Transcription Factor MSY-1 Regulates Expression of the Murine Growth Hormone Receptor Gene*

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Previous studies identified and partially characterized a 42-base pair regulatory element in the 5'-flanking region of the L1 transcript of the murine growth hormone (GH) receptor gene that interacted with both double- and single-stranded DNA-binding proteins. We present evidence that the double-stranded DNA-binding protein is NF-Y, a CCAAT box-binding protein. Experiments with a dominant negative form of NF-Y indicate that NF-Y does not play a direct role in regulating the activity of the FP42 element. A cDNA clone that specifically interacts with the upper (coding) strand of the regulatory element was isolated by screening a cDNA expression library using the Southwestern technique. DNA sequencing, electrophoretic mobility shift assay, Southwestern blot analysis, and supershift EMSA confirm the identity of the single-stranded binding protein to be MSY-1, a DNA-binding protein that is evolutionary conserved from prokaryotes to eukaryotes. Mapping of single-stranded DNA configurations reveals that MSY-1 can facilitate the formation of single-stranded DNA regions in the GH receptor 5'-flanking region. Transient transfection experiments support the role of MSY-1 as a repressor of GH receptor gene activation. Southwestern blot analysis indicates that the levels of nuclear MSY-1 are decreased in the livers of pregnant mice, suggesting a role for MSY-1 in the increased expression of the GH receptor during pregnancy.

Pituitary growth hormone (GH)
 plays a key role in the regulation of postnatal growth and metabolism of fat, protein, and carbohydrate in animals (for a review, see Ref. 1). At the tissue level, these pleiotropic actions of GH result from the interaction of GH with a specific cell surface receptor, the GH receptor. The promoter-regulatory regions of the murine (2–4), ovine (5, 6), and human (7) GH receptor have been partially characterized. Heterogeneity in the 5'-untranslated regions of the GH receptor transcripts emerges as a feature common to the GH receptor gene from these different species. The two transcripts characterized in the mouse, termed L1 and L2, arise from alternate splicing (2, 8). The L2 sequence is G + C-rich with an overall G/C content of 71%; in contrast the L1 transcript has a G/C content of 41% (8). Expression of the L1 and L2 transcripts is regulated in a tissue- and development-specific manner. Late pregnant mouse liver and placenta both express GH receptor mRNAs containing L1 and L2 sequences (8). The L1 GH receptor transcript is expressed in the liver only during pregnancy (8). In the placenta, GH receptor transcripts containing L2 untranslated regions are more abundant than transcripts containing L1 untranslated regions.

In the mouse, an enhancer element located −3 kb from the major start site of the L1 transcript interacts with the transcription factor CTF/NF-1 in COS-7 cells (3). The most distal cis-element identified to date, termed FP42, is located −3.5 kb from the major transcription start site. Previous reports from this laboratory have described studies that suggest that both double- and single-stranded DNA-binding proteins competitively regulate the expression of the L1 transcript of the murine GH receptor gene by interacting with the FP42 site (2, 4).

In the present report, we establish the identity of both the double- and single-stranded DNA-binding proteins. Based on the characteristics of the DNA-protein contact points, we deduced that the double-stranded DNA binding activity represented protein(s) belonging to the family of CCAAT box-binding proteins. These studies resulted in the identification of the double-stranded DNA-binding protein (DSBP) as NF-Y. In the case of the single-stranded DNA-binding protein (SSBP), we screened a phage cDNA expression library to identify a cDNA encoding a protein that interacts with the upper (coding) strand of the FP42 regulatory element. Further studies established the identity of this protein to be MSY-1, a member of the Y-box family of proteins. Our studies indicate that MSY-1 functions as a repressor of GH receptor gene expression and may play a role in the pregnancy-specific expression of the GH receptor gene.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The following synthetic oligonucleotides were used in these experiments (residues altered in the mutant oligonucleotide are indicated in lowercase type): FP42-US, CTGTTAGTTCTCATATGTTGTTCCACCAATAGGGTTGCAAGAC; FP42(ΔC)-Box-US, CTGTGTTAGTTCTCATATGTTGTTCCACCAATAGGGTTGCAAGAC; E(NF-Y)-US, ATTTTCGTATTGTTAAAGT; random, CCCATGTAGAATCCAGGTGTTCAAGAC; E(NF-Y)-US, ATTTTCGTATTGTTAAAGT; random, CCCATGTAGAATCCAGGTGTTCAAGAC. Complimentary sequences are designated with the suffix LS, and the corresponding DNA duplex is named with the suffix DS.

