Role of the Sp Family of Transcription Factors in the Ontogeny of Growth Hormone Receptor Gene Expression*

Jae H. Yu‡, Gary Schwartzbauer, Angel Kazlman, and Ram K. Menon§

From the Department of Pediatrics, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania 15213

The growth hormone (GH) receptor is essential for the actions of growth hormone on postnatal growth and metabolism. GH receptor transcripts are characterized by the presence of disparate 5′-untranslated exons. Factors regulating the expression of the GC rich L2 transcript of the murine GH receptor gene have hitherto remained unidentified. To characterize the mechanisms regulating expression of the L2 transcript, primer extension and ribonuclease protection assays were used to identify transcription start sites in RNA from liver of adult mice. Transient transfection experiments revealed that 2.0 kilobase pairs of the L2 5′-flanking sequence exhibited promoter activity in BNL CL2 (mouse liver) cells, CV-1 (monkey kidney) cells, and HRP.1 trophoblasts. Deletional analysis localized a major regulatory region to within 75 base pairs of the 5′ transcription start site. Sequence analysis revealed that the region contained consensus binding sites for the Sp family of transcription factors. Standard gel shift and supershift analysis using liver nuclear extracts established that Sp1 and Sp3 bound this regulatory element. Transfection of wild type but not mutant decoy oligonucleotides into BNL CL.2 cells decreased the activity of the L2 promoter. Overexpression of Sp1 and Sp3 protein in Drosophila Schneider cells established that Sp3 is more potent than Sp1 in transactivating the L2 promoter. Cotransfection experiments further established that Sp1 antagonizes the activity of Sp3 to transactivate the L2 promoter. Western blot analysis of liver nuclear extracts revealed that the levels of Sp3 increase significantly after birth, suggesting a role for the Sp family of transcription factors in controlling the fetal to postnatal increase in GH receptor gene expression.

Pituitary GH1 is essential for postnatal growth and regulation of metabolism of fat, carbohydrate, and protein in animals. At the tissue level these pleiotropic actions of GH result from the interaction of GH with the GH receptor. Hence, regulating the expression of and modulating the function of the GH receptor is critical for the action of GH in the intact animal. The promoter-regulatory regions of the murine (1–4), ovine (5, 6), bovine (7), and human (8) GH receptor genes have been partially characterized. A feature that is common to the GH receptor transcripts from these different species is the heterogeneity in the 5′-untranslated region (9). This heterogeneity in the 5′-UTR results from splicing of the various exon 1 fragments to a common splice site located 11 bp upstream of the initiating ATG. Whereas the number of 5′-untranslated exons varies among species, in all examples the location of the splice site from the initiating ATG is constant. In the human liver there are at least eight distinct 5′-UTRs (V1–V8), with the most common GH receptor variant being termed V1 (9). The identity and distribution of the 5′-untranslated exons in other tissues of the body, in which significant expression of GH receptor occurs and which are targets of GH action, such as kidney and heart, are as yet unknown. In the rat five distinct 5′-UTRs (GHR1–GHR5) have been identified (10, 11). The class of RNA containing the GHR, 5′-UTR variant is expressed only in liver, is far more abundant in females than in males, and is specifically increased during pregnancy (11). In the sheep two 5′-UTRs have been described (5, 6). Exon 1A is a liver-specific transcript and is homologous to the human V1 transcript. Another UTR termed exon 1B displays a striking GC content of 79% and is homologous to the human V2, rat GHR2, and mouse L2. In the cow three 5′-UTRs, termed 1A, 1B, and 1C, have been identified (7).

In the mouse two 5′-UTRs, termed L1 and L2, have been characterized (4). Expression of the L1 and L2 transcripts is regulated in a tissue- and development stage-specific manner. The L1-GH receptor transcript is expressed in liver only during pregnancy (3, 12). Late pregnant mouse liver and placenta both express GHR mRNAs containing L1 and L2 sequences (12). In the placenta L2-GHR UTRs are more abundant than L1-GHR UTRs. Information regarding the specific factors regulating expression of the different UTRs is at present limited to that for the mouse L1 UTR. All identified proximal and distal regulatory elements contain a core CCAAT sequence (4). An enhancer element located approximately 3 kb upstream from the transcription start site of the L1 transcript interacts with the transcription factor CTF/NF-1 in COS-7 cells (2). The most distal cis-element identified to date in the mouse promoter is located 3.6 kb 5′ to the transcription start site, and two CCAAT box-binding proteins, MSY-1 and NF-Y, regulate the activity of the L1 promoter via interaction with this cis-element (3). Furthermore, the intracellular interaction of the MSY-1 protein in the liver suggests a role for this factor in the pregnancy-specific expression of the GHR (3). In contrast to the L1 transcript, there is a paucity of information regarding the cis-elements and trans-acting factors regulating expression of the L2 transcript of the murine GH receptor gene.

This paper is available on line at http://www.jbc.org
The current report describes the identification and partial characterization of the promoter-regulatory region of the L2 transcript of the murine GH receptor gene. In this report we present data that define the location of the transcription start site, demonstrate promoter activity in the 5'-flanking region of the L2 transcript of the murine GH receptor gene, establish that the Sp family of proteins regulate expression of the L2 transcript, and provide evidence to indicate that alterations in the levels of the Sp proteins play a role in ontogenic profile of GH receptor gene expression in the liver.

