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Growth hormone receptor C-terminal domains required for growth hormone-induced intracellular free Ca^{2+} oscillations and gene transcription

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ABSTRACT **The biological effects of growth hormone (GH) are initiated by its binding to the GH receptor (GHR) followed by association and activation of the tyrosine kinase JAK2. Here we report that GH can stimulate an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cells expressing wild-type GHRs and receptor mutants lacking up to 132 amino acids of the C terminus, whereas GHRs lacking a further 52 amino acids in the C terminus are unable to induce Ca^{2+} signaling. The GH-induced rise in $[Ca^{2+}]_i$ was dependent upon extracellular Ca^{2+} and the response consisted of GH-induced Ca^{2+} oscillations of varying frequency and amplitude. GH-induced transcription of the serine protease inhibitor 2.1 gene required the same C-terminal 52-amino acid domain of the receptor as for Ca^{2+} signaling. Mutation of the four proline residues in the conserved box 1 region of the GHR, which is responsible for binding and activation of JAK2 kinase, completely abolished GH-induced gene transcription but did not affect the GH-induced rise in $[Ca^{2+}]_i$. The Ca^{2+} channel blocker verapamil prevented GH-induced Ca^{2+} signaling as well as GH-induced gene transcription in cells expressing endogenous GHRs. These findings indicate that the GHR can initiate two independent signaling pathways, one requiring the box 1 region and the other requiring the region between amino acids 454 and 506, and suggest that both of these pathways are required for GH-induced gene transcription.**

Two distinct cytoplasmic regions of the growth hormone (GH) receptor (GHR) have been identified as being important for GHR signal transduction: (*i*) the proline-rich region (box 1), which is located close to the transmembrane domain and is highly conserved among the members of the cytokine/GHR family (1, 2), and (*ii*) the C-terminal 184 amino acids. When the four proline residues in box 1 are changed to alanines, GH does not stimulate JAK2 or mitogen-activated protein (MAP) kinase activity or protein synthesis when expressed in Chinese hamster ovary (CHO) cells (3). Furthermore, the transcriptional response of the serine protease inhibitor 2.1 (Spi 2.1) promoter to GH is abolished (1). Truncation of most of the C terminus, but leaving box 1 intact, retained the ability of the GHR to transmit a mitogenic signal (4). Thus box 1 appears to be important for all of the effects of GH studied so far, presumably due to the requirement of this region for the binding of the tyrosine kinase JAK2, which becomes activated and autophosphorylated in response to GH binding (5). Although necessary, box 1 is not sufficient for all of the effects of GH. At least two domains with distinct functions have been identified. CHO cells expressing a truncated GHR lacking the C-terminal 184 amino acids (GHR 1–454) have been found to respond to GH with increased JAK2 and MAP kinase activity and increased protein biosynthesis (6), while a transcriptional response to GH of the Spi 2.1 gene could not be observed (1). Similarly, we have shown that in insulin-producing RIN 5AH cells expressing the same truncated GHR, the effect of GH on insulin gene expression is abolished (2).

It has been reported that GH can cause a relatively slow (requiring minutes), but permanent, increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in freshly isolated adipocytes (7) and in IM-9 lymphocytes (8) and that prolactin (9) and erythropoietin (10) can increase intracellular Ca^{2+} by an inositol 1,4,5-trisphosphate-independent pathway. To study the Ca^{2+} signaling property of the GHR and identify the domain of the GHR responsible for this signaling, we studied the effect of GH on $[Ca^{2+}]_i$ in single cells by using video microscopy and digital imaging processing of fura-2 fluorescence.

MATERIALS AND METHODS

GHR Mutagenesis. The *Eco*RI/*Bam*HI fragment of the GHR expression plasmid pLM108 (11) was subcloned in pUC18 and served as template for PCRs to introduce mutations at various positions in the intracellular domain of the GHR. Two rounds of PCR were performed to introduce the stop codon mutations according to Herlitze and Koenen (12). After mutagenesis the entire region generated by PCR was sequenced in both directions and the *Eco*RI/*Bam*HI fragment was reintroduced into the expression plasmid.

