

# Insulin-Like Growth Factor-I Effects on Gonadotropin-Releasing Hormone Biosynthesis in GT1-7 Cells\*

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## ABSTRACT

The immortalized GT1-7 cell line synthesizes and secretes GnRH, the key hormone of reproduction. However, GT1-7 cells lack the normal inputs from neurotransmitters, growth factors, and steroids, which are involved in the maturation and maintenance of GnRH neurons in the brain. We examined the effects of the neurotrophic factor insulin-like growth factor-I (IGF-I) on GnRH gene expression and the mechanism for these changes. Initially, effects of IGF-I on GnRH gene expression were determined by ribonuclease protection assay. In time-course experiments, IGF-I treatment caused significant increases in nuclear GnRH primary transcript levels, an index of GnRH gene transcription, 4 and 8 h after initiation of IGF-I treatment. GnRH messenger RNA (mRNA) levels in the cytoplasm were stimulated by IGF-I at 24 h of treatment. IGF-I also affected GT1-7 cell morphology, with an increase in process extension and cell-cell contacts. In contrast, GnRH peptide levels in the medium were ini-

tially stimulated and then suppressed by IGF-I, indicating an uncoupling of biosynthesis and secretion. The increase in GnRH mRNA levels induced by IGF-I is probably caused by a transcriptional mechanism, as evidenced by the increase in GnRH primary transcript levels before a change in GnRH mRNA levels, as well as our finding of a similar GnRH mRNA half-life for both control and IGF-I-treated cells. Interestingly, GT1-7 cells themselves were observed to express IGF-I immunoreactivity, suggesting the possibility of autoregulation by this neurotrophic factor. It is concluded that IGF-I is an important modulator of GnRH gene expression and release in the GT1-7 cell line. The reported stimulatory effects of IGF-I *in vivo*, and its hypothesized role in the development of GnRH neurons in the brain, suggest that IGF-I may make the GT1-7 cells line more like a mature GnRH neuron, as a model for future studies. (*Endocrinology* **139**: 1125-1132, 1998)

GnRH IS THE key factor in the control of reproduction. This decapeptide stimulates the release of gonadotropins from the pituitary gland, which then travel through the general circulation, causing the synthesis and release of steroid hormones from the gonads. Because of the GnRH neurons' scattered localization in the hypothalamus-preoptic area, cellular/molecular studies of the regulation of the GnRH neurosecretory system *in vivo* are limited. Thus, the immortalized GT1 neuronal cell lines (NCLs) have allowed investigators to elucidate the factors directly involved in the regulation of GnRH gene expression (1). These GT1 cells have many similarities to GnRH neurons, such as the synthesis, processing, and pulsatile secretion of GnRH (2-4). However, some major differences exist between the GnRH-producing cell lines and neurons in an adult animal, such as the postmitotic state of the mature GnRH neuron *in vivo*, compared with the immortalized state of the GT1 cells; a pattern of GnRH gene expression in the cell line, resembling that of neonatal but very different from that of the adult mouse (5, 6); and different responses to pharmacological agents (4). Thus, GT1 cells may be more similar to immature, neonatal GnRH neurons than to adult GnRH neurons in the brain. The absence of exposure of the GT1 cells to the normal

neuronal, glial, and steroid hormone inputs that are involved in the maturation of GnRH neurons *in vivo* may be responsible for the immature phenotype in this cell line. Thus, a more representative cell model of the mature GnRH neuron is critical for an understanding of the GnRH neuron in the adult animal.

The maturation of the neuroendocrine hypothalamus is profoundly affected by neurotrophic factors, whose synthesis and release increase dramatically during the first 2 postnatal weeks of life in rodents (7-9). This period corresponds exactly with that of the most profound alterations in GnRH gene expression in rodents, between postnatal days (P) 5 and 10 (4, 5, 10), suggesting that the maturation of GnRH neurons may be a result of an increase in neurotrophic factor input. A likely candidate for this maturation is insulin-like growth factor-I (IGF-I), which is synthesized in peripheral tissues (11) and in the brain (12, 13), and whose receptors are expressed in neurons (14) and are abundant in the median eminence (15, 16), the site of GnRH neuroterminals. Furthermore, IGF-I is strongly implicated in reproductive function, as it causes a dose-dependent increase in GnRH secretion from median eminence explants (17), and plasma IGF-I levels increase during puberty (18, 19). The temporal pattern of binding of IGF-I to its receptors in the hypothalamus, peaking between P5 and P7 (8), a period of alterations in GnRH gene expression *in vivo* (4, 5, 10), raises the possibility that this growth factor is one of the metabolic signals important or obligatory for the maturation of the neuroendocrine axis and, ultimately, the attainment of adult reproductive function.

It is hypothesized that IGF-I would cause alterations in

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GnRH biosynthesis in GT1-7 cells, such that they would become a more representative model for an adult GnRH neuron. IGF-I receptors, as demonstrated by receptor binding, are present in GT1 cells (20). Treatment with this specific growth factor produces changes in GnRH release and GT1 morphology (21, 22). Thus, in the present study, we tested the hypothesis that treatment with IGF-I would cause the maturation of the immortalized GT1-7 cell line, as evaluated by alterations in biosynthetic and morphological indices. These experiments are intended to establish a cell system that is both morphologically and physiologically more representative of the adult GnRH neuron, for future studies on the regulation of GnRH gene expression. We also report the novel observation that GT1-7 cells themselves are IGF-I-immunopositive, suggesting the intriguing possibility of autofeedback of IGF-I onto its own receptors on GnRH cells.

