Endocrine, *vol. 13, no. 1, 71–79, August 2000* 0969–711X/00/13:71–79/$12.25

Insulin-like Growth Factor-1 Causes a Switch-like Reduction of Endogenous Growth Hormone mRNA in Rat MtT/S Somatotroph Cells

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Reduction of mRNA expression from the endogenous GH gene by insulin-like growth factor 1 (IGF-1) in somatotroph-like rat MtT/S cells was measured. GH mRNA levels were reduced by 65 n*M* IGF-1 treatment in a time-dependent manner over 5 d of culture with a calculated GH mRNA half-life of 50 h, in line with previous values from primary cultures. Inhibition of inositol 3-phosphate kinase by wortmannin or LY-294,002 treatment was ineffective in blocking IGF-1 decreases in GH mRNA, as was inhibition of MAP kinase activity by PD 098059. The inhibition by IGF-1 also did not regulate Pit-1 (GHF-1) mRNA levels, which were constant during 65 n*M* IGF-1 treatment. MtT/S cells were shown to have both IGF-1 and insulin receptors as detected by Western blotting. There was also shown to be the suggestion of "hybrid" receptors containing different beta chains from each of these related heterotetrameric receptors. Analysis of the effects of IGF-1 and insulin on MtT/S cells showed that each reduced GH mRNA in a dose-dependent manner gave a calculated EC_{50} of 15.5 n*M* for IGF-1 and 0.6 n*M* for insulin, suggesting that the respective receptors for each hormone were activated. However, GH mRNA response to IGF-1 treatment was "ultrasensitive," exhibiting a switch-like effect; below 10 n*M* IGF-1, there was no decline in GH mRNA, but then maximal reduction occurred at IGF-1 concentrations above 20 n*M*. The degree of this ultrasensitive effect was calculated from the Hill equation for cooperativity, with a Hill coefficient of –4.1, greater than the classic cooperativity exhibited by hemoglobin binding to oxygen. The ultrasensitive response was specific for IGF-1, as insulin did not display this effect. These results suggest that the response evoked by the IGF-1 receptor could act as a binary molecular switch controlling GH mRNA expression in somatotrophs.

Received April 4, 2000; Revised May 16, 2000; Accepted May 16, 2000.

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Key Words: Transcription; signal transduction; mitogen-activated protein kinase; phosphatidylinositol 3-kinase; Pit-1.

Introduction

Insulin-like growth factor-1 (IGF-1) expression is induced in peripheral tissues by growth hormone (GH) and mediates the actions of GH in stimulating somatic growth *(1)*. IGF-1 acts at the levels of both the hypothalamus and anterior pituitary to reduce circulating GH levels in an important negative feedback system of the GH axis *(2)*. The mechanism of feedback has been studied with IGF-1 treatment of primary pituitary cultures, which inhibits transcription of the GH gene *(3)*. The resulting decrease in steady-state GH mRNA levels is concomitant with reduction of GH protein levels also caused by IGF-1 *(4)*. Perhaps related to the IGF-1 effect, low doses of insulin also reduce GH mRNA expression, although the physiological significance of this effect on the GH axis is unknown *(5)*.

Heterotetrameric type-1 IGF receptors (IGF-R) and related insulin receptors (insulin-R) are each expressed in the anterior pituitary and GH-producing cell lines, explaining the ability of their respective ligands to reduce GH mRNA expression *(6–8)*. IGF-1 binds the IGF-R with high affinity, but also binds the insulin-R with 50–100-fold less affinity *(9)*. Ligand binding to the IGF-R activates an intrinsic receptor tyrosine kinase domain, resulting in the stimulation of mitogen-activated protein (MAP) kinase *(10)* and phosphatidylinositol (PI) 3-kinase-dependent signaling pathways *(11)*. Signal transduction pathways from the IGF-R are presumed to decrease the activity of DNA-binding or co-activator proteins that regulate transcriptional activity of the GH promoter *(12)*, but the exact mechanism of this transcriptional regulation remains to be determined. It has been observed that IGF-1 treatment reduced the amount of mRNA for Pit-1 (also known as GHF1), a pituitary-specific transcription factor regulating GH gene expression, in primary pituitary cultures, suggesting one mechanism for IGF-1 inhibition *(13)*.

An alternative model system that obviates concern over the multiple Pit-1-expressing cell types present in whole

pituitary cultures is the somatotroph-like rat MtT/S cell line, secreting GH but not prolactin (PRL) *(14)*. These cells also uniquely express the GH-releasing hormone (GHRH) receptor gene *(15,16)*. The intracellular and extracellular levels of GH protein are reduced in MtT/S cells following treatment with IGF-1 *(17)*, and in transfected MtT/S cells, IGF-1 inhibits activity of a reporter gene driven by the GH proximal promoter *(18)*. Taken together, these results suggest the usefulness of MtT/S cells as a model for studying integrated hormonal control of GH gene expression.

Yamashita et al. *(19)* showed that the transfected human GH promoter was regulated by IGF-1 similarly to the endogenous GH gene in choriocarcinoma cells. However, it has recently been shown that the activities of the transfected GH promoter and endogenous GH gene are regulated differentially by IGF-1 in GH3 cells *(20)*. Therefore, IGF-1 regulation of endogenous GH gene expression was investigated in MtT/S cells. Measurements were performed using a novel fluorescence-labeled ribonuclease protection assay for GH mRNA. The findings suggest that the signaling pathways acting upon the endogenous GH gene may be different from those regulating the transfected GH promoter. Dose-response analysis of IGF-1 effects on endogenous GH mRNA levels showed switch-like kinetic behavior, the hallmark of ultrasensitive control, so that GH gene expression was either on or off. This "ultrasensitive" means of regulating gene expression may contribute to the pulsatile levels of circulating GH *(21)*.

