarly, there was no change in ERc concentration or relative amount of receptor in the nucleus (ERn expressed as percentage of total ER) following a 24-h incubation with β-HCH (Fig. 5, *C* and *D*). Moreover, 17β-estradiol, but not β-HCH, caused a down-regulation of total cellular ER after 24 h; the mean total ER present was 92 fmol/10^6 cells in control cultures, whereas β-HCH- and 17β-estradiol-treated cells contained 92 fmol/10^6 and 34 fmol/10^6 cells, respectively. These results indicate that *o,p'*-DDT and 17β-estradiol bind and activate the ER, facilitating its retention in the nuclear fraction. In contrast, ERs in β-HCH-treated cells are found in an unliganded, nonactivated state and are readily detected in cytosolic preparations on cell disruption. Furthermore, the fact that levels of ERn in cells treated with β-HCH for 24 h were unchanged indicates that β-HCH is not converted to an active ER-binding metabolite during an incubation period that is known to affect *pS2* gene expression (Fig. 3).

Transcription Assays. Gene transfer experiments were conducted to determine whether β-HCH stimulates the transcription of selected estrogen-sensitive genes. GH_3 cells (an estrogen-sensitive, PRL-producing, rat lactotroph cell line) were transiently transfected with a PRL promoter/enhancer reporter construct (PRL/luc). Estrogen induces transcription via activation of an ERE sequence located in the PRL enhancer (34). Transfected GH_3 cells were incubated with vehicle, 17β-estradiol, β-HCH, or *o,p'*-DDT either alone or in combination with ICI164384; luc activity in cell extracts was determined after a 24-h incubation and normalized to β-galactosidase activity. The results (Fig. 6*A*) revealed that β-HCH was as effective as 17β-estradiol or *o,p'*-DDT in stimulating PRL promoter activity (2.8-, 2.7-, and 2.8-fold, respectively), and this stimulation was blocked by inclusion of the antiestrogen.

To study the direct activation of an ERE sequence, GH_3 cells were transfected with a minimal ERE-driven reporter gene (*EREwtc/luc*). Incubation with β-HCH failed to increase luc activity in these cells, whereas 17β-estradiol induced a 3-fold increase (Fig. 6*B*). This reporter construct was also tested in transfected MCF-7 cells, and as before, β-HCH was ineffective at stimulating its transcription (results not shown).

DISCUSSION

The above studies confirmed that β-HCH may represent a new class of xenoestrogen, the intracellular mechanism of action of which is different from the classic ER-mediated pathway used by estradiol or environmental estrogens such as *o,p'*-DDT. β-HCH stimulates cell proliferation in cultured MCF-7 cells, increases the levels of *pS2* gene mRNA, and stimulates growth of MCF-7 tumor xenografts. However, we have shown that although β-HCH does not bind to or cause nuclear retention of the ER, it does require a functional ER to be present for its mitogenic actions. In studying the role of β-HCH as a transcriptional activator, we have found that β-HCH, unlike 17β-estradiol, was unable to activate a minimal promoter reporter gene containing a single ERE sequence but was as effective as estradiol in activating more complex estrogen-responsive genes.

β-HCH fails to exhibit several properties that are characteristic of true estrogens, such as competition for high-affinity ER binding sites, the ability to induce nuclear retention of ER, and *trans*-activation via an ERE sequence. Yet interestingly, the effects of β-HCH effects are antagonized by antiestrogens. Recent observations have revealed that antiestrogens also block growth and gene expression induced by nonsteroidal growth factors. Antiestrogens attenuate cell proliferation induced by insulin, insulin-like growth factor, and epidermal growth factor in cultured breast cancer cells (35) and act as anti-growth factor agents in the rat uterine cells (36, 37). Thus, our results showing that ICI164384 is a potent inhibitor of β-HCH may exemplify another non-steroid-mediated pathway that is antiestrogen sensitive. Also, the unliganded ER has been shown to be involved in non-steroid-induced transcription. In the absence of steroid, insulin-like growth factor I and epidermal growth factor induce the transcription of a transfected *ERE/cat* reporter gene in an ER-dependent manner (38). Therefore, there is precedence for ligand-independent *trans*-activation through the ER, and β-HCH may use such an alternative mechanism to be effective. On the other hand, the effects of β-HCH may be through a mechanism that is independent of ER but nonetheless sensitive to the effects of antiestrogens.

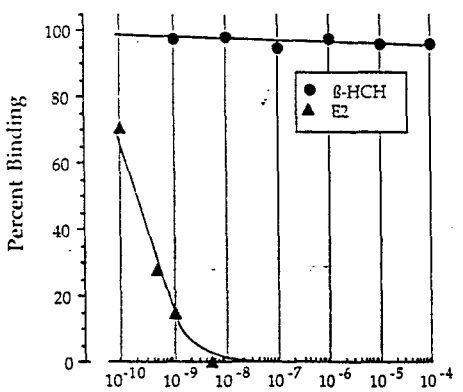

Fig. 4. Lack of effect of β-HCH in ER competitive binding assays. Cytosol was prepared from MCF-7 cells, and aliquots containing approximately 0.5 mg of protein/ml were incubated with 17β-[^{3}H]estradiol at 2.5 nM in the absence or presence of increasing concentrations of competitor, 17β-estradiol (*E2*), or β-HCH. After an 18-h incubation at 4°C, free and bound tracer were separated using the DCC technique. As expected, 2.5 nM 17β-estradiol reduced binding of the tracer by 50%, whereas β-HCH did not exhibit any appreciable competition even at 100 μM.

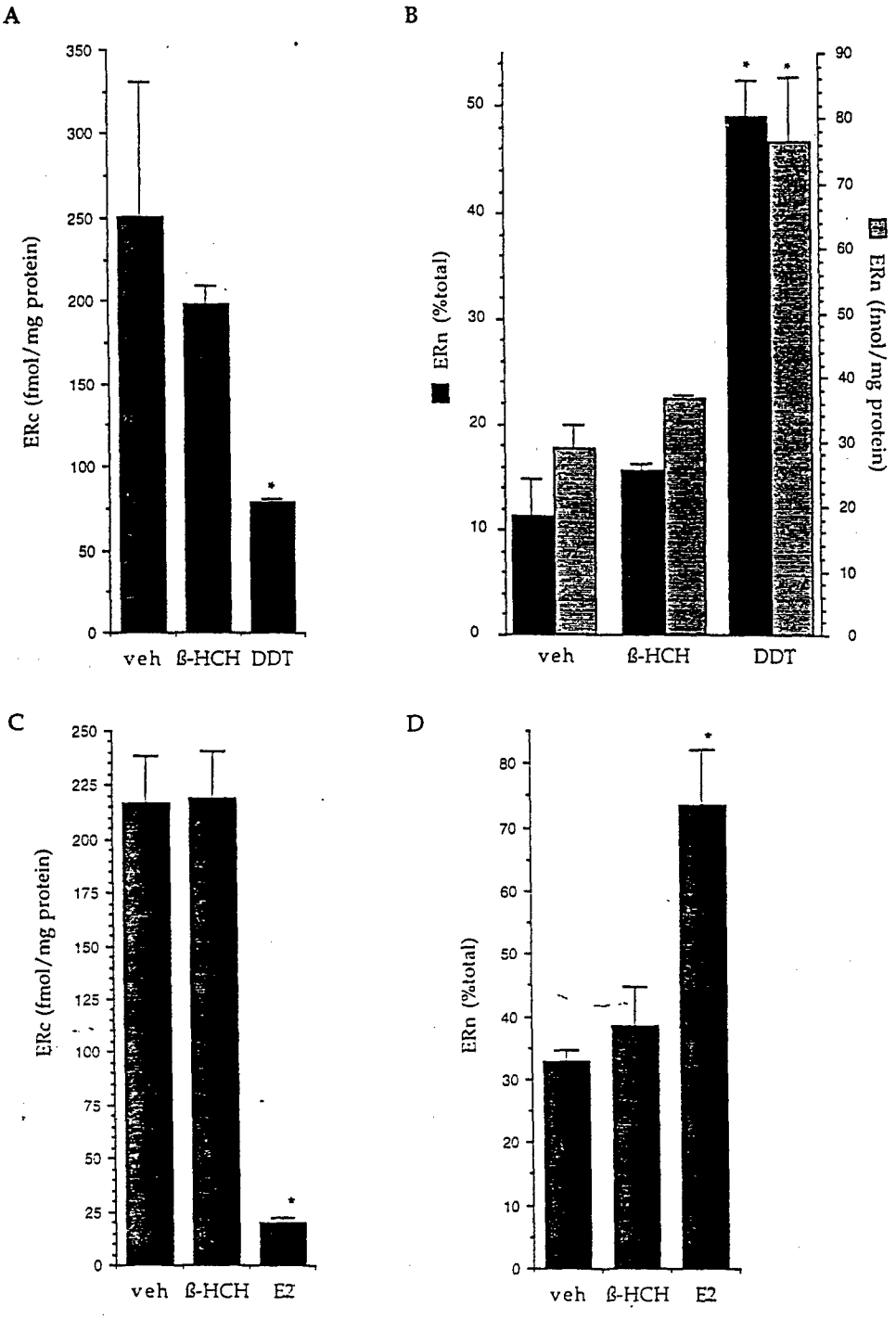

Fig. 5. Lack of effect of β-HCH on the relative cytosolic and nuclear fractionation of ER. MCF-7 cells were grown in PRF MEM plus 3% cs FBS for 1 week as described in Fig. 3. In two separate experiments cells were harvested after 2 h (*A* and *B*) or 24 h (*C* and *D*) of treatment with vehicle (*veh*), 1 μM β-HCH, 1 μM *o,p'*-DDT (*DDT*), or 1 nM 17β-estradiol (*E2*). Cells were washed four times with cmf PBS, removed from the culture dish in 10 mM EDTA, and homogenized in Tris-EDTA buffer. *o,p'*-DDT and 17β-estradiol reduced the ERc concentration (*A* and *C*) and increased the ERn relative content and concentration at 2 h (*B*) and the relative content at 24 h (*D*). Values represent the means of the maximum binding intercept from the Scatchard analysis or the means of three to six single-point determinations. *Bars*, 95% confidence intervals of the maximum binding intercept from the Scatchard analysis or SD of single-point assay determinations.

