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STAT 5 and NF-Y are involved in expression and growth hormone-mediated sexually dimorphic regulation of cytochrome P450 3A10/lithocholic acid 6β-hydroxylase

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ABSTRACT

The level of expression of a number of sexually differentiated liver proteins is primarily determined by plasma growth hormone (GH). Adult males have a pulsatile profile of GH release, while females have a relatively steady-state pattern of GH release. An important subset of these sexually differentiated hepatic proteins is certain cytochrome P450s (P450s). CYP3A10/6β-hydroxylase is a male-specific P450 that catalyzes 6β-hydroxylation of lithocholic acid, and the pattern of GH secretion is directly responsible for male-specific expression of this gene. The DNA element involved in GH-mediated regulation of CYP3A10/6β-hydroxylase promoter activity binds a member of the STAT (signal transducers and activators of transcription) family of proteins. In this study we functionally demonstrate that two members of the STAT family, STAT 5a and STAT 5b, mediate GH-dependent regulation of CYP3A10/6β-hydroxylase promoter activity. Furthermore, a neighboring DNA element binds NF-Y, a transcription factor involved in maintaining high levels of transcription of many genes and known to functionally interact with other factors. In the CYP3A10/6β-hydroxylase gene, NF-Y also modulates binding of STAT 5, thereby modulating GH-mediated activation of its transcription.

INTRODUCTION

Among the various physiological actions exerted by growth hormone (GH), some are sex-specific, such as expression of a number of sexually dimorphic hepatic proteins. The level of expression of these proteins is primarily determined by plasma GH profiles and only secondarily regulated by gonadal hormones through their effects on the hypothalamo-pituitary axis and its control of GH secretion (reviewed in 1,2). Adult males have a pulsatile profile of GH release with serum levels that vary from undetectable levels to peaks of 200–300 ng/ml, whereas females have a relatively steady-state pattern of GH release with uniform, albeit lower, levels of serum GH (10–20 ng/ml). An important subset of the sexually dimorphic hepatic proteins is certain cytochrome P450s (P450s), which are a superfamily of heme-thiolate proteins that metabolize a variety of endogenous and exogenous substrates, including bile acids.

A good insight has recently been gained into the molecular mechanisms by which GH activates gene transcription (3). GH binds to two receptor molecules causing receptor dimerization and activation of the associated JAK2 tyrosine kinase, which in turn phosphorylates both the receptor as well as JAK2 itself. JAK2 and other serine/threonine kinases then phosphorylate different STAT (signal transducers and activators of transcription) factors which are translocated into the nucleus and activate transcription by binding to specific regulatory elements (3). There are several lines of evidence to strongly suggest that different members of the STAT family of transcriptional factors are involved. First, the promoters of several GH-regulated genes, such as the serine protease inhibitor (Spi) 2.1 gene (4,5), the c-*fos* gene (6), the rat insulin 1 gene (7) and the hamster cytochrome P450 3A10/lithocholic acid 6β-hydroxylase (CYP3A10/6β-hydroxylase) gene (see below), contain a γ-interferon-activated sequence (GAS) that consists of a $TT(N)_5AA$ motif (8), which mediates transcriptional activation of these genes by GH. Second, binding of nuclear proteins to these elements is eliminated in hypophysectomized animals and restored upon GH injection (5,9). Third, GH stimulates tyrosine and serine/threonine phosphorylation of STAT 1, STAT 3 and STAT 5 (6,10–12), which is associated with translocation of STATs from the cytoplasm to the nucleus and binding to their regulatory elements. Fourth, characteristic male body growth rates and male-specific liver gene expression are decreased to wild-type female levels in *STAT 5b*$^{-/-}$ male mice, whereas female-specific liver gene products are increased to a level intermediate between wild-type male and female levels (13). However, a direct effect of any of these STATs on the activity of any promoter has yet to be demonstrated, except for a modest 2-fold induction of a heterologous promoter made of the thymidine kinase promoter and the serine protease inhibitor GAS-like element 1 brought about by overexpressing STAT 5 in CHO cells (14).

