The Regulation of Insulin-like Growth Factor 1 by Growth Hormone via Stat5b

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Abstract

The growth hormone (GH) and insulin-like factor 1 (IGF-1) growth axis is integral to growth, metabolism and tissue repair. This pathway involves binding of GH to the GH receptor and activation of the transcription factor, Stat5b, leading to nuclear translocation, interaction with DNA binding sites in chromatin and stimulation of IGF-1 transcription. Stat5b binding sites have been determined experimentally by previous research but are all found far from the IGF-1 promoters and the mechanism by which Stat5b binding initiates IGF-1 gene transcription is currently unknown. Since no system for studying this process currently exists, the goal of this project is to create a cell-based transcriptional test system by placing the rat IGF-1 locus in the form of a linearized bacterial artificial chromosome (BAC) into mammalian cell line. The transgene then will be studied for GH regulation, Stat5b binding to specific DNA sequences, and the long distance interactions between Stat5b binding sites and the IGF-1 gene promoters that are for activating IGF-1 gene transcription. If successful, this project will help to answer fundamental questions about the GH IGF-1 growth axis, more specifically the role of Stat5b.
**Introduction**

*Physiology of Growth Hormone.* Growth hormone (GH) is produced and secreted from the anterior pituitary gland.\(^1\) GH promotes growth throughout childhood and plays a role in metabolism and tissue repair throughout life.\(^2\) Having an over-abundance of circulating GH can cause disorders such as gigantism in children and acromegaly in adults.\(^3,4\) In contrast, GH deficiency in children causes severe growth defects, which can be treated with exogenous GH\(^5\). The GH-mediated growth axis is relatively well conserved among vertebrates and highly conserved among mammals.\(^6\)

*Growth Hormone Receptor and Signaling.* GH acts by binding and activating a transmembrane receptor. The growth hormone receptor (GHR) is a member of the class 1 cytokine family and it has an extracellular N-terminal domain, a single transmembrane α-helix, and a C-terminal intracellular segment.\(^7\) The intracellular part of the GHR is associated with the protein tyrosine kinase, Janus kinase 2 (JAK2). Upon binding of GH to the extracellular part of the GHR, the receptor undergoes a conformational change, which leads to activation of JAK2, and phosphorylation of multiple tyrosine residues within the intracellular part of the GHR by JAK2.\(^8\) These phospho-tyrosine residues function as docking sites for signaling proteins that mediate the biological effects of the activated GHR (Figure 1). As seen with defects in GH, mutations in the GHR also lead to growth deficiency and short stature, but these cannot be treated successfully with GH.\(^9\)

*Stats Regulate Growth Hormone–Mediated Gene Transcription.* Stats (Signal Transducers and Activators of Transcription) comprise a conserved family of seven
transcription factors in mammals (Stats 1, 2, 3, 4, 5a, 5b, and 6) that act downstream of

**Figure 1. Growth Hormone Signaling Pathway.** GH binds to the GHR activating the GHR. JAK2, covalently bound to the GHR becomes activated upon GHR activation and phosphorylates the Tyr residues in the intracellular part of the GHR. Latent proteins in the cytoplasm, including Stat5b, move to the receptors to become activated by phosphorylation. Once activated, Stat5b forms a dimer with other activated Stat5b proteins and enters the nucleus where it binds to the DNA to initiate transcription.
activated cytokine receptors, including the GHR. Stats are latent in the cytoplasm until
they are recruited to the binding sites that contain phosphorylated tyrosine residues of
activated cytokine receptors, where they undergo phosphorylation on a single critical
tyrosine residue (Figure 1). This leads to Stat activation through separate steps
including dissociation from the receptor, dimerization, and translocation into the nucleus.
In the nucleus, activated Stats bind to specific DNA sequences at regulatory sites on
target genes, setting into motion events that stimulate gene transcription. Stats all have a
general modular structure, which includes a coiled-coil region, a DNA binding domain, a
SH2 domain, and a transactivation domain (TAD) (Figure 2). The DNA binding domain is the region of the protein which allows the Stat to
bind to the DNA. The region of the protein which allows the Stats to form dimers is the
SH2 domain. The TAD is the part of the protein which recognizes specific DNA
sequences and allows it to interact with other proteins that initiate transcription. Among
the several Stats that are activated by the GHR and JAK2, Stat5b appears to be the
critical growth regulating transcription factor\textsuperscript{12} as inactivating mutations also have been found in individuals with growth deficiency and short stature.\textsuperscript{13}