### Materials and Methods

#### GT1-7 cell culture and maintenance

GT1-7 cells were maintained in DMEM (Gibco BRL; Gaithersburg, MD) with 5% heat-inactivated FCS and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and maintained at 37°C with 5% CO<sub>2</sub> (23, 24). The cells were subcultured into six-well tissue culture dishes 2-3 days before experiments and grown to approximately 70% confluency. All experiments were conducted with triplicate monolayers of  $1 \times 10^6$  cells and were repeated two to four times. Initially, effects of length of serum

deprivation were tested, and the optimal conditions for cell survival and consistency of response to treatment were determined to be serum deprivation for 1 h, followed by treatment with IGF-I or vehicle, as described below. These conditions were used for all experiments. Cells were harvested in 400 µl lysis buffer (0.3 M sucrose; 0.25% sodium deoxycholic acid; 10 mM Tris, pH 7.4; 1.5 mM MgCl<sub>2</sub>; and 0.5% Nonidet P-40). This was layered over 350 µl cushion buffer (0.4 M sucrose; 10 mM Tris; 1.5 mM MgCl<sub>2</sub>) and centrifuged at  $800 \times g$ . Subsequent isolation of cytoplasmic and nuclear RNAs for ribonuclease (RNase) protection assay are described below.

#### Experimental design

*IGF-I effects on GnRH gene expression, release, and GT1-7 cell morphology.* Dose-response experiments were initially performed to determine the optimal dose of IGF-I on GnRH gene expression. GT1-7 cells were treated with 0.1, 10, and 100 ng/ml IGF-I (Sigma, St. Louis, MO) or vehicle (the same volume of ethanol in dH<sub>2</sub>O), after 1 h of serum deprivation. Cells were harvested after 24 h, and GnRH RNA levels were measured by RNase protection assay. Then, using the 10 ng/ml dose of IGF-I, which was determined to have the most potent effect on GnRH gene expression, time-course experiments were performed: cells were treated with IGF-I or vehicle for 0, 2, 4, 8, 24, or 48 h. GT1-7 cells were harvested and RNase protection assays performed separately on GnRH messenger RNA (mRNA) in the cytoplasm, and GnRH primary transcript, an index of GnRH gene transcription (6), in the nucleus. A 1-ml aliquot of medium was collected at the time of harvest for RIA of GnRH peptide. Differences between groups were analyzed by two-way ANOVA, followed by Fisher's protected least-significant-difference *post hoc* test. Significance was set at  $P < 0.05$ .

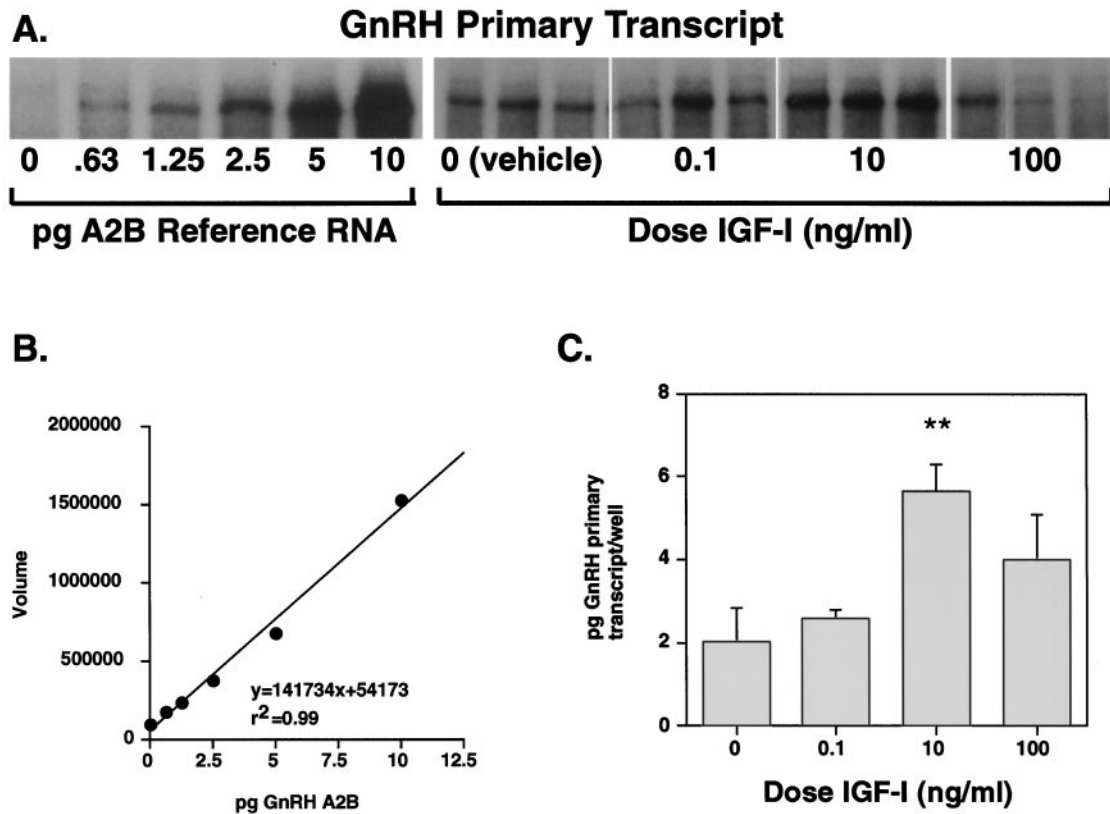


FIG. 1. Dose-response analysis of IGF-I effects on GnRH primary transcript levels. A, Representative autoradiogram of GnRH nuclear primary transcript in a standard curve (left) and in individual GT1-7 cell cultures (right), as analyzed by RNase protection assay. For GT1-7 cells, the nuclear fraction extracted from one well of a six-well tissue culture plate was loaded in each lane. B, Regression analysis of the number of picograms of standard vs. the amount of radioactivity in each lane (as expressed in Phosphor imager volume) was performed, and the corresponding analysis is presented. C, Dose-response analysis indicated that IGF-I (10 ng/ml) caused a significant increase in GnRH primary transcript levels. \*\*,  $P < 0.01$  vs. vehicle.

