The effects of growth hormone on the expression of sex-dependent testosterone 16α- and 15α-hydroxylases were studied in growth hormone-deficient Little (lit/lit) mice at the activity as well as at the mRNA levels. The male isozyme of testosterone 16α-hydroxylase ("C"-P-450,α) was repressed in the liver of male lit/lit mice, and the injection of bovine growth hormone resulted in an increase of the isozyme at both activity and mRNA levels to those seen in control lit/+ male mice. On the other hand, the female isozymes of testosterone 16α- ("T"-P-450,α) and 15α-hydroxylase (P-450,β) were increased in livers of both male and female lit/lit mice. The increased I-P-450,α, and P-450,β, in lit/lit mice were suppressed by growth hormone but only when it was injected once every 12 h. Thus, the results indicate that growth hormone acts as a masculinizing factor for testosterone hydroxylase activity by activating and inhibiting the expression of male and female isozymes of testosterone hydroxylases in mice, respectively. When growth hormone was infused to simulate a continuous secretion pattern, it showed no significant effect on the expression of hydroxylases in lit/lit mice, suggesting that growth hormone may not be a feminizing factor for testosterone hydroxylase activity in female mice. The changes of specific hydroxylase activities modulated by growth hormone in the mice correlated well with those amounts of hydroxylase mRNAs. The action of exogenous growth hormone to regulate the hydroxylases was so slow that it took 2 days to show a significant effect.

Sexual dimorphism of steroid hydroxylase activities in liver microsomes is a direct reflection of differentiated expression of cytochrome P-450s specific for the activities. Cytochrome P-450 represents a group of terminal oxidases of the membrane-bound monoxygenase system which consists of NADPH-cytochrome P-450 reductase, cytochrome b₅, and NADPH-cytochrome b₅ reductase. In inbred mice there are at least two known isozymes ("T"- and "C"-P-450,α) of testosterone 16α-hydroxylase whose expressions are sexually differentiated; I-P-450,α, is a female-specific isozyme whereas C-P-450,α, is a male-predominant isozyme (1-3). The presence of female-predominant testosterone 15α-hydroxylase activity in mice was also known, and the form of cytochrome P-450 (P-450,α) specific for the 15α-hydroxylase activity has been purified and characterized from female mice (4-6). Furthermore, we have recently demonstrated by using the hydroxylase cDNAs as hybridization probes that the sex-dependent expression of these hydroxylases is regulated pretranslationally (6, 7).

As with many liver proteins such as MUP, α₂-globulin, prolactin receptors, and drug oxidases, the sexual differentiation of steroid hydroxylase activity is under multihormonal control. In addition to sex hormones, increasing attention has been given to the role of growth hormone in regulation of sexual dimorphism of steroid hydroxylase activities in liver microsomes and other liver functions (8-10). Norstedt and Palmiter (11) reported that a pulsatile secretion of growth hormone masculinizes expression of MUP and prolactin receptor, while a continuous secretion of growth hormone feminizes their expression in mice. Here we report the use of specific inhibitory antibodies and cDNAs for the mouse hydroxylase isoforms (I- and C-P-450,α) and P-450,β) to investigate the regulatory role of growth hormone in the sex-dependent expression of these hydroxylases.

Growth hormone-deficient Little mice were used for the present study. Unlike other growth-deficient mouse strains such as Snell and Ames or hypophysectomized animals, Little mice have a defect only in growth hormone, with normal levels of other pituitary hormones such as thyroid-stimulating hormone, prolactin, luteinizing hormone, and follicle-stimulating hormone (12), providing the most suitable animal model to investigate the regulation of growth hormone (11, 13). The role of growth hormone as a masculinizing but not feminizing factor toward expression of I- and C-P-450,α, and P-450,β, in mice is discussed in this paper.

EXPERIMENTAL PROCEDURES

Animals—Two-month-old 129/J, C57BL/6J, and Little mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Treatments with Hormones—For injection, bovine growth hormone (bGH) was dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 0.9% NaCl at a concentration of 250 pg/ml. Two hundred μl/mouse of the bGH solution were administered intraperitoneally once every 12 h for 5 consecutive days. The same volume of Tris buffer was injected into control mice. For continuous infusion, a mini pump (model 2001) filled with 250 μl of bGH solution (1.5 mg/ml) was subcutaneously implanted in the back of a mouse for 7 days. The pump is designed to continuously release 1 μl of solution every hour. A pump filled with Tris buffer was implanted in each control mouse. A lyophilized somatomedin C preparation was reconstituted in 10 mM HCl at a concentration of 200 μg/ml and was diluted with 4 volumes of the Tris buffer just before injection into animals. Mice were treated by injection of somatomedin C (6 μg/mouse) once every 12 h for 4 days. The vehicle was injected as the control. The control animals received the same volumes of the buffers, and the untreated animals were not treated by any means in the present experiments.