Electromobility Shift Assay (EMSA) — Nuclear extracts from mouse liver were prepared as described by Gorski et al. (9). Protease inhibitors (2 μg/ml leupeptin, 1 μg/ml pepstatin, and 1% aprotinin) were included in the buffers used to prepare the nuclear extracts. Custom synthesized oligonucleotides were used for single-stranded DNA probes, and double-stranded DNA fragments used as probes were obtained by annealing complementary single-stranded oligonucleotides. The DNA was end-
labeled with \( {\gamma}^{32}P \)ATP and T4 polynucleotide kinase. Approximately 6 fmol of DNA was added to 1 \( \mu \)g of nuclear extract or 50–100 ng of purified recombinant MSY-1 protein in a final volume of 50 \( \mu \)l containing 1 \( \mu \)g of poly(dA-dT), 25 \( \mu \)M Hepes (pH 7.2), 75 \( \mu \)M KCl, 25 \( \mu \)M MgCl\(_2\), 250 \( \mu \)g/ml bovine serum albumin, 10% glycerol, 0.025% Nonidet P-40, and 1 mM dithiothreitol (for single-stranded DNA probes) or 1 \( \mu \)g of poly(dA-dT), 20 \( \mu \)M Tris-HCl (pH 8.0), 50 \( \mu \)M NaCl, 50 \( \mu \)g/ml bovine serum albumin, 1% Nonidet P-40, 1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol (for double-stranded DNA probes). Following incubation at room temperature for 30 min, DNA-protein complexes were resolved by electrophoresis at room temperature through a 6% non-denaturing polyacrylamide gel (acylamide:bisacrylamide ratio 19:1) with 90 \( \mu \)M Tris borate (pH 8.5), 2 mM EDTA buffer. In order to achieve better resolution, the DNA-protein complexes formed with recombinant MSY-1 protein were resolved at 4 °C on a 5% non-denaturing polyacrylamide gel (acylamide:bisacrylamide ratio of 50:1) with 90 \( \mu \)M Tris borate (pH 7.0), 2 mM EDTA buffer. Where indicated, the gels were dried and sequentially subjected to autoradiography with intensifying screens (NEN Life Science Products) at 80 °C and analysis via PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Competition experiments included the addition of excess unlabeled DNA fragments to the reaction mix. In some experiments, nuclear extracts were incubated with the indicated amounts of polyclonal antibodies against NF-YA (Rockland, Gilbertsville, PA), NF-YB (courtesy of D. Mathis and R. C. Mole, University of Cambridge, U.K.), or NF-YC (courtesy of Dr. A. Wolff) for 120 min at 4 °C before the addition to the binding reactions.

Methylation Interference Assay— Gel-purified single-stranded oligonucleotide was end-labeled with \( {\gamma}^{32}P \)ATP and T4 polynucleotide kinase. Double-stranded DNA probes uniquely labeled on one strand were obtained by annealing complementary labeled and unlabeled single-stranded oligonucleotides. The single- or double-stranded probes were modified with dimethyl sulfate for 10–40 min on ice (10). For the preparative EMSA, protein-DNA complexes were formed by incubating \( {\gamma}^{32}P \)labeled or unlabeled double-stranded FP42 DNA duplex liver nuclear extract as described above for EMSA. Following electrophoresis, DNA and protein-DNA complexes were located by short exposure of the undried gel to x-ray film, appropriate gel regions excised, eluted, and then precipitated. Base elimination and strand scission reactions at adenine and guanine (A → G) were performed as described (10). The samples were then lyophilized, resuspended in water, reolyophilized (three times), and analyzed by electrophoresis through a 12% sequencing gel. The dried gel was sequentially subjected to autoradiography and analyzed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

DNA Sequencing— Sequencing was carried out by the dyeoxynucleotide chain termination method of Sanger et al. (11) using the Sequenase 2.0 kit (U.S. Biochemical Corp.). Sequencing primers were either 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 was managed using the sequence analysis program MacVector version 5.0 (Oxford Molecular Group, PLC).

Reporter Gene Constructs— The design and construction of the luciferase reporter gene constructs containing the 3.6-kb portion of the GH receptor 5-flanking region has been reported previously (2). The reporter gene construct containing the plg2B-3.6/FP42/CBox sequence, which contains a mutation of the pentanucleotide sequence of the FP42-CCAAAT box in the context of the homologous promoter was obtained by a PCR-based strategy. All constructs were sequenced using the vector-insert junctions to verify directionality.

Transient Expression of Reporter Gene Assay— The culture media used for tissue culture experiments were obtained from Life Technologies, Inc. unless otherwise stated. BNCL2 cells (ATCC, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (with 1-glutamine, pyrdoxine hydrochloride, sodium pyruvate, and 4500 mg of glucose), 10% fetal calf serum, and penicillin G (100 units/ml) and streptomycin (100 \( \mu \)g/ml). 1 × 10\(^6\) cells were plated on 60-mm plates 24 h prior to transfection. Three micrograms of plasmid DNA was transfected per plate using the LipofectAMINE method (Life Technologies). After a 6-h incubation, the cells were washed with phosphate-buffered saline and then harvested in medium for 24 h prior to harvest for luciferase assay. For estimation of luciferase activity, the plates were rinsed twice with phosphate-buffered saline, and the cells were harvested by the addition of 400 \( \mu \)l of lysis buffer (Dual Luciferase Assay System; Promega, Madison, WI). Following a brief freeze-thaw cycle, the insoluble debris was removed by centrifugation at 4 °C for 2–3 min at 12,000 × g. The supernatant was then immediately processed for sequential quantitation of both firefly and Renilla luciferase activity (Dual Luciferase Assay System; Promega) using a Monolight 2010 Luminometer (Analytical Luminescence, San Diego, CA). All transfections were performed in duplicate. Transfection efficiency was monitored by co-transfection of 0.2 \( \mu \)g of the plasmid pRL-TK (Promega) expressing the Renilla luciferase reporter gene. All transfections were done in quadruplicate and the mean and standard deviation was calculated. Results are presented as mean ± S.E. of four independent experiments. Statistical differences between groups were determined by Student's t test, p values equal to or less than 0.05 were considered to be statistically significant.

