PROBING IUGR INDUCED ALTERATIONS IN ACID LABILE SUBUNIT

by

Lisa Joss-Moore

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SUPERVISORY COMMITTEE APPROVAL

of a thesis submitted by

Lisa Joss-Moore

This thesis has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

12-4-07

Chair: Thunder Jalili

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Date

Thunder Jalili
Chair: Supervisory Committee

Approved for the Major Department

E.Wayne Askew
Chair/Dean

Approved for the Graduate Council

David S. Chapman
Dean of The Graduate School
ABSTRACT

Intrauterine growth restriction (IUGR) is associated with a multitude of complications including an increased risk of developing adult insulin resistance. The mechanism behind this increased risk likely stems from epigenetic modifications. Acid labile subunit (ALS) is responsible for regulating the tissue bioavailability of IGF-1. IGF-1 bioavailability is vital in regulating myriad of functions, including adult glucose regulation.

In this study western blotting was used to compare serum levels of ALS in IUGR rats to that of control animals. In addition, STAT5 occupancy of GH-response elements found in association with ALS and IGF-1 was used as a means of developing a chromatin immunoprecipitation (ChIP) assay to facilitate further exploration into the interactions between transcription factors and chromatin. Serum ALS protein levels were significantly increased in female rats at day 21 and at day 120. ALS serum protein levels in males were not significantly different in control and IUGR animals. Development of a fast ChIP assay gave some preliminary insight into differential occupancy of GH-response elements, by STAT5, where it appears that in female IUGR tissue there is a shift in occupancy toward the ALS regulatory element and away from one of the IGF-1 regulatory elements. In conclusion, it appears that female IUGR animals have greater levels of serum ALS and a potential shift in STAT5 binding of the ALS and IGF-1 response elements in favor of ALS expression. The improved ChIP assay reported here should help facilitate further investigation into the transcriptional regulation of ALS, IGF-1 and other important genes.
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INTRODUCTION

Intrauterine growth restriction (IUGR) refers to a condition where a fetus fails to achieve its genetically predetermined size and as such defines a functional categorization of fetal growth. IUGR can result from many of the complications of pregnancy including uteroplacental insufficiency (UPI) and maternal malnutrition.

It has now become well accepted that the low birth weight associated with IUGR predisposes these infants to adult mortalities including coronary heart disease, stroke, hypertension and type 2 diabetes (reviewed in 1). Several human studies have also suggested a gender bias in the effect of IUGR and intrauterine undernutrition on the development of these morbidities, with males tending to be more severely affected (2).

The connection between low birth weight and adult mortalities has been described by Barker in his “Fetal Origins Hypothesis” (3, 4). Barker describes the origins of these adult diseases in terms of fetal developmental “plasticity” or the ability of the fetal genotype to respond, and adapt to, different environmental or nutritional conditions during development.

This adaptation is likely induced by changes in the expression of key genes that favor fetal survival under adverse conditions. The long term implications of this “reprogramming” appear to outlast the resolution of growth in the neonate and altered gene expression may persist into adult life. One of the means by which this altered gene expression is induced is through epigenetic modifications in chromatin structure (5-7).
There are several ways that epigenetic modifications can be made to chromatin including methylation of DNA and post-translational modification of histone tails. It is through these modifications that the interaction of DNA with the cellular transcription machinery is altered resulting in changes in gene expression and organism phenotype.

Alterations in gene expression induced by IUGR have been the subject of considerable research over the past decade, with the majority of work utilizing a rat model of IUGR. The development of a rat model of UPI induced IUGR has facilitated exploration into cellular changes occurring in the fetus as a result of UPI. Bilateral uterine artery ligation in the rat, first described by Wigglesworth (8), produces a model of IUGR that mimics late-gestation placental insufficiency in humans. The IUGR pups are asymmetrically growth restricted and are approximately 25% smaller than control animals (9). In addition, the IUGR rat pups develop insulin resistance and type 2 diabetes by midlife (10).

To date, altered gene expression patterns in IUGR rats have been reported in several tissues, some in a gender specific manner. The expression of a number of genes involved in metabolic regulation is altered in the liver of IUGR fetuses and neonates (11-13). Alterations in the regulation of p53 in the kidney and mitochondrial gene expression in the brain have been reported (14, 15). There has also been significant progress made in defining the epigenetic mechanisms associated with IUGR, including increased site-specific acetylation of lysines on histone-3 (H3) and a decrease in nuclear protein levels, and activity of, histone deacetylase 1 (HDAC1) in the IUGR rat liver (5). In the IUGR rat brain, CpG island methylation and H3 acetylation are altered as well as the expression of HDAC1 and DNA methyltransferase 1 and methyl-CpG binding protein 2 (6). Liver
glucocorticoid receptors, involved with chromatin modifications at the transcriptional level, are also altered in the IUGR rat (16).