Materials—[14C]Testosterone (60 Ci/mmol) was purchased from New England Nuclear. [3H]dCTP (3000 Ci/mmol) and nick-trans-

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Activities in Microsomes—"Little" is a mutant mouse strain probes under the conditions reported previously (3). The hybridization counting. The spots were duplicated for every poly(A+) RNA were measured by methods previously reported (1, cytochrome P-450 content and protein concentration were de-\n\nted by the methods described by Omura and Sat0 (18) and Brad-\n\nford (19), respectively.

Palmiter (11). The paper was hybridized with the radioactive cDNA lulas-The two isozymes (C- and I-P-45016,) of testosterone 16a- and 15a-hydroxylase (P-45015,) were purified from 129/J mice as \ndescribed previously (1-5).

Northern and Dot Hybridization—Poly(A*) RNA was electropho-\n\nsed on 1% agarose gel containing 5 mM potassium phosphate buffer, pH 7.25, containing 20% sucrose, 1 mM EDTA, and 2 \n\mu g/ml leupeptin. Total liver RNA was extracted by the guanidine HCl method (14) and subjected to oligo(dT)-cellulose column chromatography to enrich poly(A*) RNA (15).

Liver microsomes were prepared as described previously (1-4) and homogenized in 50 mM potassium phosphate buffer, pH 7.25, containing 20% sucrose, 1 mM EDTA, and 2 \n\mu g/ml leupeptin. Total liver RNA was extracted by the guanidine HCl method (14) and subjected to oligo(dT)-cellulose column chromatography to enrich poly(A*) RNA. Liver microsomes were prepared from each of the separate parts. Liver microsomes were as described previously (1-4) and homogenized in 50 mM potassium phosphate buffer, pH 7.25, containing 20% sucrose, 1 mM EDTA, and 2 \n\mu g/ml leupeptin. Total liver RNA was extracted by the guanidine HCl method (14) and subjected to oligo(dT)-cellulose column chromatography to enrich poly(A*) RNA (15).

RESULTS

Effect of bGH Injection on Specific Testosterone Hydroxylase Activities in Microsomes—"Little" is a mutant mouse strain raised naturally from C57BL/6J which has an autosomal recessive inheritance of growth defect (20). This growth defect is apparently due to a poor transcriptional rate of the growth hormone gene which is regulated by a locus on chromosome 6. The serum growth hormone levels were reported to be 8.5 and 0.6 \n\nng/ml in males of hetero- (lit/+ ) and homozygote (lit/\n\n), respectively, and these values in lit/+ and lit/lit females were 2.85 and 0.05 ng/ml, respectively (21).

Liver microsomes were prepared from three separate control and bGH-injected Little mice. Liver microsomes were prepared from three control and growth hormone-injected lit/+ and lit/lit males and females (see "Experimental Procedures"). I-P-45016,- and C-P-45016,-dependent testosterone 16a-hydroxylase activities and P-45015,-dependent testosterone 15a-hydroxylase activity were measured by using specific inhibitory antibodies as described in previous reports (1, 2). One-dimensional thin-layer chromatography was used to separate radioactive testosterone metabolites formed by microsomes. Panel A shows I-P-45016,-dependent activity; Panel B, C-P-45016,-dependent activity; Panel C, P-45015,-dependent activity. The values of hydroxylase activities in this figure were averaged from three separate experiments. The variation of hydroxylase assay was less than 5%.

Fig. 1 shows the activities catalyzed by each isozyme of testosterone hydroxylase in liver microsomes from lit/+ , lit/\n\nlit mice. Testosterone 16a-hydroxylase activity catalyzed by I-P-45016,- (panel A) was at least 10-fold higher in female than in male lit/+ mice. The specific activity in the lit/+ males was higher than that seen in lit/+ females. I-P-45016,-dependent activity in liver microsomes from lit/lit females was also elevated about 2.5-fold over that in the lit/+ females (Fig. 1A). Consequently, the sexual di-
Northern hybridization with cDNA probes (R17, p16a-1, and p15a-29) of total liver poly(A) RNA from various mice. Two µg of poly(A) RNA samples isolated from three mice in each experimental group (see "Experimental Procedures") were electrophoresed on 1% agarose gels containing 5 mM methyl mercury hydroxide and transferred to DBM paper. The electrophoresis was run from top to bottom. The DBM paper was hybridized with nick-translated 32P-cDNA probes (1 × 10^6 cpm of total radioactivity; R17, p16a-1 or p15a-29), and then the paper was properly washed and exposed to x-ray films. The numbers on the right-hand side of the figure indicate the sizes of P-450 mRNAs. Panel A, hybridization of I-P-450 with R17; Panel B, hybridization of C-P-450 with p16a-1; Panel C, hybridization of P-450 with p15a-29.