Results

GH mRNA Measurement by Fluorescence-Ribonuclease Protection Assay

GH mRNA was measured in MtT/S cell total RNA with a ribonuclease protection assay (RPA) that uses a fluorescent riboprobe quantified on an automated DNA sequencing instrument. A fluorescein-labeled GH anti-sense probe was prepared in vitro, used for RPA by standard methods, then the amount and size of the protected probe were determined by comparison with a fluorescence-labeled DNA ladder present in the same lane (Fig. 1A). The 245 nt molecular weight of the fluorescein-labeled peak was consistent with the size predicted for the RNase digested GH probe. Furthermore, the peak height of the band closest in size to the GH probe (200 nt) in the ROX-labeled DNA ladder was not affected by increasing amounts of GH signal (Fig. 1B). Because standards were added during the initial precipitation, peak height of the closest standard band was subsequently used for normalization so that GH signal was expressed as GH peak height divided by the peak height of the 200 nt band. Measurements with increasing input amounts of MtT/S RNA showed that normalized GH mRNA was linearly correlated ($R^2 = 0.97$) with the amount of total RNA input from 0.094 to 3.0 μg (Fig. 1B).

Time Course of IGF-1 Treatment on GH mRNA Levels

Cells were treated in CM supplemented with 65 n*M* IGF-1 for up to 5 d, total RNA was isolated, and GH mRNA levels were determined by fluorescence RPA. GH mRNA levels following treatment were expressed as a percentage of initial GH levels (Fig. 2). Results from three independent cultures analyzed by one-factor ANOVA and *post-hoc* testing indicated that GH mRNA levels were significantly affected by the time of treatment with 65 n*M* IGF-1 in the presence of serum-containing medium ($F_{4,10} = 21$; $p = 0.0001$). At 0.5, 1, 2, and 5 d of treatment, GH mRNA was significantly reduced vs initial levels as determined by *post-hoc* test ($p < 0.05$ for each, Fig. 2). The hyperbolic nature of the measured GH mRNA decrease appeared typical of a first-order reaction in which the decay rate is determined by the half-life of the GH mRNA similar to the results of Yaffe and Samuels *(22)*. Using this assumption, the calculated half-life of GH mRNA following IGF-1 treatment was 50 h (Fig. 2).

Effects of Pharmacological Inhibitors on IGF-1 Control of GH mRNA Levels

MtT/S cells were treated in CM alone, CM with 65 n*M* IGF-1, or CM with 65 n*M* IGF-1 and 1 μ*M* wortmannin, an inhibitor of PI 3-kinase. Previous studies have shown that wortmannin at this concentration prevents the effects of IGF-1 on a GH promoter-driven reporter gene in MtT/S cells *(18)*. Analysis by one-factor ANOVA indicated that different combinations of treatment with wortmannin and IGF-1 had a significant impact on GH mRNA levels ($F_{3,8} = 11$; $p = 0.0037$). Following 2 d treatment with IGF-1 alone, IGF-1 with 1 μ*M* wortmannin, or 1 h pretreatment with 1 μ*M* wortmannin followed by 2 d treatment with 1 μ*M* wortmannin and 65 n*M* IGF-1, GH mRNA levels were reduced to 40, 36, and 46% of the control ($p < 0.01$, for each treatment combination), respectively (Fig. 3A). However, combinatorial treatment with wortmannin and IGF-1, or pretreatment with wortmannin followed by treatment with IGF-1 and wortmannin did not significantly alter GH mRNA levels vs treatment with IGF-1 alone. Similarly, treatment with increasing dosages (6.25 to 100 μ*M*) of LY-294,002, a specific inhibitor of PI 3-kinase, and IGF-1 did not significantly alter GH mRNA levels vs IGF-1 alone (Fig. 3B). Increasing dosages (6.25 to 100 μ*M*) of PD 098059, an inhibitor of MAP kinase-dependent signaling, and IGF-1 did not significantly alter GH mRNA levels vs IGF-1 alone (Fig. 3C).

Effects of IGF-1 on Pit-1 mRNA Expression

To determine if IGF-1 inhibits GH mRNA levels via decreases in Pit-1 mRNA expression, MtT/S cells were treated for 2 d with or without 65 n*M* IGF-1, then Pit-1 mRNA levels were measured by chemiluminescence RPA. There was no significant difference in Pit-1 mRNA levels