Activation of the ER by estrogen rapidly enhances the affinity of the receptor for nuclear material, thereby facilitating the retention of liganded receptor in the nuclear fraction of cell homogenates. Unliganded receptor, weakly associated with chromatin, leaks out and contaminates the cytosolic preparations (33). This phenomenon can be quantified by binding assays and has been used to study the activation of the ER by steroids and other compounds (33, 39). The studies reported here determined that β-HCH does not activate the ER as evidenced by the high cytosolic and low nuclear levels of ER following treatment. This was consistent with results of the competitive binding analyses, which clearly revealed that even high concentrations (100 μM) of β-HCH failed to displace tritiated 17β-estradiol from ER preparations. However, we cannot rule out the possibility that β-HCH binds the ER, but at a site that does not interfere with estradiol binding, or that β-HCH may act indirectly on ER activity, as has been shown for cAMP analogues (37).

Coosen and van Velsen (15) also reported that β-HCH did not compete with 17β-estradiol for ER binding; however, they did find that β-HCH caused a modest decrease in ERc and an increase in ERn. It should be noted that those studies were not conducted under estrogen-free conditions (PRF media or cs calf serum); thus, the discrepancy between their results and those reported here may be due to the effect of residual estrogen in their culture medium.

It is well established that estrogen-mediated increases in gene expression are the result of enhanced transcription through the activation of ERE sequences in target genes (40). It is also likely that the

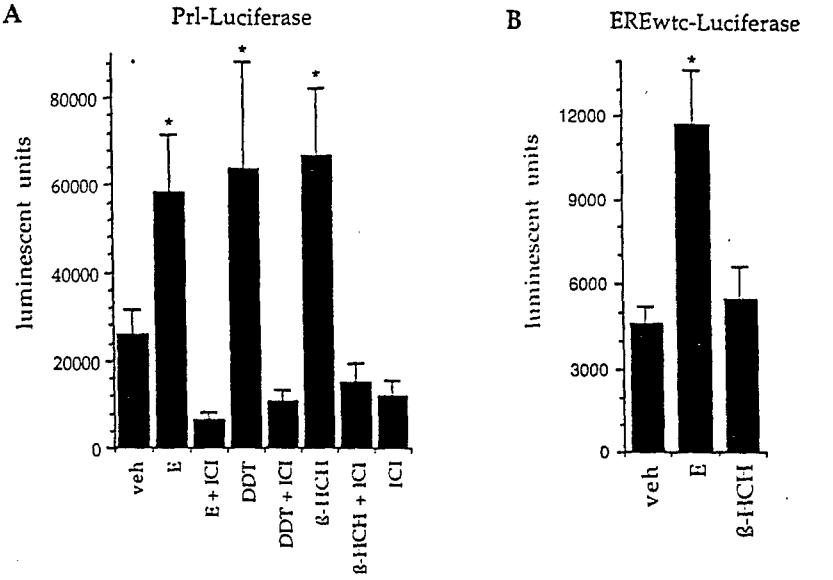

Fig. 6. Effects of β-HCH on estrogen-responsive reporter gene constructs. GH_3 cells were transfected using two different luc reporter constructs: *A*, a 2500-bp region of the *PRL* gene promoter plus the coding region of firefly *luc* (*PRL/Luciferase*); and *B*, a 15-bp wtc ERE 5′ of a minimal promoter region of the thymidine kinase gene (−105 to +36) plus the coding region of firefly *luc* (*EREwtc/Luciferase*). Cells were transfected as described in "Materials and Methods" and plated in 96-well culture plates, and the following treatments were added, vehicle (*veh*), 17β-estradiol (*E*; 10 nM), or β-HCH or *o,p′*-DDT (1 μM) alone or in combination with ICI164,384 (*ICI*, 1 μM). Results shown represent the means from a single representative experiment, with five replicate cultures per treatment group. *Bars*, SEM. Each transfection experiment was repeated three to five times with similar results.

effects of β-HCH are mediated through transcriptional regulation, albeit in a manner dissimilar to that of 17β-estradiol. This is supported by our findings that β-HCH increased the steady state levels of pS2 mRNA in MCF-7 cells and stimulated the transcription of both the PRL/luc reporter plasmid and a *pS2* gene promoter/reporter construct (data not shown), but it was unable to stimulate transcription of the minimal EREwtc/luc plasmid. It may be that β-HCH is transcriptionally active only in the context of a complex promoter in which additional *cis*-elements are required to facilitate its effects through the ERE. This type of combinatorial effect between steroid response elements and non-hormone response elements have been well documented (41, 42). The rat *PRL* gene also serves as an example of this type of functional interaction, in which binding of the POU-HOMEO domain transcription factor Pit-1 has been shown to be required for estrogen responsiveness (34) and may also be required for the observed β-HCH-induced effects. The *pS2* gene likewise has a complex promoter containing multiple *cis*-elements (32), and, analogous to the *PRL* gene, these additional elements may aid in the β-HCH-induced increase in pS2 transcription. The fact that the EREwtc/luc construct used for these studies does not contain additional *cis*-elements that might participate in such cooperative effects may account for the inability of β-HCH to stimulate its transcription. To confirm the presence of cooperative interactions, future studies will determine whether mutation of elements near the ERE in the *PRL* or *pS2* gene abolishes β-HCH transcriptional activity.

It is also possible that β-HCH transcriptional activity may not require ERE sequences but may actually be mediated through other response elements such as AP-1-binding sites. Promoter/reporter gene constructs lacking ERE sequences but including an AP-1 site can be *trans*-activated by growth factors (38) or tamoxifen (43) in an ER-dependent manner. Both the *PRL* (44) and *pS2* (32) gene regulatory regions contain putative AP-1-binding sites, which may mediate the observed increases in gene expression. Furthermore, it has been proposed that this type of ER-growth factor interaction is regulated, at least in part, by protein kinases and phosphorylation of the ER (37). Whether β-HCH acts through the ER in such an AP-1- and/or kinase-dependent pathway is under investigation.

The athymic mouse model has previously been validated as a suitable *in vivo* model for monitoring the effects of estrogens and antiestrogens on the growth of MCF-7 cells (45). Assuming that 17β-estradiol and β-HCH diffuse equally from the Silastic capsules, then β-HCH has bioefficacy equivalent to that of 17β-estradiol in stimulating the growth of these transplanted tumors. β-HCH also exerted a modest, but reproducible, stimulation of uterine weights. This differential response of tumor and normal uterine tissue to β-HCH exposure may be due to cell-specific factors found in breast tissue that enhance the proliferative effect of β-HCH, or β-HCH may synergize with other proliferative pathways that are up-regulated in neoplastic tissues.

Both *o,p′*-DDT and β-HCH are capable of stimulating breast cancer cells to proliferate. The levels of these xenobiotics measured in samples of human breast cancer tissue (22, 23) suggest that the concentrations used to stimulate cells in culture (1–10 μM) are in fact relevant. Furthermore, the observation that the breast cancer cells grown *in vivo*, *i.e.*, as a xenograft, were stimulated to a similar extent by estradiol and β-HCH indicates that high tissue levels of this xenobiotic should be of concern. Whether the relatively weak stimulation of the mouse uterus by β-HCH indicates that this compound is actually less of a threat for uterine cancer is unclear; further testing with human endometrial cancer cells in xenografts may be illuminating in this regard. The novel aspect of the estrogen-like effects of β-HCH suggest that its mode of action differs dramatically from those of other xenoestrogens. It is possible that β-HCH represents another class of compounds that produce estrogen-like effects without directly interacting with the ER. Understanding the mode of action of each type of these compounds will help in devising measures to better ensure health even in the face of their ubiquitous and persistent contamination of the environment.

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The Environmental Estrogen Bisphenol A Stimulates Prolactin Release *in Vitro* and *in Vivo**

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ABSTRACT

Environmental estrogens (xenoestrogens) are a diverse group of chemicals that mimic estrogenic actions. Bisphenol A (BPA), a monomer of plastics used in many consumer products, has estrogenic activity *in vitro*. The pituitary lactotroph is a well established estrogen-responsive cell. The overall objective was to examine the effects of BPA on PRL release and explore its mechanism of action. The specific aims were to: 1) compare the potency of estradiol and BPA in stimulating PRL gene expression and release *in vitro*; 2) determine whether BPA increases PRL release *in vivo*; 3) examine if the *in vivo* estrogenic effects are mediated by PRL regulating factor from the posterior pituitary; and 4) examine if BPA regulates transcription through the estrogen response element (ERE).

BPA increased PRL gene expression, release, and cell proliferation in anterior pituitary cells albeit at a 1000- to 5000-fold lower potency than estradiol. On the other hand, BPA had similar efficacy to estradiol in inducing hyperprolactinemia in estrogen-sensitive Fischer 344 (F344) rats; Sprague Dawley (SD) rats did not respond to BPA. Posterior pituitary cells from estradiol- or BPA-treated F344 rats strongly increased PRL gene expression upon coculture with GH_3 cells stably transfected with a reporter gene. Similar to estradiol, BPA induced ERE activation in transiently transfected anterior and posterior pituitary cells.

We conclude that: a) BPA mimics estradiol in inducing hyperprolactinemia in genetically predisposed rats; b) the *in vivo* action of estradiol and BPA in F344 rats is mediated, at least in part, by increasing PRL regulating factor activity in the posterior pituitary; c) BPA appears to regulate transcription through an ERE, suggesting that it binds to estrogen receptors in both the anterior and posterior pituitaries. The possibility that BPA and other xenoestrogens have adverse effects on the neuroendocrine axis in susceptible human subpopulations is discussed. (*Endocrinology* **138:** 1780–1786, 1997)

ENVIRONMENTAL estrogens (xenoestrogens) are a diverse group of chemicals that bind to estrogen receptors, mimic estrogenic actions, and may have adverse effects on human health (1, 2). One such compound is bisphenol A (BPA), a monomer of polycarbonate plastics. As shown in Fig. 1, BPA has two unsaturated phenol rings, but otherwise it has little structural homology with estradiol. It should be noted, however, that BPA and diethylstilbestrol (DES), a synthetic substance with potent estrogenic activity, are structurally similar. Polycarbonates are produced by condensing BPA to form the carbonate linkages of the polymer (3). Because of superior stability, toughness, and pliability, BPA-based epoxy resins are used in many consumer products, including inner coating of food cans, dental composites, and drug delivery systems. Although normally resilient, the carbonate linkages can hydrolyze at high temperatures and release BPA. BPA can also be liberated from incompletely polymerized epoxy resins (3).

Being lipophilic, many xenoestrogens can access the human body by ingestion or absorption through the skin and mucosal membranes. Of particular public health concern are two recent reports on significant amounts of BPA in foodstuff and human saliva. The first study (4) detected BPA in liquid from canned vegetables (10–20 μg/can, or 50–100 nM). The second study (5) found 20–30 μg/ml of BPA in saliva collected from subjects treated with composite dental sealants.