The CCAAT box is a widely distributed regulatory sequence present in several promoters and enhancers, and several proteins have been reported to bind this or related sequences. Among these, NF-Y, a multicomponent factor, has been shown to be involved in expression of a number of different tissue-specific genes (15). NF-Y is not a tissue-specific factor, but it functions along with an appropriate tissue-specific regulatory protein to establish the correct pattern of expression. In the case of the albumin promoter, NF-Y interacts with the liver-enriched factor hepatocyte factor-1 (16) and in the case of the major histocompatibility complex II genes, NF-Y functions synergistically with the X-box DNA binding protein and

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CIITA, a novel non-DNA binding protein (17). Recently it has been demonstrated that NF-Y plays a role in cholesterol-mediated regulation of two genes involved in maintaining cholesterol homeostasis, HMG-CoA synthase and farnesyl diphosphate synthase (18), although the factor directly involved in cholesterol-mediated regulation of transcription of these genes is the newly characterized sterol regulatory element binding protein (SREBP) (19,20).

We have previously reported the isolation of a hamster liver P450, CYP3A10/6β-hydroxylase, which catalyzes the 6β-hydroxylation of lithocholic acid (LCA) as well as some steroid hormones (21,22) and is expressed at 50-fold higher levels in males versus females (21). Hydroxylation of LCA at position 6β represents the major pathway for LCA catabolism and is important for maintenance of the bile acid pool and for prevention of cholestasis (23). We have also shown that GH is the primary hormone involved in sex-specific expression of CYP3A10/6β-hydroxylase (9). Using chimeric CYP3A10/6β-hydroxylase promoter–luciferase (Luc) reporter constructs transfected into hamster primary hepatocytes, we have shown GH-mediated induction of CYP3A10/6β-hydroxylase promoter activity and have localized a DNA element that mediates this transcriptional activation of the CYP3A10/6β-hydroxylase gene by GH. This element has the characteristics of a GAS element, and the factor that binds to it is absent in hypophysectomized hamster nuclear extracts, whereas it is restored upon injection of GH in a manner that mimics the male pattern of GH secretion, consistent with the observed induction of CYP3A10/6β-hydroxylase expression to male levels. The DNA–protein complex is supershifted by anti-STAT 5 antibodies, supporting the idea that the factor(s) involved in sexually dimorphic expression of CYP3A10/6β-hydroxylase belongs to the STAT family of transcriptional factors (9).

In the present study we have used this system to further dissect the molecular mechanisms involved in sexually dimorphic expression of liver-specific genes. We show that STAT 5 (both 5a and 5b) indeed mediates GH-dependent activation of the CYP3A10/6β-hydroxylase promoter, as suggested by the binding studies. We also demonstrate that NF-Y is involved both in basal activity of the CYP3A10/6β-hydroxylase promoter and in its GH-mediated activation.

MATERIALS AND METHODS

Plasmids

pLuc6β-821 was made as described earlier (9) and contains 821 nucleotides (nt) of the CYP3A10/6β-hydroxylase 5′-flanking region in pGL2 (Promega). pLuc6β-821 Mut –58/–57 was created by oligonucleotide-directed mutagenesis as described previously (24). pCMV-STAT 5a and pCMV-STAT 5b were created by cloning the respective STAT 5 cDNA (a gift from A.Mui) fragments into the *Eco*RI–*Xba*I site of pCMV5 (25). Orientation of the inserted fragments was confirmed by DNA sequencing. pNF-YA29 was a gift from R.Mantovani and contains the cDNA for a dominant negative mutant (26) in a mammalian expression vector.

Isolation and transfection of primary hepatocytes

Hepatocytes were prepared for primary culture by perfusion of isolated female hamster livers as described earlier (9). Cells were co-transfected by the calcium phosphate co-precipitation method, also as described earlier (9), using 5 µg test plasmid, 1 µg pCMV-RGHR and 1 µg pCMV-Gal and, where indicated, up to 3 µg corresponding expression plasmid. Human GH (National Hormone and Pituitary Program) treatment, harvesting of cells and luciferase and β-galactosidase assays were as described earlier (9). Except where otherwise noted, average values for three experiments are shown and bars indicate the SEM.