Screening of cDNA Library— 2 × 10\(^6\) plaques of a \( \lambda \) murine brain cDNA expression library (Uni-ZAP\( \alpha \) XR; Stratagene, La Jolla, CA) was screened by the Southwestern technique (12) using \( {\gamma}^{32}P \)-labeled upper (coding) strand oligonucleotide, FP42. The DNA, end-labeled with \( {\gamma}^{32}P \)ATP and T4 polynucleotide kinase, was hybridized (in a buffer containing 10 \( \mu \)M Tris-HCl (pH 7.6), 50 mM KCl, 5 mM MgCl\(_2\), 1 mM EDTA, 0.25% milk powder, and 15 \( \mu \)g/ml salmon sperm DNA) to nitrocellulose filters containing proteins induced by exposure of the \( \lambda \) clones to 20 mM isopropyl-\( \beta \)-galactopyranoside. Prior to hybridization to the DNA probe, the proteins on the nitrocellulose filters were denatured by treatment with guanidine hydrochloride (6 M guanidine hydrochloride, 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM MgCl\(_2\), and 1 mM EDTA), 0.25% milk powder, and 15 \( \mu \)g/ml salmon sperm DNA. The results of the luciferase assay are expressed in relative light units equalized for transfection efficiency. Protein concentration of the supernatant was determined using the Bradford protein assay (Bio-Rad). Results are presented as mean ± S.E. of three or four independent experiments. Mung Bean Nuclease Hypersensitivity Mapping— Mung bean nuclease was used to map hypersensitive sites in the promoter region of the human \( \alpha 1A \) subunit of the AChR gene (13). The cell pellets were washed with lysis buffer and then subjected to Mung Bean Nuclease digestion. The resulting DNA was fractionated by agarose gel electrophoresis.
ase was used as described previously to detect single-stranded regions in double-stranded probes (13). A 100-bp double-stranded probe that encompasses the FP42 site was generated by polymerase chain reaction amplification. Unique labeling of the top strand of this duplex was achieved by using a $^{32}$P-labeled oligonucleotide in the polymerase chain reaction. The labeled duplex was purified by polyacrylamide gel electrophoresis, and the eluted DNA was used in the reaction. In a final reaction volume of 10 μl, the probe (6 × 10^4 cpm) was mixed with MSY-1 (50–100 ng) and 1 μg of poly(dA-dT) in the EMSA buffer. Following incubation at 37 °C for 20 min, the reaction volume reaction was expanded 5-fold. 10 units of mung bean nuclease (New England Biolabs) were added to the reaction containing a final concentration of 1× mung bean nuclease buffer and incubated at 37 °C for varying intervals of time. The saturating concentration of the mung bean nuclease (10 units) was determined by previous titration of mung bean nuclease on the labeled double-stranded probe. The reactions were stopped by the addition of 240 μl of stop buffer (100 mM Tris (pH 8.0), 100 mM NaCl, 20 mM EDTA, 0.1% SDS, 100 μg/ml protease K, and 200 μg/ml of glycogen). The samples were then precipitated and electrophoresed through a 8% polyacrylamide gel. Labeled DNA markers and a DNA-protein complex. The Western blots were developed using the ECL enhanced chemiluminescence system (Amersham Pharmacia Biotech).

RESULTS

CCAAT Box Factor NF-Y Binds to a Distal Regulatory Element in the Murine GH Receptor 5′-Flanking Region—We had previously established that a 42-base pair regulatory element (termed FP42), located about 3.5 kb upstream of the major transcription start site for the L1 transcript of the murine GH receptor gene, bound distinct single- and double-stranded DNA-binding proteins. Chemical modification of the purines present in FP42-DS revealed that the contact points of the DSBP on both strands encompassed the CCAAT box (Fig. 1), suggesting the possibility that the murine HMC class II Ea promoter (E$_a$(NF-Y)) (19) abrogated the formation of DNA-protein complex with mouse liver nuclear proteins and $^{32}$P-labeled wild type FP42-DS (Fig. 2A). These results indicate the absolute requirement for the CCAAT motif in the formation of the DNA-protein complex with FP42-DS and suggest the possibility that the DSBP represents NF-Y. Inclusion of a 100-fold molar excess of unlabeled oligonucleotide corresponding to an NF-Y binding site derived from the mouse MHC class II Eα promoter (E$_a$(NF-Y)) (19) abrogated the formation of DNA-protein complex with mouse liver nuclear proteins and $^{32}$P-labeled wild type FP42-DS (Fig. 2A).
A or B subunits of NF-Y in an EMSA reaction with 32P-labeled FP42-DS and mouse liver nuclear extract retarded the mobility of the FP42 double-stranded DNA-protein complex, resulting in a “supershift” (Fig. 3). In contrast, antibodies against CCAAT box-binding factors CTF/NF-1 (Fig. 3) or CCAAT/enhancer-binding protein (data not shown) did not alter the mobility of the FP42 double-stranded DNA-protein complex. We conclude from these results that in liver nuclear extract the DSBD binding to FP42-DS is NF-Y.

To determine if NF-Y binding to the FP42 element affects the transcriptional activity of the murine GH receptor promoter, transient transfections were done with luciferase reporter plasmids containing 3.6 kb of 5′-flanking sequence of the murine GH receptor gene. These plasmids contained either the wild-type 3.6-kb plasmid or a mutated CCAAT box (data not shown). These results indicate that mutating the NF-Y DNA-binding site causes an increase in murine GH receptor promoter activity. In the original characterization of the FP42 element, we had demonstrated that in the context of the heterologous thymidine kinase promoter, this element exhibited enhancer activity in primary rat hepatocytes (2). The discrepancy between results obtained in the current study with the homologous promoter and the previous experiments involving a heterologous promoter illustrates a drawback of using a heterologous system to study the transcriptional ability of a putative cis-acting element. This alteration in the function of the FP42 element is unlikely to be due to the different cell types used for the transient transfection experiments (i.e. primary rat hepatocytes versus BNCL2 cells), but even in BNCL2 the FP42 element acts as an enhancer in the context of the thymidine kinase promoter (data not shown).