**Experimental Procedures**

Oligonucleotides—The following synthetic oligonucleotides were used in these experiments (residues altered in the mutant oligonucleotide are indicated in lowercase type): L2-A, TTTCCAGGTTCACG; L2-A-m, CCCTTCCCAGTTTCACCaataaCCCTTCCTCCTCCCCAGTATAGTATGGGTTTTGGGGCTTCCA; random, CACATGTTAGATCTCAGGTTACATCCAGCCGACACACATT. Where necessary double-stranded oligonucleotides were generated by annealing of synthetic oligonucleotides with the respective complimentary sequences.

**Primer Extension Reaction**—Extension reaction was carried out with 50 μg of adult mouse total RNA with synthetic oligonucleotide complimentary to a portion of the L2 transcript of the GH receptor mRNA. The 21-base oligonucleotide designated L2-REV (see Fig. 2) was complimentary to its 5'-end beginning 56 nucleotides upstream from the translational start site of the GH receptor mRNA (13). The primer was 32P end-labeled using T4 polynucleotide kinase and hybridized with RNA in 10 mM PIPES (pH 7.4), 200 mM NaCl (pH 6.4), 1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol. Following digestion with RNase, the RNase-resistant products were generated by transcription of the 5'-flanking region of the GH receptor mRNA corresponding to a portion of the L2 transcript of the murine GH receptor gene. The DNA was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. Approximately 30 fmol of DNA was added to 1 μl of nuclear extract or 1 footprint unit of recombinant Sp1 protein (Santa Cruz Biotech) in a final volume of 20 μl containing 1 μl of poly(dI-dC), 20 mM Tris-HCl (pH 8.5), 200 mM NaCl, and subjected to autoradiography with intensifying screens (NEN Life Science Products) at ~80 °C. Competition experiments included the addition of excess unlabeled DNA fragments to the reaction mixture. In some experiments nuclear extracts were incubated with the indicated amounts of polyclonal antibodies against Sp1 (sc-59x), Sp2 (sc-643x), Sp3 (sc-644x), or Sp4 (sc-646x) (Santa Cruz Biotech) for 30 min at room temperature before addition to the binding reactions.

DNA Sequencing—Sequencing was carried out by the dideoxynucleotide chain termination method of Sanger et al. (15) using either the Sequenase 2.0 kit (U. S. Biochemical Corp.) or the Fmole sequencing kit (Promega). Sequencing primers either were complementary to the canonical T3, T7, or SP6 sites flanking the multiple cloning site of the vector or were complementary to experimentally established sequences. The sequence data were managed using the sequence analysis program MacVector (Oxford Molecular Group, Inc.).

**Reporter Gene Constructs—Luciferase reporter gene constructs were engineered to contain various portions of the GH receptor 5'-flanking region. Construction of the reporter gene-GH receptor 5'-flanking region was as follows: an approximate 831-bp fragment from the 66B lambda clone (courtesy of Dr. Frank Talamanca, University of California at Santa Cruz) containing the 5'-flanking region and extending 110 bp into the L2 UTR was subcloned into the corresponding site in the polylinker of pGL3-Basic (Promega) to create pGL3B-L2[−75] and pGL3B-L2[−43] were engineered by using polymerase chain reaction; the 5'-ends of these constructs were defined by the oligonucleotides L2F4 and L2F5, respectively. pGL3B-L2[−75] and pGL3B-L2[−43] were created by incorporating the mutation defined in the oligonucleotide L2-A-m into pGL2B-L2[−75] using QuikChange (Stratagene). All constructs were sequenced through the vector-insert junctions to ensure nucleotide fidelity and verify directionality.

**Transient Expression of Reporter Gene**—The culture media used for tissue culture experiments were obtained from Life Technologies, Inc. unless otherwise stated. BNL CL.2 cells (ATCC) were maintained in Eagle's minimum essential medium (with nonessential amino acids, sodium pyruvate, and Earle's balanced salt solution), 10% fetal calf serum. The cells were allowed to grow to a confluency of 50–60% prior to commencement of transient transfection experiments. The culture media used for tissue culture experiments were obtained from Life Technologies, Inc. unless otherwise stated. BNL CL.2 cells (ATCC) were maintained in Eagle's minimum essential medium (with nonessential amino acids, sodium pyruvate, and Earle's balanced salt solution), 10% fetal calf serum. The cells were allowed to grow to a confluency of 50–60% prior to commencement of transient transfection experiments.
the role of Sp1 and Sp3 in regulating the activity of the GH receptor promoter, 12 μg of the reporter plasmids containing the GH receptor promoter fragments were co-transfected with varying amounts (0.25–16 μg) of Sp1 (pAct1-Sp1) and/or Sp3 (pAct1-Sp3) expression plasmids. 3 μg of a plasmid RSV/β-gal was included in each transfection mixture to enable monitoring of transfection efficiency. The cells were exposed to the DNA precipitate for 48 h prior to being harvested and processed for chemiluminescent assays for measurement of luciferase (Promega) and β-galactosidase activity (CLONTECH).