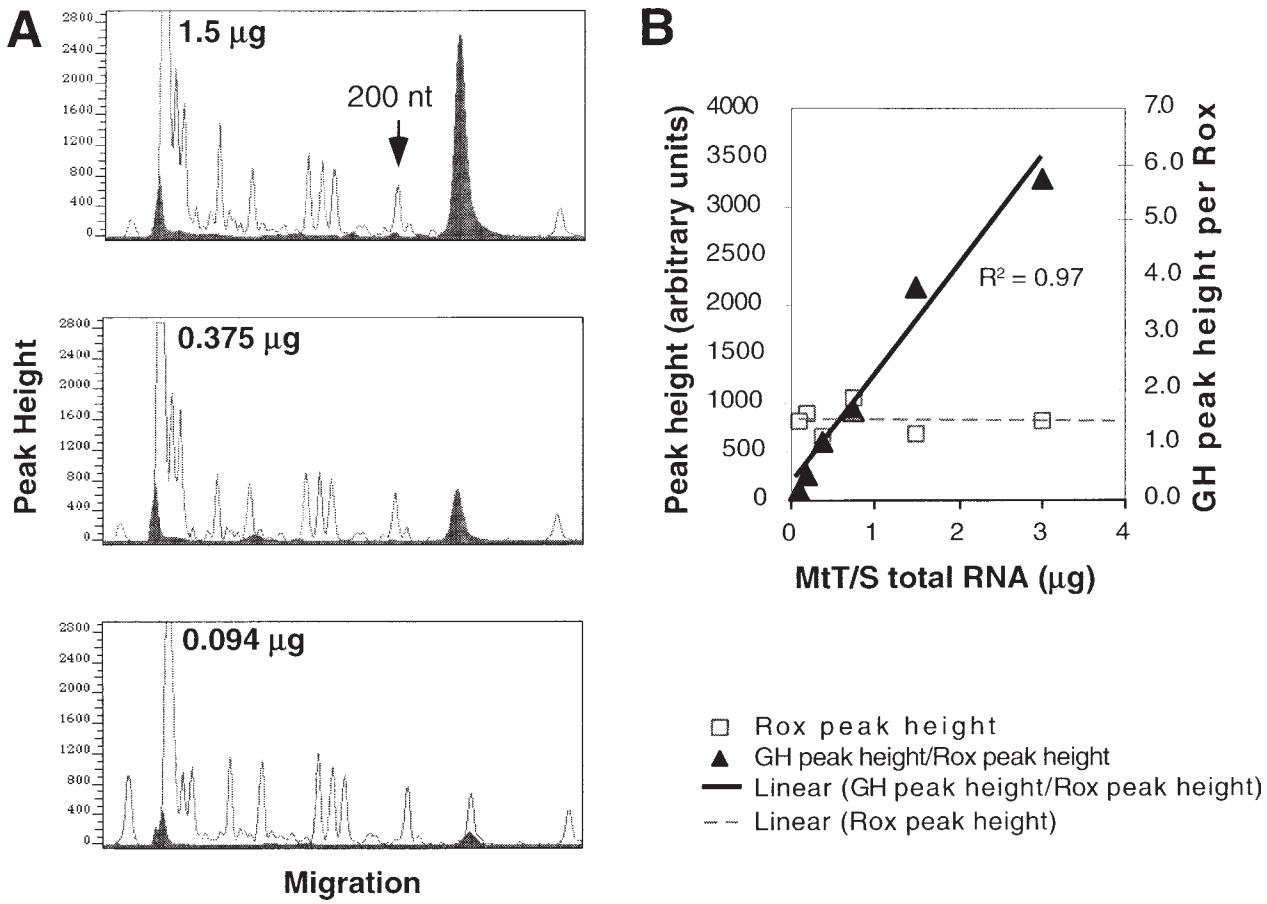

Fig. 1. GH mRNA measurement using fluorescence-RPA. The indicated amounts of MtT/S cell total RNA were assayed for GH mRNA expression as described in Methods. Fluorescence intensity profiles are shown from representative ABI 672 Genescan electropherograms (Panel A). The filled peak heights were used to determine the amount of protected fluorescein-labeled GH RNA probe, while open peak heights represent the quantity of the Rox-labeled DNA ladder. Panel B shows that the measured amounts of protected GH probe were linear with input MtT/S RNA both before and after normalization to the 200 nt Rox ladder band in each lane.

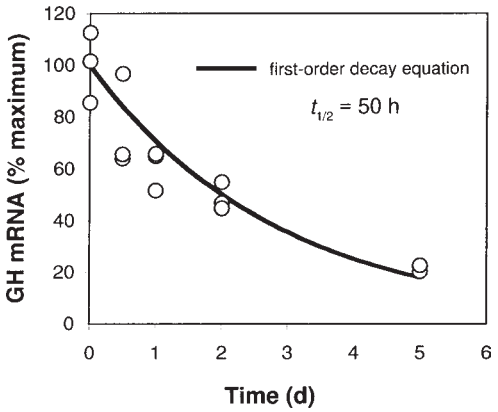

Fig. 2. Time course of IGF-1 effect on GH mRNA expression. MtT/S cells were treated with 65 n*M* IGF-1 for the indicated amount of time and GH mRNA levels were assayed by f-RPA. Results are shown as the individual values measured in each of three cell cultures. The best-fit line calculated by nonlinear regression for the first-order rate of degradation is shown. From this calculation, the measured GH mRNA half-life was determined to be 50 h.

from IGF-1-treated vs untreated control cultures (Fig. 4) although GH mRNA expression was reduced in IGF-1-treated cultures (data not shown).

Expression of Type 1 IGF and Insulin Receptors

In order to determine whether IGF-1 and/or insulin signaling in MtT/S cells occurred via their respective receptors, cell extracts were assayed by immunoprecipitation, electrophoretic separation, and immunodetection. As shown in Fig. 5 (Panel A), IGF-R β-immunoreactive species of 95 and 185 kDa were detected following immunoprecipitation with IGF-R β-specific antiserum. Interestingly, a less intense 95 kDa IGF-R β-immunoreactive species was also detected in MtT/S extracts immunoprecipitated with insulin-R β-specific antiserum (Fig. 5, Panel A). Following immunoprecipitation with insulin-R β-specific antiserum (Fig. 5, Panel B), the 95 kDa insulin-R β-immunoreactive species was detected. Analysis of insulin-R β-immunoprecipitated MtT/S extracts also revealed detectable IGF-R β-specific immunoreactivity.