To determine if the lack of binding of NF-Y was responsible for the increased activity of the pGL2B-3.6(FP42ΔC,Box), we exploited the availability of a dominant negative form of NF-YA (29) to selectively decrease the binding of NF-Y to the FP42 element. NF-Y is a heterotrimeric protein composed of at least three subunits: NF-YA, NF-YB, and NF-YC. The NF-YA subunit is essential for DNA binding of the complex. The dominant negative form of NF-YA (NF-YA29) acts by heterodimerizing with the NF-YB available in the cell to form transcriptionally defective NF-Y molecules. In BNCL2 cells, co-

**Fig. 2. Effects of mutations in the CCAAT box of the FP42 sequence on protein-DNA binding.** 32P-Labeled FP42-DS (A) or E₃(NF-Y) (B) was incubated with nuclear extracts from liver of adult mice, electrophoresed, and subjected to autoradiography as described under “Experimental Procedures.” A, 32P-labeled FP42-DS was electrophoresed either without (lane 1) or with (lanes 2–8) the addition of liver nuclear extract. Competition between labeled FP42-DS and unlabeled wild type sequence (FP42-DS, lane 3), unrelated oligonucleotide (lane 4), mutant oligonucleotide (FP42ΔC,Box)-DS, lane 5), or E₃(NF-Y) oligonucleotide (lanes 6–8) at molar ratios of 50 (lane 6), 100 (lanes 3, 4, and 6), and 200 (lane 8) is shown. The bands representing specific DSBD and the unbound 32P-labeled FP42-DS are indicated. B, 32P-labeled E₃(NF-Y) was incubated with nuclear extracts from liver of adult mice, electrophoresed, and subjected to autoradiography as described under “Experimental Procedures.” Labeled E₃(NF-Y) was electrophoresed either without (lane 1) or with (lanes 2–8) the addition of liver nuclear extract. Competition between labeled E₃(NF-Y) and unlabeled E₃(NF-Y) (lane 3), mutant oligonucleotide (FP42ΔC,Box)-DS, lane 4), unrelated oligonucleotide (lane 5), or FP42-DS (lanes 6–8) at molar ratios of 50 (lane 6), 100 (lanes 3, 4, 5, and 7) and 200 (lane 8) is shown. The bands representing the specific protein-DNA complex (C) and the unbound 32P-labeled E₃(NF-Y) are indicated.

**Fig. 3. NF-Y binds to FP42-DS.** 32P-Labeled FP42-DS was electrophoresed either without (lane 1) or presence (lane 2) of antibodies against CCAAT box-binding factors, electrophoresed, and subjected to autoradiography as described under “Experimental Procedures.” The antibodies included in the incubation mix were anti-NF-YA (lane 2), anti-NF-YB (lane 3), or anti-CTF/NF-1 (lane 4). The bands representing unbound 32P-labeled FP42-DS, DSBD, and the supershifted complex (SS) are indicated.

**Fig. 4. Mutation of the FP42 CCAAT box causes an increase in the activity of the GH receptor promoter.** Expression plasmids containing the promoterless vector (pGL2-Basic), wild type 3.6-kb (pGL2B-3.6(+53)), or mutant (pGL2B-3.6(FP42ΔC,Box)) DNA fragments were transfected into BNCL2 cells, and luciferase activity was measured as described under “Experimental Procedures.” Luciferase-specific activity in cell homogenates, equalized for transfection efficiency monitored by cotransfection of a plasmid expressing Renilla luciferase (pRL-TK; Promega), is expressed relative to that of pGL2-Basic. Results represent the mean ± S.E. of three independent transfections performed in duplicate using Student’s t test (*, p < 0.05 compared with pGL2-Basic; **, p < 0.05 compared with pGL2B-3.6(+53)).
transfection of a plasmid (pNF-YA29) expressing the dominant negative mutant NF-YA29 did not alter the activity of either the pGL2B-3.6(+53) or the pGL2B-3.6(FP42ΔC.Box) construct (Fig. 5). Previous reports had demonstrated that the mutant NF-YA29 subunit can sequester NF-YB into defective complexes that lose the ability to bind DNA (23, 24). EMSA with 32P-labeled FP42-DS and nuclear extracts from either mock-transfected BNC12 cells (lane 1) or BNC12 cells transfected with pNF-YA29 (lane 2) posited the NF-YA-DNA complex (A) and that of the free probe (F) are indicated. Bottom, the nuclear extracts indicated in the top were assayed by EMSA with 32P-labeled probe containing the consensus binding site for the ubiquitous transcription factor OCT-1. The position of the DNA-protein complex containing OCT-1 is indicated (B).