Cell Culture and Transfection. CHO K1 cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in 5% CO_2/95% air. Cells were grown to 50% confluence in 60-mm dishes. Twenty-four hours prior to transfection, the cells were washed twice with minimum essential medium (MEM) and serum-free medium, consisting of a 1:1 mixture of MEM/Ham's F-12 medium supplemented with 10 μg of transferrin per ml, 80 milliunits of insulin per ml, 2.5 mM glutamine, and nonessential amino acids. CHO cells were transiently transfected by the calcium phosphate procedure with 3 μg of pCH110 (β-galactosidase expression vector from Pharmacia), 1.5 μg of the construct containing the bacterial chloramphenicol acetyltransferase (CAT) coding sequence linked to three copies of the sequence −147 to −103 of the Spi 2.1 promoter (13, 14), and 3 μg of the different mutated GHR plasmids. The cells were subjected to a glycerol shock and fresh GC3 medium was added with or without human GH (recombinant hGH provided by Novo-Nordisk,

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Abbreviations: GH, growth hormone; GHR, GH receptor; hGH, human GH; Spi 2.1, serine protease inhibitor 2.1; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CAT, chloramphenicol acetyltransferase.

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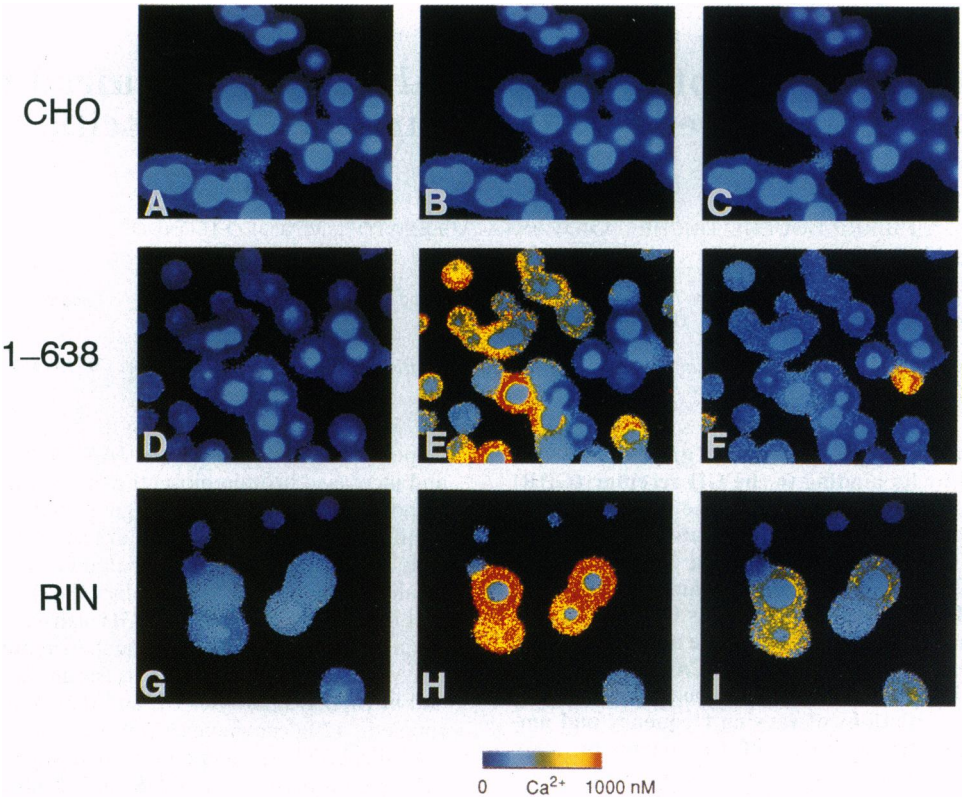

FIG. 1. GH-induced mobilization of Ca^{2+} in cells expressing GHRs. (*A–C*) Pseudo color ratio images of fura-2-labeled untransfected CHO cells. (*D–F*) CHO cells expressing the wild-type GHR 1–638. (*G–I*) Rat insulinoma RIN 5AH cells. (*A, D,* and *G*) Basal $[Ca^{2+}]_i$ before GH stimulation. (*B, E,* and *H*) Cells at maximal $[Ca^{2+}]_i$ after GH stimulation. (*C, F,* and *I*) Cells after recovery of the $[Ca^{2+}]_i$ spike. Colors represent ranges of free Ca^{2+} according to the color scale. (×300.)