**RESULTS**

**Identification of L2 Transcription Start Sites**—Primer extension was performed using reverse transcriptase after end-labeled antisense oligonucleotide (L2-REV) was hybridized to total cellular RNA from nonpregnant mouse liver. The extension of L2-REV consistently resulted in three specific bands: one major band measuring 195 nucleotides and two minor bands of 140 and 117 nucleotides in lengths (Fig. 1A). To precisely localize the transcription start sites in the 5′-untranslated region, ribonuclease protection assays were performed. 32P-Labeled antisense RNA, transcribed from the appropriate strand of pNoTA/T7/L2-mGHR14, was hybridized to total cellular RNA from nonpregnant mouse liver and digested with RNase A and T1. The major protected bands were approximately 118 and 311 nucleotides in length (Fig. 1B). The shorter band corresponds to exon 2, and the longer band corresponds to the major transcription start site mapped by the primer extension experiment at 210 bp upstream of the initiating ATG. Quantitation of the radioactivity in the bands revealed that in the liver RNA from nonpregnant mouse (Fig. 1B, lane C), the longer band representing L2 UTR containing GH receptor mRNA accounted for 56% of the total GH receptor RNA; the remaining 44% of the mRNA was in the shorter band representing non-L2 UTR containing GH receptor mRNA. In contrast in liver RNA from pregnant mouse approximately 98% of the mRNA was in the non-L2 band while 2% was in the L2 band.

**Analysis of Promoter Activity of the 5′-Flanking Region of the L2 Transcript of the Murine GH Receptor Gene**—The sequence upstream of the transcription start site is GC-rich (69% up to −213 bp from transcription start site). Comparison with the transcription factor data base TRANSFAC (16) failed to reveal consensus sequences for either a TATA or CCAAT box in the immediate upstream region from the transcription start sites (Fig. 2).
The functional role of the 5′-flanking region in the regulation of transcription of the GH receptor gene was assessed by its ability to direct expression of the luciferase gene in a transient transfection assay. A 2.0-kb fragment of the 5′-flanking region of the GH receptor gene, containing 110 bp of exon 1, was inserted upstream of the luciferase reporter gene contained in the promoterless expression vector (pGL3-basic). This fusion construct (pGL3B-L2[−2.0]) exhibited significant luciferase activity when transiently transfected into BNL CL.2 mouse liver cells (Fig. 3A). In multiple transfection experiments, the relative expression of luciferase using pGL3B-L2[−2.0] was consistently about 6–9-fold greater than the background measured with the promoterless vector and approximately equal to that observed with the positive control that contains the cytomegalovirus promoter. Primer extension experiments with RNA isolated from the transfected cells indicated that the transcription start site utilized by the L2 promoter plasmids corresponded to the major start site identified in RNA extracted from whole liver tissue (Fig. 4). Hence, we conclude that the 5′-flanking region of the L2 transcript of the murine GH receptor gene has promoter activity and that expression of the reporter gene was initiated at a site identical to that used by the GH receptor gene in liver tissue.

To localize putative cis-acting elements regulating transcription of the GH receptor gene, we engineered progressively shorter fragments of GH receptor 5′-DNA (Fig. 3A), and the expression of these deletion mutants was examined in BNL CL.2 cells by transient transfection assays. Whereas deletion of approximately 1925 bp did not result in a significant alteration in the expression of the reporter gene, deletion of the next 32 bp resulted in complete abrogation of promoter activity. These results indicate that the sequence between −75 bp and −43 bp upstream of the transcription start site is essential for basal activity of the GH receptor gene.

To examine potential cell type specificity of the GH receptor for the L2 transcript, the activities of these reporter gene constructs were assayed in CV-1 (monkey kidney) (Fig. 3B) and HRP.1 (trophoblast) (data not shown). The expression profile of these various constructs was similar to that observed in BNL CL.2 cells with the −75 to −43-bp region being necessary for basal activity. However, in CV-1 cells there was also a significant increase in activity from 0.7 kb to −210 bp, indicating the presence of a putative inhibitory regulatory element in this region. These results indicate that the basal promoter regulating expression of the L2 transcript of the GH receptor is active in a wide variety of tissues, a finding consistent with the widespread expression of the L2 transcript in mouse tissues (12). Furthermore, the results with the CV-1 cells suggest that distal promoter elements present upstream in the 5′-flanking region of the L2 UTR may play a role in the tissue-specific regulation of expression of the L2 GH receptor transcript.

**FIG. 2.** Nucleotide sequence in the vicinity of the transcription start sites for the L2 transcript of the murine GH receptor gene. Numbering was arbitrarily established with the major transcription start site designated as +1 and indicated by a box; minor transcription start sites are indicated by stars. Primers used for primer extension (L2-REV), EMSA (L2-A) and polymerase chain reaction cloning (L2F4 and L2F5) experiments are indicated. Putative binding site for Sp family of transcription factors is demarcated. Intron sequences are in lowercase letters.

**FIG. 3.** Transient expression analyses of the promoter-regulatory region of L2 transcript of the murine GH receptor gene. Luciferase expression plasmids were generated by inserting the GH receptor transcription start sites and various portions of the 5′-flanking sequence of the L2 transcript of the GH receptor gene into the promoterless luciferase plasmid pGL3-Basic. These expression plasmids were transfected into BNL CL.2 (mouse liver) cells (A) or CV-1 (African green monkey kidney) cells (B) as described under "Experimental Procedures." Luciferase-specific activity in cell homogenates, equalized for transfection efficiency monitored by co-transfection of a plasmid expressing Renilla luciferase (pRL-TK; Promega), is expressed as relative to that of the pGL3-Basic. To compare the activity of the GH receptor promoter-luciferase constructs with that of a canonical promoter-regulatory element, the activity of a control plasmid (pGL2-Control; Promega) that contains a SV40 promoter and enhancer sequences was also measured in these cells. Results represent the means ± S.E. of 3–4 independent transfections performed in duplicate. Arrows indicate orientation of the GH receptor DNA relative to direction of GH receptor gene transcription. Using analysis of variance, *, p < 0.05 compared with pGL3-Basic; #, p < 0.05 compared with pGL3B-L2[−0.7].

