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The gene for the neuropeptide gonadotropin-releasing hormone is expressed in the mammary gland of lactating rats

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ABSTRACT **The high concentration of gonadotropin-releasing hormone (GnRH) in milk of several species implies that the mammary gland is either a site of synthesis for this neuropeptide or that it is efficiently concentrated from plasma by this organ. By PCR amplification of mammary gland cDNA, we have demonstrated expression of the mRNA for GnRH. The GnRH mRNA was present in the mammary gland of pregnant and lactating rats but not of virgin rats, implying that expression of the GnRH gene is activated during pregnancy, probably by prolactin. In contrast, actin mRNA was evident in all the preparations of mammary glands. Since GnRH is also known to be synthesized by the placenta, it is likely that the placenta and the mammary gland are complementary units by which the mother exercises control over the development and the metabolism of the infant during pregnancy as well as after parturition. In addition, GnRH synthesized by the mammary gland may also affect the mother by a paracrine and/or an endocrine mechanism.**

Since our first report on the presence of gonadotropin-releasing hormone (GnRH) and thyrotropin-releasing hormone (TRH) in milk of several species (1), the existence of numerous neuropeptides such as somatostatin (SS), growth hormone-releasing hormone, vasoactive intestinal peptide (VIP), bombesin, neurotensin, oxytocin, etc., has been reported in milk in concentrations that exceed those in maternal plasma (for reviews, see refs. 2–4). This phenomenon suggests that milk is not just a nutrient source for the mammalian neonate. The presence of significant concentrations of regulatory peptides in milk may play a major role in the developmental physiology of the neonate. The high concentration of the neuropeptides in milk implies either an active concentration mechanism in the mammary gland or that this gland is an additional site of synthesis for these peptides. However, previous attempts to demonstrate synthesis of SS, VIP, and TRH by the mammary gland of rats were not successful (5, 6).

GnRH plays a pivotal role in the regulation of reproduction. This peptide is synthesized and secreted from hypothalamic neurosecretory cells (7–10), and when it reaches the pituitary gland, it induces the synthesis and secretion of follicle-stimulating hormone and luteinizing hormone (LH), which regulate gonadal functions (11). GnRH is also synthesized by the human placenta (12), T lymphocytes (13), and the rat ovary (14). The GnRH precursor protein consists of an N-terminal signal peptide followed by the decapeptide GnRH and by a 56-amino-acid peptide termed GnRH-associated peptide (7, 12).

To determine whether the GnRH gene is transcribed in the mammary gland, we have used the reverse transcription–PCR reaction to amplify the levels of endogenous GnRH mRNA that may be present in this tissue.

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MATERIALS AND METHODS

Tissue Collection and RNA Extraction. Four-month-old Wistar-derived rats were used. They were housed in air-conditioned quarters, illuminated between 05:00 and 19:00 h. Pelleted food and water were offered ad libitum. The rats were decapitated, and their mammary glands and hypothalami were immediately excised and kept in liquid nitrogen. The collected tissues were homogenized by a Polytron (Kinematica, Lucerne, Switzerland), and total RNA was extracted using the guanidinium isothiocyanate/CsCl method (15).

RT-PCR. After DNase treatment, 10 μg of the total RNA extracts was reverse transcribed, using 0.5 μg of $(dT)_{15}$ as primer together with 200 units of Moloney murine leukemia virus reverse transcriptase, according to the manufacturer's specifications (United States Biochemical). Four percent of the cDNA produced was amplified by PCR (Perkin–Elmer/Cetus) for 30 cycles using the specific GnRH oligonucleotide primers (Fig. 1*A*) 5′-GGCAGAACCCCAGAACTTC-3′ and 5′-GTAATTGTGTGGGCTTCCGC-3′ corresponding to nucleotides 3587–3605 (e3, sense) and 5125–5144 (e4, antisense), respectively (16, 17). The calculated DNA span (exon–intron) was 1558 bp while the cDNA span was 205 bp.

PCR amplification was performed under the following reaction conditions (final concentrations): 200 μM each dNTP, 100 pmol each primer, 2.5 mM $MgCl_2$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris·HCl (pH 8.8), 0.1% Triton X-100 and 2 units of Vent polymerase (New England Biolabs), in a total reaction volume of 50 μl. Each cycle entailed denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and primer extension at 72°C for 60 sec.

The PCR products were separated on a 1% agarose gel and identified by hybridization with a ^{32}P-labeled GnRH oligonucleotide probe, specific to the coding region of exon 4 (e4a: 5012–5036, antisense). Final characterization of the specific PCR bands was achieved by automated direct DNA sequencing according to manufacturer's recommendations (Applied Biosystems; model 373A).