Fig. 5. Lack of effect of dominant negative form of NF-YA on the activity of FP42. Expression plasmid for the wild type NF-YA (pGL2-Basic) or the dominant negative form of NF-YA (pNF-YA29) was co-transfected into BNC12 cells with luciferase reporter plasmids containing the promoterless vector (pGL2-Basic), wild type 3.6-kb (pGL2B-3.6(+53)), or mutant (pGL2B-3.6(FP42ΔC.Box)) DNA fragments, and luciferase activity was measured as described under “Experimental Procedures.” Luciferase-specific activity in cell homogenates, equalized for transfection efficiency monitored by cotransfection of a plasmid expressing Renilla luciferase (pGL-TK; Promega), is expressed as relative to that of the pGL2-Basic. Results represent the mean ± S.E. of three independent transfections performed in duplicate. Inset, dominant negative mutant NF-YA29 inhibits binding of endogenous NF-Y to FP42. Top, EMSA with 32P-labeled FP42-DS and nuclear extracts from either mock-transfected BNC12 cells (lane 1) or BNC12 cells transfected with pNF-YA29 (lane 2). Positions of the NF-YA-DNA complex (A) and that of the free probe (F) are indicated. Bottom, the nuclear extracts indicated in the top were assayed by EMSA with 32P-labeled probe containing the consensus binding site for the ubiquitous transcription factor OCT-1. The position of the DNA-protein complex containing OCT-1 is indicated (B).

scription factor OCT-1 in untransfected and pNF-YA29-transfected cells. These results indicate that lack of effect of NF-YA29 on the activity of FP42 occurs despite decreased binding of NF-Y to FP42-DS. We conclude from these experiments that the increase in activity of the 3.6(FP42ΔC.Box) construct is not due to lack of binding of NF-Y.

We had previously demonstrated that a DNA-binding protein with biochemical and DNA-binding characteristics distinct from the double-stranded DNA-binding protein selectively binds to the upper (coding) strand of the FP42 duplex (FP42-US) (4). Since the results with the dominant negative form of NF-Y indicated that binding of NF-Y to the FP42 element was not responsible for the functional activity of this cis-element, we therefore investigated the possibility that the single-stranded binding protein may play a role in activity of the FP42 element. Toward this goal, we next focused our efforts on the identification and characterization of the single-stranded DNA-binding protein.

Isolation of a cDNA Encoding a Protein That Specifically Binds to the Upper Strand of the GH Receptor Gene Regulatory Element FP42—To clone the cDNA for the SSBP, we initially screened a mouse liver cDNA expression library by the Southwestern procedure using as a probe radiolabeled synthetic oligonucleotide whose sequence corresponded to the upper strand of the FP42 duplex. Failing to isolate a clone that coded for a protein that specifically bound to FP42-US, we rescreened a mouse brain cDNA expression library. The brain cDNA library was chosen after confirming by EMSA that the SSBP is expressed in brain (data not shown). Following screening of the brain library, a clone (which we termed 1–3B) was identified that exhibited specific binding to FP42-US; the lower strand of FP42 did not bind to this clone. Further analysis showed that clone 1–3B contained a 1.5-kb DNA insert. Sequencing of the entire 1.5-kb DNA insert established its identity as MSY-1 as determined by BLAST alignment to GenBank™ sequences. MSY-1 is a member of the Y-box family of proteins, originally defined by the property of selective binding to a DNA sequence (Y box) that contains an inverted CCAAT sequence (21, 22).

To confirm that clone 1–3B coded for a protein that bound FP42-US, the 1–3B cDNA was subcloned into the expression vector pQE-10 (Qiagen; Chatsworth, CA), and the ability of the translated protein to bind FP42-US was tested by two methods, Southwestern analysis and EMSA. In EMSA, the recombinant 1–3B protein retarded the mobility of labeled FP42-US (Fig. 6). The specificity of this DNA-protein interaction was established by competition EMSA experiments in which the binding of 32P-labeled FP42-US by the 1–3B protein was abolished by a 20-fold molar excess of unlabeled FP42-US but not by a similar molar excess of a random sequence oligonucleotide (Fig. 6). In contrast to FP42-US, the binding of the 1–3B protein to FP42-DS was markedly reduced, and binding to FP42-LS was not demonstrable (Fig. 7). These results indicate that the 1–3B protein binds preferentially to the upper strand of the FP42 and that this interaction is DNA sequence-specific. The ability of the 1–3B protein to bind to FP42-US was also tested by Southwestern analysis. In Southwestern analysis, the translation product of the 1–3B clone was subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane before hybridization. The 1–3B protein bound to 32P-labeled FP42-US (Fig. 6) but neither to 32P-labeled FP42-LS nor to FP42-DS (data not shown). The specificity of binding was confirmed by competition experiments in which the binding reaction was carried out in the presence of either unlabeled FP42-US or an oligonucleotide with random sequence. A 100-fold molar excess of unlabeled FP42-US decreased the binding of the labeled FP42-US to the 1–3B pro-
protein, although a similar 100-fold molar excess of a random sequence oligonucleotide did not significantly alter this binding (Fig. 6). These results indicate that the binding of 1–3B protein to FP42-US is sequence-specific. Previous reports had indicated the ability of MSY-1 protein to multimerize upon binding to DNA (22, 25). We tested whether MSY-1 was able to multimerize at the FP42 site. Inclusion of increasing amounts of recombinant 1–3B protein resulted in an ascending series of shifted FP42-US, consistent with multimerization (Fig. 7). In contrast, the propensity of MSY-1 to multimerize was markedly inhibited upon binding to FP42-DS or FP42-LS (Fig. 7).

DNA sequencing had indicated that the 1–3B cDNA was identical to that for the previously described nucleic acid-binding protein MSY-1. To ascertain that the protein binding to FP42-US in liver nuclear extracts is MSY-1, we investigated the binding characteristics of recombinant MSY-1 and the FP42-US-binding protein present in liver. Having demonstrated that MSY-1 exhibits a propensity to multimerize on FP42-US, we tested whether the FP42-US-binding protein in liver nuclear extracts also exhibits this property. Thus, inclusion of increasing amounts of liver nuclear extracts in binding reaction with labeled FP42-US resulted in an ascending series of complexes that indicated the presence of multimerization (Fig. 7). To conclusively establish the identity of the proteins binding in liver nuclear extracts, we used polyclonal antibodies against MSY-1 in supershift EMSA. These experiments demonstrated that the MSY-1 antibody altered the mobility of the DNA-protein complex formed by FP42-US and liver nuclear extract (Fig. 8). The specificity of this alteration in the mobility of the DNA-protein complex was demonstrated by the lack of effect of anti-NF-YA and NF-YB antibodies (data not shown). These data support the conclusion that the 1–3B protein binds the FP42-US and that the identity of the protein is MSY-1.