\textit{Growth Hormone Signaling and IGF-I.} Insulin-like growth factor-I (IGF-I) is a 70-amino acid circulating peptide that also is critical for growth during childhood, and plays an important role in tissue maintenance and repair in the adult.\textsuperscript{14} The production of IGF-I is under control of GH, and rare defects in IGF-I also cause severe growth failure in children.\textsuperscript{15} GH promotes synthesis of IGF-I by stimulating IGF-I gene expression through induction of IGF-I gene transcription,\textsuperscript{16} a process also mediated by Stat5b. The focus of this research project will be on how GH via Stat5b activates IGF-I gene transcription.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{igf1.png}
\caption{IGF-I gene structure. The rat IGF-I gene locus is shown with its two promoters and its 6 exons. The human gene is organized similarly. The postulated Stat5b binding sites are the red dots.}
\end{figure}

\textit{IGF-I gene organization and regulation.} In humans, the \textit{IGF-I} gene is located on chromosome 12 and spans more than 80 kb of genomic DNA. The gene consists of 6 exons and 5 introns.\textsuperscript{17} IGF-I genes in other mammals are organized similarly.\textsuperscript{7} IGF-I gene (Figure 3) transcription is controlled by two adjacent promoters\textsuperscript{18}, each with its own leader exon. The transcribed IGF-I RNA undergoes alternative splicing and differential polyadenylation in the nucleus to yield multiple IGF-I mRNA species. After transport into the cytoplasm the IGF-I mRNAs are translated into two distinct IGF-I precursor proteins, which are processed into identical 70-amino acid mature IGF-I protein.\textsuperscript{19}
Main questions and project overview. The mechanism by which Stat5b regulates the transcription of IGF-1 is not clear. It is known Stat5b binds to specific DNA sequences within the IGF-1 locus (Figure 3) and that Stat5b is required for IGF-1 gene transcription, but the mechanisms connecting Stat5b binding to sites\textsuperscript{20,21} far from the IGF-1 promoters which initiate promoter activity are unknown. Also unknown is exactly how the DNA must be conformed in order to bring the Stat5b site close enough to the promoter to initiate transcription. The goal of this project will be to help answer these questions by creating a model cell-based system to study activation of the IGF-1 gene by Stat5b. This will involve placing the rat IGF-1 locus into mammalian cells in culture and studying the transgene for regulation by GH, for binding of Stat5b to specific recognition sites, and for potential interaction between each Stat5b site and the IGF-1 gene promoters. Subsequently, the Stat5b binding sites will be removed in order to determine which are most important in mediating GH stimulated transcriptional activation. We could then begin to answer questions about the mechanism of GH regulation of IGF-1 transcription and the role Stat5b plays.

Materials and Methods

BAC Preparation. A bacterial artificial chromosome (BAC) containing a fragment of rat genomic DNA spanning the \textit{IGF-1} gene in the plasmid pTARBAC2.1 was obtained from Children’s Hospital Oakland Research Institute (CH230-113M6). For digestion analysis and PCR, the DNA was isolated using a modified alkaline lysis protocol (from Colin Powers, Fruh Lab, OHSU, and buffers from Qiagen, Valencia, CA): A 10 mL culture was grown overnight in a bacterial incubator-shaker at 37°C and 275 rpm. The bacterial pellet was precipitated after centrifugation at 3000 rpm at 4°C for 20 min. The pellet was
suspended in Qiagen buffer P1 (250 µl; 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A). Qiagen buffers P2 (300 µl; 200 mM NaOH, 1% SDS) and P3 (300 µl; 3.0 M KCH₂COOH, pH 5.5) were added followed by addition of 500 µl of a phenol-chloroform-isooamyl alcohol solution (ratio of 25:24:1). Samples were rotated in a Labquake (Thermoscientific, Ashville, NC) at 30 rpm for 10 min at 4°C and separated by centrifugation at 14,000 rpm for 15 min. The aqueous layer was transferred to new centrifuge tube, followed by addition of 3M Na Acetate (100 µl) and 95% ethanol (1000 µl), and incubation at -80°C overnight. Tubes were removed from -80°C and centrifuged at 14,000 rpm for 20 min. The pellet was gently suspended in 500 µl of 70% ethanol and centrifuged at 14,000 rpm for 10 min twice. The BAC DNA was dissolved in 50 µl double deionized water (ddH₂O). As a quality control, 2 µg of each BAC DNA sample was digested with EcoRI, or BamHI. The digestion patterns are used for references of BAC presence in miniprep solutions. For obtaining BAC DNA for Cos7 transfection, the BAC was isolated using the Nucleobond BAC 100 DNA isolation kit (Machery-Nagel, Bethlehem, PA). This DNA (1-5 µg) was then transformed by electroporation (2.5kV, 25 µF and 200Ω) using the BIORAD Gene Pulser II (BIORAD, Hercules, CA) into a strain of recombination competent E.coli (SW102), plated on chloramphenicol plates. DNA from colonies was isolated by the modified alkaline lysis protocol, and analyzed by digestion and PCR. Glycerol stocks were made and stored at -80°C for future use.