Actin mRNA was also assessed in the total RNA preparations of mammary glands as a control. Two percent of the cDNA produced was amplified by PCR for 30 cycles using specific actin oligonucleotide primers: 5′-GAGACCTTCAACACCCCAGCC (sense) and 5′-GGCCATCTCTTGCTCGAAGTC (antisense) (18). The annealing temperature was set at 60°C, and the Mg^{2+} concentration was 1 mM.

RESULTS

The PCR products derived from hypothalami and mammary glands of lactating and virgin rats were hybridized with a ^{32}P synthetic oligonucleotide probe (Fig. 1*A*) specific to the coding region of exon 4 (e4a). A major 205-bp band, the predicted size of the mature spliced GnRH mRNA, was

Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; SS, somatostatin; TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal peptide.

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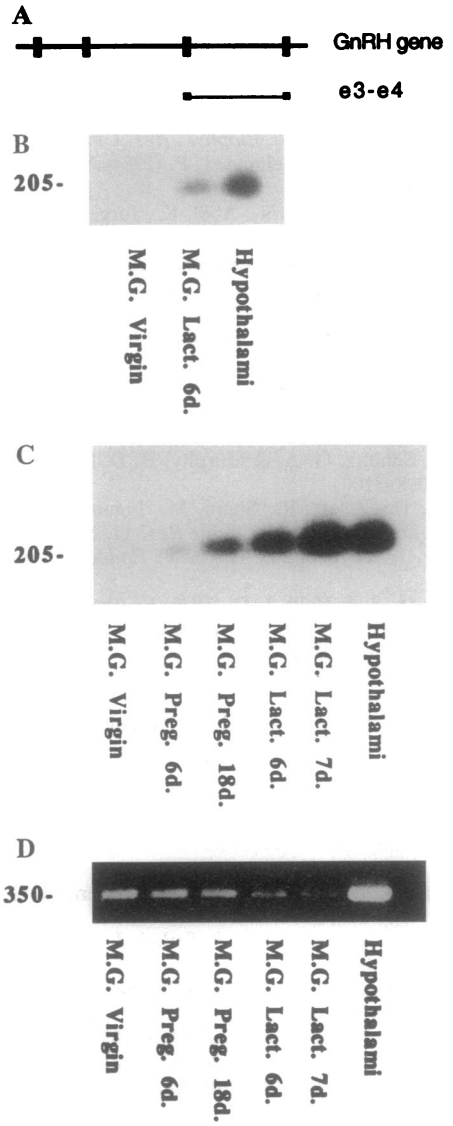

FIG. 1. Amplified GnRH cDNA fragment from mammary glands and hypothalami of rats. (*A*) Diagram of the amplified GnRH cDNA with oligonucleotide primers specific to exon 3 and exon 4. The predicted size of the mature spliced GnRH mRNA is 205 bp (of which only 112 bases are translated), whereas GnRH genomic amplification results in 1558 bp. (*B*) Amplified GnRH cDNA fragments from mammary glands (M.G.) of virgin and lactating (Lact., 6th day of lactation) rats and from rat hypothalami were hybridized to a rat GnRH ^{32}P-labeled oligonucleotide probe. pGEM DNA digested with *Hin*fI, *Rsa* I, and *Sin* I were used as size markers. (*C*) Amplified GnRH cDNA fragments from mammary glands (M.G.) of virgin, 6th day of pregnancy (Preg. 6d.), 18th day of pregnancy (Preg. 18d.), 6th day of lactation (Lact. 6d.), 7th day of lactation (Lact. 7d.) rats and from rat hypothalami were hybridized to a rat GnRH ^{32}P-labeled oligonucleotide probe. pGEM DNA digested with *Hin*fI, *Rsa* I, and *Sin* I was used as size markers. (*D*) Parallel amplification of actin cDNA, derived from the same experimental groups as of *C*, after 1% agarose gel electrophoresis and ethidium bromide staining.

detected (Fig. 1*B*) in preparations from hypothalami and from mammary glands of lactating, but not virgin, rats. To sequence the transcript, further amplification with primers e3 and e4a was performed. The amplified product thus obtained was eluted from the agarose gel, and both strands were directly sequenced. This sequence (Fig. 2) corresponded to bases 165–264 of the hypothalamic cDNA sequence of GnRH reported by Adelman *et al.* (7).