Preparation of nuclear extracts

Nuclear extracts were prepared from hamster primary hepatocytes isolated by the above procedure except that the cells were seeded onto 100 mm diameter plastic tissue culture dishes at a density of 2.3 $\times 10^6$ cells/dish (6 dishes/experimental group). The total amount of DNA transfected per dish was 20 µg. On day 3, one dish from each experimental group was harvested for luciferase and β-galactosidase activities to check transfection efficiency and fold regulation and the rest were used for preparation of nuclear extracts, essentially by the method of Sadowski and Gilman (27). Briefly, cells were first rinsed with ice-cold PBS containing 1 mM Na_3VO_4 and 5 mM NaF and then with hypotonic buffer [20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 µg/ml each leupeptin, aprotinin and pepstatin]. Ice-cold hypotonic buffer containing 0.2% Nonidet P-40 (0.5 ml) was then added directly to the dishes to lyse cells. Lysates were scraped into microfuge tubes, mixed and the nuclei pelletted (16 000 *g*, 4°C, 20 s). The nuclear pellets from each dish were resuspended in 25 µl high salt buffer (hypotonic buffer containing 320 mM NaCl, 100 mM KCl and 20% glycerol), pooled and gently rocked (4°C, 30 min). Extracted nuclear proteins were then separated from residual nuclei by spinning in an airfuge (30 p.s.i., 4°C, 5 min) and collecting the supernatants.

Experimental animals

Golden Syrian hamsters (Charles River Laboratories) were maintained on a 12 h light/12 h dark cycle, fed regular laboratory chow and killed in the middle of the dark cycle. Liver nuclear extracts were prepared by the Schibler method (28). All animal experiments were conducted in accordance with the principles and procedures of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Gel mobility shift analysis

Gel mobility shift analysis was performed as described previously (29) using the following probes: CYPGHRE (CYP3A10/6β-hydroxylase GH regulatory element), containing nt –101 to –78 from the CYP3A10/6β-hydroxylase promoter (GTTACCCAAGT-TCCTGGAAGCGTG); CYPNFY (CYP3A10/6β-hydroxylase NF-Y element), containing nt –77 to –53 from the CYP3A10/6β-hydroxylase promoter (CAAGAGGCCCTTCTACTGGCTG-CAG). For the supershift experiments affinity-purified anti-STAT 5 antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) raised against a peptide corresponding to amino acids 765–781 of mouse STAT 5b. Anti-NF-YA (monoclonal αYAMab7) and anti-NF-YB (polyclonal) antibodies (30) were a gift from R.Mantovani.

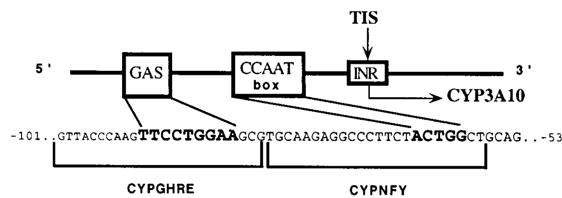


Figure 1. Schematic representation of the CYP3A10/6β-hydroxylase promoter. The two DNA elements within the CYP3A10/6β-hydroxylase promoter, the GAS element and the inverted CCAAT box, that bind the characterized *trans*-acting factors are indicated, with the nucleotide sequence (upper strand) underneath and the binding sites in bold. The initiator element (INR) and the transcription initiation site (TIS) are also indicated. The DNA probes used for the gel shift experiments are indicated under the sequence.

RESULTS

STAT 5 provides GH-mediated transactivation of the CYP3A10/6β-hydroxylase promoter

Figure 1 is a schematic representation of the CYP3A10/6β-hydroxylase promoter showing the GAS element, the inverted CCAAT box and the nucleotide sequence of the DNA probes used in the present study. In previous studies we have localized a DNA element involved in GH-mediated regulation of CYP3A10/6β-hydroxylase promoter activity (9). This element (CYPGHRE) resembles a STAT binding site (GAS site). By footprint and gel mobility shift analyses we have confirmed that expression of the protein binding to this site is regulated by GH and that the DNA–protein complex can be partially supershifted by anti-STAT 5 antibodies, indicating that the protein belongs to the STAT family of transcription factors and that it is antigenically related to STAT 5 (9). Anti-STAT 1 and anti-STAT 3 antibodies on the other hand show no specific effect on the retarded band (9).