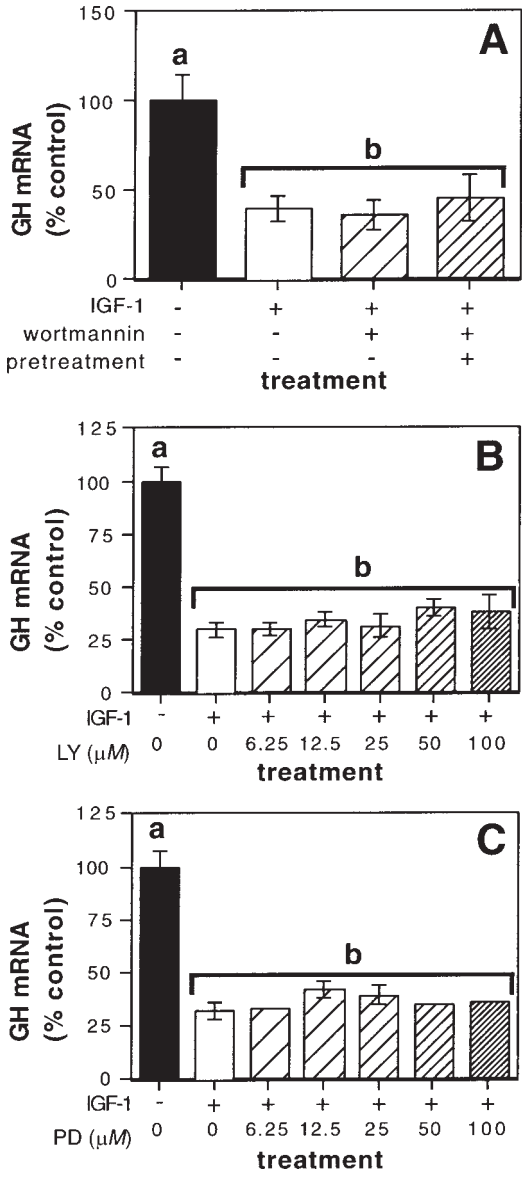

Fig. 3. Effects of pharmacological inhibitors on IGF-1 dependent suppression of GH mRNA levels. MtT/S cells were treated with 65 n*M* IGF-1 in the presence of indicated pharmacological inhibitors for 48 h and GH mRNA levels were assayed by f-RPA. Results are shown as the mean of measurements from at least three cultures, and error bars denote SEM. Wortmannin was added at 1 μ*M* concentration directly with IGF-1 treatment or 1 h prior to addition of IGF-1 (Panel A), while increasing dosages of LY-294,002 (Panel B) or PD 098059 (Panel C) were added simultaneously with IGF-1. Statistically identical measurements are grouped with brackets; these groups are significantly different ($p < 0.05$) as denoted by different letters.

Dosage-Dependent Effects of IGF-1 and Insulin on GH mRNA Levels

A dose-response curve for GH mRNA levels in MtT/s cells was determined by treatment in CM supplemented with 0, 0.02, 0.1, 0.5, 2.6, 7, 12, 25, 45, 65, and 85 n*M* IGF-1 for 5 d. As shown in Fig. 6A plotted against the logarithm of the added IGF-1 concentration, GH mRNA levels determined by f-RPA and normalized to GH mRNA levels in the absence of IGF-1 showed an "ultrasensitive" profile of decline with dose of IGF-1. Evaluation by one-factor ANOVA indicated that GH mRNA levels were significantly affected by treatment for 5 d with increasing concentrations (0–85 n*M*) of IGF-1 in the presence of serum-containing medium ($F_{10,27} = 111.36$; $p = 0.0001$). The decrease in GH mRNA with IGF-1 was not continuous; there was no effect of IGF-1 concentrations up to 7 n*M*, then treatment with 12 n*M* IGF-1 significantly reduced GH mRNA relative to untreated levels ($p < 0.05$). Treatment with 25, 45, 65, and 85 n*M* IGF-I significantly reduced GH mRNA levels further compared with 12 n*M* GH mRNA levels ($p < 0.05$, for each), but values within this group were not statistically different from each other.

Using these same methods, GH mRNA levels in MtT/S cells in response to insulin treatment were assayed in CM with 0, 0.02, 0.1, 0.5, 2.6, 12, and 65 n*M* insulin. As shown

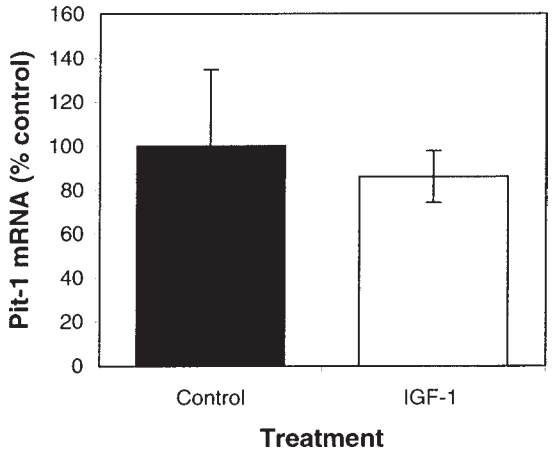

Fig. 4. Effect of IGF-1 on Pit-1 mRNA expression. MtT/S cells were treated for 48 h with or without 65 n*M* IGF-1 and Pit-1 mRNA levels were assayed by chemiluminescence RPA. Results show the mean of measurements from three cultures; error bars denote SEM.

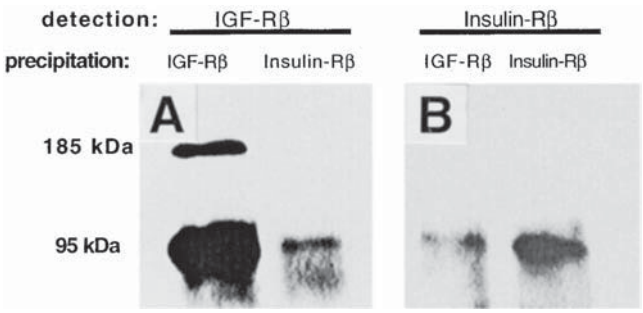

Fig. 5. Detection of IGF and insulin receptor proteins. IGF-R (left lanes) or insulin-R (right lanes) were immunoprecipitated from MtT/S cell lysates, separated by SDS-PAGE, and detected with IGF-R (Panel A) or insulin-R (Panel B) β subunit-specific antibodies using Western blotting. Molecular weights were calculated from separate marker lanes.