**Neomycin/Kanamycin cassette design.** The Neomycin/Kanamycin (Neo/Kan) cassette was designed by cloning 500 bp homology arms into neomycin and ampicillin containing plasmid PL452. The two 500 bp homology arms, termed A and B, were synthesized by PCR (see Appendix A) using primers specific to two 500 bp regions on the BAC located
3’ of the IGF-1 gene. The region of Arm A was located at relative position +84171 to +86669 and B was located at +84672 to +85220 region, relative to the +1 transcription start site of the IGF-1 gene. Arm A was added to PL452 at EcoRI and NheI restriction enzyme sites, and B was added at BamHI and NotI sites. For each step the digested DNA was combined (3:1 molar ratio insert to vector) with 1X T4 ligase buffer and T4 DNA ligase (Fermentas Glen Burnie, MD) for 5 min at 20°C. Competent E. coli were transformed with ligations and kept on ice for 30 min. The E. coli was “heat- shocked” at 42°C for 30 sec, antibiotic free media was added and E. coli were incubated for 1 hr at 37°C with 250 rpm shaking. After incubation, the E. coli were plated on agar plates containing LB media + 25 µg/ml ampicillin or kanamycin, and incubated overnight at 37°C. Selected colonies were screened using the synthetic primers specified for Arm A. The plasmid DNA was isolated from positive clones after overnight culture in 10 mL of LB containing media + 25 µg/ml ampicillin at 275 rpm and 37°C. The plasmid DNA was digested with EcoRI and NheI or BamHI and NotI to confirm Arm A or Arm B, respectively. Final confirmation of Arms A and B in PL452 was established by DNA sequencing using vector specific primers. The PL452 plasmid containing the homology arms A and B was used as a template for long-range PCR using the modified forward primer of Arm A, adjusted 50 bp towards Neo, and the reverse modified primer of Arm B, also moved 50 bp towards Neo, to isolate the cassette followed by a DpnI treatment and cleaning with the Qiagen PCR Cleaning Kit. The PCR product was electroporated (2.5kV, 25 µF and 200Ω; see Appendix B) into E.coli as described above. Clones were screened by PCR for the presence of the cassette in the BAC.
Neomycin containing BAC transformation into Cos7 cells. Passage 7 Cos7 cells were grown in antibiotic free DMEM (Life Technologies, Grand Island, NY) until 65-70% confluency. Cos7 cells (1×10^7 cells) were electroporated (300V, 950 µF, 200 Ω; See appendix B) with 1000 ng of linearized DNA of Neo containing BAC. Cells were plated and grown in antibiotic free DMEM for 48 hr at 37°C. DMEM was replaced with DMEM + G418 (200 µg/mL) and media was changed every 48 hrs for 2-3 weeks. Cells surviving G418 treatment will be harvested and screened for the integration of the Rat IGF-I locus.

Figure 4. Schematic of Recombination. The homology arms are cloned into the PL452 plasmid. The cassette containing the homology arms is separated from the plasmid using the modified primers in (B) with long range PCR and transformed by electroporation into the E.coli containing BAC.