**TABLE 1.** Transcriptional Start Sites for the L2 Transcript of the Murine GH Receptor Gene

| Start Site | Location (kb) | Activity Relative to pGL3-Basic |
|------------|---------------|-------------------------------|
| +1         | −2.0          | 1                             |
| +38        | −2.75         | 0.35                          |
| +75        | −2.25         | 0.15                          |
| +113       | −2.0          | 0.05                          |
| +151       | −1.75         | 0.01                          |
| +189       | −1.0          | 0.00                          |

**TABLE 2.** EMSA of GH Receptor Promoter Elements

| Element | Location (kb) | Activity Relative to pGL3-Basic |
|---------|---------------|-------------------------------|
| L2-A    | −2.0          | 1                             |
| L2-REV  | −2.75         | 0.35                          |
| L2F4    | −2.5          | 0.15                          |
| L2F5    | −2.0          | 0.05                          |

**TABLE 3.** Primer Extension of GH Receptor Promoter Elements

| Element | Location (kb) | Activity Relative to pGL3-Basic |
|---------|---------------|-------------------------------|
| L2-A    | −2.0          | 1                             |
| L2-REV  | −2.75         | 0.35                          |
| L2F4    | −2.5          | 0.15                          |
| L2F5    | −2.0          | 0.05                          |

**TABLE 4.** PCR Cloning of GH Receptor Promoter Elements

| Element | Location (kb) | Activity Relative to pGL3-Basic |
|---------|---------------|-------------------------------|
| L2-A    | −2.0          | 1                             |
| L2-REV  | −2.75         | 0.35                          |
| L2F4    | −2.5          | 0.15                          |
| L2F5    | −2.0          | 0.05                          |
Protein Binding Activity of the Putative Basal Regulatory Element—The loss of expression consequent on deletion of the 32-bp region located between −75 and −43 bp upstream of the transcription start site suggested the presence of basal regulatory elements within this region. Computer analysis of the sequence of this 32-bp region indicated the presence of a canonical Sp1-binding site within this region (Fig. 2). An oligonucleotide (L2-A) was designed that encompassed the putative Sp1-binding site, and its protein binding activity was tested by EMSA. Addition of nuclear extract from female mouse liver to an aliquot of 32P-labeled L2-A resulted in the formation of three protein-DNA complexes we termed C1, C2, and C3 (Fig. 5). To determine the sequence specificity of these protein-DNA complexes, competition experiments were performed. Whereas a 50-fold molar excess of unlabeled L2-A eliminated the formation of all three DNA-protein complexes, an oligonucleotide with random sequence did not affect the binding even at a 100-fold molar excess. Thus, these results demonstrate that these protein-DNA complexes are sequence-specific. To determine the identity of the proteins(s) binding to this putative regulatory element, we performed competition experiments with an oligonucleotide (Sp1, Santa Cruz Biotech) containing a consensus binding site for Sp1 protein (Fig. 6). We next tested the ability of the L2-A sequence to bind recombinant Sp1 protein. EMSA experiments wherein 32P-labeled L2-A was incubated with recombinant Sp1 prior to electrophoresis revealed that the L2-A sequence was able to bind Sp1 protein (Fig. 6, lane 5). In cross-competition experiments, unlabeled L2-A was able to abrogate the binding of recombinant Sp1 protein to both 32P-labeled L2-A and to the 32P-labeled oligonucleotide containing the consensus Sp1 binding sequence (data not shown). These results indicate that the protein(s) binding to L2A has DNA binding characteristics similar to those of the Sp1 family of proteins.

The Sp1 gene family includes at least four distinct but closely related transcription factors, Sp1–Sp4. To establish the precise regulatory element(s) within this region, we performed competition experiments with antibodies specific for different Sp proteins. The addition of antibodies specific for Sp3 in an EMSA reaction with 32P-labeled L2-A and mouse liver nuclear extracts retarded the mobility of C1 and C2 complexes (Fig. 7). In parallel
Sp1 and Sp3 Regulate Activity of the L2 Promoter—Having established that Sp1 and Sp3 bind the L2-A element, we next determined the role of these proteins in regulating the activity of the L2 promoter. Mutation of the Sp1-binding site within the L2-A element resulted in loss of activity in transient transfection assays (Fig. 3), indicating the functional significance of the binding of the Sp factors to the L2-A element. The high levels of endogenous Sp1 expression in most cell types generally invalidates the conventional strategy of overexpressing proteins to test their role in regulating the activity of a particular promoter element. Hence, we adopted the alternate strategy of using Drosophila Schneider cells (SL2) that have been previously demonstrated to be devoid of Sp1 and Sp3 proteins. In the native SL2 cells, pGL3B-L2[-75] did not exhibit significant activity when compared with the pGL3/basic vector alone. However overexpression of Sp3 in these cells resulted in significant increase in the activity of pGL3B-L2[-75] (Fig. 8A). This increase in activity was proportional to the amount of Sp3 transfected into the SL2 cells with the maximal 26–30-fold induction being achieved with 1 μg of Sp3 expression plasmid. In contrast the pGL3B-L2[-43] construct failed to exhibit Sp3-dependent induction of activity in these cells (data not shown). These results indicate that Sp3 can regulate the activity of the L2 UTR of the GH receptor gene and that this effect is dependent on the region between -75 and -43 bp upstream of the transcription start site. We next tested the effect of overexpression of Sp1 protein on the activity of the GH receptor promoter in the SL2 cells. In comparison with the results with Sp3 overexpression, induction by Sp1 was significantly less, with the maximal induction of 2–3-fold achieved with 16 μg of Sp1 plasmid DNA (Fig. 8B). These results indicate that although Sp1 is able to regulate the activity of the GH receptor promoter it is much less potent that Sp3 in its ability to activate this promoter.