The search continues for pathways and genes affected by epigenetic modifications that may be important in the development of adult morbidities such as type 2 diabetes. One candidate is insulin-like growth factor 1 (IGF-1) and its associated proteins. IGF-1 is important in linear growth, cell proliferation, cell differentiation and apoptosis. IGF-1 exhibits both endocrine and paracrine/autocrine effects. The endocrine effects of IGF-1 are mediated by circulating IGF-1 which can activate the IGF-1 receptor and, at high concentrations, can also activate the insulin receptor.

The majority of circulating IGF-1 is sequestered into a 150 kDa heterotrimeric complex consisting of IGF-1, IGF binding protein-3 (IGFBP-3) and acid labile subunit (ALS). This ternary complex is unable to cross the vascular endothelium, thereby trapping IGF-1 in the circulation and allowing high concentrations to be achieved. Once incorporated into the heterotrimeric complex, the half life of IGF-1 increases from approximately 10 minutes to 12 hours (17). Serum also contains complexes of approximately 50 kDa consisting of IGF-1 and one of the remaining IGFBP’s (-1, -2, -4 or -6); these complexes are able to cross the vascular endothelium.

Despite the potentially important role of ALS in regulating the bioavailability of circulating IGF-1, it has not received a great deal of attention until recently. ALS is an 85 kDa glycoprotein produced primarily, and possibly exclusively, in the liver (18). Synthesis of ALS appears to coincide with birth and increasing levels of growth hormone (GH) and GH receptor (GHR). Serum concentrations of ALS then continue to increase approximately 5 fold to puberty and then remain steady for the duration of life-span. Expression of ALS is
one of the last events in the development of the IGF-1 system, resulting in the transition of IGF-1 from primarily binary complexes before birth to increasing trimeric complex after birth. Serum levels of ALS in rats and humans are also modified under a variety of conditions including undernutrition, diabetes and cirrhosis (reviewed in 19).

The gene for ALS was first described in the mouse in 1996 (20), with the rat (21), human (22) and sheep (28) characterizations following. Transcription in all species involves a TATA-less promoter and mRNA of approximately 2.2 kb. Transcriptional regulation is achieved via a GH-response element in the promoter region that binds signal transducer and activators of transcription-5 (STAT5). GH mediates transcription via the JAK-STAT signal transduction pathway (reviewed in 19).

A GH-response element binding STAT5 has also been shown to be a component of the complicated and poorly understood, transcriptional regulation of IGF-1. Recent work has shown that STAT5 is a key intermediate between activated GH receptor and IGF-1 transcription (23, 24). It has been shown that IGF-1 expression is regulated by two tandem STAT5 binding sequences that both bind STAT5 and act as cis-regulatory elements capable of mediating GH activated IGF-1 transcription (25). One of the tandem sites is found in Intron 2 of the IGF-1 gene while the other element is found ~75 kb 5’ of the transcription start site in the human gene and is conserved in rat, mouse and bovine DNA (23).

Information generated by mouse models involving knockout and tissue specific deletions of key genes in the GH/IGF-1 axis supports a hypothesis by which IGF-1 bioavailability may be important in the regulation of glucose homeostasis. The reduction of liver IGF-1 production in the face of relatively normal serum ALS, as seen in the LID
mouse, results in less bioavailable IGF-1, increased GH levels and insulin resistance (26, 27). On the other hand when ALS is not present, or when its expression is down regulated, IGF-1 is more bioavailable and glucose regulation is less severely effected. This can be seen in the ALS knockout (ALSKO), combined ALSKO and LID mice and in post hepatectomy mice (28-30).

In order to understand the mechanisms by which a fetal trauma can impart long lasting alterations in cellular gene expression it is necessary to unravel the complex messages of the epigenetic code. The rat model of IUGR provides a system in which gene expression, protein levels and the epigenetic code of control animals can be compared with those who have suffered UPI and subsequent IUGR.