Gentofte). Cells were scraped from the plate after 48 hr of culture and extracts were prepared by three consecutive freeze-thaw cycles followed by centrifugation at 15,000 × *g* for 10 min. Aliquots of the supernatant were normalized for β-galactosidase activity and then assayed for CAT activity. CHO cells were stably transfected with GHR cDNA by lipofection using Lipofectin (GIBCO). Ten micrograms of the GHR-encoding plasmid was mixed with 2 μg of plasmid encoding the neomycin-resistance gene and transfected according to the manufacturer's suggestion. After 4 hr of exposure to the Lipofectin/DNA complex, the cells were washed and cultured for an additional 24 hr. The cells were split by trypsin treatment and cultured in the presence of 1 mg of G418 per ml. After 6 days of culture, colonies were picked and analyzed for GH binding. At least four different CHO clones expressing each GHR mutant were isolated and characterized. The size of the truncated GHR expressed in CHO cells was analyzed by covalent cross-linking to ^{125}I-labeled hGH (^{125}I-hGH) followed by SDS gel electrophoresis and autoradiography. All truncated GHRs migrated according to their expected molecular weight.

CAT Assay. CAT assays were performed for 3 hr using 1 μCi of [^{14}C]chloramphenicol (1 Ci = 37 GBq; Amersham). The samples were subjected to thin-layer chromatography and exposed to PhosphorImager screens. CAT activity was determined by quantitation of the radioactive spots of [^{14}C]chloramphenicol and its acetylated forms using the IMAGEQUANT program.

$[Ca^{2+}]_i$ Measurements. Cells were cultured for 2 days on thin (0.7 mm) glass coverslips coated with poly(D-lysine). Cells were loaded with 10 μM fura-2 AM in the presence of the nonionic detergent pluronic F-127 (25%) at 37°C. After 30 min an equal volume of culture medium was added and the cells were incubated for an additional 30 min to complete deesterification of fura-2 AM. The buffer for fluorescence measurement was 0.145 M NaCl/5 mM KCl/1 mM Na_2HPO_4/1 mM $CaCl_2$/0.5 mM $MgSO_4$/5 mM glucose/20 mM Hepes, pH 7.4. In experiments performed in Ca^{2+}-free medium, $CaCl_2$ was replaced by 0.5 mM EGTA. A detailed description of the procedure for $[Ca^{2+}]_i$ measurements has been described (15). Briefly, the cells were placed in a stainless steel chamber on an inverted microscope. Cells were illuminated at 340 and 380 nm alternatively using interference filters mounted on a filter wheel and observed through a 510-nm emission filter. Six to eight fields at each wavelength were averaged in real time and the background (field without cells) was subtracted. The images were captured with a low-light-level intensified charge-coupled device video camera system. Calibration of the system for free Ca^{2+} concentrations was performed by imaging the cells of interest as described (15). The ratio images (R_{340}/R_{380}) were calculated from the division of the 340-nm image by the 380-nm image on a pixel-by-pixel basis and were converted to the Ca^{2+} concentration using the calibration curve.

Nuclear Run-Off Assay. RIN 5AH cells were cultured in 150-mm cell culture dishes to 70% confluence. Cells were stimulated for 2 hr in the absence or presence of 20 nM hGH, and verapamil (100 μg/ml) was added 30 min before stimulation. Nuclei were isolated and nuclear run-off was performed as described (16). Labeled RNA was isolated from nuclei by the RNAzol (Tel-Test, Friendswood, TX) method and hybridized to nitrocellulose filters to which linearized plasmid (5 μg) containing either insulin cDNA, cyclophilin cDNA, or plasmid (pGEM) alone was immobilized. Filters were hybridized for 72 hr at 65°C in 1 ml of buffer containing 5× SSC, 0.5% SDS, 10× Denhardt's solution, 0.1 mg of tRNA per ml, 10 mM EDTA, and 2.0 × 10^6 cpm of labeled RNA. Filters were washed at 65°C in 1× SSC for 60 min and subsequently washed in 0.2× SSC

for 30 min. Filters were exposed to PhosphorImager screens for 48 hr and the intensity of the bands was quantitated using the IMAGEQUANT program. Insulin transcription rates were calculated as RNA hybridized to the insulin cDNA relative to RNA hybridized to the cyclophilin cDNA.