The estrogenic activity of BPA was accidentally discovered. After reporting that yeast produced estrogens (6), the authors realized that the estrogenic substance in the conditioned media had leached from polycarbonate flasks during autoclaving of water (7). The substance was purified by HPLC and identified by mass spectrometry as BPA. When incubated with the estrogen-responsive MCF-7 breast cancer cells, BPA induced progesterone receptors, competed with tamoxifen in binding to the estrogen receptor and promoted cell proliferation (7). However, the potency of BPA was 3–4 orders of magnitude lower than that of estradiol.

Most studies to date focused on putative carcinogenic effects of xenoestrogens, using primarily *in vitro* systems, while neglecting their potential impact on the neuroendocrine axis. The pituitary lactotroph is a well characterized estrogen-responsive cell (8, 9). Estrogens can affect PRL release by acting directly on the lactotrophs (10, 11), or indirectly via hypothalamo-pituitary factors that regulate the lactotrophs. These include dopamine, the primary PRL inhibiting factor (12, 13), and PRL regulating factor (PRF) from the posterior pituitary (14). PRF, the structure of which is yet unknown, is produced by a subset of intermediate lobe cells (15) and is the most potent inducer of PRL gene expression (16). PRF-producing cells are likely targeted by estrogens

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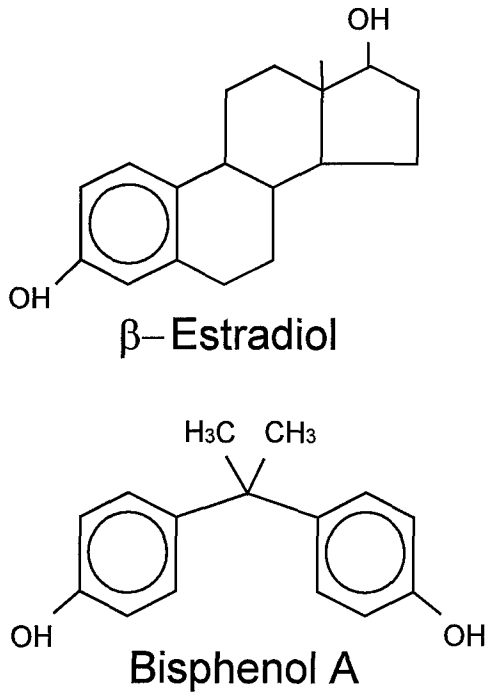

FIG. 1. Chemical structure of estradiol and BPA.

because an intact posterior pituitary is necessary for mediating estrogen-induced surges of PRL (17, 18).

The overall objective of these studies was to examine the effects of BPA on PRL release *in vitro* and *in vivo*. For the *in vitro* system, we used both primary rat anterior pituitary cells and GH_3 cells, a somatomammotroph cell line. For the *in vivo* system, we used two strains of rats: Fischer 344 (F344) and Sprague Dawley (SD). The inbred F344 rat is exquisitively sensitive to exogenous estrogens that rapidly induce hyperprolactinemia and formation of prolactinomas (19, 20). The SD rat, like other rat strains, responds to estrogens with a moderate rise in plasma PRL levels and does not readily form prolactinomas (21, 22).

The specific objectives were to: a) compare the potency of estradiol and BPA in increasing PRL gene expression and release *in vitro*; b) determine whether BPA stimulates PRL release *in vivo*; c) examine whether the stimulation of PRL release by estrogens is mediated, in part, by increasing PRF activity in the posterior pituitary; and d) investigate whether BPA regulates transcription in either anterior or posterior pituitary cells through the estrogen response element (ERE).

Materials and Methods

Animals

All animal studies were performed under an institutionally approved protocol according to the USPHS Guide for the Care and Use of Laboratory Animals. Ovariectomized (OVEX) F344 and SD rats (8–10 weeks old) were maintained on a 12-h light, 12-h dark schedule (lights on at 0700 h). SILASTIC brand (Dow Corning, Midland, MI) capsules (1 cm long) made from medical grade SILASTIC tubing (id 0.062", od 0.125") were filled with crystalline 17β estradiol (E_2; Sigma Chemical Co. St. Louis, MO) or BPA (Aldrich, Milwaukee, WI). The capsules were incubated for a few hours in PBS at 37 C before being inserted sc into the rats. Control rats received empty capsules. After 3 days, rats were decapitated, trunk blood was collected, and the serum was analyzed in duplicate for PRL by RIA, using NIDDK rat PRL kit with RP-3 as a reference preparation. The pituitary glands were removed, the anterior pituitaries weighed, and the posterior pituitaries dispersed with trypsin for use in the coculture experiment.

Estimation of the release rates of E_2 and BPA from SILASTIC capsules

Because there is no established assay for measuring BPA in body fluids, we compared the release rates of estradiol and BPA under simulated *in vitro* conditions. SILASTIC capsules filled with crystalline estradiol or BPA were incubated in PBS for 3 days at 37 C. Daily aliquots were fractionated on reversed phase HPLC, eluted isocratically with acetonitrile-water (40:60) and monitored at 254 nm, as described (7). Quantitation was based on peak height. The results showed that BPA and estradiol diffused from the capsules at the approximate rates of 40–45 μg/day and 1.2–1.5 μg/day, respectively.

Pituitary cell cultures

GH_3 cells were maintained in F-10 media supplemented with 15% horse serum and 2.5% FBS (Life Technologies, Grand Island, NY) and were plated in protamine precoated 96-well plates (NUNC, Copenhagen, Denmark) at 2.5×10^4 cells/well as described (23). The cells were first incubated for 48 h in phenol red-free, serum-free media (SFM) composed of DMEM/F-10 (50/50; vol/vol) and supplemented with 1% ITS + Premix (Collaborative Research, Bedford, MA) and penicillin/streptomycin and then incubated with the test substances for 7 days. Stock solutions of BPA, E_2 or testosterone (T; Sigma) were made in ethanol and serially diluted in SFM; final ethanol concentration was 0.001% or less. Media aliquots were analyzed in duplicate for PRL by RIA. At different times during culture, cell number in parallel plates was estimated using the MTT optical density method (15). Anterior pituitaries, removed from OVEX F344 rats, were trypsinized and the cells plated as above at 2.5×10^4 cells/well. After 4 days in SFM, the cells were washed and incubated with different concentrations of E_2 or BPA for 3 days. Media aliquots were analyzed in duplicate for PRL.

Stimulation of PRL gene expression using stably transfected GH_3 cells

GH_3 cells were transfected by electroporation with 5 μg PRL/luciferase plasmid containing 2.5 kb of the 5′ flanking region of the rat PRL gene placed upstream of the luciferase coding sequence (a gift from Dr. R. Maurer, Oregon Health Sciences University) and 0.5 μg pcDNA3 neomycin expression vector (Invitrogen, San Diego, CA). Positive clones were selected using 300 μg/ml geneticin (G418; Promega, Madison, WI), and the resulting stably transfected cells were maintained in 50 μg/ml of G418. The GH_3/*luc* cells were plated at 2.5×10^4 cells/well and preincubated in SFM for 48 h. The cells were then incubated with E_2 (1 pM), BPA (1 nM) or TRH (1 nM) for 8 or 24 h. Luciferase activity (designating induction of the PRL promoter) was determined in cell lysate by luminometry (16).

Determination of PRF activity using a coculture approach

PRF activity was determined by a bioassay that measures the ability of posterior pituitary cells to increase PRL gene expression when cocultured with the GH_3/*luc* cells. Posterior pituitaries (neurointermediate lobes) were removed from OVEX F344 and SD rats pretreated for 3 days with E_2 or BPA as described above. The cells were dispersed with trypsin, plated at 1×10^4 cells/well and incubated for 4 days in SFM. The GH_3/*luc* cells, preincubated for 48 h in SFM, were then added at 2×10^4 cells/well, and cocultured with the posterior pituitary cells for 24 h. Luciferase activity, determined in cell lysate by luminometry as above, was normalized for cell density that was determined in parallel plates using the MTT assay.

Determination of estrogen receptor expression in the pituitary gland by RT-PCR

Anterior and posterior pituitaries were pooled from 2–3 OVEX F344 and SD rats. Total RNA was isolated using Tri-Reagent (Molecular

Research Center, Cincinnati, OH), and 5 μg were reverse transcribed using SuperSript II reverse transcriptase (Life Technologies, Grand Island, NY) and random hexamers. For the PCR reaction, 10% of the RT products were used. The samples contained intron-spanning primers for either the ligand binding domain of the estrogen receptor gene (ER-1 5′-GCTCCTAACTTGCTCTTGGACA-3′ and ER-2 5′-ATCTCCAGCAGCAGGTCATAGA-3′), or for the POMC gene (MP-2 5′-TCCTGCTTCAGACCTCCATAGA-3′ and MP-3 5′-GGAAGTGACCCATGACGTACTT-3′), a marker for intermediate lobe melanotrophs. All PCR reactions also had primers for ribosomal protein L19 (RPL19–1 5′-AGTATGCTTAGGCTACAGAAG-3′ and RPL19–2 5′-TTCCTTGGTCTTAGACCTGCG-3′), a housekeeping gene serving as an internal standard. Expected product sizes are 500, 415, and 209 bp for RPL19, ER, and POMC, respectively. PCR reactions were denatured at 94 C for 30 sec, annealed at 57 C for 30 sec, and extended at 72 C for 30 sec for 25 cycles. Products were separated on a 1.5% agarose gel containing ethidium bromide, and the photograph was scanned and analyzed using Scion Image software. The number of cycles and annealing and extension temperatures were optimized, resulting in a linear relationship between band density and RNA amounts (data not shown). Band densities for ER and POMC were corrected for those for RPL19.

Transient transfection of anterior and posterior pituitary cells with ERE/luciferase reporter gene

Anterior and posterior pituitary cells from OVEX F344 rats were plated in 24 well plates at 6–7 × 10^4 cells/well, with 3–4 wells per treatment, and cultured for 4 days in SFM. Using calcium phosphate precipitation (Life Technologies) the cells were cotransfected with 5 μg ERE/luciferase plasmid, containing a single Xenopus vitellogenin A2 ERE sequence (GGTCACAGTGACC) placed 5′ to a minimal TK promoter driving the expression of the luciferase gene (a gift from Dr. E. Holler, Regnsburg, Germany), and 0.5 μg CMV-β-galactosidase plasmid. After 18 h, media were changed and the cells incubated with E_2 (10 nM) or BPA (1 μM) for 24 h. Luciferase activity was normalized for β-gal activity, determined using Galacto-Lite (Tropix, Bedford, MA).