Although the high levels of endogenous Sp1 protein did not allow us to test the effect of overexpression of Sp proteins on the activity of the L2-A promoter, we performed experiments designed to decrease the levels of endogenous Sp proteins in BNL CL.2 cells and studied the effect of this manipulation on the activity of the L2 promoter. We achieved this goal by employing the decoy nucleotide strategy. In this approach high concentrations of a double-stranded oligonucleotide encoding the recognition motif for the transcription factor of interest is cotransfected into the cell with the reporter constructs. This decoy oligonucleotide will compete with the DNA-binding site in the promoter element being tested and could thus result in a decrease in the protein available to bind to the promoter element. This approach of decreasing the functional levels of an endogenous protein has been previously used to study the functional role of Sp1 (17) and other transcription factors such as c-myc and cdc2 (18). As shown in Fig. 9, cotransfection of double-stranded oligonucleotides with the wild type L2-A sequence or a consensus Sp1-binding site resulted in a decrease in the activity of the pGL3B-L2[-75] construct. This effect was proportional to the amount of the oligonucleotide transfected with a 25 and 30–50% decrease in activity with 0.9 and 1.8 μg, respectively. In contrast, oligonucleotides that did not contain an Sp1 consensus binding site (random sequence) or with mutations in the Sp1-binding site of either the L2-A sequence (L2-A-m) or the consensus Sp1 oligonucleotide (Sp1-m) failed to significantly alter the activity of the pGL3B-L2[-75] construct. We conclude from these experiments that Sp1 and Sp3 regulate the activity of the L2-A promoter, and this regulation is dependent on the presence of the L2-A element.

Functional Interaction between Sp1 and Sp3—Previous reports have documented that Sp1 and Sp3 can interact with each other to either synergize or antagonize each other’s activity at any given DNA-binding site (19–22). Upon demonstrating that both Sp1 and Sp3 bind to and alter the activity of the L2-A element in the 5’-flanking region of the murine GH receptor gene, we next determined whether there is a functional interaction between Sp1 and Sp3 at the L2-A site. For this purpose varying amounts of Sp1 and a fixed amount of Sp3 experiments addition of antibody specific for Sp1 also resulted in supershift of C1 and C2 complexes, although the amount of the protein-DNA complex supershifted was significantly less than with the Sp3 antibody (Fig. 7, inset). In contrast, antibodies against Sp2 and Sp4 did not alter the mobility of C1 and C2 protein-DNA complexes, and none of the Sp antibodies altered the mobility of the C3 complex. These results indicate the presence of Sp3, and to a lesser extent Sp1, in the C1 and C2 protein-DNA complexes formed with mouse liver nuclear proteins and the L2-A sequence.

Determination of Sp1 and Sp3 Interaction at the L2-A Element—To analyze the interaction between Sp1 and Sp3 at the L2-A element, we cotransfected Sp1 and Sp3 expression plasmids with a luciferase reporter plasmid containing the L2-A element in the 5’-flanking region of the murine GH receptor gene, cotransfected into the cell with the reporter constructs. This decoy oligonucleotide will compete with the DNA-binding site in the promoter element being tested and could thus result in a decrease in the protein available to bind to the promoter element. This approach of decreasing the functional levels of an endogenous protein has been previously used to study the functional role of Sp1 (17) and other transcription factors such as c-myc and cdc2 (18). As shown in Fig. 9, cotransfection of double-stranded oligonucleotides with the wild type L2-A sequence or a consensus Sp1-binding site resulted in a decrease in the activity of the pGL3B-L2[-75] construct. This effect was proportional to the amount of the oligonucleotide transfected with a 25 and 30–50% decrease in activity with 0.9 and 1.8 μg, respectively. In contrast, oligonucleotides that did not contain an Sp1 consensus binding site (random sequence) or with mutations in the Sp1-binding site of either the L2-A sequence (L2-A-m) or the consensus Sp1 oligonucleotide (Sp1-m) failed to significantly alter the activity of the pGL3B-L2[-75] construct. We conclude from these experiments that Sp1 and Sp3 regulate the activity of the L2-A promoter, and this regulation is dependent on the presence of the L2-A element.
were cotransfected into SL2 cells, and the activity of the pGL3B-L2[−75] construct was monitored. As illustrated in Fig. 10A, these results indicate that Sp1 inhibited the stimulatory activity of Sp3 in a dose-dependent manner. The inhibition of the transactivation potential of Sp3 by Sp1 was maximum at 8 μg of Sp1 and resulted in a decrease in the activation potential of Sp3 to 10–15% of that observed with Sp3 alone. It is noteworthy that this decrease in the transactivation potential of Sp3 was not due to decreased expression of Sp3 consequent to co-expression of Sp1 (Fig. 10B). We conclude from these results that Sp1 antagonizes the ability of Sp3 to transactivate the GH receptor L2 promoter.