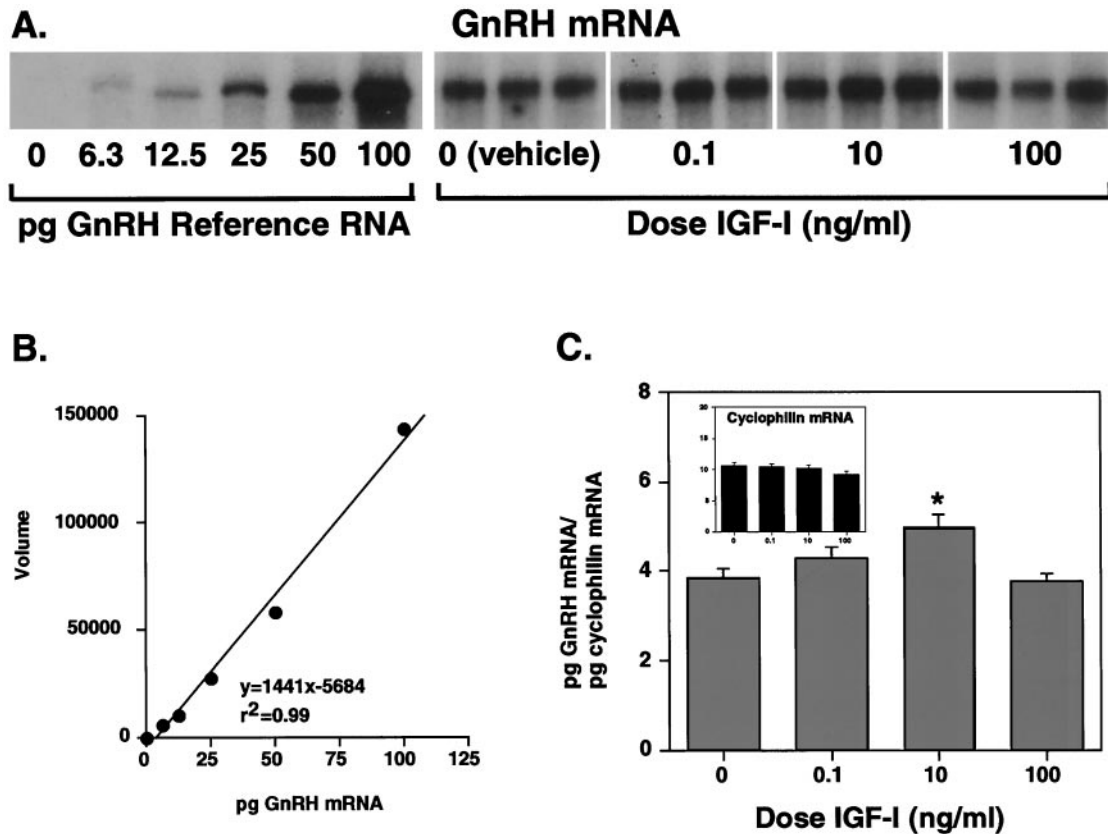


FIG. 2. Dose-response analysis of IGF-I effects on GnRH mRNA levels. A, Representative autoradiogram of GnRH cytoplasmic mRNA in a standard curve (left) and in individual GT1-7 cell cultures (right), as analyzed by RNase protection assay. For GT1-7 cells, 0.5  $\mu$ g of total RNA was loaded in each lane. B, Regression analysis of the number of picograms of standard vs. the amount of radioactivity in each lane (as expressed in Phosphor imager volume) was performed, and the corresponding analysis is presented. C, Dose-response analysis indicated that IGF-I (10 ng/ml) caused a significant increase in GnRH mRNA levels. Cyclophilin mRNA levels (inset) were unaffected by IGF-I. \*,  $P < 0.05$  vs. vehicle and 100 ng/ml.

**IGF-I effects on GnRH mRNA half-life.** To determine whether IGF-I affects GnRH mRNA half-life, indicating a posttranscriptional mechanism of action of IGF-I on GnRH mRNA levels, GT1-7 cells were serum-deprived for 1 h. Then, at  $t = -2.5$  h, cells were treated with IGF-I or vehicle. At  $t = 0$  h, cells were treated with the RNA polymerase II inhibitor 5,6-dichloro-1- $\beta$ -ribofuranosylbenzimidazole (DRB; 100  $\mu$ g/ml final concentration, as described previously) (24). Cells were harvested at  $t = 0, 2, 4, 8, 16,$  or 24 h after DRB treatment. The GnRH mRNA half-life was determined by performing a regression analysis on the changes in GnRH mRNA levels for both control and IGF-I-treated cells.