Results/Discussion
Project Overview. There is no system currently in place to study the mechanism of the GH regulation of IGF-1 via Stat5b. The goal of this project (Figure 5) is to create a model system in which this mechanism can be closely studied. This will involve transfecting the about 250 kb of the IGF-1 gene locus into a cell line and stabilizing it by making it essential to the cell. To do this, antibiotic resistance to Geneticin (G418) will be added to

![Diagram](develop-a-model-system-of-gh-regulation-of-igf-1-transcription)

- **Develop a model system of GH regulation of IGF-1 transcription**
- **Study mechanisms of GH regulation of IGF-1 gene expression through Stat5b**

Figure 5. Project overview. These are the main objectives in the project.

the gene prior to transfection. The selection pressure will be applied throughout the experiment to ensure only those cells containing the integrated transgene are able to survive the treatment. Once this model system is created, it will allow for a system in which experiments to be conducted to study the Stat5b mediated GH regulation of IGF-1 transcription.

Addition of selectable marker. Before the BAC can be placed into cell culture, it must be modified so cells which integrated the BAC into their genomic DNA can be distinguished from the cells which did not. The most efficient way to do this is by addition of a
selectable marker which is a gene that allows for resistance against antibiotics, in this case *Neomycin*, which is complete with its own prokaryotic and eukaryotic promoters. These promoters allow the antibiotic selection to be used in both *E. coli* and Cos7 cell culture. Inserting this gene into the BAC was achieved by recombination based gene engineering, or recombineering (Figure 6). Recombineering is a method using modified *E. coli* strains which contain genes, *exo*, *bet* and *gam* from λ-phage technology. These genes are under the control of a temperature sensitive promoter which when activated, produces proteins that allow foreign DNA to be inserted by homology into another DNA fragment. Homology arms, small DNA fragments made from the BAC from the desired location for the insert of the gene, are inserted into either side of the gene creating a

![Diagram of recombineering process](image-url)

**Figure 6. Recombineering.** The process of recombineering involves selecting the location of the gene insertion, amplifying the region by PCR and inserting them around the gene of interest. By adding the isolated cassette to the BAC in the special strain of *E. coli* which contains the genes required for foreign DNA insertion, the new gene can be inserted.
cassette (Figure 7). The cassette is then recombined into the BAC providing antibiotic resistance to Geneticin (G418) when in cell culture and Neomycin with in *E. coli*. When this BAC is modified in *E. coli*, the clones can be grown on neomycin selection plates and only the ones containing this marker will survive the treatment. The process is similar in cell culture because only the cells which integrate the BAC into their genome will survive the G418 treatment. The successful integration of the gene be confirmed after the selection step by screening for the gene by PCR. The PCR screen in Figure 8B shows the successful integration of the neomycin gene into the BAC using primers oriented as in Figure 8A.

Figure 7. Neomycin cassette. The neomycin cassette is made up of its two promoters, one that is functional in prokaryotic organisms (pGK) and the other functional in eukaryotic organisms (pGH). Surrounding the gene are the A and B homology arms (Blue).
**Figure 8. PCR Screen for Presence of Neo Cassette in the BAC.** (A) The schematic for the positions of the screening primers for Arms A and B. (B) PCR screen for the presence of Arm A (Labeled 1), Arm B (Labeled 2), BAC (positioned at -10kb from exon 1 on the Igfl gene) (Labeled 3) and Neo cassette (positioned within the Neo gene) (Labeled 4). M is the 1kb ladder.

**BAC Transformation into the Cos7 cell line.** After successfully integrating the resistance marker to the DNA, the BAC DNA was linearized and transfected into Cos7 cells. The cells will then be grown in G418 for 2-3 weeks, harvested and screened by PCR for the integration of the IGF-1 gene. If the integration of the IGF-1 gene can be confirmed, we can then begin to take the next steps to confirm the system works by stimulating it with GH. If the system works, IGF-1 will be produced and we will be able to start designing experiments to study the mechanism of GH regulated IGF-1 transcription. From this system we can study the amount of IGF-1 protein produced compared to normal levels of IGF-1 protein production when stimulated by GH. The structure and the amount of
mRNA can also be studied. We can compare the sequences of the mRNA produced to that of the known structure of the two predominant IGF-1 mRNA transcripts. The postulated long distance interactions between the Stat5b binding sites and the promoters can be studied using methods known as chromosome conformation capture (3C) or circular chromosome conformation capture (4C). Both methods involve stimulating the cells with GH for various amounts of time and then essentially freezing the DNA in its conformation during transcription. The DNA can then be digested, ligated, and tested for interaction. The 4C method is more of a screening tool in which any DNA interacting with the promoter will be identified. The 3C method is more of a confirmation tool that is positive only if there are interactions between the known regions of DNA, in this case the promoter and Stat5b enhancer regions.

