Genetic differences in the IGF-I gene among inbred strains of mice with different serum IGF-I levels

K Iida, C J Rosen1, C Ackert-Bicknell1 and M O Thorner

Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Virginia, Charlottesville, Virginia, USA
1The Jackson Laboratory, Bar Harbor, Maine, USA

(Requests for offprints should be addressed to M O Thorner, Box 800466, Department of Internal Medicine, University of Virginia, Charlottesville, Virginia 22908, USA; Email: mot@virginia.edu)

Abstract

There is significant heterogeneity in serum IGF-I concentrations among normal healthy individuals across all ages and among inbred strains of mice. C3H/HeJ (C3H) mice have 30% higher serum IGF-I concentrations over a lifetime than C57BL/6J (B6), even though body size and length are identical. The underlying mechanism for this disparity remains unknown although several possibilities exist including altered GH secretion, resistance to GH action, or impaired IGF-I secretion from the liver or peripheral tissues. To study this further, we evaluated mRNA levels of pituitary GH, and of IGF-I, GH receptor (GHR) and acid-labile subunit (ALS) in liver and skeletal muscle of male C3H and B6 strains. mRNA levels of hepatic IGF-I paralleled serum IGF-I levels, whereas pituitary GH mRNA expression was significantly lower in C3H than B6. In addition, reduced hepatic mRNA levels of ALS and GHR in B6 suggests hepatic GH resistance in B6. In contrast, mRNA levels of IGF-I and GHR in skeletal muscle were not different between B6 and C3H. There was a single sequence repeat polymorphism (SSR) in the promoter region of both GHR and IGF-I genes in mice; the SSR in the IGF-I gene was significantly different between the two strains. The SSR in the IGF-I gene corresponds to the E2F binding site, which is critical for regulating IGF-I gene expression. These results suggest that the SSR in the promoter region of the IGF-I gene may be partially responsible for differences in serum IGF-I levels between B6 and C3H strains.

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Introduction

Insulin-like growth factor-I (IGF-I) is a ubiquitous peptide that has multiple physiological functions acting through endocrine, paracrine and autocrine pathways (D’Ercole et al. 1984, Stewart & Rotwein 1996). Several lines of evidence suggest that genetic determinants may regulate human serum IGF-I concentrations. Studies in healthy twin children indicated marked genetic influences on serum IGF-I levels (Kao et al. 1994, Harrela et al. 1996). There are also epidemiological reports that serum IGF-I levels vary greatly even between normal individuals and between ethnic groups regardless of their serum growth hormone (GH) levels (Platz et al. 1999). Recent evidence suggests that the serum IGF-I phenotype is a complex heritable trait with multiple genetic determinants, some of which may be GH-independent (Rosen et al. 2000). The association of serum IGF-I concentrations with glucose tolerance (Sandhu et al. 2002), bone mineral density (BMD) (Langlois et al. 1998) or risk of cancers (Giovannucci 1999) makes identification of genetic determinants of serum IGF-I levels important.

In humans, there are several cases of inherited syndromes with low serum IGF-I levels, which include congenital GH deficiency (Procter et al. 1998), bioinactive GH (Takahashi et al. 1996, 1997), mutations of the protein-coding region of the GH receptor (GHR) gene (Laron 2004), a mutation of signal transducer and activator of transcription (STAT) 5b protein (Kofoed et al. 2003), IGF-I gene deletion (Woods et al. 1996), and inactivation of the acid-labile subunit (ALS) gene (Domene et al. 2004). These cases suggest that genes encoding the molecules involved in the GH–IGF-I axis play a critical role in determining serum IGF-I levels. Furthermore, polymorphisms in the promoter region of the human IGF-I gene are associated with variation of serum IGF-I levels (Rosen et al. 1998).