Levels of Sp3 Increase in the Liver Postnatally—The expression of the L2 transcript of the murine GH receptor gene increases significantly after birth. To investigate whether changes in the levels of Sp3 play a role in this ontogenic profile of GH receptor expression, we compared the levels of Sp3 expression in livers of fetal and adult mice by Western blot and EMSA. As shown in Fig. 11, Western blot analysis of liver nuclear extracts from adult mouse revealed three specific bands that correspond to the previously described isoforms of Sp3 (23). In contrast to the adult liver nuclear extracts, the levels of Sp3 protein were distinctly less in the fetal liver nuclear extracts. The decrease in levels of Sp3 in the fetal liver was specific and not an artifact of the nuclear extract preparation, because in the same fetal nuclear extract preparation the concentration of the ubiquitously expressed nuclear protein YY1 was not similarly altered. These results were also confirmed by supershift EMSA with labeled L2-A probe in which the binding of Sp1 but not that of Sp3 could be detected in fetal liver nuclear extracts. It should be noted, however, that the Sp1 antibody failed to alter the electrophoretic mobility of a significant portion of the protein-DNA complex. This result could represent the binding of other GC box-binding factors and the role(s) of these yet to be identified protein(s) is unclear at the present time. Nonetheless, this profile of binding of Sp factors to the L2-A element is in contrast to those obtained with adult liver nuclear extracts wherein binding of Sp3 to the L2-A element was much more abundant than the binding of Sp1 protein. We conclude from these results that the levels of Sp3 increase in the liver after birth.

DISCUSSION

Heterogeneity in the 5′-untranslated regions of the GH receptor transcripts is a feature common to the GH receptor gene from different species (9). In the mouse two transcripts termed L1 and L2 have been identified. We have previously reported on the factors regulating expression of the L1 transcript of the GH receptor gene (1–3, 14). The current study was undertaken to identify and characterize cis-elements and cognate trans-acting factors that regulate expression of the L2 transcript of the murine GH receptor gene. In this report we define the location of the transcription start site, demonstrate that the 5′-flanking region of the L2 transcript of the murine GH receptor gene exhibits promoter activity, identify the minimal promoter, establish that the Sp family of proteins regulates expression of the L2 transcript, and provide evidence to indicate that alterations in the levels of the Sp proteins play a role in ontogenic profile of GH receptor gene expression in the liver.

Our results using ribonuclease protection assay with a probe specific for the L2 UTR indicate that approximately 50% of the GH receptor mRNA in the mouse liver contains L2. The remaining 50% contains non-L2 mRNA and in conjunction with the prior observation that L1 is not expressed in nonpregnant liver (3) predicts the presence of hitherto unidentified UTRs. These results are in agreement with 5′-rapid amplification of cDNA ends analysis of liver cDNA, wherein sequencing of 31
clones indicated that 24/31 (77%) of the clones contained the L2 transcript (data not shown). The rest of the clones contained other UTRs that have yet to be characterized. The identification of putative novel UTRs for the murine GH receptor gene has also been previously described in a preliminary report (24).

In contrast to the L1 transcript that is not GC-rich and has a TATA box (14), the L2 transcript is GC-rich and is devoid of a TATA box. In general, GC-rich promoters are usually considered to be a target for regulation by zinc finger transcription factors. Thus TATA-less promoters have been shown to be particularly sensitive to regulation by the Sp family of proteins. Sp1, originally identified as a cellular transcription factor necessary for SV40 gene expression, is an ubiquitous nuclear protein that activates the transcription of a wide variety of viral and cellular genes (25). Further work has expanded our understanding of this protein by revealing the existence of a family of zinc-finger (HMG-Cys) transcription factors which includes Sp1, Sp2, Sp3, Sp4, and two distantly related proteins termed BTEB and BTEB2 (25). Analysis of the DNA binding activities of Sp3, Sp4, BTEB, and BTEB2 proteins recognize DNA motifs with specificity and affinity that are very similar to those of Sp1. In contrast, Sp2 binds GC boxes with significantly lower affinity than the other members of the Sp family do. It is postulated that the property of Sp1 to make strong contacts with individual components of the basal transcriptional machinery and the apparent independence of Sp1 from requiring a TATA box-binding protein TBP to activate transcription make it uniquely suited to activate the TATA-less promoters by by-passing selective steps in assembly of the core transcription machinery. This predilection of Sp family of transcription factors to regulate GC-rich promoters is also evident in the case of the L2 transcript of the GH receptor gene. Hence results of the deletional analysis indicated that the sequence between −73 and −45 bp is essential for activity of the L2 promoter and represents the minimal promoter. Computer analysis of the intervening sequence revealed the presence of a canonical hexanucleotide (CCGCC) binding site for the Sp family of proteins. EMSA experiments established that the Sp consensus element, which we termed L2-A, bound recombinant Sp1 protein and nuclear proteins from adult mouse liver. Using antibodies against the various Sp proteins in supershift EMSA experiments, our results indicate that the L2-A element preferentially binds Sp3 and to a lesser extent Sp1 protein; the L2-A element did not bind Sp2 or Sp4 protein. Whereas both Sp1 and Sp3 are ubiquitously expressed in various tissues, Sp4 expression appears to be restricted to certain cell types of the brain. To determine whether the apparent lack of Sp4 binding to the L2-A element was the result of the absence of Sp4 protein in liver nuclear extracts, we assayed for binding to the L2-A element with brain nuclear extracts. In results similar to that obtained with liver nuclear extracts, only binding of Sp1 and Sp3 and not that of Sp4 could be demonstrated with brain nuclear extracts (data not shown), suggesting that the observed Sp protein binding profile of the L2-A element was not solely determined by the composition of the nuclear extracts being assayed.