**Expression of IGF-I-immunoreactivity in GT1-7 cells.** To determine whether IGF-I-like immunoreactivity is present in GT1-7 cells, immunocytochemistry for IGF-I was performed in GT1-7 cells using the rabbit polyclonal IHC-7296 antibody (Peninsula Laboratories, Belmont, CA). Cells were subcultured on sterile 22-mm tissue culture cover slips (Nunc, Naperville, IL) in six-well tissue culture plates for 2-3 days. Then, cells were fixed in 4% paraformaldehyde, 0.12 M sucrose for 20 min at 37 C. Cells were washed three times in PBS, then permeabilized with 0.25% Triton X-100 for 5 min at room temperature. Cells were washed three times in PBS, then pretreated with 10% BSA/PBS for 1 h at 37 C. This was removed, and the primary antibody (1:500 dilution) was applied. For control experiments, the antibody was preabsorbed with 100 ng, 500 ng, or 1  $\mu$ g of the antigen (IGF-I, Sigma), or the primary antibody was omitted. GT1-7 cells were incubated with the primary antibody overnight at 4 C, then washed three times in PBS. The biotinylated secondary antibody (goat antirabbit IgG; Vector, Burlingame, CA), at 1:200, was applied and then incubated for 1 h at 37 C. Cells were washed three times in PBS, then treated with a Vector ABC kit according to the directions;

the DAB reaction was allowed to proceed for 10 min. Cells were washed three times in PBS, then dehydrated in graded ethanol series and mounted with Gurr's Fluoromount (BDH Laboratories, Poole, England).

#### RNA extraction

GT1-7 nuclear RNA, harvested above, was treated with deoxyribonuclease (DNase) I (40 U) for 5 min at 37 C. Both cytoplasmic and nuclear fractions were treated with proteinase K at 45 C, extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and then with chloroform:isoamyl alcohol (24:1), and precipitated with 2.5 vol ethanol (nuclear) or 1.5 vol isopropanol (cytoplasmic) at  $-20$  C (23, 24). Both fractions were centrifuged and washed with 70% ethanol, and the pellets were resuspended in diethylpyrocarbonate-water. An aliquot from each cytoplasmic fraction was used to determine the total RNA content using an optical density reading at 260 nm. Aliquots of 0.5  $\mu$ g were resuspended in 20  $\mu$ l hybridization solution (0.1 M EDTA, 4 M guanidine thiocyanate) for RNase protection assay of GnRH mRNA. The nuclear fraction underwent a DNase I treatment for 30 min at 37 C with 60 U DNase I, extracted as above, and precipitated with ethanol. It was then centrifuged and washed with 70% ethanol, and the pellets were resuspended in 20  $\mu$ l hybridization solution for RNase protection assay of GnRH nuclear RNA transcripts.

#### RNase protection assay

Cytoplasmic and nuclear RNA samples were incubated overnight at 30 C with 5  $\mu$ l of probe (1 ng) labeled to low (600,000 cpm/ng: GnRH cDNA and cyclophilin) or high (1,300,000 cpm/ng: GnRH A2B) specific

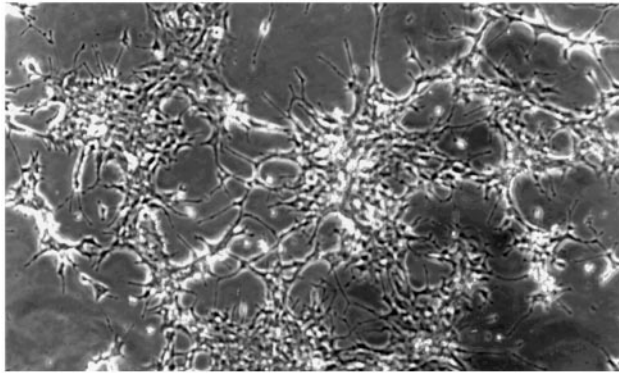
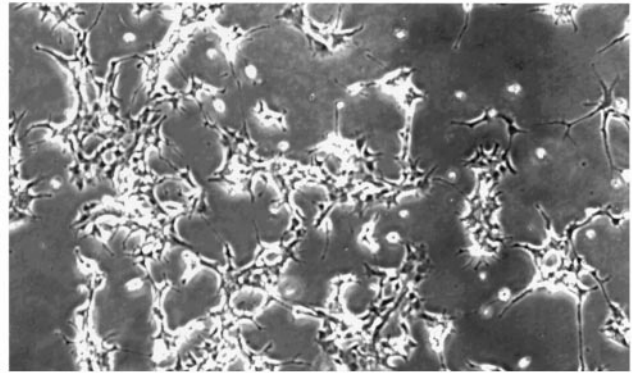
**A. IGF-1****B. Vehicle**

FIG. 3. Effects of IGF-I (A, 10 ng/ml) or vehicle (B) for 24 h on GT1-7 cell morphology. Cells seemed to have a greater extension of processes and a broader, flatter cell body, after treatment with IGF-I.

activity with [ $\alpha$ - $^{32}$ P]uridine 5'-triphosphate. The probe was also incubated with increasing concentrations of sense RNA to produce a standard curve. Samples and standards were allowed to hybridize with the probe for 16–18 h at 30 C. The remainder of the RNase protection assay was performed exactly as described previously (23, 24). Samples were electrophoresed through 6% nondenaturing polyacrylamide gels, which were exposed to x-ray film for 18–48 h to produce an autoradiogram, then exposed to a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) for 24 h for quantitation. The amount of radioactivity in each sample, as determined by the phosphorimager, was compared with the amount of reference RNA, as calculated by regression analysis, and used to calculate the amount of RNA in each sample.

### Probes

The following probes were used: for GnRH mRNA, a 443-bp mouse GnRH cDNA clone spanning the *Eco*O109I and *Xba*I restriction sites and subcloned into a Bluescript SK(+) vector (23); for cyclophilin, a 111-bp cDNA clone spanning the *Pst*I and *Xmn*I restriction sites and subcloned into a Bluescript KS(+) vector (25); and for GnRH primary transcript, a mouse GnRH clone, complementary to the intron A-exon 2-intron B (A2B) region of the proGnRH gene, spanning the *Spe*I and *Hind*III restriction sites and subcloned into a Bluescript SK(+) vector (24).