Based on the above results and with the availability of two murine STAT 5 cDNAs, STAT 5a and STAT 5b (31), we were interested to see whether GH-dependent activation of the CYP3A10/6β-hydroxylase promoter could indeed be mediated by STAT 5. We co-transfected hamster primary hepatocytes with expression plasmids for STAT 5a (pCMV-STAT 5a) or STAT 5b (pCMV-STAT 5b) and the growth hormone receptor (pCMV-RGHR), together with a luciferase reporter construct containing the CYP3A10/6β-hydroxylase promoter, pLuc6β-821 and pCMVGal (to normalize for transfection efficiency). pCMV5 served as the negative control. Cells were co-transfected with different amounts of STAT 5b and treated with or without GH (500 ng/ml medium) (Fig. 2A) or co-transfected with 3 µg STAT 5a or STAT 5b with increasing amounts of GH (Fig. 2B). As seen in Figure 2A, STAT 5b brought about induction of promoter activity only in the presence of GH. Both STAT 5a and STAT 5b worked similarly well as GH-dependent transactivators and they brought about a 3- to 5-fold induction on GH-mediated promoter activity as compared with the control pCMV5 (Fig. 2B). This induction reflected increasing concentrations of GH added, reaching a plateau at ~100 ng GH/ml. Since STAT 5b brought about a slightly better induction than STAT 5a we decided to use STAT 5b for all further experiments.

Having shown that STAT 5 does mediate GH-dependent activation of the CYP3A10/6β-hydroxylase promoter, we wanted to see if this was reflected in binding of STAT 5 to the CYPGHRE. Hamster primary hepatocytes were co-transfected with pLuc6β-821, pCMV-STAT 5b, pCMV-RGHR and pCMV-Gal, treated with or without GH (500 ng/ml medium) and then harvested for preparation of nuclear extracts. Gel mobility supershift assays (Fig. 3), using as a probe a 24 bp (–101 to –78) fragment from the CYP3A10 promoter that contains the already localized CYPGHRE and using antibodies to STAT 5, revealed that anti-STAT 5 supershifted the DNA–protein complex only in cells that had been transfected with both STAT 5b and the GH receptor cDNA and then treated with GH (lanes 8, 10 and 12).

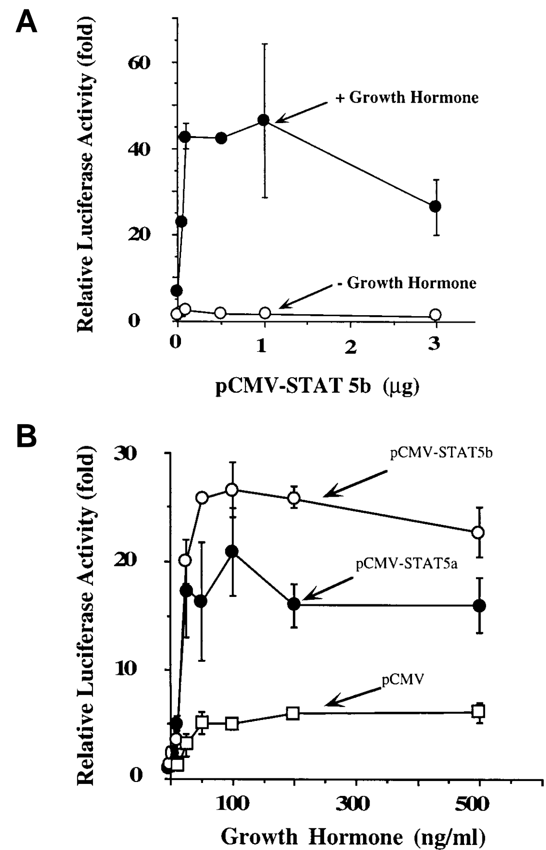


Figure 2. Overexpression of STAT 5a or STAT 5b enhances GH-mediated activation of the CYP3A10/6β-hydroxylase promoter. (**A**) Female hamster primary hepatocytes were co-transfected with pLuc6β-821, the indicated amounts of pCMV-STAT 5b, pCMV-RGHR and pCMV-Gal, as described in Materials and Methods. Transfected cells were incubated with or without GH (500 ng/ml medium) and harvested after 48 h. (**B**) Female hamster primary hepatocytes were co-transfected with pLuc6β-821, pCMV-STAT 5a, pCMV-STAT 5b or pCMV (as control), pCMV-RGHR and pCMV-Gal, as described in Materials and Methods. Transfected cells were then treated with the indicated concentrations of GH for 48 h and harvested for luciferase and β-galactosidase assays. Relative activities were determined by normalizing luciferase activity to β-galactosidase activity. Average values for three experiments relative to the values for cells grown in the absence of GH are shown. Bars indicate the standard errors of the means.