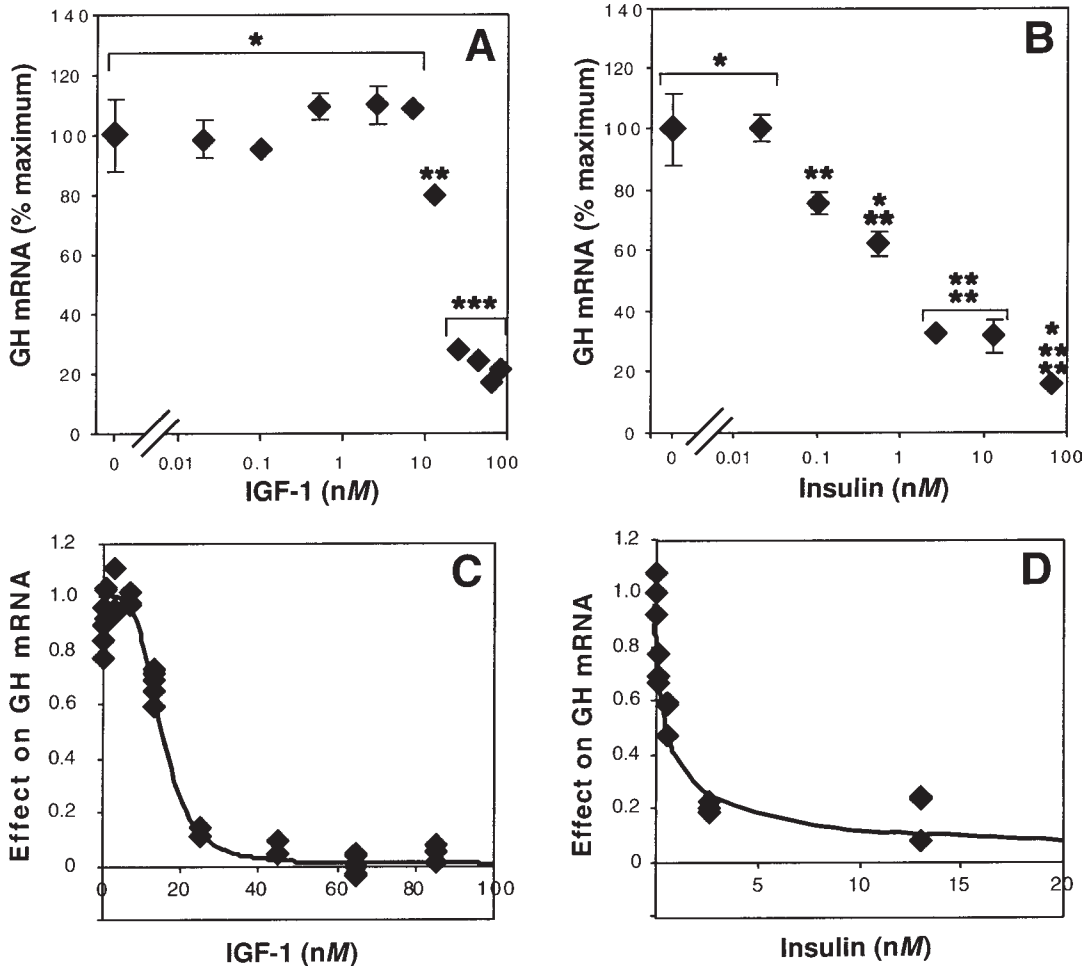

Fig. 6. Dose-dependent effects of IGF-1 or insulin on GH mRNA expression. MtT/S cells were treated for 5 d with the indicated concentrations of IGF-1 or insulin and GH mRNA levels were assayed. Results are the mean of measurements from at least three cultures and are expressed as a percentage of the control with error bars denoting SEM (A and B). Statistically identical measurements are grouped with brackets, while significantly different groups ($p < 0.05$) are denoted with different letters. Individual measurements were then normalized to a value between 1 and 0 representing the maximum and minimum GH mRNA levels following hormonal stimuli and plotted vs dose of IGF-1 (Panel C) or insulin (Panel D). The best-fit line is shown for the Hill equation, which was solved simultaneously for the Hill coefficient and EC_{50} using nonlinear regression.

in Fig. 6B, GH mRNA levels following treatment with increasing concentrations of insulin did not display an "ultrasensitive" response, but decreased continuously with insulin dose. Evaluation by one-factor ANOVA indicated that GH mRNA levels were significantly affected by increasing concentrations (0–65 n*M*) of insulin in the presence of CM ($F_{5,12} = 115.75$; $p = 0.0001$). Treatment with 0.1, 0.52, 2.6, 13, and 65 n*M* insulin reduced GH mRNA levels to 76, 62, 33, 32, and 16% of levels in the CM controls without added insulin (Fig. 6B). As determined by *post-hoc* test, all values were significantly different from each other ($p < 0.05$) with the exception of the 2.6 and 13 n*M* treatments.

This switch-like reduction of GH mRNA expression over a narrow range of IGF-1 concentrations suggests an ultrasensitive response to IGF-1. In order to process these data using the Hill equation describing cooperative processes, the relative effect on GH mRNA of the concentration of each compound was calculated by subtracting the minimal GH mRNA level observed at maximal inhibition from GH mRNA levels at each concentration tested, then dividing that result by the maximal mRNA levels (i.e., no treatment). The calculated value of GH mRNA levels will then vary between 1, indicating no effect, and 0 for maximal effect. From these normalized data for IGF-1 (Fig. 6C) and insulin treatments (Fig. 6D), the Hill coefficient (n_H) and the EC_{50} of the response were then calculated by solving the Hill equation using nonlinear regression. Analysis of IGF-1 dose-effect on GH mRNA expression by nonlinear regression determined n_H to be –4.1 with a 95% confidence level that the value is between –5.3 and –2.9. The inhibition constant (EC_{50}) for IGF-1 was calculated as 15.6 n*M* with a 95% confidence level that the value is between 14.5 and 16.7 n*M*. For comparison, analysis of

insulin effect on GH mRNA expression yielded a value of $n_H = -0.7$ with a 95% confidence level that the value is between –0.9 and –0.5, while the EC_{50} for insulin was calculated to be 0.6 n*M* with a 95% confidence level that the value is between 0.3 and 0.8 n*M*.

Discussion

MtT/S cells are a somatotroph-like cell line expressing high levels of GH *(14)*. The use of these cells to analyze GH mRNA responses to physiologic regulatory signals would provide a novel model of the GH transcriptional complex in cell culture *(12)*. Because MtT/S cells normally maintain a high level of GH mRNA transcripts, the use of inhibitors of GH mRNA expression may be the most productive approach to this model. However, rather than employing transfected GH promoter constructs *(18,19)*, the response of the endogenous GH promoter in MtT/S cells to treatment with IGF-1 was studied.