To goal of this work is to further the understanding of the manner in which IUGR alters the regulation of expression of key components of the IGF-1/GH axis and thus the mechanisms by which IUGR sets the stage for adult onset impaired glucose regulation. Analysis of serum ALS in the adolescent and adult control and IUGR rats should provide a significant piece of the puzzle. In addition, development of assays that can be used to examine binding of transcription factors to DNA, such as STAT5 binding to GH-regulatory elements, will facilitate further exploration in the field.
METHODOLOGY

Animals

All procedures were approved by the University of Utah Animal Care Committee and are in accordance with the American Physiological Society's guiding principles (31). The surgical procedures have been described previously (7, 32). Briefly, on day 19 of gestation, pregnant Sprague-Dawley rats were anesthetized with intraperitoneal xylazine (8 mg/kg) and ketamine (40 mg/kg), and both inferior uterine arteries ligated (IUGR). Control animals underwent identical anesthetic procedures (CON). After recovery rats were given ad libitum access to food and water.

Day 0 (d0) pups were delivered by caesarian section at term, 2.5 days after the bilateral uterine artery ligation. Genotyping of d0 pups, using PCR of the spermatogenic gene (Sby) from the Y chromosome (33), was performed to determine sex. Twenty-one day old (d21) animals were sacrificed after a four hour separation from their dams. Animals raised to 120 days were given ad libitum access to food and water and sacrificed without fasting. For all animals, livers were quickly removed after sacrificing, flash frozen in liquid nitrogen, and stored in −80°C. Serum harvested at d21 and d120 was also frozen in liquid nitrogen.
Western Blotting

Western Blotting was used to determine relative levels of ALS protein in the serum of IUGR rats compared to CON rats. Serum samples obtained from nonfasted rats at d21 and d120 were thawed and centrifuged briefly to separate fat. After removal of the fat layer, serum was diluted fifteen fold in distilled water and samples were then assayed in triplicate for protein concentration using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Standard western blotting procedure for used with the following specifics. Gel samples containing equal amounts of total protein (5 µg) were then prepared from the diluted serum and run on 10% bis-tris XT Criterion gels (Bio-Rad Laboratories, Hercules, CA) in MOPS buffer at 190 V for 1 hour. Proteins were then transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in 5% milk-TBST for 1 hour before addition of primary antibody (anti-mouse ALS (AF1436), R&D Systems, Minneapolis, MN) at a final concentration of 0.1 µg/ml, also in 5% milk-TBST, and incubated for 1 hour at room temperature (RT). Owing to the high sequence homology between mouse and rat ALS proteins (92%) and the lack of availability of anti-rat ALS, anti-mouse ALS was used. After thorough washing, membranes were incubated in anti-goat secondary antibody conjugated with horseradish peroxidase, (Santa Cruz Biotechnology, Santa Cruz, CA), in 5% milk, for an additional hour. The inclusion of 5% milk-TBST with the secondary antibody was necessary to reduce significant background resulting from high levels of serum proteins. After washing, signal was determined using enhanced chemiluminescence (ECL) according to the manufacturer’s instructions.
**ChIP Assay**

Investigation of the relative STAT5 occupancy of the GH-response elements of ALS and IGF-1 was used to optimize a chromatin immunoprecipitation (ChIP) assay for future analysis of transcription factor-chromatin interactions with multiple samples.

A revised ChIP protocol, based primarily on the methods of Farnham and Bomstztyk (34, 35), was developed. Briefly, whole d0 livers from male and female CON and IUGR animals, treated simultaneously, were thawed, weighed and chopped on ice. Tissue weights ranged from 190-280 mg. Chromatin isolation was performed as follows; tissue was fixed in 1% formaldehyde in PBS for 10 minutes at room temperature, after which the reaction was stopped by the addition of glycine to a final concentration of 125 mM. Samples were then centrifuged, washed twice with cold PBS and resuspended in PBS with added protease inhibitor cocktail (PIC) at manufacture's recommended concentration (Complete, Mini. Roche, Indianapolis, IN). At this point tissue was disaggregated manually and centrifuged. Resulting cell pellets were resuspended in lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Igepal) with PIC and dounced on ice using a tight pestle at least 20 times. After centrifuging, pelleted nuclei were resuspended in nuclei lysis buffer (50 mM Tris-Cl, pH 8.1, 10 mM EDTA, 1% SDS) with PIC, incubated for 20 minutes on ice and split into aliquots of approximately 200 μl (in 1.5 ml tubes) for sonication. Due to the importance of the sonication step in producing relatively uniform chromatin fragments while maintaining protein integrity for subsequent immuoprecipitation (IP), significant optimization was performed. Sonication was performed on ice using a Fisher Scientific Model 100 sonicator with microtip attachment (Fisher Scientific, Pittsburgh, PA). Each
sample was pulsed 10 times with the micotip placed near the base of the tube and retuned to ice, this was repeated a total of 10 times. Foaming of samples was avoided as much as possible. After centrifuging, chromatin containing supernatants for each of the 4 samples were frozen at -80°C.