NF-Y is a positive transcription factor for the CYP3A10/6β-hydroxylase promoter and modulates GH-mediated regulation by STAT 5

Our previous experiments with 5′-deletion constructs (9) and specific mutants indicated that a DNA element important for CYP3A10/6β-hydroxylase promoter activity was located between nt –186 and –62 of the promoter. DNase I footprint analysis and

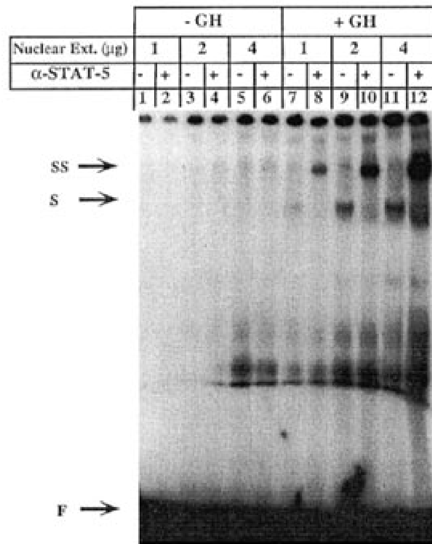
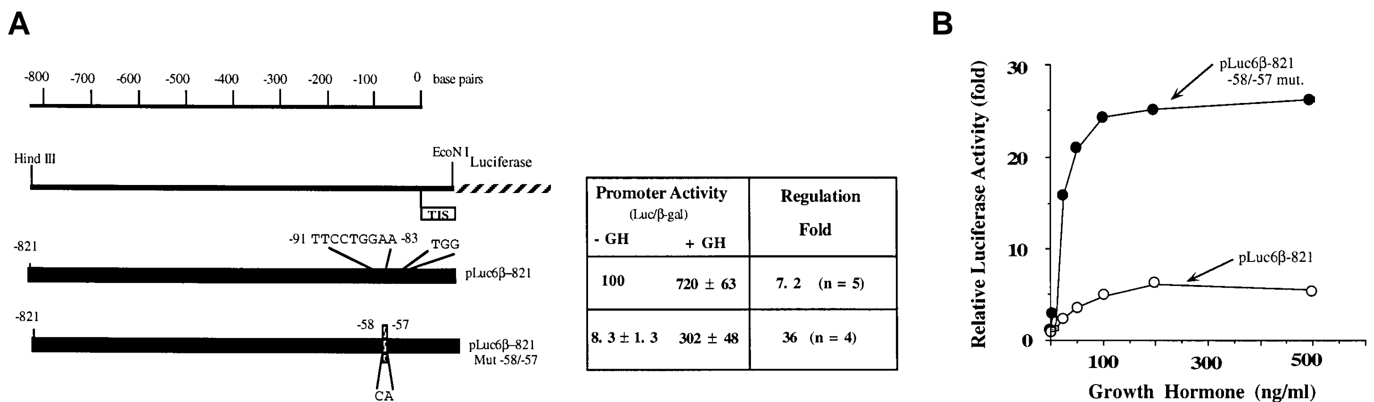


Figure 3. Antibodies to STAT 5 supershift the GAS–nuclear protein complex in extracts made from GH-treated primary hepatocytes co-transfected with pCMV-STAT 5. Female hamster primary hepatocytes were co-transfected with pLuc6β-821, pCMV-STAT 5b, pCMV-RGHR and pCMV-Gal, as described in Materials and Methods. Transfected cells were incubated with (lanes 7–12) or without (lanes 1–6) GH (500 ng/ml medium) for 48 h and harvested for preparation of nuclear extracts. Increasing amounts (1, 2 and 4 µg protein) of nuclear extract were used in a gel shift experiment, performed as described in Materials and Methods, in the presence (lanes 2, 4, 6, 8, 10 and 12) or absence (lanes 1, 3, 5, 7, 9 and 11) of 0.2 µg anti-STAT 5 antibodies. The –101 to –78 fragment from the CYP3A10/6β-hydroxylase promoter was used as probe. Arrows indicate the shifted (S) and supershifted (SS) complexes and the free probe (F).

