

# Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Kofoed EM, Hwa V, Little B, et al. Growth Hormone Insensitivity Associated with a *STAT5b* Mutation. N Engl J Med 2003;349:1139-47.

## **Supplementary Appendix 1**

Recombinant human growth hormone was a generous gift from Genentech. Other reagents were recombinant human interferon- $\gamma$  (Roche), prolactin (Sigma), enhanced chemiluminescence system (Perkin-Elmer Life Sciences), and pcDNA3.1, a mammalian expression vector (Invitrogen Life Technologies).

### **Cell Culture**

Cell cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10 percent fetal-calf serum, 100 U of penicillin per milliliter, and 100  $\mu$ g of streptomycin per milliliter (Gibco–Invitrogen). For all experiments, cells between passages 6 and 12 were seeded at a density of 50,000 cells per milliliter, grown to approximately 85 percent confluency, and deprived of serum overnight before being incubated with growth hormone (500 ng per milliliter), prolactin (100 ng per milliliter), or interferon- $\gamma$  (5 ng per milliliter). COS-7 cells (American Type Culture Collection), maintained in Dulbecco's modified Eagle's medium with 10 percent fetal-calf serum, were transiently transfected with pcDNA3.1 carrying STAT5b complementary DNA (cDNA) with the use of the transection agent TransIT-LT1 (Mirus).

### **Western Immunoblotting**

Cells were solubilized in immunoprecipitation lysis buffer (phosphate-buffered saline, 1 percent Nonidet P-40, 0.5 percent sodium deoxycholate, 0.1 percent sodium dodecyl sulfate, 10 mg of phenylmethylsulfonyl fluoride per milliliter, 100 mM sodium orthovanadate, and a cocktail of protease inhibitors [Roche, catalogue no. 1836170]). Equal quantities of protein (Bio-Rad protein assay) in 0.1 M of dithiothreitol were fractionated according to size on 8 percent or 13 percent sodium dodecyl sulfate–polyacrylamide gels and electroblotted onto nitrocellulose membranes. Western blots were processed with the appropriate primary and secondary antibodies, according to the manufacturers' protocols, and evaluated with the use of an enhanced chemiluminescence system (ECL, Perkin-Elmer Life Sciences).

### **Analysis of Genomic and Complementary DNA**

Genomic DNA was obtained from whole blood, as previously described.<sup>1</sup> Total RNA was extracted from primary fibroblast cells (RNeasy, Qiagen) and used in Northern blotting analysis and real-time quantitative PCR (SuperScriptII Rnase H<sup>-</sup> reverse transcriptase kit, Invitrogen). Primers were designed for the amplification of cDNAs for growth hormone receptor,<sup>1,2</sup> STAT5b, insulin-like growth factor I (IGF-I), and 18S. Both strands of the growth hormone receptor cDNA and STAT5b cDNA encoding the open reading frame were sequenced. In addition, exon 15 of STAT5b was amplified from 100 samples of normal genomic DNA (Coriell) and sequenced.

### **Real-Time Quantitative PCR for IGF-I Transcripts**

Total RNA samples were extracted from duplicate cultures of primary fibroblast cells that had been exposed to 500 ng of human growth hormone per milliliter for up to 180 minutes. Two independent experiments were performed. The forward primer for IGF-I was 5'TGCCCGGCTAATTTTTTGG3', the reverse primer was 5'CATGCCTGTAATCCCAGCAA3', and the labeled probe was

FAM:5'56-FAM/TTTTACCAATGTTGGCCAGGTTGGACTCA/36TAMTph3'.

Real-time quantitative PCR for each sample was performed in triplicate, and the results were compared with those for 18S, which were used as a standard. Real-time quantitative PCR was carried out on a Prism Sequence Detector (model 7700, Applied Biosystems) with the use of TaqMan Universal PCR Master Mix (Roche Molecular Systems). Cycling conditions were as follows: 95°C for 15 seconds and 60°C for 1 minute, for a total of 40 cycles. The resultant data were analyzed with Sequence Detection Systems version 1.6 software (Perkin Elmer ABI) and Excel 98 (Microsoft).

### **Northern Analysis of IGF–Binding Protein 3**

Cells were grown to 80 percent confluency, deprived of serum for 24 hours, and incubated with growth hormone (500 ng per milliliter) for 24 and 48 hours. Total RNA was collected, as described above, and 5- $\mu$ g samples were fractionated according to size with the use of 1.2 percent agarose–formaldehyde-gel electrophoresis. Northern blots were probed with full-length IGF–binding protein 3 cDNA labeled with [ $\alpha$ P<sup>32</sup>]deoxy-cytidine triphosphate as previously described.<sup>3</sup>  $\beta$ -Actin transcripts were used as a standard for densitometric analyses of transcripts of IGF–binding protein 3.

### **References**

1. Woods KA, Fraser NC, Postel-Vinay MC, Savage MO, Clark AJ. A homozygous splice site mutation affecting the intracellular domain of the growth hormone (GH) receptor resulting in Laron syndrome with elevated GH-binding protein. *J Clin Endocrinol Metab* 1996;81:1686-90.
2. Woods KA, Dastot F, Preece MA, et al. Phenotype: genotype relationships in growth hormone insensitivity syndrome. *J Clin Endocrinol Metab* 1997;82:3529-35.
3. Tsubaki J, Hwa V, Twigg SM, Rosenfeld RG. Differential activation of the IGF binding protein-e promoter by butyrate in prostate cancer cells. *Endocrinology* 2002;143:1778-88.