### RIA

GnRH in the medium was measured in 150- $\mu$ l aliquots by RIA in a single assay using antiserum LR-10, kindly provided by Dr. R. Guillemain. Synthetic GnRH (Richelieu Laboratory, Montreal, Canada) was used for the radiolabeled antigen and the reference standard. The antigen-antibody complex was precipitated with a goat antirabbit  $\gamma$ -globulin (Calbiochem, La Jolla, CA). The sensitivity of the assay was 0.5 pg/tube in a final vol of 500  $\mu$ l without secondary antibody. The intraassay coefficient of variation was 4%.

## Results

### IGF-I effects on GnRH gene expression, release, and GT1-7 cell morphology

Dose-response measurements were performed initially to determine the optimal dose of IGF-I on GnRH gene expression. Representative autoradiograms of GnRH primary transcript and cytoplasmic mRNA in individual samples and standards are shown in Figs. 1A and 2A, respectively, and the regression analyses of the standard curve data are presented in Figs. 1B and 2B. Quantitation of levels of GnRH primary transcript indicated a significant effect of IGF-I ( $P < 0.01$ ), with an inverted-U dose-response curve; the maximal re-

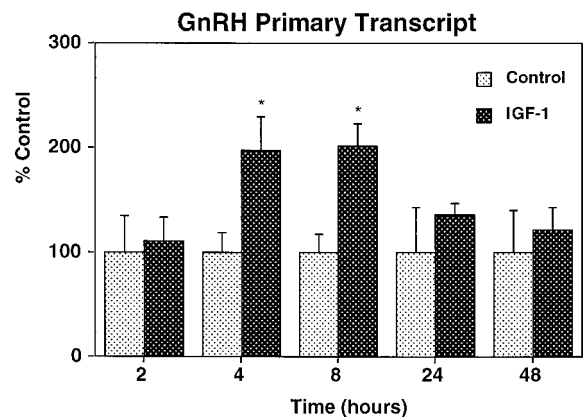


FIG. 4. Time-course effects of IGF-I on mean GnRH primary transcript levels. A significant effect of IGF-I was observed at 4 and 8 h. \*,  $P < 0.05$  vs. corresponding control.

sponse occurred at the 10 ng/ml dose ( $P < 0.01$  vs. vehicle; Fig. 1C). A significant effect of IGF-I ( $P < 0.05$ ), with a similar pattern, was observed for cytoplasmic GnRH mRNA levels, with a significant increase observed using the 10 ng/ml dose ( $P < 0.05$  vs. vehicle and 100 ng/ml; Fig. 2C). Qualitative analysis of changes in GT1-7 cell morphology indicated that the 10 ng/ml dose also caused the most profound changes, with an increased extension of processes, an appearance of more connections between cells, and flattened and broader cell bodies, similar to other preliminary reports (Fig. 3) (22, 26). Thus, this 10 ng/ml dose was used for all subsequent experiments. Cyclophilin mRNA levels, measured in the same cytoplasmic fraction as GnRH mRNA levels, were not affected by IGF-I (Fig. 2C, inset), indicating that the response is specific to the GnRH system. Thus, for all subsequent experiments, GnRH mRNA levels were normalized to cyclophilin mRNA levels measured in the same assay, as an internal control.

Using the 10 ng/ml dose of IGF-I, time-course experiments were performed on cells treated with IGF-I or vehicle for 0, 2, 4, 8, 24, and 48 h. Fig. 4 shows levels of GnRH primary transcript in GT1-7 cells, treated with IGF-I or vehicle. GnRH primary transcript levels were significantly affected by IGF-I

treatment ( $P < 0.05$ ; Fig. 4). *Post hoc* analysis indicated that IGF-I caused a significant increase in GnRH primary transcript levels at 4 and 8 h of treatment ( $P < 0.05$  for both treatments). GnRH primary transcript levels returned to control levels by the 24-h time point. A significant increase in GnRH cytoplasmic mRNA levels also was induced by IGF-I treatment ( $P < 0.05$ ; Fig. 5), and a representative composite autoradiogram is shown. Levels of GnRH mRNA were significantly elevated only at the 24-h time point ( $P < 0.05$ ). Cyclophilin mRNA levels were unaffected by IGF-I treatment (data not shown).

GnRH peptide levels in the medium measured by RIA were found to be significantly affected by IGF-I treatment ( $P < 0.01$ ; Table 1). Initially, at the 2-h time point, a significant increase in GnRH peptide levels was measured ( $P < 0.01$ ). However, beginning at 4 h and throughout the rest of the 48 h time-course, GnRH peptide levels were significantly decreased by IGF-I treatment ( $P < 0.01$  for the 4- and 48-h time points;  $P < 0.05$  for the 8- and 24-h time points).

#### IGF-I effects on GnRH mRNA half-life

To determine whether GnRH mRNA half-life is altered by IGF-I, suggesting that a posttranscriptional mechanism contributes to the increase in GnRH mRNA levels after IGF-I

treatment, cells were pretreated with IGF-I or vehicle for 2.5 h; then, the RNA polymerase II inhibitor DRB was added, and cells were harvested after 0, 2, 4, 8, 16, or 24 h of exposure to DRB. It was observed that GnRH mRNA half-life was 31 h in vehicle-treated control cells (Fig. 6A), as was previously observed (24). IGF-I treatment resulted in a similar GnRH mRNA half-life of 26 h (Fig. 6B).