RESULTS AND DISCUSSION

A GH-induced increase in $[Ca^{2+}]_i$ was observed in CHO cells transfected with the wild-type GHR cDNA and an identical Ca^{2+} response was observed in RIN 5AH cells, which express endogenous GHRs (Fig. 1). More than 200 nontransfected CHO cells were analyzed and in no case did we observe any effect of GH on $[Ca^{2+}]_i$. The Ca^{2+} response to GH was further characterized and found to be dependent upon extracellular Ca^{2+}, since no response was seen in Ca^{2+}-free medium (Fig. 2*D*), and addition of calcium by itself did not induce a rise in $[Ca^{2+}]_i$ (Fig. 2*A*). The effect was prevented by the noncompetitive Ca^{2+} channel blocker *l*-verapamil (Fig. 2*B*) and by the competitive Ca^{2+} channel blocker Co^{2+} (Fig. 2*C*). In addition, another Ca^{2+} channel blocker, nitrendipine at 10 μM, completely inhibited the GH-induced rise in $[Ca^{2+}]_i$ (P.B., unpublished data). These data indicate that GH activates a voltage-dependent L-type Ca^{2+} channel in the plasma membrane. The Ca^{2+} response was dose-dependent with a minimal effective GH dose of 50 ng/ml and a maximal response observed at 500 ng/ml (Fig. 2*E*). A complex Ca^{2+} response was observed in most cells, consisting of a delayed response varying from 10 to 120 s, with a typical response consisting of $[Ca^{2+}]_i$ oscillations of varying amplitude and frequency. Four typical recordings from single cells are shown in Fig. 2 *F–I*.

CHO cells expressing mutant forms of the GHR were then analyzed for GH-induced changes in $[Ca^{2+}]_i$. In CHO cells expressing the proline-to-alanine (P → A) mutated GHR, a Ca^{2+} response similar to that observed in cells expressing the