In mice, serum IGF-I levels vary considerably between strains. C3H/HeJ (hereafter C3H) mice have 30% higher serum IGF-I concentrations than C57BL/6J (hereafter B6) mice although there are no differences in body length and weight between the two strains (Rosen et al. 1997). C3H mice with higher serum IGF-I levels have increased BMD compared with B6. B6 mice have higher glycosylated
hemoglobin levels (Kaku et al. 1989) and demonstrated poor glucose tolerance with a lower insulin response to i.p.
glucose administration compared with C3H (Kayo et al.
2000). B6 mice are also susceptible to atherosclerosis,
whereas C3H are resistant (Ishida et al. 1991, Shi et al.
2000). On the other hand, primary cultured hepatocytes
from C3H are susceptible to hepatocarcinogenesis whereas
those from B6 are resistant (Yoshie et al. 1998). Although
these two inbred mice have been extensively studied as
models of genetic influences on metabolism of glucose,
lipid and bone or carcinogenesis, it is still unknown
whether genetic differences between strains may exist in
the genes involved in the GH–IGF-I axis. We hypothe-
sized that characteristics of B6 might be explained in part
by reduced GH action: either low GH synthesis in the
pituitary gland or GH resistance in peripheral tissues.
Alternatively, a GH-independent factor such as alteration
of IGF-I gene per se may account for low serum IGF-I
levels in B6.

In this study, we first characterized mRNA expression
of pituitary GH in B6 and C3H using quantitative
real-time RT-PCR to elucidate whether low GH syn-
thesis is responsible for low serum IGF-I levels in B6. We
then examined mRNA levels of hepatic IGF-I, ALS and
GHRK to clarify whether B6 is characterized by reduced
hepatic GH action. We also quantified IGF-I as well as
GHRK mRNA levels in skeletal muscle to elucidate the
tissue-specificity in GH action between the two strains.
Finally, we examined sequence differences in the pro-
moter and protein-coding region of GHR, as well as IGF-I
genes between B6 and C3H mice and identified single
sequence repeat polymorphisms (SSRs).

Materials and Methods

Animals

Male C57BL/6J (B6) and C3H/HeJ (C3H) inbred mice
of 8 weeks of age were used in this experiment. The
mouse colonies were maintained and housed in an animal
care facility at the Jackson Laboratory (Bar Harbor, ME,
USA). They were maintained under conditions of 14 h
light:10 h darkness and an ambient temperature of
20-2°C. Water (acidified with HCl, pH 2.8–3.2) and an
autoclaved NIH31 pellet diet (18% protein, 6% fat, 1.27%
Ca, 0.92% P, trace mineral and vitamin fortified; Purina,
Madison, WI, USA) were freely available. The body
weights of B6 and C3H were not different, as described
previously (Rosen et al. 1997). We previously reported
that serum IGF-I concentrations as measured by a specific
RIA were 30% higher in C3H than B6 at 4 and 8 weeks
in both male and female mice (Rosen et al. 1997). The
mice were killed at 8 weeks and pituitary gland, liver,
skeletal muscle (quadriceps femoris) from B6 and C3H
(n=10/group) were collected and flash-frozen in liquid
nitrogen and then stored at -80°C for subsequent
mRNA analysis. Use of mice in this research project was
reviewed and approved by the Institutional Animal Care
and Use Committee of the Jackson Laboratory.

Serum IGF-I measurements

Serum IGF-I in mouse sera was measured by RIA after
removal of IGF-binding proteins as described previously
(Rosen et al. 1997).

Total RNA preparations

The RNA extraction was performed using Tri Reagent
(Molecular Research Center, Inc., Cincinnati, OH, USA)
followed by an RNeasy Mini Kit (Qiagen, Inc., Valencia,
CA, USA) with DNase treatment according to the
manufacturers’ instructions. The quantity of extracted
total RNA was determined using the RiboGreen RNA
Quantitation Kit (Molecular Probes, Eugene, OR, USA)
with a Genios multi-detection reader (Phenix Research
Product, Hayward, CA, USA).