The increased expression of I-P-450 and C-P-450 mRNA that hybridized with the probes was seen in male and female lit/lit mice, and this was consistent with the increases of the hydroxylase activities catalyzed by these isozymes. The amounts of C-P-450 mRNA were higher in males than in females in these mice (Fig. 2B), which also agreed with the C-P-450-dependent activities in the mice.

The expression of I-P-450 and C-P-450 mRNA by growth hormone were consistent with those of C-P-450, and P-450, in the mice, which is consistent with the female-specific expression of hydroxylase activities catalyzed by these isozymes. The amounts of C-P-450 mRNA were higher in males than in females in these mice (Fig. 2B), which also agreed with the C-P-450-dependent activities in the mice.

Liver microsomes and total liver poly(A) RNA were prepared from bGH-infused and control lit/lit mice. Two mice were used for each group. Testosterone 16α-hydroxylase activity in microsomes was measured in the presence and absence of anti-I-P-450, or anti-C-P-450, antibodies. The isozyme-specific activity was calculated by subtracting the activity with the antibody from the activity without the antibody. The assay conditions were described under "Experimental Procedures." The mRNA contents for the hydroxylases are expressed by the average radioactivity recovered from the two separate dot hybridizations of poly(A) RNA with the appropriate cDNA probe: R17 for I-P-450, and p16α-1 for C-P-450, (see "Experimental Procedures").

**TABLE I**

| Control | bGH infusion |
|---------|--------------|
| Activity | mRNA | Activity | mRNA |
| P-450<sub>lit/lit</sub> | P-450<sub>lit/lit</sub> |
| Male | 0.16 | 1960 | 0.17 | 1390 |
| Female | 0.12 | 1334 | 0.18 | 1685 |
| Male | <0.01 | 53 | 0.07 | 296 |
| C-P-450<sub>lit/lit</sub> | C-P-450<sub>lit/lit</sub> |
| Male | 0.16 | 1505 | 0.12 | 1394 |
| Female | 0.10 | 1375 | 0.07 | 1228 |
| Male | 0.45 | 6300 | 0.038 | 713 |

(data not shown). Pretranslational regulation is most likely a mechanism of the growth hormone-dependent modulation of these hydroxylases.

**FIG. 3. Linear correlation between I-P-450<sub>lit/lit</sub> activity and mRNA amounts in Little mice.** The amounts of specific mRNA hybridized with the R17 cDNA probe were measured by dot hybridization. One µg of poly(A) RNA from the mice was dotted and hybridized with 32P-labeled R17 cDNA. The radioactivity recovered from the three hybridizations for one sample was averaged to obtain the amounts of I-P-450<sub>lit/lit</sub> mRNA in these mice. I-P-450<sub>lit/lit</sub>-dependent testosterone 16α-hydroxylase activity was calculated from the activity inhibited by the I-P-450<sub>lit/lit</sub> antibody. The values used here were obtained from control C57BL/6J, lit/+ and lit/lit and bGH-injected lit/lit female and male mice, which were used in Figs. 1 and 2.
mone happened to feminize the hydroxylase activities in male mice.

During infusion experiments, it was noticed that the implantation of a minipump into Little mice caused stress to the animals and that stress itself was affecting hydroxylase activity. In particular, I-P-450_16α-dependent activity was drastically decreased from that in untreated lit/lit mice (compared with the activity in Fig. 1).

**Time-dependent Decreases of I-P-450_16α, mRNA and Activity by bGH in Lit/lit Males**—The time-dependent repression of I-P-450_16α by bGH in lit/lit males is shown in Fig. 4. A significant decrease of I-P-450_16α was seen 2 days after the first injection, and the maximum bGH effect was obtained 3 days later. This slow effect of bGH is in sharp contrast to the rapid induction of cytochrome P-450 mRNAs by phenobarbital and polycyclic aromatic hydrocarbons. Maximum induction by these inducers was achieved within 20 h after administration (22, 23). Due to the slow effect of growth hormone on the expression of hydroxylases and since somatomedin C has been reported to mediate growth hormone activity in genetically small mice such as Little (24), we tested to see if somatomedin C could mimic the activity of bGH. No effect on the hydroxylases (C-P-450_16α, I-P-450_16α, and P-450_15α) was observed after injection of somatomedin C once every 12 h for 5 consecutive days into lit/lit males (data not shown).