homology searches with DNA elements known to bind *trans*-acting factors indicated the presence of a putative site between nt –63 and –58 of the promoter (ACTGGC) which resembled an inverted CCAAT box with the potential to bind the transcription factor NF-Y (32). Given the proximity of the already localized CYPGHRE to this potential NF-Y binding site we wanted to see if NF-Y plays any role in expression and/or GH-mediated regulation of the CYP3A10/6β-hydroxylase gene. A mutant construct (pLuc6β-821 Mut –58/–57) was made where nt –58 and –57 (GG), which are in the core of the putative NF-Y binding site, were mutated (Figs 1 and 4A). Both the wild-type and mutant constructs were independently tested for their ability to provide GH-mediated induction of promoter activity at 500 ng GH/ml medium. As can be seen in Figure 4A, transfection with the mutant brought about a 10-fold drop in basal activity of the promoter while enhancing GH-mediated induction of promoter activity by ~5-fold over the wild-type. Figure 4B shows a GH concentration curve for the wild-type and mutant constructs. Both constructs showed induction of GH-mediated promoter activity, reaching a plateau at ~100 ng GH/ml medium. As expected, GH (500 ng/ml medium) brought about a 25-fold regulation of the mutant, as compared with 6-fold regulation of the wild-type promoter.

To prove that the NF-Y protein in fact binds to nt –63 to –58, we performed gel mobility supershift assays using the CYPNFY probe and two different antibodies raised against two of the subunits of NF-Y, NF-YA and NF-YB (Fig. 5). A shifted band (S) was seen (lane 1) that was partially supershifted (SS) by anti-NF-YA (lanes 2–4) and fully supershifted by anti-NF-YB (lanes 5–7). Note that most of the antibody–NF-Y–DNA complex stays at the very top of the gel, as expected if NF-Y were to bind to that site. As a control we used another probe where nt –58/–57 at the core of the NF-Y binding site were mutated. No binding was seen to this probe (data not shown).

To further explore the role of NF-Y in expression and GH-mediated regulation of CYP3A10 promoter activity, we over-expressed a dominant negative mutant of NF-YA, named NF-YA29, in our tissue culture system (26). Figure 6 shows that increasing amounts of the expression plasmid produced a strong decrease (9-fold) in CYP3A10 promoter activity with no significant change in regulation by GH at each amount of pNF-YA29 tested. To demonstrate the specificity of this effect we transfected a plasmid containing the mouse albumin enhancer/promoter in front of the luciferase gene (pLucAEP) (9). No effect was observed at any amount of pNF-YA29 co-transfected, as expected, since the albumin promoter and enhancer have no NF-Y sites.



Promoter Activity (Luc/β-gal) - GH	+ GH	Regulation Fold
100	720 ± 63	7. 2 (n = 5)
8. 3 ± 1. 3	302 ± 48	36 (n = 4)

Figure 4. Mutation of the NF-Y binding site in the CYP3A10/6β-hydroxylase promoter inhibits transcription while enhancing GH-mediated induction of promoter activity. (**A**) The wild-type sequence with the GAS and NF-Y sites and the mutated NF-Y site are indicated. The wild-type (pLuc6β-821) and mutant (pLuc6β-821 Mut –58/–57) plasmids were independently transfected into female hamster primary hepatocytes and incubated with or without GH (500 ng/ml medium), as described in Materials and Methods. The data were then normalized to the activity produced by the wild-type in cells grown in the absence of GH and represent the average of *n* experiments ± SEM. (**B**) Hamster primary hepatocytes transfected with the wild-type CYP3A10/6β-hydroxylase promoter construct (pLuc6β-821) or NF-Y mutant (pLuc6β-821 Mut –58/–57) were incubated with the indicated concentrations of GH for 48 h and promoter activities quantified as described.