Real-time RT-PCR

Primers for quantification of murine GH, GHR, IGF-I
and ALS mRNAs are listed in Table 1. The reaction
of RT-PCR, was described previously (Iida et al. 2004a).
Briefly, 1 µg total RNA from the liver and skeletal muscle
and 100 ng total RNA from the pituitary gland were
reverse transcribed in a total volume of 10 µl using the
iScript cDNA Synthesis Kit (Bio Rad Laboratories,
Hercules, CA, USA) according to the manufacturer’s
instruction. A 1:20 dilution of the resultant cDNA was
prepared and 4 µl of this template were used in the
real-time PCR protocol. The iCycler iQ Real-Time PCR
detection system (Bio-Rad Laboratories) was used for
sample cDNA quantification. The PCR was performed in
a total volume of 20 µl and SYBR Green I (Molecular
Probes) was used for detection of PCR products.

Quantification

We constructed the plasmid including the PCR product of
the target gene and the DNA concentration of constructed
plasmid was determined as described previously (Iida et al.
2004a). These plasmids were used for standards of quan-
tification. A standard curve was generated by amplifying
serial dilutions of a known quantity of plasmid DNA. The
standards and cDNA samples were then co-amplified in
the same reaction plate. The standard curve displayed a
linear relationship between cycle threshold (Ct) values
and the logarithm of input plasmid copy number. The
amount of product in a particular sample is determined by

interpolation from a standard curve of Ct values generated from the plasmid dilution series. The 18S ribosomal RNA (rRNA) was used as a housekeeping gene.

Gene analysis

Total DNA was isolated from liver or kidney of mice using a DNeasy Tissue Kit (Qiagen) according to the manufacturer’s instruction. The P1 promoter region and the GH-responsive element in intron 2 of the IGF-I gene (HS 7) (Woelfle et al. 2003), and the L1 and L2 promoters including the downstream region of the GHR gene were amplified with primer pairs listed in Table 1. For analysis of the coding region of GH, GHR and IGF-I gene, RT-PCR was performed with total RNA from pituitary or liver as a template. Platinum Pfx DNA polymerase (Clontech, Palo Alto, CA, USA) was used for PCR to obtain results with high-fidelity. Sequence analysis was performed using a DNA sequencer (Model 3100; Applied Biosystems, Foster, CA, USA).

Statistical analysis

Results of gene expression were corrected for 18S rRNA amplified and are presented as means ± s.e. Differences were determined by an unpaired t-test and coefficients of linear correlation (Pearson’s) were calculated using GraphPad Prism version 4.00 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Differences of allelic frequencies were determined by a chi-square test using GraphPad Quickcalcs software (GraphPad Software Inc). P<0.05 was considered significant.

Table 1

<table>
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<th>Sequence (5′→3′)</th>
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<td>GGTGGACCCAGGCTGGGCGTCT</td>
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<tr>
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<tr>
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Polymorphisms of IGF-I gene in mouse strains · K IIDA and others
Results

In the pituitary gland, GH mRNA levels of C3H were 66% of those of B6 mice ($P=0.02; n=10$ for each strain) (Fig. 1). Serum IGF-I levels in C3H were 609.3 ± 16.0 ng/ml whereas those in B6 were 455 ± 14.0 ng/ml ($P<0.001$). Hepatic IGF-I mRNA levels of C3H were 146% of those of B6 ($P=0.01; n=10$ for each strain). Hepatic ALS mRNA levels of C3H were 249% of B6 ($P<0.005; n=10$ for each strain). Hepatic GHR mRNA levels of C3H were 148% of those of B6 ($P=0.03; n=10$ for each strain) (Fig. 2).