#### Expression of IGF-I-immunoreactivity in GT1-7 cells

Immunocytochemistry studies indicated that GT1-7 cells express IGF-I-like immunoreactivity (Fig. 7). IGF-I staining was observed in the cytoplasm of many GT1-7 cells, al-

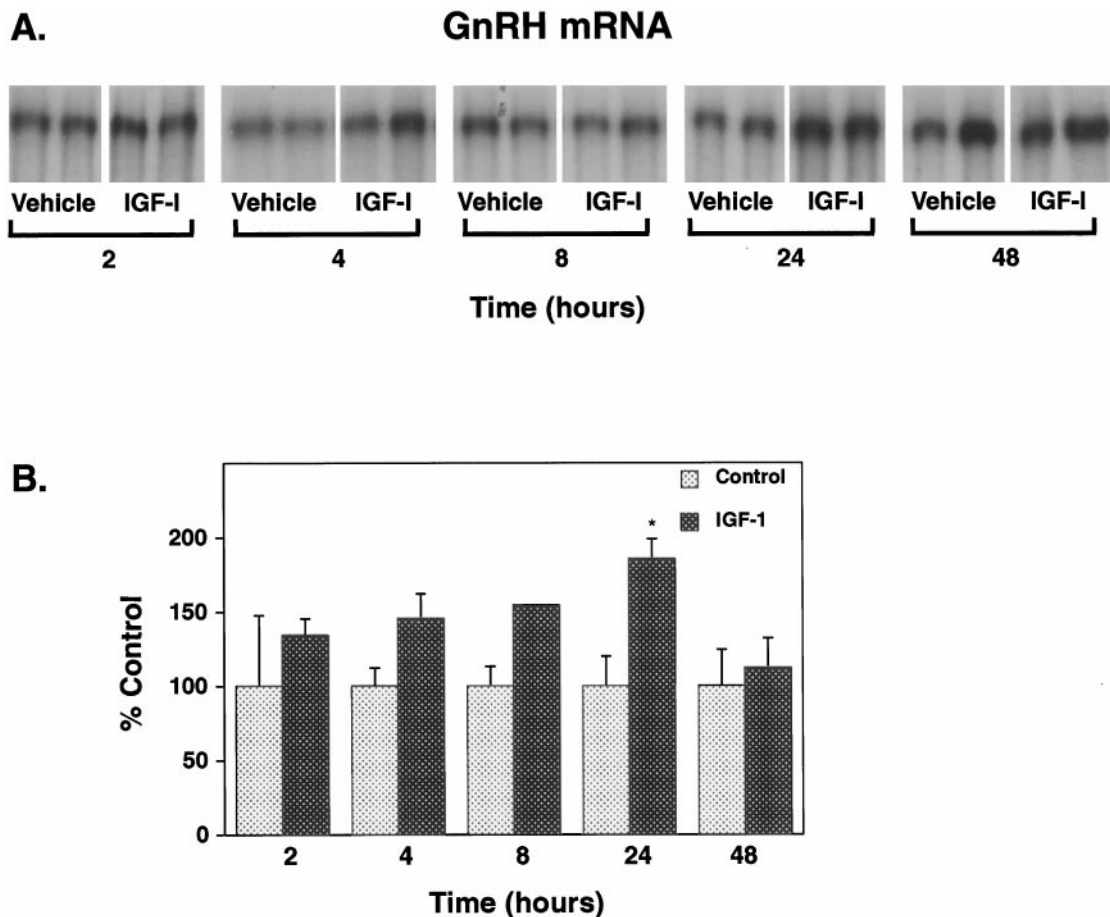
**TABLE 1.** Effects of IGF-I or vehicle on GnRH peptide levels in the medium of GT1-7 cells

Time (h)	Control	IGF-I	% Control
2	0.94 ± 0.05	1.31 ± 0.27 <sup>a</sup>	139%
4	2.82 ± 0.40	1.37 ± 0.18 <sup>a</sup>	48%
8	5.29 ± 0.80	3.47 ± 0.60 <sup>b</sup>	66%
24	3.67 ± 1.16	1.74 ± 0.52 <sup>b</sup>	47%
48	3.51 ± 0.81	2.12 ± 0.13 <sup>a</sup>	60%

Data are expressed in pg/150  $\mu$ l as measured by RIA.

<sup>a</sup>  $P < 0.01$  vs. corresponding control.

<sup>b</sup>  $P < 0.05$  vs. corresponding control.



**FIG. 5.** Time-course effects of IGF-I on mean GnRH mRNA levels. A, Representative composite autoradiogram of GnRH mRNA, in response to IGF-I or vehicle for 2, 4, 8, 24, or 48 h. B, GnRH mRNA levels were significantly stimulated by IGF-I at 24 h. \*,  $P < 0.05$  vs. corresponding control.