In EMSA, three specific protein-DNA complexes were formed with L2-A probe and liver nuclear extracts. Whereas we were able to determine that Sp3 and Sp1 proteins were present in the two slower migrating complexes, the identity of the proteins in the protein-DNA complex with the fastest electrophoretic mobility (C3) remains unknown. The results of the competition experiments with Sp consensus sequence oligonucleotides suggests that the DNA binding specificity of this protein(s) is similar to that of the Sp family of proteins. BTEB (basic transcription element-binding) protein is a protein of smaller size than Sp1 with DNA binding specificity similar to that of Sp1. Thus, BTEB or a related protein could represent the protein forming the C3 complex with the L2-A element. The precise role of the proteins involved in the formation of this protein complex will have to await the identification of this protein(s).

In general, Sp1 and Sp4 function as transcriptional activators. In contrast, Sp3 is a bifunctional protein with independent domains that can both activate and repress transcription with the predominant Sp3 function being dependent upon both the promoter and the cellular milieu (26). The activation potential of Sp3 is distributed over two glutamine-rich N-termini regions. Both glutamine-rich domains of Sp3 can stimulate transcription as efficiently as the corresponding Sp1 glutamate domains. Thus both Sp1 and Sp3 proteins have an N terminus glutamine-rich region that functions as a transferable activation domain. The Sp3 transactivation repressor domain has been mapped to a small amino acid region adjacent to the zinc finger domain (26, 27). In addition it has been demonstrated that the Sp3 gene encodes at least three proteins by different transcription initiations. Studies have revealed that the three variants of molecular sizes 115, 80, and 78 kDa are abundantly expressed in a broad range of tissues, and these variants could also play a role in enabling Sp3 to assume the dual function of activator/repressor of transcription (23). Our results indicate that both Sp1 and Sp3 act as transactivators of the L2 promoter of the GH receptor gene. However there is differential sensitivity of the L2 promoter to these two proteins with Sp3 being a potent activator and Sp1 a weak activator. Sp1 and Sp3 proteins have been shown previously to directly interact with each other to modify the transactivation potential of each individual protein with Sp3 antagonizing the transactivation potential of Sp1 (19–22). Our results indicate that Sp1 antagonizes the ability of Sp3 to transactivate the L2 promoter. In addition to competition for DNA binding, steric hindrance,
squelching, quenching, and direct repression are putative mechanisms that could explain the negative effect of transcription factors on gene expression. Because Sp1 is an activator, albeit a weak activator, of L2 promoter expression in SL2 cells, direct repression is an unlikely mechanism to explain the effect of Sp1 on Sp3 activation of the L2 promoter. We propose that consistent with prior reports of effects of Sp1 and Sp3 on the ornithine decarboxylase (21), uteroglobin (20), HIV-1 (28), and c-myc (29) promoters, the Sp1 repression of Sp3 activation of the L2 promoter is mediated by competition for DNA binding at the L2-A site of the L2 minimal promoter. However, our data do not exclude the possibility of squelching or steric hindrance playing a role in this interaction between Sp1 and Sp3 proteins. The Sp proteins are subject to post-translational protein modifications including phosphorylation and glycosylation (25). The role of post-translational modifications in regulating the actions of Sp proteins on the GH receptor L2 promoter is not clear. Phosphorylation is unlikely to play a major role in modifying the ability of Sp factors to bind to the L2-A element because inhibition of phosphatase activity by okadaic acid did not alter the DNA binding activity of the L2-A element (data not shown).

GH receptor gene expression displays a distinct ontogenic pattern with expression being minimal in the fetus and increasing significantly after birth (4). The paucity of GH receptor during fetal life correlates with the observation in humans that intrauterine growth is, for the most part, GH-independent (30). The molecular mechanisms controlling this development-specific expression of the GH receptor remain obscure. A previous report from our laboratory had identified a developmentally regulated enhancer element in the promoter-regulatory region of the L1 transcript of the murine GH receptor gene (14). However, because it is now known that the predominant expression of L1 occurs only during pregnancy (3, 12), it is unlikely that this enhancer element and its cognate trans-acting factors (3) plays a significant role in the fetal to postnatal transition of expression of the GH receptor gene. Our data from the ribonuclease protection assays indicate that approximately 60% of the transcripts in the adult mouse liver contain the L2 UTR. This observation argues for factors regulating the expression of the L2 transcript to be playing a significant role in the regulating the post-natal increase in expression of the GH receptor gene. Our results indicate that the L2 promoter of the GH receptor gene is activated by Sp3 and that levels of Sp3 in the liver increase significantly after birth. Hence these results would be compatible with a model wherein Sp3 regulates the fetal-postnatal increase of expression of the GH receptor gene (Fig. 12). Additionally, our studies suggest an indirect role for Sp1 in facilitating the fetal to postnatal transition of GH receptor gene expression. It is known that levels of Sp1 are elevated in fetal liver compared with the adult liver (31). Whereas by itself Sp1 is a weak activator of the L2 promoter, our results indicate that Sp1 interacts with Sp3 to antagonize the transactivation potential of Sp3 on the L2 promoter. Hence the pattern of increasing Sp3 and declining Sp1 concentrations during the transition from fetal to postnatal life supports a role for these proteins in conjointly regulating the fetal to postnatal increase in GH receptor gene expression (Fig. 12).