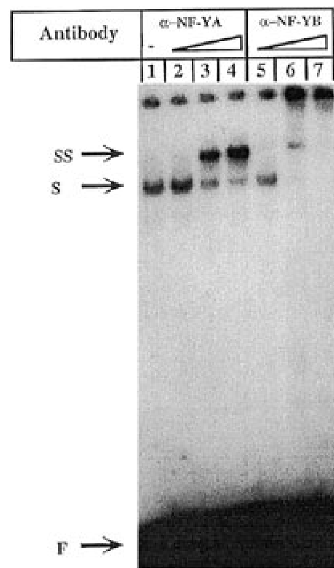


Figure 5. Anti-NF-Y antibodies supershift the CYPNFY–nuclear protein complex. Gel shift experiments were performed as described in Materials and Methods, using 3 µg male hamster liver nuclear protein in the absence of any antibody (lane 1) or in the presence of increasing amounts (0.04, 0.2 and 0.4 µg) of anti-NF-YA or increasing amounts (0.02, 0.1 and 0.2 µg) of anti-NF-YB. The –77 to –53 fragment from the CYP3A10/6β-hydroxylase promoter was used as a probe. Arrows indicate the shifted (S) and the supershifted (SS) complexes and the free probe (F).

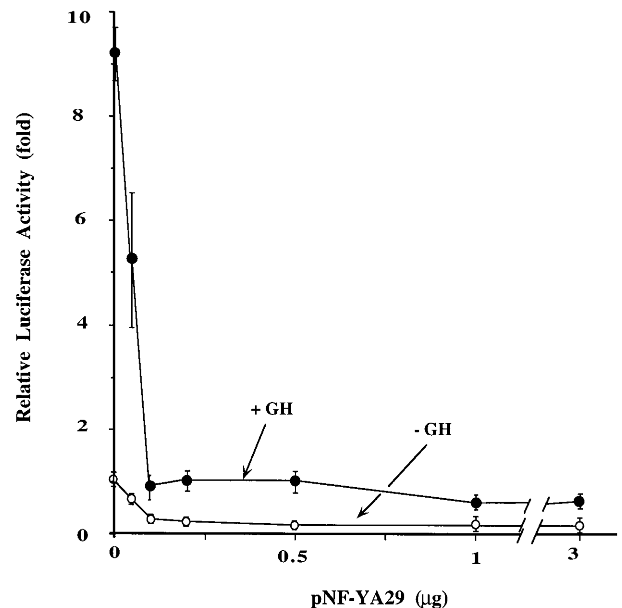


Figure 6. Overexpression of NF-YA29, a dominant negative mutant of NF-YA, suppresses activity of the CYP3A10/6β-hydroxylase promoter. Female hamster primary hepatocytes were co-transfected with pLuc6β-821, pCMV-NF-YA29, pCMV-RGHR and pCMV-Gal, as described in Materials and Methods. Transfected cells were then treated with 500 ng GH/ml medium for 48 h and harvested. Relative transcription was determined by normalizing luciferase activity to β-galactosidase activity. Average values for three experiments relative to the values for cells with no pNF-YA29 transfected and in the absence of GH are shown.

DISCUSSION

We have previously reported that the CYP3A10/6β-hydroxylase gene is expressed at 50-fold higher levels in male than in female hamsters (21) This sex-specific expression was shown to be mediated by the differential pattern of GH secretion and, based on DNA–protein binding experiments, we suggested that the factor involved in GH-mediated induction of CYP3A10/6β-hydroxylase transcription was related to STAT 5 (9). In this report we demonstrate for the first time a direct effect of a STAT factor on the promoter of a GH-regulated gene and show that both STAT 5a and STAT 5b are indeed involved in this regulation. We also show that NF-Y, a factor that has been shown to interact with other regulatory factors, such as SREBP (18) and the T3 receptor (33), and is thus involved in maintaining high levels of transcription of many genes, is involved in transcription of the CYP3A10/6β-hydroxylase gene and also modulates GH-mediated regulation of its transcription.

The direct effect of STAT 5a and STAT 5b was shown by co-transfection of expression vectors containing the cDNAs for murine STAT 5a and STAT 5b. Both factors greatly enhanced GH-mediated induction of CYP3A10/6β-hydroxylase promoter activity as compared with control experiments where the vector alone was co-transfected (Fig. 2). This induction correlated with a major increase in STAT 5 protein in the nucleus (Fig. 3). These results illustrate the diversity of factors that mediate GH-mediated regulation of gene transcription depending on the gene and/or cell type involved. For example, STAT 5b seems to be exclusively the factor involved in sex-specific expression of several mouse liver genes, such as the major urinary protein CYP15α/2A4, prolactin receptor and testosterone-6β-hydroxylase, since sex-specific expression of all these genes is lost in *STAT 5b*$^{-/-}$ mice (13). On the other hand, in L cells GH induces tyrosyl phosphorylation of STAT 5a and its association with the GH receptor but has no effect on STAT 5b (34). Yet, in 3T3 F442A, CHO and COS cells, GH stimulates tyrosyl phosphorylation and DNA binding activity of both STAT 5a and STAT 5b (35), as measured by binding to the GAS-like element in the β-casein promoter. Interestingly, GH-mediated regulation of female-specific CYPC12 is not mediated by any STAT factor but by hepatocyte nuclear factor 6, which is also induced by the female pattern of GH release. The factor(s) responsible for sex-specific expression of other genes, such as CYPC11, is still unidentified.