As can be observed in Fig. 1*B*, expression of the GnRH gene occurs in the mammary gland of lactating, but not virgin,

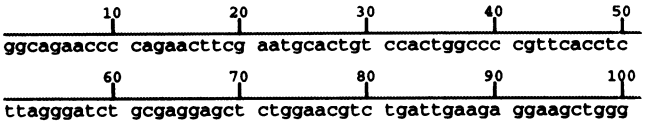

FIG. 2. The nucleotide sequence of the amplified lactating mammary gland GnRH cDNA obtained with e3 and e4a primers (see *Materials and Methods*). The 100-bp product is identical to nucleotides 165–264 of hypothalamic GnRH cDNA (7).

rats. This phenomenon raises the question as to the mechanism that activates GnRH gene expression and mRNA splicing. To determine whether GnRH gene expression is regulated by suckling itself or by placental or pituitary hormone(s), we have processed mammary glands from rats during pregnancy and lactation. As shown in Fig. 1*C*, expression of the GnRH gene was evident even on the 6th day of pregnancy but not in the preparation derived from virgin rats. In contrast, actin mRNA was evident in all mammary gland preparations (Fig. 1*D*).

DISCUSSION

In this study we have demonstrated expression of mRNA for GnRH in mammary glands of pregnant and lactating, but not virgin, rats. The mammary gland is a target for several steroid and peptide hormones, such as glucocorticoids, insulin, and prolactin, that interact during pregnancy to induce mammary gland growth and differentiation, which are essential in its preparation for lactation. The observation that initiation of GnRH gene expression in the mammary gland was evident already on day 6 of pregnancy (first day after implantation) suggests that the surges of plasma prolactin that occur during the afternoon and night of each day throughout the first half of pregnancy (19–21) may be responsible for this activation. Indeed, prolactin had been demonstrated to regulate gene expression of casein in the mammary gland (22), of prolactin-inducible protein in human breast cancer cells (23), and of some other proteins in several tissues (24). After cessation of pituitary prolactin surges at midpregnancy, the GnRH gene in the mammary gland may be regulated by a prolactin-like hormone, the rat placental lactogen (25). During the lactation period, GnRH gene expression in the mammary gland is probably regulated by the massive discharges of pituitary prolactin that are brought about by suckling-induced neuronal stimulation (26, 27). The inability of previous studies to demonstrate the presence of mRNA for SS, VIP, and TRH (5, 6) may be due to the use of detection methodologies of low sensitivity in a large and defused tissue like the mammary gland. The ability to amplify the mRNA transcripts by the PCR technique makes it possible to study the expression of genes for GnRH and other peptides in the mammary gland.

GnRH that is synthesized by the lactating mammary gland could have multiple physiological roles and may assume biological activities that are still unknown. Thus, for instance, SS functions as a neurohormone in the pituitary gland, as a neurotransmitter in the cerebral cortex, and as a paracrine agent in the pancreas (28). Similarly, the neuropeptide VIP has recently been demonstrated to act as a growth factor in embryos (29). GnRH that is synthesized by the mammary gland may exert its bioactivity on the suckling pups as well as on the mother. When suckling was prevented, serum LH concentrations dropped to about 30%, and when suckling was allowed to resume, the pup's serum LH concentrations were restored within 1 h (1). These observations suggest that at least part of the milk GnRH is absorbed from the gastrointestinal tract of the suckling pup in a biologically active form. Indeed, the gastrointestinal tract of the neonatal mammals is largely permeable and allows the transport of peptides and proteins across the intestinal epithelium (2–5,

30). Similar results were also obtained in another study that demonstrated that milk GnRH may have a modulatory role on the development of the infantile rat ovary. The available ovarian GnRH receptors in rat pups were increased after a period of fasting and returned to basal levels after resumption of suckling (31). Another study indicated that GnRH may be involved in the process of sexual differentiation. Male rats that were treated, during the first week of life, with an antiserum to GnRH exhibited bisexual behavior at maturity (32).

In the mother, GnRH synthesized by the mammary gland may function as a paracrine agent within the mammary gland or on the anterior pituitary of the mother. Preliminary results indicate that the total amount of GnRH that can be synthesized by the mammary gland of the lactating rat is far larger than that synthesized by the hypothalamus. Indeed, the concentration of GnRH in different preparations of milk that we have analyzed are in the nanomolar range (1, 33).

Besides being expressed in the mammary gland, GnRH transcripts are also present in the placenta (12). Although the exact function(s) of this neuropeptide during embryogenesis and following delivery is still unknown, its existence in the placenta and in the mammary gland during these critical phases of life suggest that it is biologically significant. It seems that the placenta and the mammary gland serve as complementary organs in a mechanism by which the mother exercises control over the infant's development and metabolism. Milk provides the mechanism by which regulatory information is transferred from the mother to the progeny.

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