Pharmacological inhibitors were utilized to determine which IGF-1-dependent signal transduction pathways mediate decreases of endogenous GH mRNA levels. The failure of wortmannin to block the reduction of GH mRNA levels by IGF-1 suggests that PI 3-kinase is not involved in this process. Although wortmannin blocked the action of IGF-1 on a transfected GH promoter construct *(18)*, this inhibition did not occur in the MtT/S cells. These findings suggest that the regulation of the transgenic GH promoter may not be the same as the endogenous GH mRNA levels in MtT/S cells. Supporting this concept is the fact that additional studies with the more specific PI 3-kinase inhibitor LY-294,002 also showed no effects on decline in GH mRNA because of IGF-1 treatment. The failure of PD 098059, a potent inhibitor of MAP and Erk kinase (MEK) activation, to inhibit the effects of IGF-1 on GH mRNA expression suggests that MAP-dependent pathways are not involved in this process. This is consistent with earlier reports that although phorbol esters activate MAP kinase in GH-producing cells *(10)*, phorbol esters do not cause inhibition of GH transcription rate or GH mRNA levels *(23,24)*. However, it will be essential to directly test transfected vs endogenous GH expression to verify whether different signaling pathways exist in the somatotroph-like cells. It is possible that the MtT/S line may have characteristics unlike a normal somatotroph that give rise to these findings.

Additional investigation of the signaling pathway for IGF-1 in the MtT/S cells was performed by assessing Pit-1 mRNA, a pituitary-specific DNA-binding factor required for transcription of the GH gene *(25)*. Although IGF-1 suppresses Pit-1 mRNA levels in primary pituitary cultures *(13)*, and Pit-1 protein levels in other GH-producing cells *(20)*, there was no change in MtT/S cells. The lack of change in MtT/S cells with suppressed GH mRNA suggests that IGF-1 action on the GH promoter does not require inhibition of Pit-1 steady-state mRNA expression. It is unknown whether the Pit-1 mRNA changes in primary cultures were somatotroph-specific because of the mixed cell population *(13)*. Furthermore, the absence of Pit-1 mRNA changes in MtT/S cells treated with IGF-1 have a precedent. Deletion of the Pit-1 response elements from the GH promoter does not reduce the effect of IGF-1 on the activity of a reporter gene in transfected MtT/S cells, indicating that reduction of Pit-1 DNA-binding activity is not required for the effects of IGF-1 on the transfected proximal GH promoter *(18)*.

IGF-1 signal pathways were investigated by assessing expression of the IGF-1 type 1 receptor. Two species of IGF-R β-immunoreactive receptor were detected, the precursor peptide 185 kDa and the 95 kDa final processed receptor subunit. To assess whether insulin receptors were also present, the 95 kDa insulin-R β-immunoreactive species was assayed, and found to be present in the MtT/S cells. Previously, ligand-binding studies showed that the MtT/S cells have active IGF-R capable of binding IGF-1 *(18)*, and RT-PCR analysis revealed expression of IGF-R mRNA in these cells *(18)*. The present immunological detection results are the first direct demonstration of the presence of IGF-R and insulin-R in these cells.

Coexpression of IGF-R and insulin-R is found in a wide variety of tissues, and some fraction of the IGF-R and insulin-R αβ half-receptors are assembled via disulfide bonds into hybrid receptors *(26–29)*. These hybrid receptors bind IGF-1 with high affinity and insulin with low affinity *(30,31)*, and are capable of signaling as measured by receptor autophosphorylation *(28,31)*. Coimmunoprecipitation of insulin-R β with IGF-R β antiserum, as well as coimmunoprecipitation of IGF-R β with insulin-R β antiserum, also suggests the presence of hybrid receptors, although these results could be due in part to antibody cross-reactivity. Thus, these results suggest that hybrid receptors may be present in MtT/S, and perhaps other GH-producing, cells. However, the unequivocal demonstration of the presence and importance of these hybrid receptors for physiologic GH regulation has not been established.

Demonstration of the presence of IGF and insulin receptors in MtT/S cells was important for understanding the measured responses to IGF-1 and insulin. The calculated 15.5 n*M* EC_{50} for IGF-1-dependent inhibition of GH mRNA expression suggests that this response is mediated by the IGF-R, which binds IGF-1 with high affinity ($K_d \approx 1.5$ n*M*) but insulin with 100–1000-fold lesser affinity *(32)*. In contrast, the insulin-R binds insulin with a high affinity and IGF-1 with a 50–100-fold less affinity *(9)* so that the 0.6 n*M* EC_{50} for insulin-dependent inhibition of GH mRNA expression suggests that this response is mediated by the insulin receptor. Thus, these kinetic data agree with previous analysis of these receptors and indicate that both IGF-R and insulin-R proteins are present and functional in MtT/S cells.

Transfected GH promoter-driven reporter genes have provided much insight into the DNA response elements and transacting factors that control GH gene expression *(12,25)*. It has been reported that a human GH transgene was regulated by IGF-1 and insulin in parallel with the endogenous GH gene in JEG-3 cells *(19)*. However, it has been proposed that in some models the transfected proximal GH promoter and the endogenous GH gene are differentially regulated by IGF-1 *(20)*. Specifically in GH3 cells, IGF-1 causes a reduction of GH mRNA levels with no alteration in GH mRNA stability, whereas the transfected GH promoter activity is not affected by IGF-1 *(20)*. As the transfected GH promoter has recently been shown to be regulated by IGF-1 in the MtT/S cell line *(18)*, analysis of endogenous GH gene expression was warranted.

This analysis of GH mRNA expression following IGF-1 treatment revealed a hyperbolic decrease from the initial time point typical of a first-order degradation of GH mRNA. The 50 h half-life of GH mRNA in IGF-1 treated MtT/S cells is consistent with the 50-h half-life of GH mRNA measured in the GH-producing GC cell line by pulse-chase labeling *(22)*. Previous studies revealed that IGF-1 treatment of MtT/S cells causes a reduction of reporter gene activity driven by the GH promoter, but IGF-1 does not alter the stability of GH mRNA in GH3 or GH4C1 cells *(18,20)*. Although transcription was not directly measured, these data suggest that the IGF-1-induced decreases of endogenous GH mRNA levels in MtT/S cells result from a sustained decrease in GH transcription rate followed by uniform mRNA degradation throughout the time course studied.