Data analysis

Data were analyzed by analysis of variance, followed by Dunnett's test.

Results

BPA stimulates PRL release from primary anterior pituitary cells

We first tested whether BPA has estrogenic action *in vitro* using primary anterior pituitary cells harvested from untreated OVEX F344 rats. The cells were first cultured for 4 days to enable cell attachment and recovery and then incubated with different concentrations of estradiol and BPA for 3 days. As shown in Fig. 2, as little as 1 pM estradiol stimulated PRL release, exhibiting dose dependency up to 1 nM. The magnitude of release in response to estradiol was not large, however, and even at 10 nM (data not shown) the rise in PRL did not exceed 2.5-fold. Like estradiol, BPA increased PRL release in a dose-dependent manner, but at a 1000- to 5000-fold lower potency.

BPA increases PRL release and cell proliferation in GH_3 cells

GH_3 cells, plated in SFM, were incubated with 10 nM estradiol, 1 μM BPA, or 10 nM testosterone for 7 days. Both estradiol and BPA increased PRL release 2- to 3-fold in a time-dependent manner while testosterone was inactive (Fig. 3, *left panel*). The effect of BPA on cell proliferation was examined next. Within 3–5 days, cell number increased 50–60% in response to estradiol or BPA whereas testosterone was ineffective (Fig. 3, *right panel*). Because GH_3 cells are quiescent without serum, the mitogenic effects of both compounds is significant, albeit small.

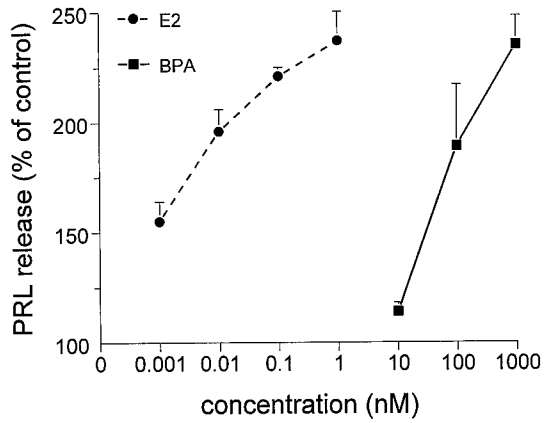

FIG. 2. Concentration-dependent stimulation of PRL release from primary anterior pituitary cells by E_2 and BPA. Cells were harvested from OVEX F344 rats and plated at 2.5 × 10^4 cells/well. After culturing in SFM for 4 days, the cells were incubated with BPA or E_2 for 3 days. Control cells were incubated with vehicle. Media aliquots were analyzed in duplicate for PRL by RIA. Each value is a mean ± SEM of five replicates. Data shown are representative of three experiments.

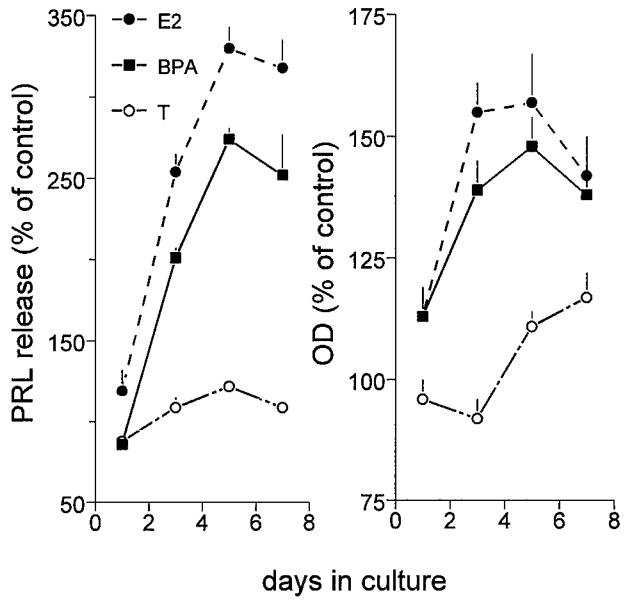

FIG. 3. Time-dependent stimulation of PRL release (*left panel*) and cell proliferation (*right panel*) by E_2 and BPA but not testosterone (T). GH_3 cells, plated at 2.5 × 10^4 cells/well, were preincubated in SFM for 2 days and then incubated with E_2 (10 nM), BPA (1 μM) or T (10 nM) for 7 days. Control cells were incubated with vehicle. Cell number was determined in parallel plates using an optical density (OD) method. See Fig. 2 for other details.

A low dose of BPA induces PRL gene expression

The efficacy and time dependency of estradiol and BPA in stimulating PRL gene transcription were compared. GH_3/*luc* cells were incubated with estradiol (1 pM), BPA (1 nM), or TRH (1 nM), and luciferase activity was determined after 8

and 24 h. As shown in Fig. 4, luciferase activity increased 1.5- to 2.5-fold in response to either estradiol or BPA; higher doses of either compound did not further increase PRL promoter activity (data not shown). Note that 1 nM TRH, a well characterized inducer of the PRL gene, caused a 6- to 8-fold rise in PRL gene expression, whereas estradiol induced only 2-fold elevation. This suggests that the overall effect of estrogenic compounds may involve both direct and indirect effects on PRL synthesis and release.

BPA induces hyperprolactinemia in F344, but not SD, rats

The objective was 2-fold: a) to determine if BPA increases PRL release *in vivo*, and b) to compare the effects of BPA and estradiol on PRL release and anterior pituitary weight in F344 and SD rats. To minimize potential negative feedback effects of elevated PRL, a short term exposure of 3 days was chosen. As shown in Fig. 5, basal serum PRL levels were 40 and 25 ng/ml in F344 and SD rats, respectively. Within 3 days, estradiol increased serum PRL levels 10-fold in F344 rats ($P < 0.05$) but only 3-fold in SD rats ($P < 0.5$). BPA increased serum PRL levels 7- to 8-fold over controls in F344 rats ($P < 0.05$) and was without effect in SD rats. As evident in Fig. 6, estradiol doubled the anterior pituitary weight in F344 rats within 3 days of treatment ($P < 0.05$) but caused no significant increase in pituitary weight of SD rats. Unlike its marked effect on PRL release in F344 rats, BPA did not alter anterior pituitary weight in either F344 or SD rats.

Induction of PRF activity by estradiol and BPA in F344 rats

One mechanism by which estrogens might stimulate PRL release *in vivo* is by increasing PRF activity. Therefore, we examined whether either compound increases PRF activity, using a coculture approach. Posterior pituitary cells from the above rats were incubated for 4 days in SFM and then cocultured with the stably transfected GH_3/luc cells for 24 h. Posterior pituitary cells from untreated rats of either strain increased luciferase activity 3- to 5-fold, indicating basal PRF

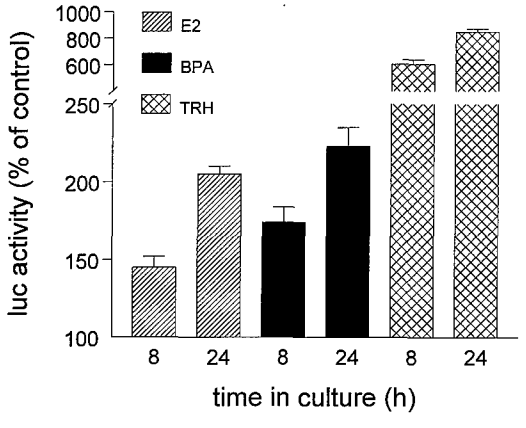

FIG. 4. Stimulation of PRL gene expression by E_2, BPA, and TRH. Stably transfected GH3/*luc* cells, plated at 2.5×10^4 cells/well, were preincubated in SFM for 2 days and then incubated with E_2 (1 pM), BPA (1 nM), or TRH (1 nM) for 8 or 24 h. Control cells were incubated with vehicle. Luciferase activity was determined in cell lysate by luminometry. Each value is a mean ± SEM of four replicates. Data shown are representative of two experiments.

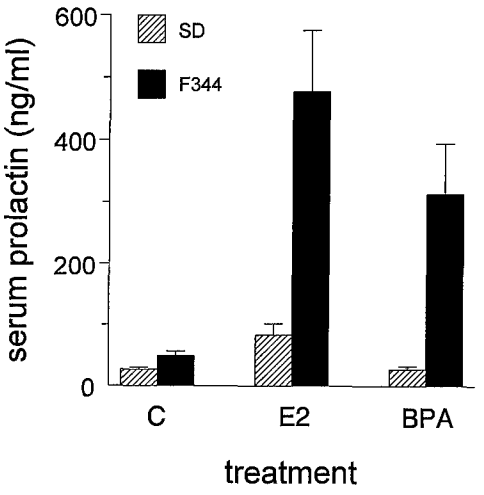

FIG. 5. Induction of hyperprolactinemia in F344 but not SD rats by E_2 and BPA. OVEX rats were implanted with SILASTIC capsules containing crystalline E_2 or BPA for 3 days; controls (C) had empty capsules. Trunk blood was analyzed in duplicate for PRL by RIA. Each value is a mean ± SEM of 12 rats/treatment for F344 rats and 8 rats/treatment for SD rats.

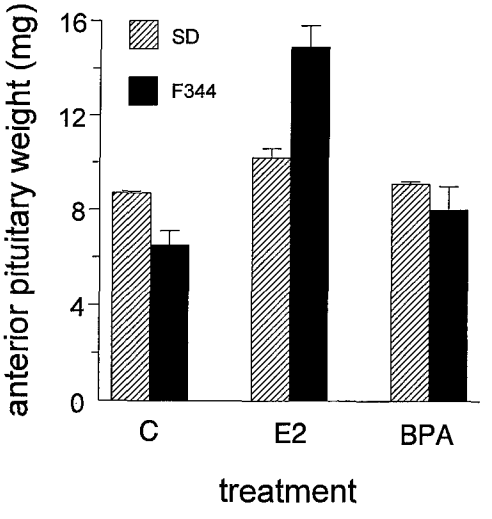

FIG. 6. Effects of E_2 and BPA on anterior pituitary weight in F344 and SD rats. See Fig. 5 for other details.

activity (Fig. 7). The stimulation by cells harvested from estradiol- or BPA-pretreated F344 rats was 15- to 17-fold ($P < 0.05$), whereas PRF activity in posterior pituitary cells from SD rats was unchanged by the estrogens. Note that the estrogen-induced PRF activity was retained by the cells after several days in culture.