**MSY-1 Regulates the Activity of FP42**—Having established the identity of the SSBP binding to FP42-US, we next determined the effect of MSY-1 on the activity of the FP42 regulatory element by testing its ability to alter the activity of FP42 in the context of the homologous promoter contained in the luciferase reporter vector pGL2-Basic (Promega). Overexpression of MSY-1 was achieved by cloning the 1–3B DNA into the
overexpression experiments using BNCL2 cells, pGL2B-3.6(+53) exhibited 2-fold more activity over the activity of the vector alone. Whereas cotransfection of the expression plasmid pFLAG-CMV alone did not alter the activity of the pGL2B-3.6(+53) plasmid, cotransfection of the pFLAG-CMV-(1–3B) plasmid resulted in a 50–60% decrease in the luciferase activity of the pGL2B-3.6(+53) reporter plasmid (Fig. 9). There was no effect of MSY-1 overexpression on the basal activity of the pGL2-Basic vector. Importantly, overexpression of MSY-1 did not have an effect on the activity of the pGL2B-3.6(FP42A.Box) construct, which contains a mutation of the pentanucleotide sequence of the FP42-CCAAT box in the context of the homologous promoter. To confirm that the mutation in the CCAAT box motif is essential for this action, we used EM SA to test the ability of FP42A.Box-US to bind recombinant MSY-1. In EM SA, the addition of MSY-1 protein failed to retard the mobility of 32P-labeled FP42A.Box-US oligonucleotide, demonstrating the inability of FP42A.Box-US to bind MSY-1 (data not shown). These results indicate that the increased activity of the pGL2B-3.6(FP42A.Box) construct can be attributed to lack of binding of MSY-1. In conjunction with the results obtained with the dominant negative form of NF-Y, these results support a model wherein MSY-1 is the active factor and NF-Y does not play a direct role in regulating the activity of the FP42 element. These data support the conclusion that MSY-1 regulates the activity of the FP42 element by acting as a repressor of its activity and that the CCAAT box motif is essential for this action.

MSY-1 Facilitates Formation of Single-stranded Configuration at the FP42 Site—In EMSA, MSY-1 exhibited high affinity for the upper strand of the FP42 duplex, while not binding to the lower strand. We used mung bean nuclease to examine the possibility that MSY-1 could facilitate the formation of single-stranded configuration within this region. Mung bean was chosen for this purpose, since it has a much lower affinity for double-stranded DNA than the single-stranded nuclease S1 (13). Incubation of the double-stranded probe that encompasses the FP42 site with MSY-1 resulted in the appearance of a mung bean nuclease-sensitive region adjacent to the CCAAT box motif (Fig. 10). This result suggests that MSY-1 either brings about the melting of this region or stabilizes the inherent tendency of the DNA to assume a single-stranded configuration.

Levels of Nuclear MSY-1 Decrease in the Liver during Pregnancy—The L1 transcript of the murine GH receptor is expressed in the liver only during pregnancy (see Fig. 11 and Ref. 8). To investigate whether MSY-1 plays a role in this pregnancy-specific expression of the growth hormone receptor, we determined levels of MSY-1 mRNA in liver. As shown in Fig. 12, Northern blot analysis of liver total RNA indicated that there is no significant change in the level of MSY-1 RNA in nonpregnant compared with pregnant mice. A straightforward interpretation of this result is that MSY-1 does not play a significant role in pregnancy-specific hepatic expression of the GH receptor. However, recent studies have demonstrated that one of the mechanisms by which Y-box proteins regulate gene expression is by nuclear translocation (26, 27). To examine this mechanism, we used Southwestern analysis to determine the levels of MSY-1 in nuclear and cytoplasmic compartments of the liver from pregnant and nonpregnant mice. As shown in Fig. 12, there was a decrease in the levels of MSY-1 in the nucleus. The decrease in nuclear MSY-1 levels in the pregnant mouse liver was specific, since determination of the levels of a ubiquitously expressed transcription factor Sp1 were not altered in these samples (Fig. 12). It is noteworthy that Southwestern blot analysis also revealed a protein of about 40–45 kDa that in-

![Graph showing relative luciferase activity](image)

**Fig. 9.** Overexpression of MSY-1 represses activity of the GH receptor promoter. Luciferase expression plasmid containing the 3.6-kb (pGL2B-3.6(+53)) or mutant (pGL2B-3.6(FP42A.Box)) DNA fragments were cotransfected into BNCL2 cells with either expression plasmid pFLAG-CMV-(1–3B) or the vector pFLAG-CMV-2. Luciferase activity was measured as described under "Experimental Procedures." Luciferase-specific activity in cell homogenates, equalized for transfection efficiency monitored by cotransfection of a plasmid expressing Renilla luciferase (pRL-TK; Promega), is expressed as relative to that of pGL2B-3.6(+53) plus pFLAG-CMV-2. Results represent the mean ± S.E. of three independent transfections performed in duplicate using Student's t-test (*, p < 0.05 compared with pGL2B-3.6(+53) plus pFLAG-CMV).