The EC_{50} constants for IGF-1 and insulin were not calculated in the previous study of the transfected GH promoter in MtT/S cells *(18)*. Although the activity of the GH promoter-driven reporter gene was reduced by IGF-1 in MtT/S cells, the effect of IGF-1 was temporally biphasic reaching a minimum at 18 h but returning to control levels following 36 h of treatment *(18)*. This is not consistent with the long-term reduction of GH protein levels reported for primary pituitary cultures and MtT/S cells *(4,17,33)* or for the time course of GH mRNA degradation observed in the current study. Thus, IGF-1 treatment reveals distinct differences in the expression of the transfected GH promoter and the endogenous GH mRNA in MtT/S cells.

The switch-like inhibition in response to a narrow range of IGF-1 concentrations suggests ultrasensitive regulation of GH mRNA expression in MtT/S cells. The Hill equation describes the degree of ultrasensitivity in a system in terms of the Hill coefficient, n_H. In a nonultrasensitive or hyperbolic system n is equal to 1, but as a system becomes more switch-like or ultrasensitive, the absolute value of n becomes greater than 1 *(34)*. The calculated n_H value for the response of GH mRNA expression to IGF-1 is –4.1, implying that the response of GH mRNA expression to IGF-1 is more ultrasensitive than the response of hemoglobin binding to oxygen (n_H = 2.8), a benchmark of ultrasensitive systems *(35)*. The mechanism of ultrasensitivity appears to be specific to IGF-1 signaling pathways because insulin inhibits GH mRNA expression in a non-ultrasensitive manner (n_H = –0.7). The ultrasensitivity observed for MtT/S mRNA levels may have physiological importance. These "switch-like" kinetics would prevent up to ≈10 n*M* IGF-1 from affecting GH mRNA levels, but allow large changes in GH expression with slightly higher concentrations. This sensitive control mechanism may be relevant to feedback inhibition of somatotrophs during postnatal growth or during the formation of acromegalic tumors when IGF-1 levels are elevated *(36)*.

In sum, these results illustrate that IGF-1 reduces endogenous GH mRNA levels in MtT/S cells through pathways that are independent of PI 3-kinase and MEK. Both immunologic and kinetic data suggest that the IGF-R and insulin-R mediate the inhibition of GH mRNA expression by their respective hormone ligands. Importantly, detailed kinetic analysis revealed that IGF-1 treatment specifically produced an ultrasensitive reduction of GH mRNA levels in MtT/S cells. Because this switch-like response may have physiological implications in the control of GH expression, further studies will be required to determine the underlying mechanisms regulating the response, and the role of these mechanisms in normal somatotroph cells.

Materials and Methods

Preparation of Media and Hormone Stocks

MtT/S cells were routinely cultured in serum-containing Complete Medium (CM) as previously described *(14)*. CM was composed of DMEM/F12 (1:1) medium with 3151 mg/L D–glucose, 365 mg/L L–glutamine, and 2.438 g/L sodium bicarbonate supplemented with 50 units/mL penicillin G sodium, 50 μg/mL streptomycin sulfate, 100 μg/mL kanamycin sulfate (Life Technologies, Grand Island, NY), 10% donor horse serum, and 2% certified fetal bovine serum (Hyclone Laboratories, Logan, UT). Recombinant human IGF-1 (Peninsula Laboratories, Belmont, CA) and pancreatic bovine insulin (Sigma, St. Louis, MO) were resuspended in 0.01 *M* sterile acetic acid at concentrations of 65 μ*M* and 1.75 m*M*, respectively. Wortmannin, LY-294,002, and PD 098059 (LC Laboratories, San Diego, CA) were resuspended in DMSO at concentrations of 1 m*M*, 10 m*M*, and 30 m*M*, respectively.

Maintenance of MtT/S Cell Cultures

MtT/S rat somatotroph cells were obtained live from the Riken Cell Bank (Tsukuba Science City, Japan). Following expansion of cultures, cells were slow-frozen in CM supplemented with 5% DMSO and stored under liquid nitrogen. Cells were routinely plated at 2 million cells per 75 cm^2 tissue culture flask (Corning) in 10 mL CM and incubated

at 37°C in 5% CO_2 and 100% humidity. Cells were passaged every 7 d after reaching a density of 6–8 million cells/flask. All experiments were performed with cells at passages 3-10 after thawing.

Isolation of Total RNA

Cells were removed from wells by gentle pipeting, and the media were removed following centrifugation at 500*g* for 3 min. Total RNA was isolated from cell pellets using phenol/chloroform extraction in the presence of 14 *M* guanidine salts and urea (Ultraspec reagent, Biotecx, Houston, TX). After resuspension in sterile ultrapure water, spectrophotometric absorbance at 260 nm was used to determine yield of total RNA, which averaged 45–55 μg/well.

Treatment with Hormones and Pharmacological Agents

At the beginning of plateau phase (9 d of culture, ≈1 million cells/well), medium was removed from wells by pipeting. Cells were washed three times with 5 mL serum-free medium (SFM) taking care not to dislodge loosely attached cell clusters. Wash medium was discarded without centrifuging to reclaim detached cells, and 5 mL of serum-containing medium (CM) with test compounds or carrier was added to each well for subsequent incubation.

Measurement of mRNA Levels by Ribonuclease Protection Assay

Regions of the GH and the Pit-1 gene were amplified by polymerase chain reaction from rat anterior pituitary cDNA. PCR products were cloned into pCR 2.0 plasmid vector (InVitrogen, Carlsbad, CA), and confirmed by sequencing using standard automated protocols (Applied Biosystems, Inc., Foster City, CA). Rat GH template was linearized with Xmn I restriction enzyme (Life Technologies) before in vitro transcription, which yields a 325 nucleotide (nt) probe that would be reduced to a 245 nt protected fragment after RNase digestion. Pit-1 template was linearized with Dra I restriction enzyme (New England Biolabs, Beverly, MA), producing a 546 nt probe that would be reduced to a 461 nt protected fragment. Control rat β-actin template was commercially supplied (Ambion, Austin, TX). Antisense GH RNA probes for fluorescence detection were generated by in vitro transcription using SP6 RNA polymerase (Life Technologies) to incorporate fluorescein-UTP (Molecular Probes, Eugene, OR). Antisense probes for chemiluminescence detection of Pit-1 and β-actin were generated by in vitro transcription using SP6 polymerase to incorporate biotinylated CTP (BIOTINscript Kit, Ambion). Probes were purified by gel electrophoresis and aliquots were stored at –80°C.