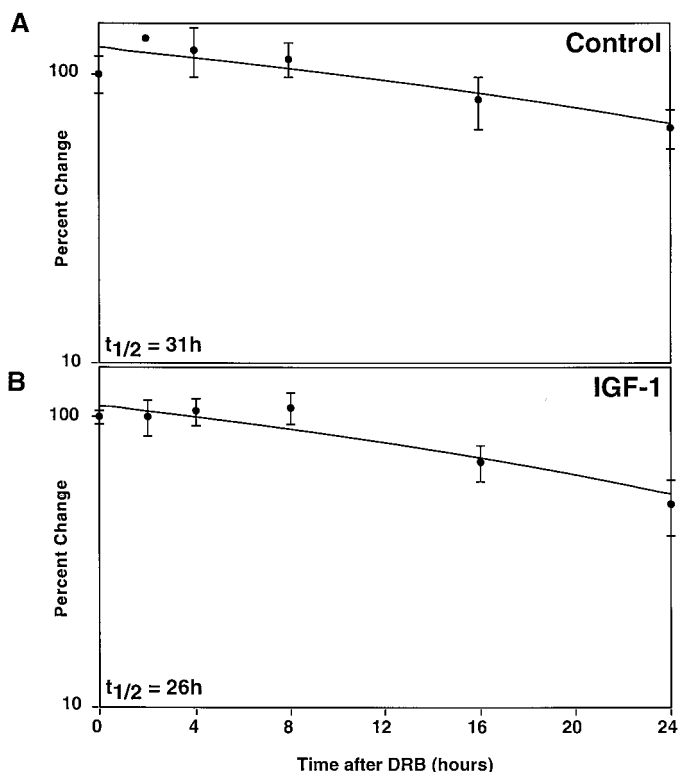


FIG. 6. Half-life of GnRH mRNA, as determined by RNase protection assay. In vehicle-treated GT1-7 cells, the half-life was determined to be 31 h (A). In IGF-I-treated GT1-7 cells, the half-life was 26 h (B).

though its staining seemed heterogeneous across cell populations. In control experiments, preincubation of the IGF-I antibody with the antigen, or omission of the primary antibody, eliminated the detection of IGF-I-like immunoreactivity in the GT1-7 cells (Fig. 7, inset).

### Discussion

The present results indicate that the growth factor IGF-I has profound effects on GnRH gene expression and release in GT1-7 cells. Treatment with IGF-I caused a significant increase in GnRH nuclear primary transcript levels, an index of GnRH gene transcription (6), and in GnRH cytoplasmic mRNA levels in GT1-7 cells. An inverted U-shaped dose-response curve was observed for both transcripts, with a peak response induced by the 10 ng/ml dose. Interestingly, a similar range of doses was reported to be effective in stimulating GnRH release from median eminence explants of rats (17). Time-course analyses of IGF-I's effects in GT1-7 cells indicated that GnRH primary transcript levels were maximally stimulated at 4–8 h of IGF-I treatment, with an increase of approximately 200% at these times. GnRH mRNA levels increased more modestly and with a longer latency, with a significant response occurring only at 24 h. The effects of IGF-I on GnRH gene expression in GT1-7 cells were specific, because the amount of cyclophilin mRNA was unaffected by this growth factor.

The timing of events caused by treatment with IGF-I, with an initial increase in GnRH primary transcript and a subsequent increase in GnRH mRNA levels, suggests that the

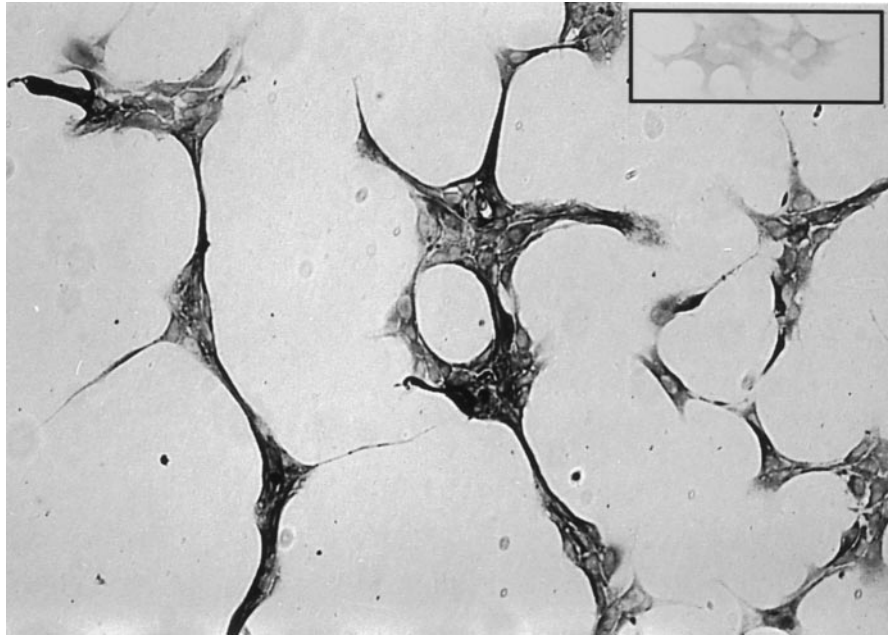
elevation in GnRH mRNA is a consequence of stimulated GnRH gene transcription. This is consistent with a report in a human hypothalamic NLT cell line in which GnRH gene transcription, as measured by luciferase reporter activity, was stimulated by IGF-I (27). Such a transcriptional mechanism for the increase in GnRH mRNA levels is supported by our observation that the GnRH mRNA half-life did not change substantially after IGF-I treatment. Studies on the effects of second-messenger activators on GnRH gene expression also have indicated significant changes in GnRH gene transcription (28–30), suggesting that this is an important mechanism for the regulation of GnRH mRNA levels in hypothalamic NCLs. This does not seem to be the case in the animal, however, in which most of the regulation of GnRH mRNA levels seems to occur at a posttranscriptional level, such as mRNA stability (25, 31; reviewed in Ref. 4).

To our knowledge, this is the first report of a substance stimulating GnRH gene expression in GT1-7 cells. Stimulation of the protein kinase A (PKA) or C (PKC) second-messenger systems has been reported to cause rapid and substantial decreases in GnRH gene expression (23, 24, 28, 30, 32, 33). In contrast, it has been reported that neurotransmitters (such as glutamate) stimulate GnRH gene expression in the animal (25, 34, 35) but not in GT1-7 cells (A. C. Gore and J. L. Roberts, unpublished observation). It is possible that IGF-I represents an input to GnRH neurons that is obligatory for a stimulatory effect on the GnRH gene to occur.