Estrogen receptor expression in anterior and posterior pituitaries from F344 and SD rats

The difference in estrogen responsiveness between the two rat strains could be due to dissimilar expression of pituitary estrogen receptors. The minute size of the rat pituitary, especially the posterior pituitary, hinders receptor characterization by classical methods. Analysis by RT-PCR revealed no significant difference between F344 and SD rats in the level of expression of estrogen receptors in either the pos-

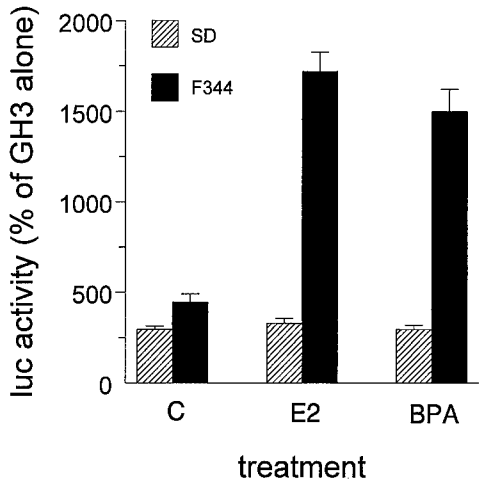

FIG. 7. Increased PRF activity in posterior pituitary cells from F344, but not SD rats, pretreated with E_2 or BPA for 3 days. Posterior pituitary cells, harvested from rats treated as in Fig. 5, were plated at 1×10^4 cells/well in SFM. After 4 days, the cells were cocultured for 24 h with GH3/*luc* cells (2×10^4 cells/well). Luciferase activity was determined in cell lysate and normalized for cell density, determined in parallel plates by MTT assay. Increased luciferase activity above GH_3 cells incubated alone indicates basal PRF activity. Each value is a mean ± SEM of 12 determinations from three separate experiments.

terior or anterior pituitaries (Fig. 8). The estrogen receptors were strongly expressed in the anterior pituitary with a significantly lower expression in the posterior pituitary, possibly due to the scarcity of PRF-producing cells. As expected, POMC expression was much higher in the posterior than anterior pituitaries.

BPA activates ERE in both anterior and posterior pituitary cells

We next examined whether BPA regulates transcription through an ERE. Anterior and posterior pituitary cells harvested from OVEX F344 rats were transiently transfected with ERE/luciferase reporter gene and incubated with estradiol (10 nM) or BPA (1 μM) for 24h. Luciferase activity was expressed as relative light units (RLU) after correction for β-gal. As shown in Fig. 9, like estradiol, BPA stimulated

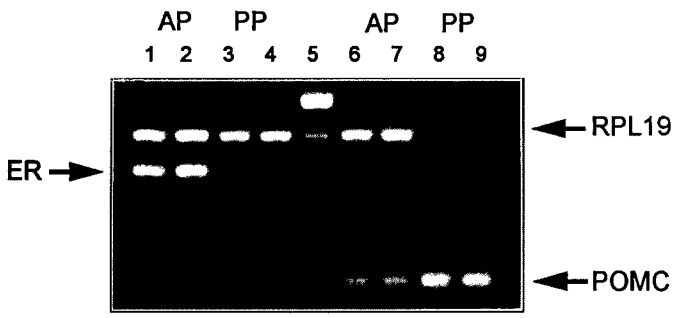

FIG. 8. Comparison of estrogen receptor (ER) expression in anterior (AP) and posterior (PP) pituitaries from OVEX F344 and SD rats. Total RNA was extracted from tissue pools and subjected to RT-PCR. Expected product sizes are 500, 415, and 209 bp for ribosomal protein L19 (RPL19), ER and POMC, respectively. Lanes 1, 3, 6, and 8, tissues from SD rats; lanes 2, 4, 7, and 9, tissues from F344 rats; lane 5, 100 bp standards.

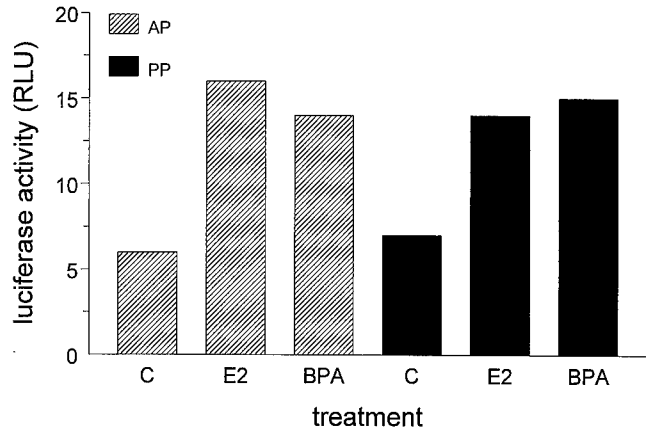

FIG. 9. Activation of estrogen response element (ERE) in anterior (AP) and posterior (PP) pituitary cells by E_2 and BPA. Cells from OVEX F344 rats were transiently transfected with ERE/luciferase reporter gene and incubated with E_2 (10 nM) or BPA (1 μM) for 24 h. Luciferase activity is expressed as relative light units (RLU) after correction for β-gal. Each value is a mean of two determinations. Data shown are representative of two independent experiments.

ERE-dependent gene expression, suggesting its binding to estrogen receptors in both tissues.

Discussion

We are reporting that BPA, a monomer of plastics that is abundant in the environment, mimics estradiol in stimulating PRL secretion both *in vitro* and *in vivo*. Like estradiol, BPA induced hyperprolactinemia in an estrogen-sensitive rat, but had only weak estrogenic activity *in vitro*. The *in vivo* actions of estradiol and BPA in F344 rats were mediated in part by increasing PRF activity in the posterior pituitary. BPA appears to regulate transcription through an ERE, suggesting that it binds to estrogen receptors in both the anterior and posterior pituitaries.

Our data show that BPA mimicked estradiol in inducing PRL gene expression, release, and cell proliferation in both primary anterior pituitary cells and GH_3 cells. Similar to its action on MCF-7 cells (4, 5, 7), the potency of BPA *in vitro* was 1000- to 5000-fold lower than that of estradiol. In contrast, BPA was rather effective in stimulating PRL release *in vivo*, albeit only in F344 rats. The discrepancy between the efficacy of BPA *in vitro* and *in vivo* could be due to a combination of factors. First, under simulated *in vitro* conditions, BPA diffused from the capsules 30–35 times faster than estradiol; this alone, however, cannot explain its increased efficacy *in vivo*. Second, the pharmacokinetics of BPA may differ from that of estradiol because of higher resistance to degradation, lesser binding to sex-hormone binding proteins, or retention in fat tissues. All of these possibilities should be examined. Third, BPA *in vivo* may form metabolites, *e.g.* 5-hydroxy bisphenol and bisphenol o-quinone (24), that are either more active than BPA or synergize with it. As reported recently, combinations of two weak xenoestrogens can be 100 to 1000 times as potent in activating estrogen receptors as each substance alone (25).

We also explored the mechanism underlying the estrogen-sensitivity of F344. Previous reports suggested that the genetic susceptibility of F344 rats to estrogens resides in the

pituitary because uterine growth in response to estrogen is normal (19). Furthermore, only pituitaries from F344, but not other strains, increased in size when grafted to the kidney capsule of estrogen-treated recipients (19). Other reports suggested increased neovascularization in the pituitary gland (26) and elevated production of basic fibroblast growth factor in F344 rats in response to estrogens (21). The present data confirmed rapid induction of hyperprolactinemia in F344, but not SD, rats by estrogens. This could be due either to altered estrogen receptors and/or estrogen-responsive gene(s) that affect the lactotrophs in F344 rats. Our results suggest that while functional estrogen receptors are present in both the anterior and posterior pituitaries (Fig. 9), there were no apparent differences in their expression between F344 and SD rats (Fig. 8). Still, the difference between the rat strains could be attributed to the presence of estrogen receptor splice variants (27) or estrogen receptor β (28). We have preliminary evidence that both the anterior and posterior pituitaries express a truncated estrogen receptor product (TERP) as well as estrogen receptor β. These findings are presently being confirmed and expanded. Alternatively, the difference between the rat strains could reside in factors downstream of the receptor, *e.g.* coactivators, repressors, or sequence and binding affinity of ERE on target genes. It would be of interest to further investigate these possibilities.

The coculture data clearly show that estrogens increase PRF activity in F344 rats. We previously reported that PRF is produced by a subpopulation of intermediate lobe cells (15, 29), is distinct from other PRL secretagogues (30, 31), and is a strong inducer of the PRL gene (16, 32). Further, we suspected that PRF-producing cells are targeted by estrogens because an intact posterior pituitary is necessary for mediating the acute estradiol-induced rise in PRL (17) and for generating the full pattern of the PRL surge on proestrus (18). This notion was supported by Frawley *et al.*, reporting that estrogen induced a mammotropic factor (presumably αMSH) that rapidly recruited additional PRL secretors into the secretory pool (33). Further, the posterior pituitary expresses estrogen receptors (34 and Fig. 8), and like the uterus, estrogen induces c-*fos* expression in this tissue (35).

Although basal PRF activity was similar in both rat strains (Fig. 7), pretreatment with estradiol or BPA increased PRF activity only in posterior pituitary cells from F344 rats. This suggests that the estrogen sensitivity of F344 rats is attributed, at least in part, to increased responsiveness of PRF-producing cells to estrogens. Because the structure of PRF is yet unknown, identification of PRF cells awaits the sequencing of PRF and generation of cellular and molecular probes. Of interest, BPA stimulated PRL release but did not increase the pituitary weight in F344 rats (Fig. 6). This suggests that BPA does not mimic all of the *in vivo* actions of estradiol. Indeed, tissue-selective estrogenic activity has been reported for several estrogenic compounds (36).

Because humans are exposed to significant amounts of BPA through canned food and dental devices (4, 5), the present findings may have implications to human hyperprolactinemia. Although oral contraceptives do not normally induce hyperprolactinemia (37), women who used oral contraceptives for menstrual irregularities rather than for prevention of pregnancy, have a 7- to 8-fold higher incidence of prolactinomas (38). This suggests that exogenous estrogens may stimulate incipient prolactinomas to grow or are more mitogenic in women with reproductive disorders. Whether this is related to the expression of multiple splice variants of the estrogen receptor by human prolactinomas (39) remains to be determined.

In conclusion, we demonstrated estrogen-mimicking activity of BPA both *in vitro* and in an animal model. BPA and other xenoestrogens constitute an unsuspected source of compounds capable of altering the natural hormonal balance. Perhaps there is a human homolog to the F344 rat, *i.e.* only individuals with altered estrogen receptors and/or estrogen responsive genes are predisposed to the effects of xenoestrogens. To better evaluate potential hazards posed by such compounds to human health, more information is needed on their exposure, pharmacokinetics, synergistic interactions, and ability to activate a variety of estrogen-responsive genes.