IP of each chromatin sample was performed on half of the original material. For each sample a mock IP was prepared in parallel. Input chromatin, 53-77 μg, was made up to 500 μl with IP buffer (150 mM NaCl, 50mM Tris-Cl, pH 7.5, 5 mM EDTA, 0.5% NP-40 and 1.0% Triton X-100). STAT5 (C-17) antibody (Santa Cruz Biotechnology) was added to sample tubes (final concentration 1.6 μg/ml) and 3 μl of anti-IgG (concentration not specified by manufacturer) (Cell Signaling Technology, Beverly, MA) added to mock tubes. Treatment of all sample and mock tubes was identical from this point on. Tubes were placed in a sonicating water bath at 4°C for 30 minutes to facilitate antibody binding. After centrifugation, the cleared chromatin supernatant was added to 20 μl of prewashed IgG-sepheroxide beads (MP Biomedicals, Aurora, OH) and rotated at 4°C for 45 minutes. After washing beads 6 times in IP buffer, 100 μL of a 10% slurry of Chelex 100 (Bio-Rad) was added directly to each tube. Samples were then vortex and boiled for 10 minutes to release bound proteins. After cooling, Protinase K (20 μg) was added, tubes were vortexed and shaken at 55°C for 30 minutes and boiled again for 10 minutes. Samples were then centrifuged and supernatant, containing DNA fragments, collected. In order to ensure maximum yield, distilled water (120 μL) was added to each tube of beads which were then vortexed and centrifuged again. The supernatant was removed and combined with the previous. The resulting DNA was used directly in the real-time PCR.
Real-time PCR

Real-time PCR was performed on chromatin samples as previously described (35) with the following modifications. PCR reactions were done in a 384-Well Optical Reaction Plate (Applied Biosystems, Foster, CA) with a reaction volume of 20 µl. Reactions contained 10 µl of 2X SYBR Green PCR Master mix (Applied Biosystems), 4 µl of DNA template generated using the fast ChIP assay and primers and probes at a final concentration of 0.2 µM each. Primers and probes were designed using ABI Primer Express Software (Applied Biosystems), sequences are shown in Table 1. Primers were tested on genomic DNA using standard PCR prior to use in real-time PCR to ensure satisfactory priming. Amplification, data acquisition and analysis were done using the 7900HT Real-time PCR system and SDS Enterprise Software (Applied Biosystems).

Data are expressed as a signal ratio, R, derived from the following relationship

\[ R = \exp (C_T^{\text{mock}} - C_T^{\text{sample}}) \]  

where \( C_T^{\text{mock}} \) and \( C_T^{\text{sample}} \) are mean threshold cycles of PCR done in triplicate on samples and mock IP’s.
### Table 1.
Primer Sequences\(^a\) for STAT5 ChIP PCR.

<table>
<thead>
<tr>
<th>Site</th>
<th>Primer Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALS Promoter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GGCCCTGCCAGGTGTTC</td>
<td>59</td>
</tr>
<tr>
<td>Reverse</td>
<td>CAGCCTCTCTGTGACCTCTAGGT</td>
<td>60</td>
</tr>
<tr>
<td>Probe</td>
<td>TAGAAGAGGCCCCTGGAGG</td>
<td>69</td>
</tr>
<tr>
<td><strong>IGF-1 5’flanking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CCAGAGAGAGCTGGTAAGAGAATTTT</td>
<td>59</td>
</tr>
<tr>
<td>Reverse</td>
<td>AATGGCAGCCTGTGTTTTACA</td>
<td>58</td>
</tr>
<tr>
<td>Probe</td>
<td>TCCCCCTCTATCAGACTT</td>
<td>69</td>
</tr>
<tr>
<td><strong>IGF-1 Intron 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>AATACCCTGGTGACTCTTTCC</td>
<td>58</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCGGTTCACCCCTTTTCAG</td>
<td>59</td>
</tr>
<tr>
<td>Probe</td>
<td>TGTTATTGAAAGCATGTGTC</td>
<td>68</td>
</tr>
</tbody>
</table>

\(^a\) all sequences presented 5’ → 3’ orientation
RESULTS

Serum ALS Levels

Western Blotting was used to determine the relative amounts of ALS protein in the serum of IUGR versus CON rats at d21 and d120. In males no significant difference between CON and IUGR could be detected at either time point. IUGR females, however, displayed a significant increase in serum ALS. At d21 female IUGR rats had serum ALS levels 190 ± 34* % of CON, with elevated ALS levels persisting to d120 (135 ± 16* %) P<0.05. Figure 1 shows the relative levels of serum ALS in CON and IUGR animals at d21 and d120 (expressed in arbitrary ECL units/5 ng total serum protein) with corresponding representative western blots.