**DISCUSSION**

Testosterone 16α-hydroxylase activity in mouse liver microsomes is the sum of two sexually modulated isozymes, the female-specific I-P-450_16α and the male-predominant C-P-450_16α. Testosterone 15α-hydroxylase activity in liver microsomes from female mice is due to the presence of the female-predominant isozyme called P-450_15α. We have demonstrated in this report that when growth hormone is injected once every 12 h for 5 consecutive days to simulate a pulsatile pattern of circulating hormone, it inhibits the expression of I-P-450_16α and P-450_15α and the expression of C-P-450_16α in mice. On the other hand, growth hormone infusion to mimic a continuous secretion pattern has no effect on the modulation of sexually regulated testosterone hydroxylases in mice. Thus, it can be concluded that growth hormone acts as a masculinizing factor by inhibiting expression of the female isozymes and by activating expression of the male isozyme presumably at the pretranslational level(s) of the hydroxylase in male mice, whereas growth hormone alone may not be a feminizing factor for expression of the hydroxylases in female mice.

According to the report by Norstedt and Palmiter (11), male-predominant expression of prolactin receptor is repressed in lit/lit female and male mice to a level below that in the lit/+ female. The infusion, but not injection, of bovine growth hormone increased the receptor level in lit/lit males to a concentration higher than that seen in lit/+ females. However, an infusion experiment with lit/lit female mice was not described in their report. Nonetheless, the authors concluded that growth hormone, when it is secreted continuously, is a feminizing factor for expression of the receptor in female mice. A similar conclusion has been reached by Gustafsson and co-workers (25) to explain female-specific expression of 5α-androstane-3α,17β-diol-3,7-disulfate 15β-hydroxylase activity in rat liver microsomes. In their experiments, hypophysectomized rats were used to elucidate the role of growth hormone in feminization of this hydroxylase activity (25). A shortcoming of hypophysectomized animals is that they lose not only growth hormone but a source of all pituitary hormones as well. Therefore, growth hormone may not be the only supplement necessary to reflect what is happening in normal female animals. Our results which show that the levels of female-predominant hydroxylases (I-P-450_16α and P-450_15α) are higher in lit/lit females than those in lit/+ females suggested that growth hormone has no positive role in the sex-dependent expression of I-P-450_16α and P-450_15α in female mice. This conclusion does not agree with that drawn by other researchers (8–11). The reason for this difference is not certain at the present time. One possibility, however, is that there are growth hormone-dependent and -independent mechanisms regulating sexual dimorphism of liver proteins in female mice.

The male-predominant expression of C-P-450_16α in males is regulated by the pulsatile, but not continuous, secretion of growth hormone in mice. This observation is consistent with the previous findings of Norstedt and Palmiter (11) concerning male-predominant MUP expression and of Gustafsson and co-workers (8) concerning the expression of male-predominant steroid 16α-hydroxylase in rats. The feminization of hepatic steroid and drug hydroxylase activities in liver microsomes by a continuous infusion of growth hormone in male rats was originally reported by Kremer and Colby (26) and Mode et al. (27) and recently by Jellinick et al. (28). It is now evident that the repression of male-predominant hydroxylase activities such as testosterone 16α-hydroxylase and catechol estrogen formation in growth hormone-infused male rats does not provide direct evidence of a continuous secretion of growth hormone as a feminizing factor, but rather due to the disruption of the pulsatile secretion of growth hormone by the infusion which is a positive regulatory factor for expression of the hydroxylase activity in male animals.

As expression and repression of sex-dependent testosterone hydroxylases (I- and C-P-450_16α, and P-450_15α) are regulated pretranslationally by growth hormone, it is postulated that the hormone modulates sex-dependent transcription of the hydroxylase genes. A transcriptional regulation of α2-globulin gene by multihormones, including growth hormone, was directly demonstrated by measuring nuclear runoff RNA in vitro (29). The processes by which the pulsatile pattern of growth hormone concentrations in serum transmits its information to hydroxylase genes through the cell membrane and how the genes receive this information are still undescribed.
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