The correlations between hepatic IGF-I and pituitary GH, between hepatic IGF-I and ALS and between hepatic IGF-I and GHR mRNA in the two strains were examined. There was an inverse correlation between pituitary GH and hepatic IGF-I mRNA (Fig. 3a). There was a significant linear correlation between hepatic IGF-I and ALS mRNA (Fig. 3b) in both strains. There was also a significant correlation between hepatic IGF-I and GHR mRNA (Fig. 3c), consistent with our previous report (Iida et al. 2004a). In contrast in skeletal muscle, IGF-I mRNA levels of C3H were comparable with those of B6 ($n=10$ for each strain). GHR mRNA levels of C3H were comparable with those of B6 as well ($n=10$ for each strain). There was no significant correlation between IGF-I and GHR mRNA in skeletal muscle (Fig. 4).

From the results of gene expression of hepatic IGF-I as well as ALS mRNA, we conclude that B6 is characterized by reduced GH action in liver despite having a greater expression of pituitary GH mRNA compared with C3H. Reduced mRNA levels of GHR in B6 suggest that reduced hepatic GH action in B6 may be at least in part due to the down-regulation of the expression of GHR gene. Therefore, we next examined the genetic differences in the GHR as well as IGF-I genes including promoters, GH-responsive elements (HS 7), and protein-coding regions between the two strains. We also examined the protein-coding region of the GH gene to exclude the possibility of bioinactive GH in B6.

We identified the GT SSR (GT-SSR) downstream of the L2 promoter of the GHR gene (Fig. 5, Table 2). In addition, we identified the polyguanine repeat polymorphisms (poly G-SSR) in the P1 promoter region of the IGF-I gene (Fig. 5, Table 2). The GT-SSR in the GHR gene, however, was not significantly different between B6 and C3H. On the other hand, the length of the poly G-SSR of the IGF-I gene was significantly longer in B6 than in C3H ($P<0.0001$) (Table 2). No polymorphisms
were identified in either the protein-coding region or L1 promoter region of the GHR gene. No differences were observed in the protein-coding region or HS 7 of the IGF-I gene. Bioinactive GH in B6 was excluded as well.

**Discussion**

We identified the genetic differences in the promoter region of IGF-I gene between B6 and C3H mice. In addition, we believe that this is the first report in terms of characterization of gene expressions involved in the GH–IGF-I axis between B6 and C3H. We also demonstrated that there were no differences of IGF-I and GHR mRNAs in skeletal muscle between the two strains in contrast to the observations in liver, suggesting the tissue-specific alteration of IGF-I and GHR gene expression in liver in mouse strains.

We first showed that B6 mice had significantly greater GH mRNA levels in the pituitary gland than observed in the C3H strain (Fig. 1), whereas no difference was observed in single-point serum GH level (data not shown). We suggest that the greater GH mRNA in B6 compared
with C3H pituitary reflects greater overall GH secretion in B6, because single-point serum measurement might not provide an accurate reflection of GH synthesis or release due to the pulsatile pattern of GH secretion. In this study, serum IGF-I level as well as hepatic IGF-I mRNA was significantly higher in C3H than B6. As discussed below this probably reflects that hepatic IGF-I synthesis largely determines serum IGF-I; serum IGF-I is primarily responsible for negative feedback on GH synthesis and secretion. Thus, our results suggest that the phenotype of B6 is not due to insufficient GH synthesis.

Next, we examined the hepatic IGF-I, ALS and GHR mRNAs to clarify whether the reduced serum IGF-I level in B6 is GH-dependent or due to specific impairment of hepatic IGF-I synthesis per se. We used IGF-I and ALS mRNA levels as a marker of GH action, since both genes possess a STAT 5 binding element in the regulatory region and gene expression in both cases is regulated transcriptionally by GH (Ooi et al. 1998, Woelfle et al. 2003). The expression level of GHR is a critical factor in determining GH sensitivity in tissues. Our results demonstrated that IGF-I, ALS and GHR mRNA levels in liver were significantly reduced in B6 compared with C3H. Furthermore, we identified a significant correlation between hepatic IGF-I and ALS and between hepatic IGF-I and GHR mRNA levels in individual mice of both B6 and C3H strains. Taken together, our results suggest that low serum IGF-I levels in B6 compared with C3H are, at least in part, due to reduced GH action in the liver.