**Fig. 10.** MSY-1 promotes single-stranded configuration of the FP42 duplex. 32P-Labeled FP42-US was incubated with (lanes 2 and 4) or without (lanes 1 and 3) 100 ng of recombinant MSY-1, followed by exposure to mung bean nuclease for 30 min (lanes 1 and 2) and 60 min (lanes 3 and 4). The reactions were electrophoresed through an 8% polyacrylamide gel and subjected to autoradiography. An increase in the intensity of a respective band in the presence of mung bean nuclease indicates the presence of a hypersensitive site. The precise location of the hypersensitive region (indicated by the arrow) was determined by concurrently electrophoresing a DNA sequencing ladder.
and lose membrane. The nitrocellulose membranes corresponding to lanes 1
SDS-polyacrylamide gel electrophoresis and transferred to nitrocellu-
Northern analysis was performed using 20 m
or cytoplasmic (described under “Experimental Procedures.”
lane 1 from either male (lane 1), nonpregnant (lane 2), or pregnant (lane 3), mouse liver tissue. Immobilized RNA was sequentially hybridized to
described pregnancy-specific expression of the GH receptor gene.
during pregnancy suggests that MSY-1 plays a role in the
scription in conjunction with decreased levels of nuclear MSY-1
identity of this band remains to be elucidated. The demonstra-
creased in the liver nuclear extracts from pregnant mice. The
autoradiography as described under “Experimental Procedures.”

In a previous report, we had identified a 42-bp region in the
5′-flanking region of the murine GH receptor gene that exhib-
regulated regulatory activity. We had previously established that in
liver nuclear extracts both double- and single-stranded DNA-
linking proteins bind to this 42-bp element. In this report, we
establish the identity and investigate the functional role of
both of these proteins; the double-stranded binding protein is
NF-Y, and the single-stranded DNA-binding protein is MSY-1.

Three lines of evidence indicate that the liver nuclear protein
binding to the FP42-DS is NF-Y: first, the inclusion of residues
within the CCAAT motif critical for DNA recognition by the
DNA-binding protein; second, the demonstration of the absolute
requirement of the CCAAT sequence for DNA-protein in-
teraction; and third, the ability of anti-NF-Y antibodies to alter the
mobility of the FP42-DS-protein complex. The core binding
site for NF-Y at the FP42 element is in agreement with the
consensus binding site, PuPuCCAAT(C/A)/G/G/G reported for
NF-Y (28). The CCAAT box is a widely distributed cis-regulatory
element found in the promoter and enhancer region of a
large number of genes. This pentanucleotide is usually positioned
near (within 60–80 base pairs of) the transcription start site. However, this proximal location is not an invariant fea-
ture, as demonstrated by the presence of functional NF-Y bind-
ing sites approximately 700–800 bp upstream of the transcrip-
tion start sites of the human myeloperoxidase and CYP1A1
genes (29, 30). Our studies indicate that this is also the case for
the GH receptor gene where the NF-Y site characterized in the
present study is located about 3.5 kilobases upstream from the
major transcription start site.

NF-Y is an ubiquitous heterotrimeric complex composed of at
least three subunits: NFY-A, NFY-B, and NFY-C. The three
subunits contain protein domains that are highly conserved
across species. The exact mechanism for the transcriptional
activation of NF-Y is not clear. Although NF-Y is not able to
directly activate transcription, it appears to increase the activity
of neighboring enhancer elements. Possibly, this cooperative
function is in part mediated by indirect facilitation of
protein-protein interactions with other transcription factors
(31–33) brought about by NF-Y-induced bending of the DNA
(28).

We had previously established that the upper strand of the
FP42 element bound a DNA-binding protein. To determine the
identity of this single-stranded DNA-binding protein, we suc-
cessfully employed the Southwestern protocol to screen a cDNA
expression library to isolate a cDNA clone (which we termed
MSY-1) that specifically interacted with the upper strand of
FP42. DNA sequencing of this clone established the identity of
the clone to be MSY-1, and both Southwestern blot analysis
and EMSA established that MSY-1 bound to FP42-US in a
sequence-specific manner. Three lines of evidence support the
conclusion that the protein encoded by 1–3B clone represents
the protein in mouse liver nuclear extract that is bound by
FP42-US: first, similarities in the DNA-binding characteristics
of the protein in mouse liver and MSY-1, including the ability
to multimerize on FP42-US; second, the fact that antibodies
against MSY-1 alter the mobility of the DNA-protein complex;
and third, the ability of recombinant MSY-1 to alter the func-
tion of the FP42 element. Thus, in transient transfection ex-
periments, overexpression of MSY-1 functions as an inhibitor
of GH receptor gene transcription. This inhibitory function is
mediated in large part through the distal FP42 element, since
mutation of this site abolishes the repressive effect of MSY-1 on
GH receptor gene transcription. The inhibitory role of MSY-1 is
compatible with previous studies that have shown a similar
role for MSY-1 in the regulation of other genes (25). Our results
with recombinant MSY-1 protein indicate that concentration-
dependent multimerization occurs upon binding to FP42-US.