Acknowledgments

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Xenoestrogens: The Emerging Story of Bisphenol A

Nira Ben-Jonathan and Rosemary Steinmetz

Xenoestrogens are chemicals with diverse structure that mimic estrogen. Bisphenol A (BPA), a monomer of polycarbonate and epoxy resins, has been detected in canned food and human saliva. BPA stimulates cell proliferation and induces expression of estrogen-responsive genes in vitro, *albeit with a relatively low potency.* In vivo, *BPA increases prolactin release and stimulates uterine, vaginal and mammary growth and differentiation. BPA shares similarities in structure, metabolism and action with diethylstilbestrol (DES), a known human teratogen and carcinogen. This review considers the hypothesis that BPA is converted* in vivo *to hydroxylated metabolite(s) with enhanced estrogenicity and genotoxicity.*

• The Controversy Over Xenoestrogens

Xenoestrogens are pesticides and industrial by-products with little or no structural homology to estradiol that are common in the environment and act as agonists or antagonists of estrogens. Whether they pose harm to animals or humans is highly controversial. Several studies have linked xenoestrogens to malformations and reduced fecundity in wildlife (Colborn 1995). For example, poor egg survival and precocious puberty were observed in salmon from lakes polluted with PCBs and pesticides. Bald eagles and mink, feeding on contaminated fish, had high offspring mortality, while herring gull chicks developed abnormal oviducts and testicular feminization. A large spill of dicofol in Lake Apopka in Florida resulted in a low hatching rate of alligators and a drastically reduced penis size in the juvenile males (Guillette *et al.* 1995). Male rainbow trout collected near a sewage treatment plant that discharged nonyl- and octyl-phenols had elevated levels of vitellogenin, an estrogen-inducible protein expressed normally in females only (Sumpter and Jobling 1995).

Whether exposure of wildlife to moderate amounts of xenoestrogens causes reproductive abnormalities is uncertain. Because wildlife is exposed to a mixture of pollutants, the effects of individual substances or their interactions cannot be verified. Although often implied, there are no solid data linking xenoestrogens to reduced fertility or increased cancer incidence in humans. Furthermore, a common argument is that xenoestrogens pose no harm to humans because of their poor binding affinity to the estrogen receptor (ER) and low abundance in the environment, especially when compared with the potential counter-effects of dietary phytoestrogens. Here, we describe the estrogenic effects of bisphenol A (BPA), an industrial product that shares several characteristics with diethylstilbestrol (DES). We propose potential mechanisms that might explain an apparent discrepancy between the *in vitro* and *in vivo* bioactivity of BPA.

• Lessons Learned from Human Exposure to DES

DES, produced in 1938 as the first potent synthetic estrogen, was prescribed to several million pregnant women to prevent miscarriage. An astute observation by a few physicians in the early 1970s, who noticed increased incidence of a rare vaginal cancer in adolescent women, drew attention to its

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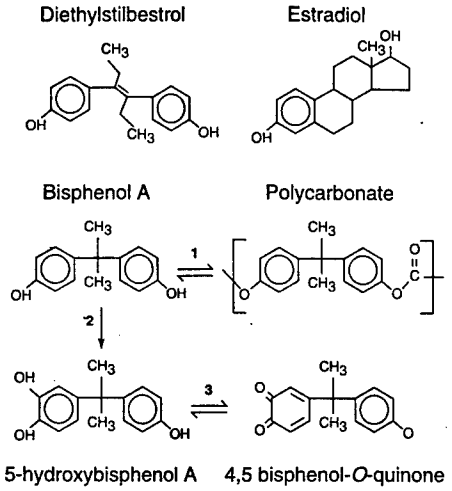

Figure 1. Comparison of the chemical structure of estradiol, diethylstilbestrol (DES) and bisphenol A (BPA), and the proposed metabolic pathway of BPA. 1: polymerization of BPA to polycarbonate and depolymerization at high temperature; 2: 5-hydroxylation of BPA to the catechol compound 5-OH-BPA; 3: formation of bisphenol-*O*-quinone via a semiquinone intermediate (not shown). Modified from Atkinson and Roy (1995a).

deleterious effects (reviewed in Marselos and Tomatis 1992). In retrospect, there was no good reason for treating pregnant women with DES, except that it was readily available and considered safe. Like thalidomide, DES had little impact on the women themselves, confirming that drug effects in the developing fetus cannot be extrapolated from adult responsiveness (Newbold 1995). During the 1960s and 1970s, DES was used as a growth promoter in 70–80% of poultry and livestock in the USA (Marselos and Tomatis 1993). Because this practice was so widespread, the consequences of human exposure to DES via meat consumption cannot be evaluated.

Prenatal exposure to DES caused multiple structural and functional abnormalities in men and women (Marselos and Tomatis 1992). A large percentage of the daughters of mothers prescribed DES had benign anomalies of the reproductive tract, with increased incidence of infertility and ectopic pregnancy. The most common cancer was vaginal clear cell adenocarcinoma, affecting 0.1–0.15% of exposed women. Structural abnormalities in the sons included cryptorchidism, malformed urethras and epididymal cysts, often accompanied by decreased sperm count, increased sperm deformities and prostatic inflammation. Whereas congenital malformations caused by thalidomide were recognized at birth, the teratogenic effects of DES were subtle and delayed.

Unlike thalidomide, which is not teratogenic in most laboratory animals, DES exerts its effects in many species, including non-human primates (Marselos and Tomatis 1993). In addition to the abnormalities seen in humans, DES-treated fetal or neonatal rodents developed latent tumors of the pituitary, mammary gland and uterus. Notably, DES is not an environmental estrogen but a potent pharmaceutical compound. Yet, its striking endocrine-disruptive effects underscore the ability of exogenous estrogens to induce congenital malformations and cancer, and illustrate the dual action of estrogens as morphogenic hormones during embryogenesis and as cellular regulators in adulthood.

- **Structure, Usage and Prevalence of BPA**

BPA is produced by an acid-catalyzed reaction of phenol and acetone and is composed of two unsaturated phenol rings (Fig. 1). Over 1.6 billion pounds were produced in 1996, with a yearly increase in production of 5–6%. Most BPA is used in the manufacture of polycarbonate (60%) and epoxy resins (30%), with the remaining 10% being used for the production of polyester resins. Polycarbonate, composed of BPA monomers, has many desirable commercial qualities such as transparency, moldability and high impact strength (Lazear 1995). The carbonate linkages are rather stable but can be hydrolyzed at high temperature and neutral to alkaline pH, resulting in release of BPA. Epoxy resins containing BPA diglycidylether (BADGE) have superior adhesive properties. When reacted with a hardener they become crosslinked and can be used in coating or bonding applications (Brotons *et al.* 1995). BPA diglycidyl methacrylate (bis-GMA) is a constituent of dental sealants used to replace tooth structures. Their polymerization (curing) reaction is photo-initiated by UV or visible light. Incompletely polymerized resins may contain 5–10% of free BPA (Olea *et al.* 1996).

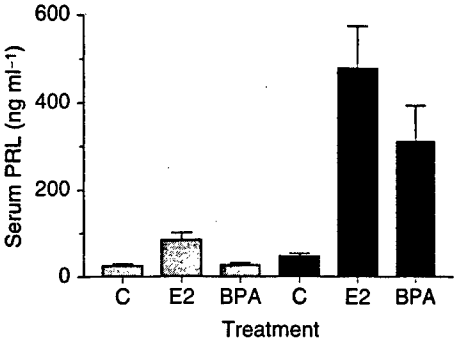

Figure 2. Induction of hyperprolactinemia in Fischer 344 (F344) (closed bars) but not Sprague Dawley (SD) (shaded bars) rats by estradiol (E2) and bisphenol A (BPA). Ovariectomized rats were implanted with silastic capsules containing crystalline E2 or BPA for three days; controls (C) had empty capsules. Trunk blood was analyzed for prolactin (PRL) by radioimmunoassay. Each value is a mean ±SEM of eight to 12 rats. Modified from Steinmetz *et al.* (1997).

Human exposure to BPA is not insignificant, given that BPA-based polycarbonate and epoxy resins are common in food utensils. For example, beverage bottles and baby food containers that are heated before use are made of polycarbonate. Epoxy resins are used widely in the food packaging industry for the inner coating of food cans to prevent corrosion. Leaching of BPA can be facilitated when canned food is cooked or sterilized at high temperatures. BPA-based resins are also used as susceptors to achieve food browning in some packages designed for microwave cooking (Sharman *et al.* 1995).

- **Estrogenic Activity and *In Vitro* Potency of BPA**

The estrogenic activity of BPA was discovered serendipitously. Investigators from Stanford University identified an estrogen-binding protein in yeast and then investigated whether yeast have an endogenous ligand. After first reporting that yeast produce estradiol (Feldman *et al.* 1984), they found that the estrogenic activity did not come from the yeast, but from culture media

that were prepared with water autoclaved in polycarbonate flasks. With binding to ER used as a bioassay, the estrogenic compound was purified and identified as BPA (Krishnan *et al.* 1993). Approximately 2–3 μg l^{-1} of BPA was detected in the autoclaved water. Next, they tested whether authentic BPA is estrogenic, using four criteria: (1) binding to ER, (2) proliferation of MCF-7 breast cancer cells, (3) induction of progesterone receptors, and (4) reversal of estrogenic action by tamoxifen. BPA satisfied all criteria as an estrogenic compound, with the lowest effective dose being 10–20 nM.

Intrigued by the discovery of BPA in autoclaved water, another group analyzed liquid from 20 different brands of canned vegetables (Brotons *et al.* 1995). The BPA concentration ranged from 0 to 33 μg per can, with the variability attributed to the type of polymer, sterilization procedure and food variety. For instance, an alkaline or fatty food should favor increased leaching of BPA during heating. They also examined dental sealants for estrogenicity and identified the active compounds as BPA and bis-GMA. Interestingly, as much as 3–25 mg ml^{-1} of BPA was detected in saliva of patients treated with dental sealants (Olea *et al.* 1996).

Subsequent studies revealed that BPA also affects the pituitary lactotrophs, as judged by increased prolactin (PRL) gene expression and release, and enhanced cell proliferation (Steinmetz *et al.* 1997). Similar to its action on MCF-7 cells, BPA was 2000–5000 times less potent than estradiol in stimulating PRL release *in vitro*. Other reports confirmed that BPA interacts directly with the ER, with a 1000–5000 lower binding affinity than estradiol (Dodge *et al.* 1996). The binding affinities of BPA relative to estradiol, using *in vitro* translated ERα and ERβ proteins, were 0.05 and 0.33, respectively (Kuiper *et al.* 1997). Thus, by all criteria, BPA behaves as a rather weak estrogen *in vitro*.