Pilot ChIP

A revised ChIP protocol was developed to minimize experiment time, tube transfers and DNA loss. The new protocol reduced the experiment time from 3-4 days to one day. In addition, a reduction in the number of tube transfers and elimination of the phenol precipitation results in reduced DNA loss and improved final yield. The revised ChIP protocol yielded chromatin fragments concentrated about 500bp. Figure 2 shows 4% agarose gel of a sample of prepared hepatic chromatin from a female CON.
Figure 1. Serum ALS protein levels. The graph shows the relative levels of serum ALS, for males and females at d21 and d120 of life with corresponding representative western blots below. Solid bars are CON, hatched bars IUGR. All data are expressed as arbitrary ECL units/5 ng total serum protein and error bars are standard error of mean n=5. *P<0.05.
Figure 2. Sheared hepatic chromatin. Left lane shows 100bp DNA ladder, right lane shows sample of prepared sheared chromatin centered about 500bp indicating that chromatin preparation is comprised of primarily dinucleosomes. Sample prepared from female CON.

Real-time PCR

Real time PCR was used to examine relative STAT5 occupancy of three GH-response elements in CON and IUGR hepatic chromatin; one ~250bp 5' of transcription start of ALS, one in Intron 2 of IGF-1 and one ~210 kbp 5' of transcription start of IGF-1. Hepatic chromatin fragments were precipitated with STAT5 antibodies and DNA isolation continued using the fast ChIP protocol. Real time PCR was performed directly on the resulting DNA without further purification.
The real-time PCR results showed that the product of the fast ChIP assay is suitable for use in real-time PCR. Table 2 shows $C_T$ values and standard deviations of triplicate measures for female CON and IUGR samples.

Calculation of a binding ratio, $R$, (given by Equation 1), using the $C_T$ value of the sample and that of a mock IgG control, allows comparison of the STAT5 binding ratios for each response element on each template (i.e. female CON, female IUGR, etc). Comparison of the relative occupancy of the three GH-response elements under investigation suggests that in female IUGR animals STAT5 tends to occupy relatively more ALS response elements and less IGF-1 5' flanking response elements than CON animals. Figure 3 shows a graph of the relative occupancy. The ratio $R$ is expressed as a % of the total occupancy of the three response elements.
**Table 2.** Real-time PCR C<sub>T</sub> values for female hepatic CON and IUGR anti-STAT5 ChIP DNA prepared using the fast ChIP protocol. Results are presented as average ± std deviation.

<table>
<thead>
<tr>
<th></th>
<th>ALS</th>
<th>IGF-1 5' Flanking</th>
<th>IGF-1 Intron 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female CON</td>
<td>31.12 ± 0.110</td>
<td>32.79 ± 0.099</td>
<td>31.21 ± 0.169</td>
</tr>
<tr>
<td>Female IUGR</td>
<td>29.79 ± 0.071</td>
<td>33.55 ± 1.5</td>
<td>31.96 ± 0.362</td>
</tr>
</tbody>
</table>
**Figure 3.** Relative STAT5 occupancy of GH-response elements in ALS and IGF-1 for female CON and IUGR hepatic DNA (see text for details). The hatched bars are ALS, the solid bars are IGF-1 Intron 2 and the checkerboard bars are IGF-1 5’flanking.
Barkers Fetal Origins Hypothesis (1, 4) is consistent with the notion that adjustments in gene expression made in utero can persist throughout life. When these adjustments are triggered by a nutritional deficit, such as that induced by UPI, fetus are "reprogrammed" to survive in an environment with limited nutritional availability. In the case of the IUGR rat however, the post-natal life is associated with adequate nutrition and the previously compensatory adjustments in gene expression become detrimental and the animal may develop adult onset diseases such as type 2 diabetes.

Epigenetic modifications of chromatin have been shown to take place in the IUGR rat (5, 6) and provide a plausible mechanism for the fine-tuning of gene expression in response to a fetal insult such as UPI. In order to isolate important pathways in the development of adult onset disease, to identify altered gene expression and protein levels, and finally, to explain the mechanism leading to the altered gene expression one requires a suitable experimental model. The UPI induced IUGR rat provides such a model.