For analysis of GH mRNA expression, 1.5 μg MtT/S cell total RNA, 5 fmol ROX-labeled DNA sizing ladder (Applied Biosystems, Inc.), fluorescein-labeled GH probe, 5 μg yeast carrier RNA, and "Blue" co-precipitant (Ambion) were coprecipitated. For analysis of Pit-1 mRNA expression, 10 μg MtT/S cell total RNA, biotinylated Pit-1 probe, biotinylated actin probe, and 5 μg yeast carrier RNA were coprecipitated. RNA protection assays were then performed according to the manufacturer's directions (Ambion).

For fluorescence detection, protected RNA pellets were denatured at 95°C for 5 min in loading buffer containing 80% formamide, 10 m*M* EDTA, and 10 mg/mL blue dextran, then separated by electrophoresis through an 8 *M* urea, 5% polyacrylamide gel (LongRanger, FMC Bioproducts, Rockland, ME) and quantified using a 373A automated sequencing instrument and GeneScan 672 software (Applied Biosystems, Inc.). The ROX-labeled DNA ladder was used for calibration and varied less than 5% (SEM) between samples analyzed on a single gel.

For chemiluminescence detection of Pit-1 probe, protected RNA pellets were resuspended in loading buffer containing 95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 *M* EDTA, 0.025% SDS (Ambion), denatured at 95°C for 5 min, and separated by electrophoresis on an 8 *M* urea, 5% polyacrylamide gel (Life Technologies) for 45 min at 200 V. Samples were electrotransferred to a positively charged nylon membrane (Ambion) for 2 h at 300 mA, then nucleic acids were covalently bound by exposure to 120 mJ 254 n*M* UV light (Stratalinker 2400, Stratagene, La Jolla, CA). Chemiluminescence detection was performed using streptavidin-conjugated alkaline phosphatase and CDP-Star substrate (Ambion). Multiple exposures were recorded on BioMax Light Film (Kodak, Rochester, NY). Developed films were placed on a constant intensity transilluminator (FotoDyne, Madison, WI) and images were digitally captured using a CCD video camera (Hamamatsu C2400) and a Macintosh Quadra 950 computer (Apple Computer). Bands were identified and the integrated optical density (OD) of each band was compared using Gel Pro Analyzer 2.0.1 software (Media Cybernetics, Silver Spring, MD). For analysis of Pit-1 expression, the actin signal was used as a control for handling variation during the RPA and loading of the gel. Pit-1 intensity in each lane was expressed as the ratio of the integrated OD of the Pit-1 band divided by the actin band.

Western Blotting and Immunodetection

Approx 24 million MtT/S cells were pelleted and resuspended in 1 mL of lysis buffer (1% Triton X-100, 1% bovine hemoglobin, 1 m*M* iodoactetamide, 0.2 units/mL aprotinin, 1 m*M* phenylmethylsulfonyl fluoride, 14 m*M* sodium chloride, 0.025% sodium azide, 10 m*M* Tris-HCl, pH 8.0). After incubation for 1 h at 4°C, nuclei were removed from the lysate by centrifugation at 3,000*g* for 10 min. Particulates were removed from the supernatant by centrifugation at 10,000*g* for 30 min. The supernatant was then incubated with 20 μL of Protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C to remove nonspecifically bound proteins. Following removal of protein A-agarose by centrifugation, protein concentration was measured using a modified Bradford assay (Bio-Rad) and aliquots were stored at –80°C until use.

Immunoprecipitation was performed using 500 μg of protein lysate, 200 ng rabbit polyclonal serum specific for the IGF-R β-subunit or the insulin-R β-subunit (Santa Cruz Biotechnologies). Following incubation for 1.5 h at 4°C, 20 μL of protein A-agarose (Santa Cruz Biotechnologies) was added and incubation was continued for 1.5 h at 4°C with gentle shaking. Protein A-agarose complexes were collected by centrifugation and washed repeatedly. Specifically bound proteins were eluted from the protein A-agarose in SDS-sample loading buffer under reducing conditions.

Proteins were separated by electrophoresis through a 10% polyacrylamide gel containing SDS and then electrotransferred to a PVDF membrane (Micron Separations, Westboro, MA). Nonspecific protein binding was blocked using a solution of purified casein containing low alkaline phosphatase activity (Tropix, Bedford, MA). Membranes were then incubated with rabbit polyclonal serum specific for the IGF-R β subunit or the insulin-R β subunit (Santa Cruz Biotechnologies). Following extensive washing, the primary antibody was detected using anti-rabbit IgG serum coupled to alkaline phosphatase and CSPD chemiluminescent substrate (Tropix). Following exposure to membranes, BioMax light film was developed according to manufacturer's instructions (Kodak).

Statistical and Mathematical Analysis

Statistical differences in measurements from at least three cultures for each experimental condition were determined using ANOVA followed by Student–Newman–Keuls *post-hoc* test when warranted (SuperANOVA software, Abacus Concepts, Berkley, CA). Nonlinear regression was used to determine the best-fit parameters and 95% confidence intervals for the Hill equation and the first-order reaction rate equation (Mathematica 3.0, Wolfram Research, Champaign, IL) from the equations below:

Hill equation:

$$\text{response} = \frac{[\text{hormone}]^{n_H}}{[\text{hormone}]^{n_H} + \text{EC}_{50}{}^{n_H}} \quad (1)$$

First-order reaction rate equation:

$$\text{rna}_t = (\text{rna}_0)e^{-\frac{\ln 2t}{t_{1/2}}} \quad (2)$$

Acknowledgments

The authors wish to thank Ms. Carol Carlton for primer syntheses, Dr. Akira Arimura for technical advice on the MtT/S cell line, and Dr. Patrick Wojtkiewicz for helpful discussions concerning the use of the ABI 373A instrument. T.M.M. performed this work as part of her Senior Honor's Thesis project. This study was supported by a grant from the Tulane/Xavier Center for Bioenvironmental Research (DWSA project) and NSF CAREER Award IBN-9600805 to D.L.H.

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