GH, rather than IGF-I, is the major regulator of liver growth (Behringer et al. 1990). The previous report demonstrated that GH-deficient little mice with the background of C3H showed comparable susceptibility to hepatocarcinogenesis compared with those with a B6 background; these results contrast with those in wild-type, GH-deficient, C3H mice, which were significantly more susceptible compared with B6 (Bugni et al. 2001). These results support reduced hepatic GH action in B6 mice.

The finding of reduced GHR mRNA levels in B6 led us to assume that low hepatic GHR expression might be responsible for hepatic GH resistance in B6. We therefore examined the sequence differences of GHR as well as IGF-I genes including the protein-coding, the GH-responsive element, and the 5’ flanking regions after excluding the possibility of bioinactive GH in B6 by sequencing. We detected no sequence differences between B6 and C3H in the protein-coding region of the GHR. We identified a GT-SSR downstream of the L2 promoter region of the GHR gene (Table 2), which can act as an enhancer of transcription. There are several distinct 5’ untranslated region (exon 1) variants in mouse (L1–L5) as well as human GHR (V1–V9), and expression of each transcript is regulated by a different promoter (Edens & Talamantes 1998, Menon et al. 2001). Several polymorphisms in the promoter region of GHR gene were reported in species including humans (Hadjiyannakis et al. 2001), Angus steers (Hale et al. 2000) and Angus cattle (Ge et al. 2003), although hitherto there has been no report in mice. Interestingly, the polymorphisms in humans located at 87 bp upstream of V9 (Hadjiyannakis et al. 2001), homologous to mouse L3, correspond to those we found in mice. On the other hand, the polymorphisms identified in Angus steers or Angus cattle are located at the liver-specific promoter region, which is homologous to

**Table 2** Allelic frequencies of the single sequence repeat (SSR) in the GHR and IGF-I genes

<table>
<thead>
<tr>
<th>GT-SSR in GHR gene</th>
<th>Poly G-SSR in IGF-I gene***</th>
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<tr>
<td>Length (bp)</td>
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<td>68</td>
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</table>

***P<0.0001 between B6 and C3H.
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In the GHR gene in mice, there were no significant differences between B6 and C3H in this region. Although we identified GT-SSR in the GHR gene in mice, there were no significant differences between B6 and C3H. Therefore, it is unlikely that this GT-SSR is the exclusive determinant of hepatic GHR expression between B6 and C3H. It is unclear why the hepatic expression of GHR in B6 was reduced compared with C3H. Since we searched a limited region of the whole GHR gene, it is possible that other unidentified polymorphisms may play a role in regulating hepatic GHR expression. Our previous report showed that there is a significant correlation between hepatic IGF-I and GHR mRNA levels in bovine GH transgenic and GH-deficient lit/lit mice (Iida et al. 2004a), consistent with the results in this study.

In the IGF-I gene, we also identified poly G-SSR in the P1 promoter region. Importantly, we could not detect any differences either in the GT-SSR in the P1 promoter, where polymorphisms were identified in humans (Rosen et al. 1998), or in the HS 7 region, which Woele et al. (2003) pointed out as a critical regulatory region of IGF-I gene expression. On the other hand, the length of poly G-SSR was significantly longer in B6 than in C3H. This region corresponds to an E2F binding sequence. Li & Baserga (1996) reported that the IGF-I promoter is constitutively active when the E2F binding sequence in the IGF-I promoter is mutated, suggesting the importance of this region for regulating IGF-I gene expression.