- ***In Vivo* Effects of BPA**

The *in vivo* effects of BPA on the neuroendocrine system were examined in two strains of rats: Fischer 344 (F344) and Sprague Dawley (SD). The inbred F344 rat is highly sensitive to exogenous estrogens, which induce hyperprolactinemia and prolactinoma formation (Wiklund *et al.* 1981, Burgett *et al.* 1990). To achieve a constant low exposure level, rats were implanted with silastic capsules containing crystalline estradiol or BPA. Under simulated *in vitro* conditions, these capsules release 50 μg of BPA per day and 1 μg of estradiol per day. Within three days, estradiol and BPA produced a ten- and sevenfold increase, respectively, in serum PRL levels in F344 rats. In contrast, PRL in SD rats was unaffected by BPA (Fig. 2). Unlike estradiol, BPA did not increase pituitary weight or induce prolactinoma formation (Steinmetz *et al.* 1997).

Single injections of high doses of BPA stimulated cell proliferation in the uterus and vagina that followed a similar time-course to that induced by estradiol (Steinmetz *et al.* in press). On the other hand, the BPA-induced expression of c-*fos* in the vagina was longer in duration than that caused by estradiol (Fig. 3). Treatment of F344 rats for three days with capsules that supplied much lower doses of BPA (0.3 mg kg^{-1} per day) resulted in hypertrophy, hyperplasia and mucus secretion in the uterus, and hyperplasia and cornification of the vaginal epithelium. As was the case with PRL, the reproductive tract of SD rats did not respond to this treatment with BPA. Unlike estradiol, BPA did not increase uterine wet weight in either strain of rats, suggesting cellular selectivity (Steinmetz *et al.* in press). Profound effects of relatively low doses of BPA were also reported on the growth of the mammary gland of Noble rats, where treatment with BPA increased lobular maturation and altered cell cycle kinetics of mammary epithelial cells (Colerangle and Roy 1997).

Collectively, the *in vivo* studies revealed three intriguing findings: higher than expected bioactivity of BPA, partial agonist activity and rat strain differences in susceptibility to its effects. As discussed below, conversion of BPA to a more active metabolite and polymorphism in one or more of the metabolic enzymes might, in part, explain these observations.

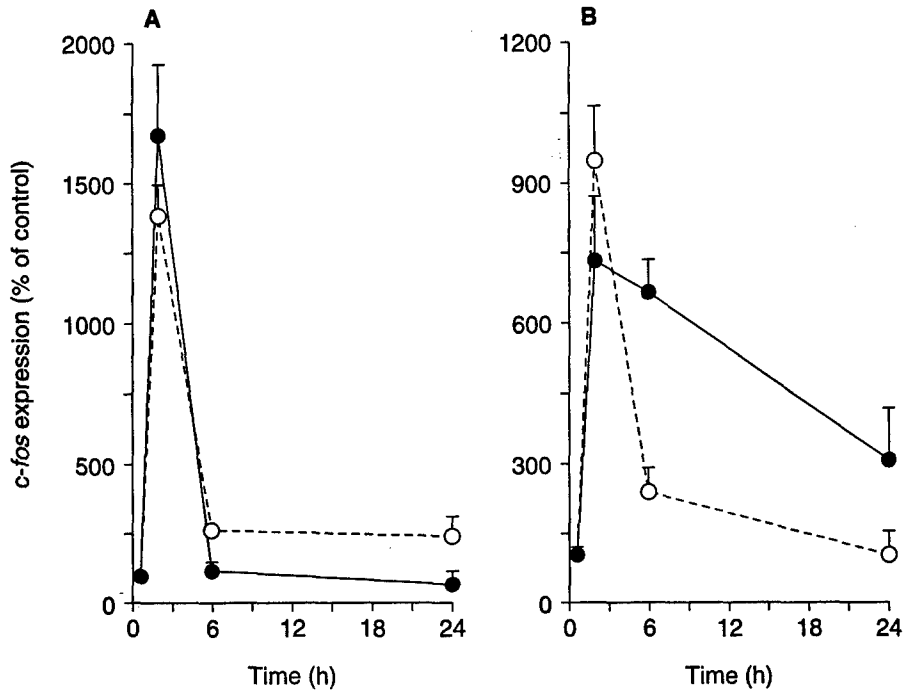

Figure 3. Induction of c-*fos* expression by estradiol (E2) (open circles) and bisphenol A (BPA) (closed circles) in the uterus (**A**) and vagina (**B**). Ovariectomized Fischer 344 rats were injected with E2 (10 μg kg^{-1}) or BPA (50 mg kg^{-1}) and sacrificed after 0, 2, 6 or 24 h. Levels of c-*fos* mRNA were determined by quantitative reverse transcription–polymerase chain reaction. Each value represents a mean ±SEM of four to six rats. Modified from Steinmetz *et al.* (1998).

• **Metabolism of BPA**

Little is known about the metabolic activation, or inactivation, of xenoestrogens. Presumably, their metabolism follows similar pathways to those of 17β-estradiol (Yager and Liehr 1996). Oxidation of estradiol at the C-2 and C-4 positions form 2-3 and 3-4 catechols, which retain significant, although reduced, estrogenic activity. Several cytochrome P450 (CYP) monooxygenases, both hepatic and extrahepatic, have been implicated in estrogen metabolism, including CYP1A1, 1A2, 3A3, 3A4, 3A5 and 2C11 (Guengerich 1991, Martucci and Fishman 1993). Recently, CYP1B1, a newer member of the CYP1 family that is not highly expressed in the liver but is abundant in uterine and breast tissue, was found to catalyze the 4-hydroxylation of 17β-estradiol (Hayes *et al.* 1996, Hakkola *et al.* 1997). Polymorphism in CYP enzymes is rather common among various human populations, resulting in alterations in their ability to detoxify many drugs and pollutants (Puga *et al.* 1997).

Conversion of BPA to a hydroxylated metabolite (Knaak and Sullivan 1966) and redox cycling between the semiquinone and quinone have been reported (Roy *et al.* 1997). In the latter studies, rat liver microsomes converted BPA to a catechol (5-OH-BPA) that was further converted to bisphenol-*O*-quinone (Fig. 1). Because this activity was inhibited by α-naphthoflavone, an inhibitor of CYP1 enzymes, a member of this family could be responsible for BPA hydroxylation.

Hydroxylation of an estrogen can alter its binding to the receptor in an unpredictable manner. Indeed, the binding affinity and K_i of 4-OH-tamoxifen for ERα and ERβ are 25 and 60 times higher, respectively, than those for tamoxifen, the parent compound. In contrast, 4-OH-estradiol has an eight to 15 times lower binding affinity for these receptors than estradiol (Kuiper *et al.* 1997). In a recent report, BPA was only 20–50 times less potent than estradiol in activating a human hepatoma cell line transfected with ERα (Gould *et al.* 1997). Because hepatocytes are rich in CYP enzymes, it is possible that increased BPA bioactivity in these cells is due to conversion to an active metabolite(s).

• **Genotoxicity Versus Estrogenicity**

Some estrogens undergo CYP-mediated redox cycling to quinones. Quinones are reactive molecules that can bind covalently to DNA, as well as to nuclear proteins, such as DNA and RNA polymerases. DES is catalyzed by CYP1A1 to DES-quinone, which is reduced by quinone reductase. Administration of DES induces DNA adduct formation in the liver, kidney, uterus and testes, presumably by interaction of DES-quinone with guanine bases (Liehr 1990). When pregnant hamsters were treated with DES, DNA adducts were also detected in the fetal liver (Gladek and Liehr 1991). Like DES, BPA forms quinones and DNA adducts *in vitro* (Atkinson and Roy 1995a) and *in vivo* (Atkinson and Roy 1995b). In addition, both DES and BPA have been shown to interfere with the assembly of microtubules (Metzler and Pfeiffer 1995), a process by which DES is thought to promote the induction of aneuploidity and chromosomal aberrations (Liehr 1990).

Notable differences exist between estrogenicity and genotoxicity. The actions of an estrogen are dose dependent, receptor mediated and reversible, whereas those of a genotoxin are usually irreversible and delayed (Wells and Winn 1996). Furthermore, a genotoxin is often a minor product reflecting the balance between elimination, bioactivation and detoxification processes. Thus, a relatively weak estrogen can still be a potent genotoxin. Given that quinones and other reactive molecules have short half-lives and the adverse effects of DES occur primarily in estrogen-responsive tissues, it is plausible that conversion to quinones and formation of DNA adducts occur within tissues that bind and retain DES. Formation of DNA adducts in reproductive tissues during organogenesis might induce genetic instability, mutations and cellular aberrations with lasting consequences for the adult.

In a recent study, pregnant mice were fed with relatively low doses of BPA or octylphenol (2 and 20 mg kg^{-1} per day) from Day 11 to Day 17 of pregnancy. Prenatal treatment with either dose of BPA, but not octylphenol, resulted in a significant increase in prostate weight of adult male offspring (Nagel *et al.* 1997). These findings are important for several reasons. First, they provide evidence that microgram (or even sub-microgram) quantities of BPA can exert genotoxic/teratogenic actions in the developing fetus. Second, they demonstrate that BPA is both absorbed and active after oral ingestion.

• **Conclusions and Perspectives**

BPA resembles DES in structure and metabolic processing except that its binding affinity to the estrogen receptor is significantly lower. Indirect evidence suggests that *in vivo* BPA might be converted to a more potent estrogen, as is the case with 4-OH-tamoxifen. Experiments should be designed to test this hypothesis and answer the following questions: (1) Is conversion of BPA performed by the liver or by target reproductive tissues and which candidate CYP enzyme is involved? (2) Does hydroxylation of BPA increase its binding affinity to ERα, ERβ or both? (3) What is the mechanism of partial agonist activity of BPA? (4) Can polymorphism in oxidative or detoxifying enzymes account for individual predisposition to the estrogenic actions of BPA? (5) Does orally ingested BPA, the most likely route for human exposure, retain significant estrogenic activity? The pursuit of these questions should provide a challenging endeavor.