Although IGF-I is stimulatory to GnRH gene expression in GT1-7 cells, it was found to have an overall inhibitory effect on levels of GnRH decapeptide in the medium. IGF-I treatment caused an initial stimulation of GnRH peptide levels at the 2-h time point. However, beginning at 4 h and for the rest of the 48-h time-course, GnRH peptide levels were significantly suppressed, compared with control peptide levels. This result differs from that of another laboratory, which reported no effect of 2 h of exposure to IGF-I on GnRH secretion (20). Furthermore, preliminary reports from another group indicated a stimulatory effect of 12-h exposure to IGF-I on GnRH release in the GT1-1 cell line (22). Differences between laboratories are attributed to different cell lines (GT1-1 vs. GT1-7) and possible so-called drift in the phenotype of the GT1 cells between laboratories with repeated passaging. It was reported that GT1 cells can alter the expression of certain molecules over time, and in fact, these cells even can express glial markers, depending on the passaging technique (36). However, it should be noted that preliminary studies in our laboratory indicated no expression of glial fibrillary acidic protein, an astroglial marker, in our GT1-7 cell cultures (K. M. Longo and A. C. Gore, unpublished observations). Differences between our and other studies may also involve the effects of serum deprivation, because in our study, GnRH peptide levels increased and then decreased over time after serum deprivation (Table 1), whereas other studies used different cell culture and serum starvation conditions.

The observation that GnRH levels in the medium were decreased under those conditions in which increases in GnRH gene expression were observed suggests an uncoupling of synthesis-secretion events. Such an uncoupling was observed when GT1 cells were treated with phorbol ester,

FIG. 7. Immunocytochemistry of IGF-I in GT1-7 cells. Cells were found to express IGF-I-like immunoreactivity, although the intensity of staining was heterogeneous. In control experiments (*inset*), a large reduction in labeling is seen in cells in which the antibody was preabsorbed with the antigen.



which causes decreases in GnRH gene expression concomitantly with increases in GnRH release (24, 28, 30, 32, 33). In the case of IGF-I treatment, it seems that there is a shift in favor of biosynthesis, perhaps at the expense of translational, posttranslational events and/or release. Moreover, it is unknown whether the overall decrease in GnRH decapeptide levels is caused by a decrease in release of GnRH peptide, or by an increase in the degradation of GnRH peptide.

Concomitantly with the effects of IGF-I on GnRH gene expression, a qualitative change in the morphology of GT1-7 cells also was observed, similar to the finding by other laboratories (22, 26). Interestingly, the most profound changes induced by IGF-I on GT1-7 cell morphology occurred at the same dose (10 ng/ml) that caused the greatest increases in GnRH gene expression. When IGF-I was added, the cells formed longer and more extended processes. It previously has been reported that GT1-7 cells form gap junctions with one another (37); it is therefore possible that IGF-I affected the number of gap junctions, because the number of contacts between GT1-7 cells seemed to increase. However, it is necessary to perform quantitative analyses of such changes in future studies to prove this hypothesis. Interestingly, PKC activators, which have opposite effects to IGF-I on GnRH gene expression and peptide release, also have opposite morphological effects to IGF-I on GT1-7 cell morphology: PKC caused the cell body to become more condensed and processes to retract (30), whereas IGF-I caused the cell body to become more flattened, with an appearance of an enlarged surface area, and an extension of processes. It also may be relevant that during postnatal development, GnRH neurons undergo morphological changes that make them become more irregular in shape (38), suggesting an increase in cell surface area, with the potential for increased synaptic input. A similar effect seems to be induced by IGF-I treatment in GT1-7 cells, suggesting a recapitulation of events occurring during the development of GnRH neurons *in vivo*.

Immunocytochemical studies indicated the novel obser-

vation that GT1-7 cells are immunoreactive for IGF-I. This is not the only growth factor synthesized in GT1-7 cells, which were reported to produce basic fibroblast growth factor as well (39). It is possible that these cells are under a tonic autofeedback loop from the endogenous IGF-I that they are producing. This is consistent with the report that in hypothalamic neuronal and glial cultures, IGF-I can down-regulate its own receptors (40). However, our observation that IGF-I stimulates GnRH gene expression in GT1-7 cells suggests that the amount of IGF-I released into the medium may not be sufficient to down-regulate the IGF-I receptors on these cells. Furthermore, changing the GT1-7 cells' medium before IGF-I treatment may resensitize them to IGF-I. In future studies, we will measure IGF-I levels in the medium to determine the exposure of GT1-7 cells to endogenous IGF-I under basal conditions. The expression of IGF-I in GT1-7 cells also suggests the intriguing possibility that GnRH neurons *in vivo* may synthesize their own IGF-I. The report that embryonic day-17 rat neuronal primary cultures express IGF-I mRNA (41) supports the idea that neurons are capable of synthesizing this neurotrophic factor. Studies are currently underway in our laboratory to determine whether GnRH neurons *in vivo* are IGF-I immunopositive.

The present findings suggest that IGF-I may be directly involved in alterations in the gene expression and morphology of the immortalized GT1-7 cell line. These studies are the first crucial steps in producing a more representative model of the adult mammalian GnRH neuron. Regarding *in vivo* models, it was reported that IGF-I knockout mice have a smaller central nervous system size and do not undergo reproductive development (42). It is not known whether this latter effect is caused by a primary central nervous system, pituitary, or gonadal deficiency. However, the preliminary observation in our laboratory that GnRH mRNA levels are substantially lower in IGF-I knockout mice (A. C. Gore, unpublished observation) suggests that the lack of exposure of developing GnRH neurons to IGF-I results in a more imma-

ture phenotype, in fact, similar to that observed in GT1-7 cells. Future studies will examine changes in IGF-I input to the GnRH system of developing mice.

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