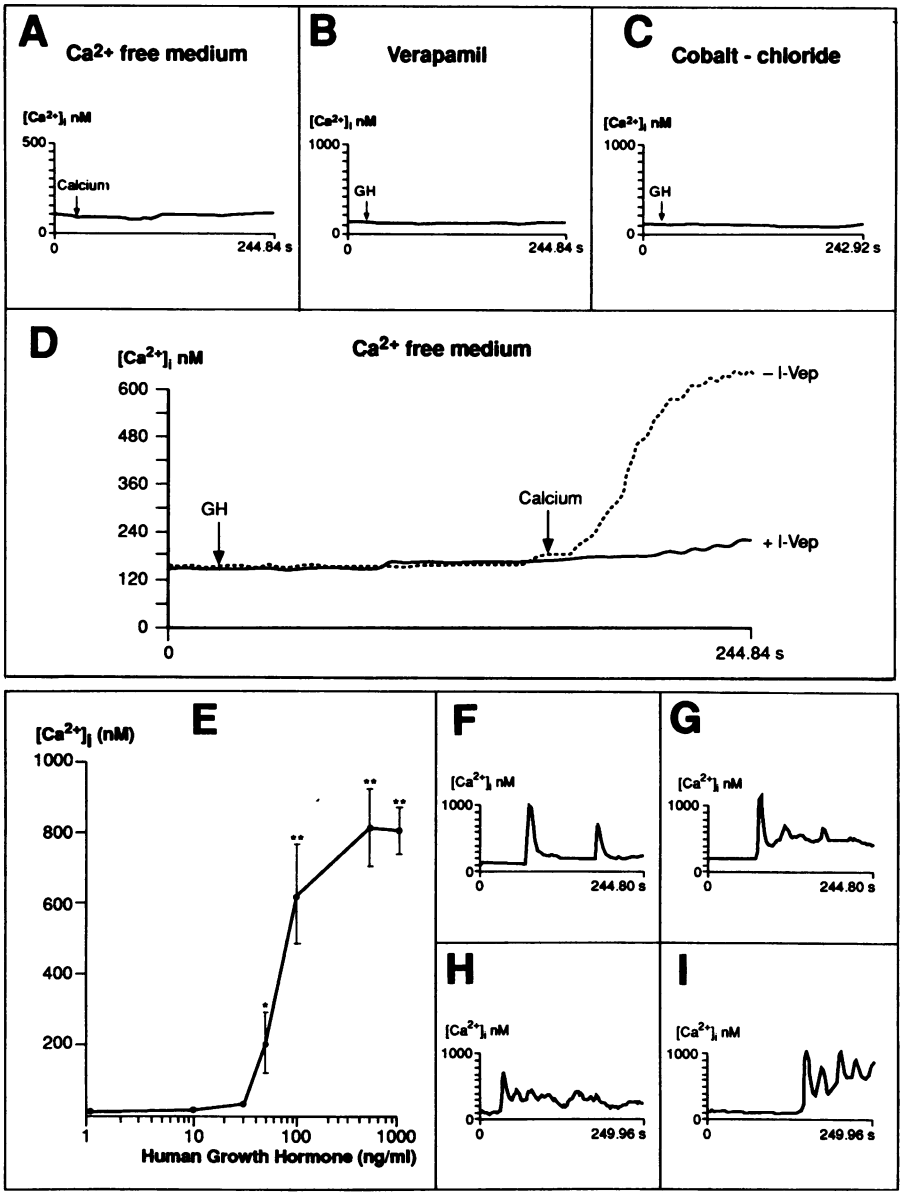

FIG. 2. Characterization of GH-induced $[Ca^{2+}]_i$ in CHO cells expressing GHRs. (*A*) Effect of addition of $CaCl_2$ on $[Ca^{2+}]_i$ in Ca^{2+}-free medium. (*B* and *C*) Effect of GH on $[Ca^{2+}]_i$ in the presence of 100 μM verapamil (*B*) or 30 mM $CoCl_2$ (*C*). (*D*) Effect of GH on $[Ca^{2+}]_i$ in CHO cells in Ca^{2+}-free medium in the presence of an inactive (+) or active (−) isomer of *l*-verapamil. $CaCl_2$ at a final concentration of 1 mM was added as indicated. (*E*) Dose–response curve of the effect of GH on $[Ca^{2+}]_i$. (*F–I*) Time course of GH-stimulated $[Ca^{2+}]_i$ from four representative CHO cells expressing the wild-type GHR. GH was added 10 s after the recording was initiated. Representative traces of 50 single cell recordings from 20 different experiments are shown. Cells were analyzed for 250 s with $[Ca^{2+}]_i$ recordings every 2 s.