The ability to multimerize is a property common to the Y-box
family of proteins (22, 25). Studies with the Y-box protein,
FRGY, suggest that the “tail domain” at the C-terminal region
of the protein facilitates both homo- and heteromultimeriza-
tion. This propensity of MSY-1 to multimerize on FP42 DNA
may provide an explanation for its extended DNA footprint
(34). The actual significance of the multimerization remains
speculative at this time, but it may facilitate interactions with
other transactivating factors.
The Y-box family of proteins, of which MSY-1 is a member, represent a multigene family that has been structurally and functionally conserved during evolution (21, 22). An 80-amino acid sequence in the amino-terminal half of the protein is conserved among Y-box proteins. This region corresponds to the DNA-binding domain, known as the “cold shock domain” because of the high degree of homology with the cold shock proteins of *E. coli*. Adjacent to the cold shock domain is a hydrophilic domain (“tail domain”) that extends up to the carboxyl terminus, in which there is an alternation of basic and acidic groups of amino acids that may contribute to protein-protein interactions. Y-box proteins modulate gene expression by a variety of different mechanisms. As DNA-binding proteins, they preferentially bind single-stranded DNA, although binding to double-stranded DNA can also be achieved, albeit with lower affinity (21, 22). These proteins can directly activate or repress transcription from prokaryotic and a diverse group of eukaryotic promoters (21, 22). As RNA-binding proteins, they play a role in the blocking (“masking”) of translation of paternal mRNA in mouse spermatocytes and maternal RNA in *Xenopus* oocytes (35, 36).

The significance of the overlap of the binding sites for MSY-1 and NF-Y is not clear. Interaction between MSY-1 and NF-Y has been demonstrated in the regulation of the MHC class II gene I-Aq (37). Overlapping MSY-1 and NF-Y DNA recognition sites did not allow us to use mutational analysis of the binding site as a strategy to isolate the effects of NF-Y and MSY-1 at the FP42 site. NF-Y binds exclusively to the double-stranded FP42 element and does not bind the individual strands of the FP42 element. In contrast, MSY-1 binds much more strongly to single-strand (FP42-US) DNA than to the FP42 duplex. In addition, the results of the mung bean nuclease experiments indicate that MSY-1 acts to induce or stabilize a single-stranded DNA configuration at this region. One model to explain the significance of the overlap of the binding sites for NF-Y and MSY-1 is that MSY-1 acts to inhibit transcription of the GH receptor gene, and NF-Y modulates access of MSY-1 to the single-stranded DNA-binding site by maintaining the DNA in a double-stranded configuration. However, the transient transfection experiments with the dominant-negative form of NF-Y failed to reveal a functional role for NF-Y in modulating the function of the FP42 element. Chromatin structure plays a crucial role in the control and regulation of eukaryotic gene transcription (38). This phenomenon is partly attributed to the remodeling of nucleosomes in a dynamic manner by a number of multicomponent complexes that participate in enzymatic modification of chromatin structure. In particular, enzymes that acetylate or deacetylate specific N-terminal lysines in the core histone proteins play an important role in this regulation. Recent evidence suggests that NF-Y possesses histone acetyltransferase activity through physical association with the related histone acetyltransferase enzymes, GCN5 and p300/CPB-associated factor (39). It is postulated that these associated histone acetyltransferases serve to modulate the transactivation potential of NF-Y by causing disruption of local chromatin structure and allowing NF-Y access to its DNA-binding site. The data obtained in the present study supporting the nonfunctionality of NF-Y binding at the FP42 site were obtained by experiments using episcopal reporter gene constructs. Hence, if the role of NF-Y is to regulate the access of MSY-1 to the binding site by remodeling of the chromatin structure, then this function will not be apparent in these episcopal studies. A comprehensive dissection of the functional role of the NF-Y at this DNA-binding site may thus require experiments with stable cell lines or transgenic models in which there is retention of the chromatin structure.

The expression of the GH receptor in the liver is significantly increased during pregnancy. In the mouse, this increase in GH receptor gene expression is because of an increase in expression of the L1 transcript. The demonstration that MSY-1 inhibits GH receptor gene transcription and that levels of nuclear MSY-1 protein decrease in the liver of pregnant mice compared with nonpregnant mice suggests that MSY-1 plays a role in the pregnancy-specific expression of the GH receptor. Our results indicate that nuclear translocation is one of the mechanisms contributing to the alteration in MSY-1 levels in the nucleus during pregnancy. This mechanism for modulating the function of MSY-1 has been previously reported with other genes. Thus, increased levels of YB-1 in the nucleus as a consequence of nuclear translocation results in increased expression of the *MDR1* gene in primary human breast cancers (26). Similarly, ultraviolet irradiation was shown to result in nuclear translocation in human epidermoid cancer cells despite a lack of alteration in the total cellular content of YB-1 (27). Whereas the current report suggests a role for MSY-1 in the pregnancy-specific expression of the GH receptor gene, the precise role of this protein in this phenomenon remains to be elucidated. Thus, the role of NF-Y, the possible involvement of chromatin remodeling, and the role of post-translational modifications of MSY-1 in modulating its activity (35) constrain the inferences that can be drawn from this study regarding the role of MSY-1 in the pregnancy-specific expression of the GH receptor gene. In addition, the identity and the role of the 40–45-kDa protein(s) in liver of pregnant mice that bind FP42-US on Southwestern blot remain to be clarified. Since the L1 transcript is only expressed in the liver during pregnancy, the precise role of the FP42 element and that of MSY-1 in regulating the activity of the GH receptor gene in the intact animal will have to await studies using transgenic mice.

In summary, this report is the first to establish the identity of factors that regulate the transcription of the GH receptor gene. Our studies establish that MSY-1 serves as an inhibitor of the murine GH receptor gene transcription and suggest a potential role for NF-Y in this interaction. A role for MSY-1 in the pregnancy-specific expression of the GH receptor gene is suggested by the decrease in levels of nuclear MSY-1 in the livers of pregnant mice. These findings will allow for the examination of the functional role of these factors in the pregnancy-specific expression of the GH receptor in intact animal models.

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