The presence of an inverted CCAAT box just 3′ of the STAT binding site (Fig. 1) suggested to us that NF-Y could be involved in transcription and/or regulation of the CYP3A10/6β-hydroxylase gene. Supershift experiments using antibodies against two of the subunits of the NF-Y protein (Fig. 3) demonstrated that NF-Y indeed binds to the CYP3A10/6β-hydroxylase promoter. Further confirmation was obtained by showing lack of a DNA shift when a DNA probe was used where guanidines –59 and –60 of the inverted CCAAT box were mutated (data not shown). The NF-Y binding element in the CYP3A10/6β-hydroxylase promoter (ACTGGC) is slightly different from the consensus binding element (ATTGGC) (32), and to our knowledge this is the first time that this site has been shown to bind NF-Y. Activity of the mutated CYP3A10/6β-hydroxylase promoter was decreased by ~10-fold in the absence of GH (Fig. 4A). Interestingly, the mutated promoter was much more sensitive to GH, since its activity in the presence of GH was only half that of the wild-type promoter activity (Fig. 4A and B). These data suggest that NF-Y

is not only involved in positive transcription of the CYP3A10 promoter but also in its GH-mediated activation. There are other cases described where NF-Y has been shown to interact with other regulatory factors and thus play a crucial role not only in expression of many genes but also in their regulation. For example, NF-Y plays a role in cholesterol-mediated regulation of HMG-CoA synthase and farnesyl diphosphate synthase (36). Also, NF-Y is required for thyroid hormone regulation of rat liver *S14* gene transcription (33). Our data indicate that in the case of the CYP3A10 promoter NF-Y plays an important role in GH-mediated regulation by modulating access of STAT 5 to its binding site located just 5′ of the inverted CCAAT box. Supershift experiments using a DNA probe expanding both STAT 5 and NF-Y sites showed that more STAT 5 protein was capable of binding to its site when NF-Y site was mutated (data not shown). This greater binding of STAT 5 results in higher GH-mediated activation of the CYP3A10/6β-hydroxylase promoter with the NF-Y site mutated (Fig. 4A and B).

The role of NF-Y in the activity and regulation of the CYP3A10/6β-hydroxylase promoter was further demonstrated by co-transfection experiments with pNF-YA29 (Fig. 6), a dominant negative mutant of NF-YA defective in its DNA binding capability (26). Low amounts of this mutant produced a strong decrease in the level of CYP3A10A/6β-hydroxylase promoter activity, supporting the idea that NF-Y plays a positive role in its transcription. Unexpectedly, overexpression of pNF-YA29 failed to reproduce the higher sensitivity to GH that we observed when the inverted CCAAT box was mutated (Fig. 4). We believe that this reflects the fact that nuclear extracts prepared from cells transfected with this NF-Y mutant still maintain binding activity to the inverted CCAAT box (18), probably because under transient transfection conditions only a relatively small number of cells are transfected. The fact that the level of expression of pNF-YA29 was enough to dramatically reduce expression of the CYP3A10/6β-hydroxylase promoter without significantly affecting GH-mediated regulation indicates that NF-Y and STAT 5 do not physically interact with each other to mediate regulation and that a total absence of NF-Y binding is necessary in order to see its effect on GH regulation.

In conclusion, GH-mediated sex-specific expression of the CYP3A10/6β-hydroxylase promoter is mediated by both STAT 5a and STAT 5b. NF-Y is a crucial factor involved both in positive transcription and in GH-mediated regulation and seems to function by controlling the level of potential occupancy of the STAT 5 binding site. Further experimentation should clarify the role played by NF-Y in this regulation.

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