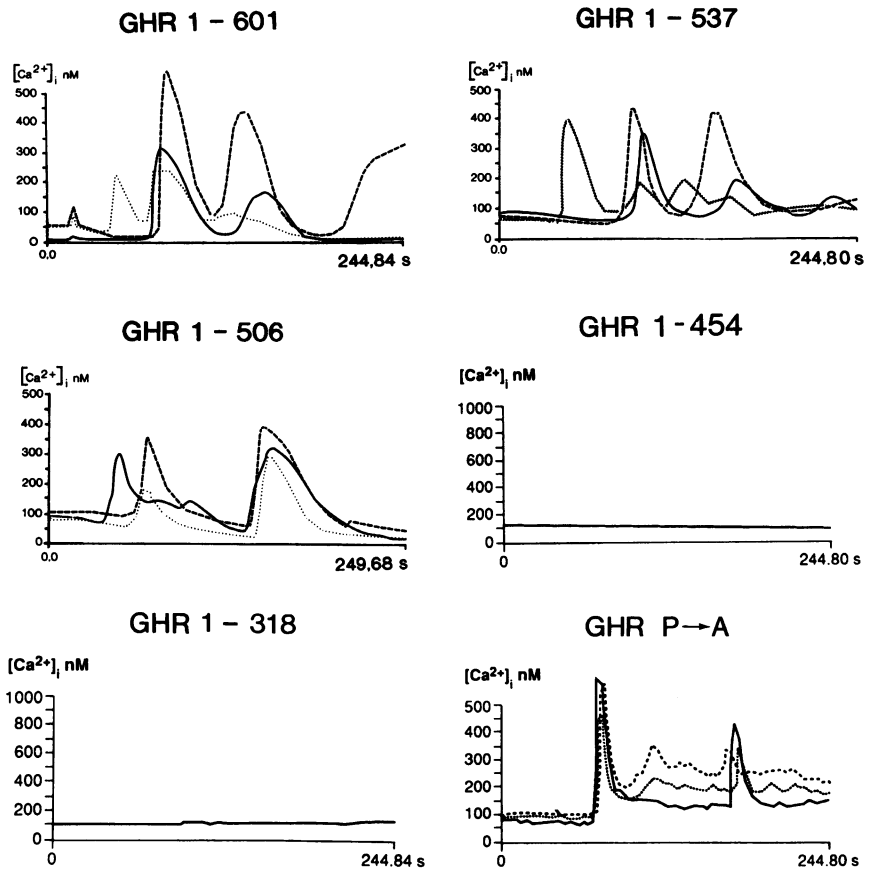

FIG. 3. Effect of GH on $[Ca^{2+}]_i$ in CHO cells expressing mutated GHRs. The numbering of the truncated GHRs refers to the first and last amino acid of the GHR. Four different CHO clones expressing the GHR P → A mutant and two different clones expressing each of the truncated GHRs were analyzed. The GHR P → A clone shown here expresses the GH P → A mutant receptors at a level similar to that of the CHO cells expressing the wild-type receptor. CHO cells were stimulated with 20 nM GH and $[Ca^{2+}]_i$ was measured as described in the text.

wild-type GHR was observed (Fig. 3). Four different clones of CHO cells expressing different levels of the P → A mutated GHR all responded similarly to GH. CHO cells expressing a GHR mutant in which the entire box 1 region from amino acid 297 to 311 was deleted also exhibited a normal Ca^{2+} response to GH (N.B. and P.B., unpublished data). In cells expressing the GHR 1–601, GHR 1–537, and GHR 1–506, a Ca^{2+} response was observed in >50 cells from 20 different experiments (Fig. 3), but no Ca^{2+} response was observed in cells expressing the GHR 1–454 or GHR 1–318 (Fig. 3), indicating the importance of the region between amino acids 454 and 506 in mediating the increase in $[Ca^{2+}]_i$. These experiments show that the box 1 region, and thus JAK2 activation, is not required for this calcium response, which is in accordance with the lack of inhibition of the Ca^{2+} response to GH in IM-9 cells by protein kinase inhibitors (8). For comparison we studied the effect of the same receptor mutations on the transcriptional activation of the Spi 2.1 promoter. When expressed transiently in CHO cells, the GHR truncated at position 601 was fully active in mediating GH-induced transcription. Truncations at positions 537 and 506 of the GHR resulted in a reduced activity; however, these receptors were still able to mediate a significant transcriptional response to GH. The GHR 1–454, however, showed no significant activity (Fig. 4). The GHR in which the four proline residues in the box 1 region were mutated to alanines was also inactive, as reported previously (1). These results indicate that in addition to box 1, a domain between amino acids 454 and 506 of the GHR is required for stimulation of Spi 2.1 transcription, and this domain coincides with the domain required for Ca^{2+} signaling.

To determine the functional significance of the GH-induced rise in $[Ca^{2+}]_i$, GH-stimulated insulin gene transcription was measured. Because of the long-term toxicity of verapamil, it was not possible to study the effects of this Ca^{2+} channel blocker on GH-induced transcription of the Spi 2.1 gene using the CAT assay since a minimum of 24 hr is required for GH induction. Instead, GH-stimulated insulin transcription was measured in RIN 5AH cells cultured in the absence or presence of verapamil. We showed previously that GH-stimulated insulin transcription requires the same 184 amino acids of the GHR as does stimulation of Spi 2.1 gene transcription (2). A 2.0-fold increase in insulin transcription was observed in control cells, whereas this effect was abolished in cells preincubated in the presence of verapamil (Fig. 5). This thus demonstrates that a rise in $[Ca^{2+}]_i$ is required for GH-stimulated insulin gene transcription. It has previously been reported that verapamil can blunt glucose-induced insulin gene transcription in normal islet cells (17).

It is thus apparent from this report that activation of JAK2 kinase and intracellular calcium are both essential for GH's ability to stimulate transcription of certain genes, but there is still very little known about the downstream signaling events that result in the various cellular effects of GH. The effect of GH on $[Ca^{2+}]_i$ is comparable to that observed with other growth factors and hormones (18). The basal $[Ca^{2+}]_i$ of 100–200 nM is within the normal range. The GH-stimulated $[Ca^{2+}]_i$ of up to 1 μM is comparable to that observed following stimulation with other hormones known to activate intracellular Ca^{2+} signaling mechanisms (19). The pathways involved in regulating $[Ca^{2+}]_i$ are numerous and include mobilization of intracellular stores by inositol 1,4,5-trisphosphate or cyclic ADP-ribose or by the opening of Ca^{2+} channels in the plasma membrane. Although it is apparent from these studies that the opening of voltage-dependent L-type Ca^{2+} channels is re-