Here we have focused on a component of the GH/IGF-1 axis, the ALS subunit, which is potentially important in the regulation of IGF-1 bioavailability and hence possibly the onset of impaired glucose regulation. A revised ChIP protocol is also described with a preliminary examination of the binding of STAT5 to the ALS and IGF-1 GH-response elements.

Serum ALS levels were compared in CON and IUGR rats at d21 and d120,
corresponding to adolescence and adulthood respectively. Female IUGR rats displayed a significant increase in serum ALS levels at d21 and at d120 (190 ± 34%, 135 ± 16%, d21 and d120 respectively) shown in Figure 1. This increase in serum ALS levels is consistent with the idea that an increase in ALS could lead to reduced IGF-1 bioavailability and the subsequent onset on impaired glucose regulation seen in the IUGR animals.

On the other hand, male rats showed no significant difference between CON and IUGR at either time point. This is possibly due to a greater disruption in expression of other key components of the IGF-1 system, including IGF-1 itself and IGFBP3. It is known that liver IGF-1 and IGFBP3 expression and serum IGF-1 protein levels are reduced in IUGR animals (Lane, unpublished observation) and ALS binds only to the IGF-1:IGFBP-3 binary complex with no affinity for free IGF-1 (36). As a result male IUGR animals could still have a reduced IGF-1 bioavailability with a relative excess of ALS in the face of greatly reduced IGF-1 and/or IGFBP3. Relative timing of gene expression in relation to development could also be a factor in these results. It is known that ALS levels begin to rise at birth and continue to do so rapidly through adolescence until finally stabilizing in the adult. It is possible that the timing of increased expression is altered in IUGR animals.

In order to begin to understand the origins of altered gene expression in IUGR animals it is necessary to understand the triggers for altered expression at the level of chromatin. One way to begin to unravel this complex set of questions is to examine the binding of transcription factors to DNA response elements; a process typically facilitated by a ChIP assay using an antibody to the transcription factor of interest and real-time PCR to quantify relevant genes. Unfortunately the traditional ChIP assay is very time consuming, taking 3-4 days to complete, not amenable to studying large sample numbers.
simultaneously and involving steps that tend to result in large losses of DNA, such as phenol extractions. Nelson et al., (35) greatly enhanced the ChIP assay with cultured cells by the use of Chelex-100 resin. Chelex-100 is an ion-exchange resin that chelates divalent metal ions and has typically been used to isolate PCR ready DNA from forensic specimens.

Here the ChIP assay has been further modified to optimize treatment of chromatin from hepatic tissue. In order to produce chromatin fragments of the most uniform length possible, the sonication step was optimized (as described in the methods section). A 4% agarose DNA gel (Figure 2) shows that the resulting fragments were clustered about 500bp, indicating that the average size corresponds to a dinucleosome. In addition the protocol can be performed in one day, can be done on multiple samples simultaneously and results in minimal DNA losses.

DNA prepared using this fast ChIP assay was successfully used in real-time PCR with primers and probes against the three GH-response elements. Table 2 shows C_T values for female CON and IUGR samples. Consistent values and generally low standard deviations indicate successful PCR results.

Although preliminary in nature, the ChIP results for female hepatic tissue suggest a redistribution of STAT5 binding with IUGR. Figure 3 shows the relative occupancy of each of the three response elements on the female CON and IUGR templates. It appears that STAT5 occupies increasingly more ALS GH-response element in IUGR and correspondingly less of the extreme 5' flanking GH-response element of IGF-1. This is consistent with the observed increase in ALS protein levels seen in female IUGR serum at d21 and d120. Data from male rats was not consistent at the PCR stage and hence not examined further.
This work sets the stage for further examination into the interaction of transcription factors with chromatin; those involved with the IGF-1 system as well as others. A pattern of transcription factor binding in the context of chromatin in CON and IUGR animals will be useful in identifying the effects of epigenetic modifications.

In conclusion, the IUGR rat fetus experiences an inhospitable environment resulting from decreased placental blood flow and this results in fine modifications in gene expression, with many of these modifications persisting into adulthood. It appears that increased serum levels of ALS in female IUGR rats, and the resulting reduced IGF-1 biavailability, could be an important part of the impaired glucose regulation seen in the adult animals. Further investigation will be needed to assess details of ALS expression and the influence of STAT5 on this expression.
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