Besides the polymorphisms identified in the GHR and IGF-I genes, however, it is possible that other genetic factors may affect the expression of hepatic IGF-I and GHR as well. In this regard, we previously identified chromosomal locations or quantitative trait loci (QTL) linked to serum IGF-I concentrations. The QTL that mapped to the largest effect on circulating IGF-I concentration is on chromosome (Chr) 6, and the second largest one is on Chr 10, where murine IGF-I gene is located, whereas murine GH and GHR genes are mapped on Chr 11 and 15 respectively (Rosen et al. 2000).

Another relevant finding in this study is that expression patterns of IGF-I and GHR mRNAs differed between skeletal muscle and liver (Fig. 4). There was no correlation between IGF-I and GHR mRNA in skeletal muscle in contrast to the results in liver. This tissue-specific regulation of IGF-I and GHR mRNA is consistent with our recent report using GH-deficient lit/lit mice and bovine GH transgenic mice (Iida et al. 2004b). Our current results of tissue-specific regulation of IGF-I mRNA suggest that serum IGF-I concentration is not necessarily a surrogate of local IGF-I mRNA expression in all tissues. Serum IGF-I reflects primarily hepatic IGF-I synthesis. It acts to provide negative feedback to GH secretion.

LID or LI-IGF-I−/− mice are a mouse model of selective knockout of hepatic IGF-I synthesis; these animals have a low serum IGF-I concentration (Sjogren et al. 1999, Yakar et al. 1999). Although LID and LI-IGF-I−/− mice show extremely low serum IGF-I levels, they have no resistance to GH in peripheral tissues and have high serum GH levels (Sjogren et al. 1999, Yakar et al. 1999, Wallenius et al. 2001). Furthermore, only a modest growth retardation was observed in LID mice despite their having an extremely low level of serum IGF-I (Yakar et al. 2002), suggesting that the serum IGF-I level is not a critical determinant of body growth. Therefore, it is not surprising that the body weight and height of B6 and C3H are identical, despite having different levels of serum IGF-I (Rosen et al. 1997). Interestingly, LID mice demonstrated muscle insulin resistance with high serum insulin concentration (Yakar et al. 2001), hypercholesterolemia (Sjogren et al. 2001) and reduced cortical periosteal bone growth (Sjogren et al. 2002). Taken together, serum IGF-I level may play a relevant role in determining normal glucose, lipid and bone metabolisms rather than body growth, consistent with the phenotype between B6 and C3H. Inhibition of GH action improved insulin sensitivity in LID mice (Yakar et al. 2004), suggesting that increased GHR signaling in skeletal muscle plays a critical role in reducing insulin action. Our results in this study showed that B6 demonstrated relatively low GHR mRNA levels in liver, but the opposite trend was observed in skeletal muscle. The relatively increased GH action in skeletal muscle may be one of the explanations for the susceptibility to the development of diabetes in B6 compared with C3H (Kaku et al. 1989). Normal GHR and IGF-I receptor signaling in skeletal muscle may also explain the normal growth in B6 compared with C3H. The mechanism underlying the differences of gene expression of IGF-I and GHR between liver and skeletal muscle despite having the identical polymorphism between tissues is unclear. The use of different promoters of genes may regulate gene expression in a tissue-specific manner (Moffat et al. 2000). Alternatively, tissue-specific factors may contribute to the regulation of IGF-I and/or GHR expression. In this regard, Gowri et al. (2003) demonstrated that a repressome complex at the L2 promoter of the GHR gene played a role in differential regulation of GHR between liver and kidney in insulinopenic diabetes resulting in down-regulation of GHR in liver but up-regulation of GHR in kidney. Further studies are required to clarify the tissue-specific regulation of GHR as well as IGF-I between liver and skeletal muscle.

In conclusion, we have elucidated the profile of gene expressions of IGF-I and GHR in two inbred strains of mice with different serum IGF-I levels. The genetic differences between these inbred strains affected not only IGF-I but also GHR mRNA levels in a tissue-specific manner. We also identified the sequence differences in the 5′ flanking region of IGF-I gene in mice. Our results may in part account for the phenotypic differences between C3H and B6 mice.
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