In summary, this report identifies and partially characterizes the promoter-regulatory region controlling expression of the L2 transcript of the murine GH receptor gene. Our studies establish that the 5′-flanking region of the L2 transcript of the murine GH receptor gene exhibits promoter activity and delineate the extent of the minimal promoter necessary for transcription of the L2 UTR containing GH receptor transcripts. We identify and characterize a regulatory element in the minimal L2 promoter that interacts with the Sp family of proteins. A role for the Sp family of proteins in the ontogenic profile of GH receptor gene expression in the liver is suggested by the interaction of Sp1 and Sp3 proteins at the L2 promoter and the profile of expression of Sp1 and Sp3 proteins in fetal and adult liver.

Acknowledgments—We gratefully acknowledge the support and encouragement provided by Dr. Mark A. Sperling. The generosity of Drs. F. Talamanes (lambda clone 66B), E. Seto (pAct-SP1), and G. Suske (pPacUSp3) in providing the respective reagents is gratefully acknowledged.

REFERENCES

1. Menon, R. K., Cheng, H., and Singh, M. (1997) Mol. Endocrinol. 11, 1291–1304
2. Zou, L., and Menon, R. K. (1995) Endocrinology 136, 5236–5239
3. Schwartzbauer, G., Yu, J. H., Cheng, H., and Menon, R. K. (1998) J. Biol. Chem. 273, 24760–24769
4. Schwartzbauer, G., and Menon, R. K. (1998) Mol. Genet. Metab. 63, 243–253
5. O’Maloney, J. V., Brandon, M. R., and Adams, T. E. (1984) Mol. Cell. Endocrinol. 101, 129–139
6. Adams, T. E., Baker, L., Fiddes, R. J., and Brandon, M. R. (1990) Mol. Cell. Endocrinol. 73, 135–145
7. Jiang, H., Okamura, C. S., and Lucy, M. C. (1999) J. Biol. Chem. 274, 7893–7900
8. Zou, L., Burmeister, L., and Sperling, M. A. (1997) Endocrinology 138, 1771–1774
9. Edens, A., and Talamanes, F. (1998) Endov. Rev. 19, 559–562
10. Domene, H. M., Cassorla, F., Werner, H., Roberts, C. T., Jr., and LeRoith, D. (1995) DNA Cell Biol. 14, 185–204
11. Baumbach, W. R., and Bingham, B. (1995) Endocrinology 136, 749–760
12. Southard, J. N., Barrett, B. A., Bikbulatova, L., Ilkbahar, Y., Wu, K., and Talamanes, F. (1995) Endocrinology 136, 2913–2921
13. Smith, W. C., Kuniyoshi, J., and Talamanes, F. (1989) Mol. Endocrinol. 3, 984–990
14. Menon, R. K., Stephan, D. A., Singh, M., Morris, S. M., Jr., and Zou, L. (1995) J. Biol. Chem. 270, 8851–8859
15. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
16. Hennevey, M., Wangender, E., Reuter, T., Hermjakob, H., Kel, A., Kel, O., Ignatieva, E., Ananko, E. A., Podkolodnaya, N. L., Kolpakov, F. A., Podkolodny, N. L., and Kolchanov, N. A. (1998) Nucleic Acids Res. 26, 364–370
17. Liang, Y., Schaufele, F., and Gardner, D. G. (1999) Endocrinology 140, 1695–1701
18. Morishita, R., Gibbons, G. H., Ellison, K. E., Nakajima, M., Zhang, L., Kaneda, Y., Ogihara, T., and Drazin, V. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5855–5859
19. Bigger, C. B., Melnikova, I. N., and Gardner, P. D. (1997) J. Biol. Chem. 272, 25976–25982
20. Hagen, G., Muller, S., Beato, M., and Suske, G. (1994) EMBO J. 13, 3843–3851
21. Kumar, A. P., and Butler, A. P. (1997) *Nucleic Acid Res.* **25**, 2012–2019
22. Kwon, H. S., Kim, M. S., Edenberg, J. H., and Hur, M. W. (1999) *J. Biol. Chem.* **274**, 29–28
23. Kennet, S. B., Udvadia, A. I., and Herwitz, J. M. (1997) *Nucleic Acids Res.* **25**, 3110–3117
24. Moffat, J. G., Edens, A., and Talamantes, F. (1998) 80th Annual Meeting of the Endocrine Society, New Orleans, June 24–27, 1998, Endocrine Society, New Orleans, Louisiana
25. Lania, L., Majello, B., and De Luca, P. (1997) *Int. J. Biochem. Cell Biol.* **29**, 1313–1323
26. Majello, B., De Luca, P., and Lania, L. (1997) *J. Biol. Chem.* **272**, 4021–4026
27. Dennig, J., Beato, M., and Suske, G. (1996) *EMBO J.* **15**, 5659–5667
28. Majello, B., De Luca, P., Hagan, G., Suske, G., and Lania, L. (1994) *Nucleic Acids Res.* **22**, 4914–4921
29. Majello, B., De Luca, P., Suske, G., and Lania, L. (1995) *Oncogene* **10**, 1841–1848
30. Gluckman, P. D. (1986) *Reviews in Reproductive Biology* (Clarke, J. R., ed) pp. 1–60, Oxford University Press, Oxford
31. Saffer, J. D., Jackson, S. P., and Annarella, M. B. (1991) *Mol. Cell Biol.* **11**, 2189–2199