**Future Implications.** This model system is vital to answering questions about the mechanism by which Stat5b mediated GH regulation of IGF-1 transcription functions. Once this system can be established further modification of this system may help to answer different questions. A long term goal of this project would be isolate the key Stat5b site(s) needed to promote transcription. These sites could be isolated using a method known as chromosome immunoprecipitation (ChIP) which shows protein-DNA interactions. After confirming the sites which Stat5b binds, the all the sites can be either removed completely, all can be removed except one, or only one site can be removed at a time. After the levels of IGF-1 transcription have been established by the native system, this data can be used to compare to other systems in which the Stat5b binding sites are removed or transclocated. There could be no change in the transcription levels indicating the removed site may not be the key site or the amount of transcription may be reduced
indicating it may be a part of multiple sites required for transcription. It could also completely stop the production of IGF-1 protein meaning that it may be a critical site. If the important site(s) can be narrowed down, the structure of both the protein and the mRNA can be compared to the native model system. It is possible the removal of the sites may have an effect on the structure of the IGF-1 proteins and mRNA. The long distance interactions between the site and the promoter can also examined to see if the interaction still occurs, or if the site being removed or translocated has an effect on how the DNA conforms upon GH stimulation. As has been described, if this system can be established, many questions about this mechanism can be studied.

**Conclusion**

The GH regulation IGF-1 transcription is a process integral to growth, tissue repair, and metabolism. The exact mechanism by which Stat5b mediates this process is still not clear though it has been established is critical to initiate transcription by previous research. Since there is no model system in place to study this mechanism, one will be created which will provide the material necessary to closely examine Stat5b. A BAC containing the IGF-1 gene will be modified with an antibiotic selection marker, linearized, and inserted into a cell line. This cell line will select for the integrated gene using antibiotic selection and a stable cell line will be created. If this model system can be established, questions about the mechanism of Stat5b interaction with the DNA can be examined to help elucidate the pathway. Longer term goals include removing the putative Stat5b binding sites from the BAC to help isolate the most important sites. If successful, this project can help to answer larger questions about the role of Stat5b in GH/IGF-1 growth axis in humans.


Appendices

Appendix A. Polymerase Chain Reaction (PCR). The purpose of PCR is to create an abundance of a small stretch (500 bp-4kb for genomic DNA\(^1\)) of DNA. In this case, it was used in two ways: To make template for downstream applications and to confirm the presence of insert. To make template, primers must be designed that surround the desired region on DNA to be replicated. For our purposes, there was also restriction sites added so cloning in the fragment was possible. CH0113M6 BAC was used as a template for the arms to be cloned into the PL452 vector. PCR was also used as a confirmation tool as shown in Figure 5B. The only possible way for a signal to be produced is if the template is present exactly as shown. If there is no template then there will be no signal.

Appendix B. Electroporation. The technique of electroporation is used in homologous recombination and for integrating large fragments of DNA. In this case, it was used for recombining the Neo cassette into recombinant E. coli and for transfecting the Cos7 cells with the linearized Neo containing M6 BAC.

Appendix A. The Schematic of Polymerase Chain Reaction (PCR)

Original DNA Template

Step 1. Melting of the DNA. The DNA strands are separated from each other (94°C)

Step 2. Annealing of the primers. The primers align with the separated DNA strands (55-60°C; This temperature depends on the GC content of the primers)

Step 3. Extension of the primers. The dNTP’s are aligned along the separated DNA strands replicating the template (72°C)

Repeat cycle 25-30x

New synthesized DNA
Appendix B. Electroporation

E. coli + Neo Cassette

Linearized CH0113-M6+Neo BAC

2.5 kV
25 μF
200 Ω

Shock

300 V
950 μF
200 Ω

E. coli + CH0113-M6+Neo BAC

into the genome

Cos7 Cell

CH0113-M6+Neo BAC

integrated into the genome