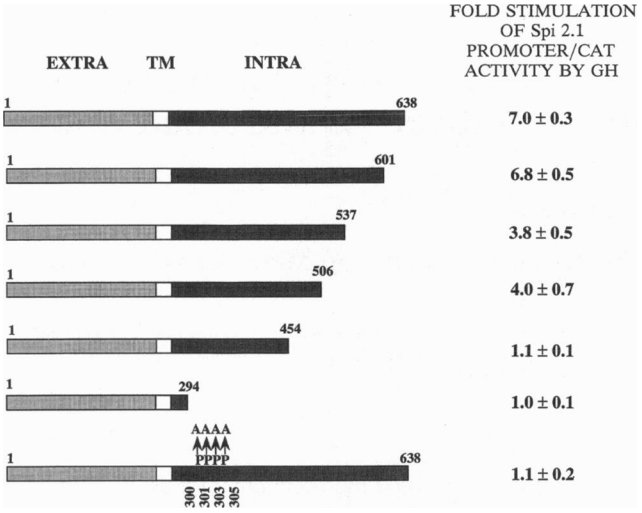

FIG. 4. GH-induced expression of Spi 2.1 promoter/CAT construct in CHO cells expressing mutated GHRs. CHO cells were transiently transfected by the calcium phosphate method using a β-galactosidase-encoding plasmid (as internal control), a plasmid encoding CAT under the transcriptional control of three copies of the sequence −147 to −103 from the Spi 2.1 gene in front of a minimal thymidine kinase promoter, and the various mutated GHR-encoding plasmids. After 48 hr of culture in the absence or presence of 20 nM hGH, CAT activity was measured and the fold induction by GH was calculated. Results are shown as the mean ± SD for four separate experiments.

quired for the GH-induced rise in $[Ca^{2+}]_i$, the involvement of intracellular Ca^{2+} stores in the maintenance of the observed $[Ca^{2+}]_i$ oscillations cannot be ruled out. Ca^{2+} signaling has been found to regulate gene expression in several cases (18). Ca^{2+} uptake is involved in glucose-induced insulin transcription (17) and transcription of the glucagon gene is directly induced by increases in $[Ca^{2+}]_i$ (20).

Activation of the JAK2 kinase by interferon γ has been shown to result in tyrosine phosphorylation and subsequent

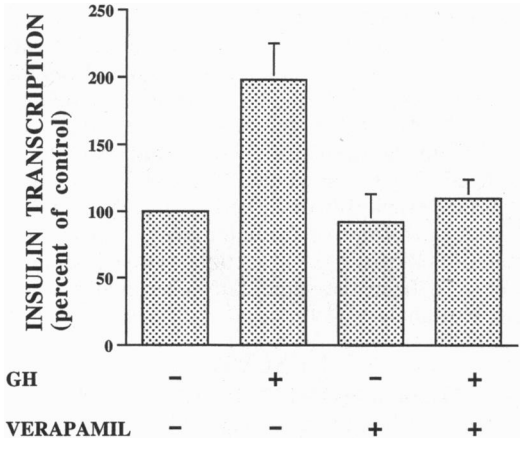

FIG. 5. Inhibition of GH-induced insulin transcription by verapamil. Insulin-producing RIN 5AH cells were stimulated with 20 nM GH for 2 hr. Verapamil at a concentration of 100 μg/ml was added 30 min before stimulation. Nuclear run-off analysis was performed to measure the rate of insulin transcription using cyclophilin transcription as an internal control. Nuclear run-off blots were quantitated after exposure to PhosphorImager screens by the IMAGEQUANT program. Results are shown as the mean ± SD for three separate experiments.

activation of the transcription factor STAT 1 (21, 22). A STAT protein is also phosphorylated and activated in response to GH, and this activation has been found to be associated with increased transcription of the *fos* gene (23). Another STAT family transcription factor, MGF, has been found to mediate prolactin-induced transcription of certain genes (24). Therefore this pathway may be activated only in the presence of box 1, which is present in several cytokine and growth factor receptors and which is also sufficient for a proliferative response to these factors. GH-induced transcription of cell-specific genes such as insulin and Spi 2.1 requires an additional signal mediated by another receptor domain. The present results suggest that this signal is Ca^{2+} and it can be speculated that a mechanism exists by which GH stimulates tyrosine phosphorylation of a STAT protein, which, in a Ca^{2+}-dependent manner, will form a functional transcription factor complex resulting in the stimulation of GH-responsive genes such as insulin and Spi 2.1.

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