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Heparin-Binding Property of Human Prolactin: A Novel Aspect of Prolactin Biology

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ABSTRACT. Prolactin (PRL) shares several characteristics with growth factors and cytokines, many of which are known to bind to heparan sulfate proteoglycans. In this study we examined the heparin-binding properties of selected members of the PRL/GH family, using heparin affinity columns followed by gel electrophoresis/Western blotting. Purified human PRL and its cleaved 16K fragment, but not human GH or placental lactogen, were retained on the heparin column and were displaced by 0.5M NaCl. Native PRL in human pituitary extracts and amniotic fluid showed a similar binding affinity to heparin as the purified hormone. None of the other hormones tested, e.g., rat, ovine and bovine PRL, glycosylated ovine PRL or rat GH, bound to heparin. Two consensus heparin-binding sequences are present in human PRL but not in the other hormones included in this study. We postulate that the heparin-binding capability of PRL affects its biological activity as a growth factor and the angiostatic actions of its 16K fragment.

PRL is a multifunctional protein hormone secreted by the pituitary lactotrophs. An indistinguishable species of PRL, synthesized by the human decidua, is present at very high concentrations in the amniotic fluid (1). In most species, PRL exists in several variant forms which are believed to fulfill different functions (2). PRL shares many characteristics with growth factors, including multiple sites of synthesis, wide distribution of receptors, homology of the receptors to those of cytokine/hematopoietic growth factor family, similarities in signal transduction pathways and mitogenic and morphogenic actions (for review, 3). Recently, several studies have established that a cleaved form of PRL, named 16K PRL, has a unique anti-angiogenic activity (4,5). One of the features that is common to most angiogenic/ angiostatic factors and some growth factors and cytokines is their ability to bind heparin/heparan sulfate chains of cell associated proteoglycans (6,7).

The classical pituitary polypeptide hormones are not known as heparin-binding proteins. However, we were intrigued by the role of PRL as a growth factor and the angiostatic activity of 16K PRL and wished to explore: a) whether purified PRL and its 16K fragment bind heparin, b) if members of the PRL/GH family from different species bind heparin, and c) the heparin-binding properties of pituitary and amniotic fluid PRL.

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Materials and Methods

Materials. All purified hormones and the anti-hPRL antiserum (IC-5) were a kind gift of the NIDDK. Hormone preparations include: human PRL (hPRL, AFP8982C), ovine PRL (oPRL-20), bovine PRL (bPRL-B1), rat PRL (rPRL-B7), human GH (hGH-B1), rat GH (rGH-1-6), human placental lactogen (hPL-1), and glycosylated oPRL (gly-oPRL-AFP-5200). Frozen human pituitaries were also obtained from the NIDDK. Amniotic fluid samples were collected by amniocentesis for genetic counseling from pregnant women at 18-25 weeks of gestation at the University of Cincinnati hospital according to a protocol approved by the institutional review board.

Sample preparation. Human pituitaries were homogenized in 10 mM phosphate buffer, pH 7.0 at 4C and centrifuged at 25,000xg for 15 min. Aliquots of the supernatant were diluted in the same buffer prior to use. Amniotic fluid samples, cleared by centrifugation, were similarly diluted. 16K PRL was generated from purified 23K hPRL

by thrombin (Sigma, St. Louis, MO) proteolysis as reported for hGH (8).

Heparin affinity chromatography. 1ml pre-packed heparin-Sepharose columns (Pharmacia Biotech, Piscataway, NJ) were equilibrated with 10 mM phosphate buffer, pH 7.0. Purified hormones (10 μg), amniotic fluid (200 μg protein) and human pituitary extracts (200 μg protein) were diluted to 2 ml of the same buffer and applied onto the columns. After collecting the flowthrough (unbound) fractions, the bound samples were displaced by a sequential application of 0.5M and 1.0M NaCl in the same buffer. Fractions were desalted and concentrated using centrifugal-filters with a 5K cut off (Millipore Corp, Bedford MA).

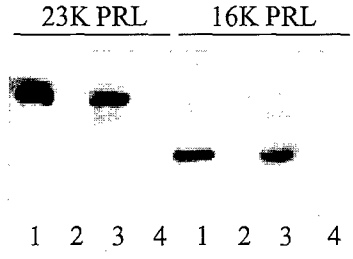

Fig. 1. Affinity chromatography of intact (23K) and cleaved (16K) human PRL on heparin-Sepharose column. Samples were applied to the column in phosphate buffer and were eluted stepwise with 0.5M and 1M NaCl. Aliquots were resolved by SDS-PAGE and silver-stained. For each run, lane 1: input; lane 2: flowthrough; lane 3: 0.5M NaCl fraction; lane 4: 1M NaCl fraction.

Gel electrophoresis and Western blotting. The desalted fractions were subjected to SDS-PAGE analysis (15% gel) under reducing conditions (5% 2-mercaptoethanol) followed by silver staining using a Sigma Silver Stain Kit. For the amniotic fluid and pituitary extracts, gel electrophoresis was followed by electrotransfer on nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and incubation with anti-hPRL antiserum (1:2000). The PRL bands were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Each experiment was repeated 2-3 times (five times in the case of hPRL and rPRL) and whenever possible, parallel runs were performed. Bound and unbound fractions of each sample were subjected to SDS-PAGE at equivalent sample loads.

Results

As revealed by gel electrophoresis, purified 23K human PRL and its 16K fragment have an affinity for heparin (Fig. 1). In both cases, the bound PRL was completely displaced from the heparin-Sepharose column with 0.5M NaCl without any detectable PRL in the flowthrough fractions.

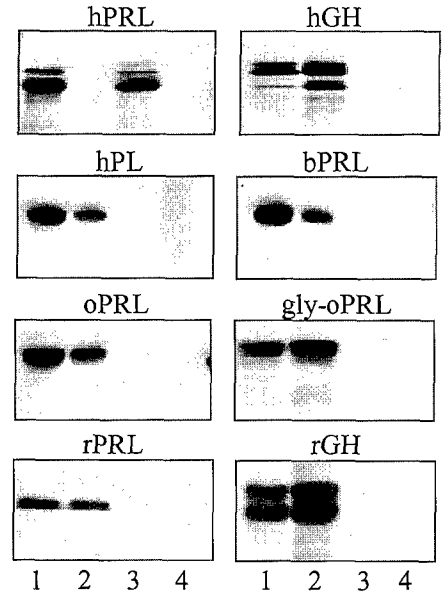

Fig.2.Comparison of the heparin-binding properties of various members of the PRL/GH family. See Fig. 1 for details.

To determine whether this feature is conserved across species, we tested rat, bovine and ovine PRL. Surprisingly, none bound to the heparin column (Fig. 2). The study was then extended to other members of the GH/PRL superfamily. Again, only human PRL, but not human GH or placental lactogen, was retained on the column. To check if glycosylation facilitates binding to heparin, glycosylated ovine PRL was tested. However, similar to the non-glycosylated species, it showed a complete lack of affinity for heparin (Fig. 2).

Our next objective was to compare the heparin-binding properties of purified and native human PRL. As shown by Western blotting (Fig. 3), PRL in human pituitary extracts as well as in amniotic fluid was retained on the heparin column and was displaced by 0.5M NaCl. However, in both cases, small amounts of the hormone were also detected in the unbound fractions. Heat denaturation of either purified or native human PRL did not affect its binding to heparin (Fig. 3, right panel).

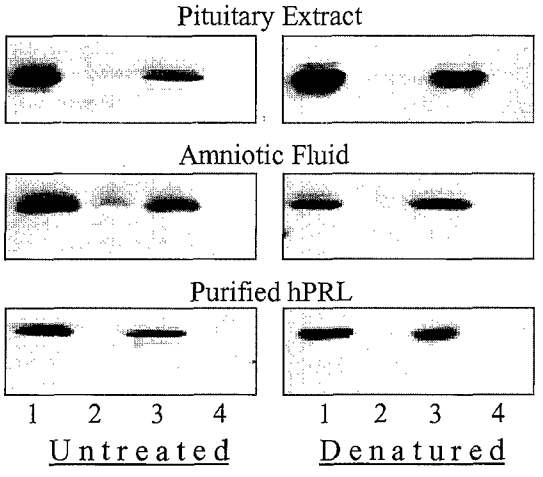

Fig. 3. Comparison of heparin binding of untreated and heat denatured (boiling for 5 min) human PRL in pituitary extracts (top), amniotic fluid (middle) and purified hormone preparation (bottom). PRL in pituitary extracts or amniotic fluid was detected by Western blotting. See Fig. 1 for other details.

Discussion

We are reporting that human PRL binds to heparin. To our knowledge, this is the first pituitary polypeptide hormone identified as a heparin-binding protein. This property, however, is not shared by PRL from other species or by other members of the PRL/GH superfamily, including human GH and placental lactogen. Whereas the significance of this finding remains to be elucidated, it brings a new dimension to the biology of PRL.

As shown in Fig.3, the heparin-binding property of purified hPRL is shared by both pituitary and amniotic fluid PRL, suggesting that it is not an artifact of purification. Further, binding of native PRL was similar to that of the purified hormone, as both eluted from the column with 0.5M NaCl. The presence of traces of PRL in the unbound fractions of pituitary homogenates and amniotic fluid suggests the presence of some non-heparin binding forms of PRL. This was not due to overloading of the column, which has a binding capacity of several mg proteins. Interestingly, a rat decidual protein, named dPRP, with less than 40% sequence homology to pituitary PRL, has been reported to have a high binding affinity to heparin (9).

Two putative 'consensus sequences' implicated in heparin binding are XBBXBX or XBBXXBX, where B is a basic amino acid (Arg, Lys and infrequently His) and X is any hydropathic amino acids (10). Such sequences are not always contiguous but can be brought into proximity by protein folding. We have identified two such sequences in human PRL, one between residues 41-47 (Asp-Lys-Arg-Tyr-Thr-His-Gly) and the other between residues 175-181 (Leu-Arg-Arg-Asp-Ser-His-Lys). Such sequences are absent in the primary structure of human GH or placental lactogen. Except for rat PRL which carries the second site, all hormones included in this study lack consensus heparin sequences. The presence of heparin-binding motifs in the primary structure of human PRL explains the retention of its binding properties upon heat denaturation, which unfolds the polypeptide chain.

Heparin-Sepharose chromatography is a widely accepted method for identifying proteins that bind to cell surface proteoglycans. Indeed, several growth factors (11) and angiogenic/angiostatic factors (12) were purified based on their ability to bind heparin. However, the inability of other PRL and GH species to bind heparin, observed in the present study, does not rule out the possibility of their binding to other proteoglycans. Binding of proteins to extracellular matrix proteoglycans can confer protection from inactivation, enhance binding to membrane receptors, or facilitate cleavage by proteolytic enzymes (13). Whether any of the above is pertinent to the biological activity of human PRL is presently unknown.

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